CHAPTER 1

Retrograde flush following topical cooling is superior to preserve the non-heart-beating donor lung

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ABSTRACT

Objective
The use of non-heart-beating donors (NHBD) has been propagated as an alternative to overcome the scarcity of pulmonary grafts. Formation of microthrombi after circulatory arrest, however, is a major concern for the development of reperfusion injury. We looked at the effect and the best route of pulmonary flush following topical cooling in NHBD.

Methods
Non-heparinized pigs were sacrificed by ventricular fibrillation and divided in 3 groups (n=6/group). After 1 hour of in situ warm ischemia and 2.5 hours of topical cooling, lungs in group I were retrieved unflushed [NF]. In group II, lungs were explanted following an anterograde flush [AF] through the pulmonary artery with 50ml/kg Perfadex (6°C). Finally, in group III lungs were retrieved after an identical but retrograde flush [RF] via the left atrium. Flush effluent was sampled at intervals to measure hemoglobin concentration. Performance of the left lung was assessed during 60 minutes in our ex vivo reperfusion model. Wet-to-dry weight ratio (W/D) of both lungs was calculated as an index of pulmonary edema. IL-1β and TNF-α protein levels in bronchial lavage fluid from both lungs were compared between groups.

Results
Hemoglobin concentration (g/dl) was higher in the first effluent in RF versus AF (3.4 ± 1.1 versus 0.6 ± 0.1) (p < 0.05). Pulmonary vascular resistance (dynes*sec*cm⁻⁵) was 975 ± 85 [RF] versus 1567 ± 98 [AF] and 1576 ± 88 [NF] at 60 minutes of reperfusion (p < 0.001). Oxygenation (mmHg) and compliance (ml/cmH₂O) were higher (491 ± 44 versus 472 ± 61 and 430 ± 33 [NS]; 22 ± 3 versus 19 ± 3 and 14 ± 1 [NS], respectively) and plateau airway pressure (cmH₂O) was lower (11 ± 1 versus 13 ± 1 and 13 ± 1 [NS]) after RF versus AF and NF, respectively. No differences in cytokine levels or in W/D ratios were observed between groups after reperfusion. Histology demonstrated microthrombi more often present after AF and NF compared to RF.

Conclusion
Retrograde flush of the lung following topical cooling in the NHBD results in a better washout of residual blood and microthrombi and subsequent reduced pulmonary vascular resistance upon reperfusion.

INTRODUCTION

The scarcity of suitable donor organs is the main limiting factor for widespread application of lung transplantation. Only 15-30% of the brain dead donors have lungs that are deemed transplantable [1]. As a result of the disparity between the growth in demand and the inadequate organ supply, a renewed interest in the use of lungs from non-heart-beating donors (NHBD) is emerging [2]. There is now experimental [3-6] and clinical [7,8] evidence that a limited period of warm ischemia does not compromise the performance of the pulmonary graft from the NHBD and that topical cooling is an effective method to protect the pulmonary graft inside the cadaver [5].

The formation of microthrombi after circulatory arrest and the subsequent development of primary graft dysfunction resulting from ischemia-reperfusion injury, however, are still a concern for the use of lungs from NHBD. From a recent study, we have data suggesting that retrograde flush of the lungs after 1 hour of warm ischemia is better to preserve graft performance compared to anterograde pulmonary flush or no flush [9]. However, no experimental data on the effect of pulmonary flush following additional topical cooling in the cadaver are available in the literature.

The aim of this study was to investigate the benefit and the most effective route (anterograde versus retrograde) of pulmonary flush following topical cooling after warm ischemia using our isolated porcine lung reperfusion model.

MATERIAL AND METHODS

Animal Preparation
Domestic pigs (n=6/group; weight: 37.2 ± 1.1 kg) were used, given their physiological and anatomical similarity to man. All animals received human care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (NIH Publication No. 85-23, Revised 1996). The study was approved by the institutional review board on animal research at the Katholieke Universiteit Leuven.
Animals were premedicated with an intramuscular injection of Xylazine (5 ml Xyl-M® 2%, V.M.D. nv/sa, Arendonk, Belgium) and Zolazepam/Tiletamine (3 ml Zoletil® 100, Virbac s.a., Carros, France). The animals were installed in a supine position and intubated with an endotracheal tube 7.5 (Portex Tracheal Tube, SIMS Portex, Ltd. Hythe, Kent, UK) and ventilated with a volume-controlled ventilator (Titus®, Dräger, Lübeck, Germany) with an inspiratory oxygen fraction (FiO₂) of 0.5 and a tidal volume of 10 ml/kg body weight. Respiratory rate was adjusted to achieve an end-tidal CO₂ of 40 mmHg. Positive end-expiratory pressure was set to 5 cmH₂O. Anaesthesia was maintained with isoflurane 0.8 – 1% (Isoba® Vet, Schering – Plough Animal Health, Harefield, Uxbridge, UK) and muscle relaxation with intermittent bolii of pancuronium bromide (Pavulon 2 mg/ml, Organon, Teknika, Boxtel, The Netherlands). A 14 G catheter (Secalon® T, Becton Dickinson Ltd., Singapore) was placed in the right common carotid artery for measurement of the systemic arterial pressure (SAP) and sampling of arterial blood. A 7.5 F Swan-Ganz thermodilution catheter (Baxter Healthcare Corp., Irvine, CA, USA) was inserted through the right external jugular vein into the pulmonary artery. With this catheter, hemodynamic parameters including pulmonary artery pressure (PAP) and pulmonary capillary wedge pressure (PCWP) were monitored. Hemodynamic parameters (SAP, PAP and PCWP) and aerodynamic parameters (plateau airway pressure and compliance) were continuously monitored and stored.

Pigs were sacrificed by inducing ventricular fibrillation with a subxyphoidal needle puncture using a square-pulse generator (amplitude range +15 to -15 V, current < 300 mA, frequency 50 Hz). After cardiac arrest, the endotracheal tube was disconnected from the ventilator and left open to the air. The cadavers were left untouched for 1 hour at room temperature followed by a 2.5 hour interval of topical cooling (Figure 1.1A). Therefore, 2 chest drains were inserted in each pleural cavity (one superficial, one deep). Lungs were then cooled with cold saline in a closed circuit. To ensure that the lungs were well immersed in the fluid, the superficial drains were connected to an overflow system of 5 cmH₂O. Temperature of the lung was measured via a probe in the endotracheal tube and rectal temperature was monitored. After that interval of topical cooling, sternotomy was performed. The thymic tissue was excised and the pericardium and pleural cavities were widely opened. The lungs were inspected. The pulmonary artery, ascending aorta and caval veins were encircled. Gross microthrombi in the pulmonary artery and left atrium were evacuated as much as possible.

Experimental Groups

Eighteen domestic pigs were randomly divided in 3 groups (n=6/group; weight: 37.2 ± 1.1 kg). In the first group the lungs were explanted without flush (NF). In the second group the pulmonary artery was cannulated (DLP Inc, Grand Rapids, MI, USA) through the right ventricular outflow tract and secured with a purse-string. The caval veins were ligated and the ascending aorta was clamped. The pulmonary artery was isolated from the right ventricle by a ligature around the tip of the catheter just distal to the pulmonary valve. The right and the left atrium were incised for venting of the heart. The lungs were flushed in an anterograde (AF) (Figure 1.1B) way with 50 ml/kg cold (6°C) Perfadex® (Vitrolife, Göteborg, Sweden) buffered with Trometamol (0.3 ml/l, 2 g/5 ml, Addex-THAM) and CaCl₂ (0.6 ml/l, 11mEq). During the flush, ventilation was restarted with a low tidal volume and a small frequency to avoid cold lung injury related to mechanical stress. Finally, in the third group the left atrium was cannulated (MOD Cannula 18 Fr, International Medical Products NV/SA, Brussels, Belgium) through a purse-string and the lungs were flushed in an identical but retrograde manner (RF) (Figure 1.1C). The anterior aspect of the main pulmonary artery was incised for drainage of the flush solution. The perfusion
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Preparation of the Heart-Lung Block
The lungs in all 3 groups were then explanted and prepared in the same way for ex vivo evaluation in the isolated reperfusion system. The right lung was separated from the heart-lung block and used as a control for morphological and biochemical analysis. The pulmonary artery was cannulated through the right ventricular outflow tract using a 36 Fr cannula and isolated with a ligature around the catheter distal to the pulmonary valve. A small catheter was placed in the pulmonary artery for measurement of PAP. The ascending aorta was clamped. The left atrium was cannulated through the apex of the left ventricle with a second 36 Fr cannula and secured with a purse-string. Finally, an endotracheal tube nr. 8 was placed in the trachea for ventilation of the graft.

Preparation of the Perfusate
Autologous blood (1200 ml) was rapidly withdrawn from each animal at the moment of sacrifice via the catheter in the right external jugular vein and collected in a sterile bag containing 5000 IU of heparin (NAtrium Heparine B. Braun, 25000 IU/5 ml, B. Braun Medical SA, Jaén, Spain). This whole blood was centrifuged with a Cell Saver (Sequestra 1000, Medtronic Inc., Parker, CO, USA) and washed with saline for 12 minutes at 5600 rpm. Leukocytes were sequestered using a leukocyte filter (Imugard III-RC, Terumo Europe N.V., Haasrode, Belgium). The remaining red blood cells (350 ml) were then diluted to a hematocrit of 15% with a low potassium dextran solution (Perfadex®) and human albumin (final concentration: 8%, CAF-DCF, Brussels, Belgium). The perfusate was finalized by adding CaCl₂ (2.4 ml/l, 100 mg/ml), heparin (10000 IU/l) and sodium bicarbonate (45 ml/l, 16.8 g/250 ml Baxter, Lessines, Belgium). The total volume of the perfusate was 1400 ml.

Isolated Reperfusion Circuit
The ex vivo reperfusion system consisted of a hardshell reservoir (Minimax Hardshell reservoir, Medtronic, Minneapolis, MN, USA), a centrifugal pump (Bio-medicus, Medtronic), a heater/cooler system (Bio-Cal, Heater Cooler Model 370, Medtronic, Minneapolis, MN, USA) and a hollow fiber oxygenator (Capiox®SX, Terumo, MI, USA) with integrated heat exchanger. The heating element of the gas exchanger was connected to the heater/cooler system. The left lung and the heart were then placed in a specially designed evaluation box and mounted in the reperfusion system. The cannula in the pulmonary artery was connected to the inflow tubing and the outflow tubing was connected to the cannula in the left atrium.

Technique of controlled reperfusion and ventilation
Reperfusion of the left lung was started with normothermic (37°C) oxygenated perfusate (O₂: 0.4 l/min) after de-aring of the inflow tubing. Pulmonary artery pressure was gradually increased to a maximum of 15 mmHg and the left atrial pressure on the outflow was kept at 0 mmHg by adjusting the height of the blood reservoir. This resulted in warming up of the lung and a gradual increase in pulmonary artery flow. Ventilation with a FiO₂ 0.5 was started when the temperature of the outflowing perfusate reached 34°C and slowly increased to a tidal volume of 140 ml, a frequency of 14 breaths/min and PEEP of 5 cmH₂O. At that moment, the perfusate was partially deoxygenated to a PO₂ of 50 – 60 mmHg with a gas mixture of CO₂ (8%), O₂ (6%) and N₂ (86%).

Assessment of the Graft
Thirty-five minutes after the onset of reperfusion the temperature of the lung parenchyma reached 37.5°C. At this moment functional graft parameters were recorded up to one hour (Figure 1.1). Pulmonary artery pressure (PAP) (mmHg) was measured via an 18 Gauge catheter inserted in the main pulmonary artery. The pressure in the left atrium (LAP) (mmHg) was measured on the outflow line. An electromagnetic flow probe (FF 100T 10 mm probe, Nihon Kohden, Tokyo, Japan) was inserted in the tubing on the inflow line for continuous measurement of the pulmonary artery flow (PAF) (l/min). Pulmonary vascular resistance (PVR) was calculated using the formula: PVR = [PAP – LAP] x 80/PAF and expressed in dynes x cm⁻⁵. Dynamic lung compliance (Compl) (ml/cmH₂O) and plateau airway pressure (Plat AwP) (cmH₂O) were recorded. PO₂ and PCO₂ were continuously measured in the perfusate via probes (Terumo CDITM, 500 shunt sensor, Leuven, Belgium) on the outflow tubing using an inline blood gas analyzer (CDITM 500, Terumo, Borken, Germany). Oxygenation capacity was calculated using the formula PO₂/FiO₂ (mmHg).

Temperature (°C) of the inflowing and outflowing perfusate was continuously measured, the last being considered as the graft temperature. All data were recorded online and stored on a central server (Datex AS/3 and S5 collect 3.0 Software respectively, Datex-Ohmedia, Helsinki, Finland).
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Measurement of IL-1ß and TNF-α
Bronchial lavage was performed in the right lung after explantation and in the left lung immediately at the end of the reperfusion. Twenty-five ml of sterile saline at room temperature was instilled in the bronchus and aspirated with gentle suction after 1 minute in a standardized way. The supernatant was collected for further analysis and stored at -80°C.

Swine IL-1ß and TNF-α protein levels were measured in bronchial lavage supernatant from both lungs using the commercially available ELISA kits (BioSource Europe SA, Nivelles, Belgium). The sensitivity was 15 pg/ml for IL-1ß and 3 pg/ml for TNF-α.

Histology
Tissue samples were obtained from the non-perfused right lung and from the perfused left lung. Specimens were fixed in 6% formaldehyde, dehydrated and stained with phosphotungstic acid hematoxylin (PTAH) to detect fibrin deposits. Histological analysis was performed by one experienced pathologist (E.V.) who was blinded to the experimental set-up.

STATISTICAL ANALYSIS
Data were analyzed using GraphPad Prism 4 (San Diego, CA, USA). All data are expressed as mean ± standard error of the mean (SEM). Graft parameters between study groups were compared using a one-way analysis of variance with multiple comparisons. An unpaired t-test was used to look for significant differences in flush time and in hemoglobin concentration between AF and RF. A p-value < 0.05 was considered as significant.

RESULTS

Study groups
Baseline parameters in the three animal groups prior to sacrifice are listed in Table 1.1. There were no significant differences in animal weight, PAP, Plat AwP, Compl and PaO₂/FiO₂ between the 3 groups.

<table>
<thead>
<tr>
<th>Group (n=6/group)</th>
<th>Animal weight (kg)</th>
<th>PAP (mmHg)</th>
<th>Plat AwP (cmH₂O)</th>
<th>Compl (ml/cmH₂O)</th>
<th>PaO₂/FiO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>35 ± 2</td>
<td>10 ± 1</td>
<td>15 ± 1</td>
<td>31 ± 2</td>
<td>626 ± 33</td>
</tr>
<tr>
<td>AF</td>
<td>36 ± 2</td>
<td>11 ± 1</td>
<td>15 ± 1</td>
<td>38 ± 2</td>
<td>619 ± 11</td>
</tr>
<tr>
<td>RF</td>
<td>40 ± 2</td>
<td>11 ± 1</td>
<td>16 ± 1</td>
<td>36 ± 3</td>
<td>623 ± 39</td>
</tr>
<tr>
<td>p-value</td>
<td>0.11</td>
<td>0.76</td>
<td>0.65</td>
<td>0.16</td>
<td>0.99</td>
</tr>
</tbody>
</table>

NF: no flush; AF: anterograde flush; RF: retrograde flush; PAP: pulmonary artery pressure; Plat AwP: plateau airway pressure; Compl: compliance

Values are expressed as mean ± SEM.

Graft characteristics in the three study groups are compared in Table 1.2. There were no statistically significant differences among the 3 groups regarding warm and cold ischemic intervals, graft temperature at the end of the cold ischemic period and time needed to complete the flush. Warming up the lung to 34°C in the ex vivo circuit took significantly longer in NF compared to RF and AF (p = 0.011).

<table>
<thead>
<tr>
<th>Group (n=6/group)</th>
<th>WIT (min)</th>
<th>CIT (min)</th>
<th>Graft temperature* (°C)</th>
<th>Flush time (sec)</th>
<th>Warming up (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>60.0 ± 0</td>
<td>180.2 ± 0.2</td>
<td>7.1 ± 0.4</td>
<td>-</td>
<td>30 ± 2*</td>
</tr>
<tr>
<td>AF</td>
<td>60.3 ± 0.3</td>
<td>180.0 ± 0.0</td>
<td>6.9 ± 0.3</td>
<td>560 ± 63</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>RF</td>
<td>60.2 ± 0.2</td>
<td>180.0 ± 0.0</td>
<td>6.5 ± 0.6</td>
<td>620 ± 30</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>p-value</td>
<td>0.56</td>
<td>0.39</td>
<td>0.60</td>
<td>0.41</td>
<td>0.011</td>
</tr>
</tbody>
</table>

NF: no-flush; AF: anterograde flush; RF: retrograde flush; WIT: warm ischemic time; CIT: cold ischemic time
* at end of topical cooling; * p = 0.011 NF versus AF and RF

Values are expressed as mean ± SEM.

Pulmonary graft function

Pulmonary vascular resistance
During the whole evaluation period, PVR (dynes x sec x cm⁻⁵) of the left lung was lower in RF compared to AF and NF becoming significant at 50 minutes (947 ± 67 versus 1614 ± 110 and 1665 ± 121, respectively; p = 0.0002) until the end of the reperfusion (975 ± 85 versus 1567 ± 97 and 1576 ± 88, respectively; p = 0.0003) (Figure 1.2A). There was no significant difference in PVR between AF and NF.

Dynamic lung compliance
During the assessment period, dynamic lung compliance (ml/cm H₂O) was higher after RF compared to AF and NF (20 ± 3 versus 16 ± 3 and 13 ± 2 at 35 minutes (p = 0.26); and 22 ± 3 versus 19 ± 3 and 14 ± 1 at 60 minutes (p = 0.096), respectively) (Figure 1.2B).
Plateau airway pressure

There were no significant differences in plateau airway pressure. However, Plat AwP (cmH2O) was lowest in RF compared to AF and NF (11 ± 0 versus 13 ± 1 and 13± 1 at 60 minutes; p = 0.38, respectively) (Figure 1.2C).

Oxygenation capacity

During reperfusion, PO2/FiO2 (mmHg) was lower in RF compared to AF and NF at the start of the assessment. Towards the end, oxygenation capacity improved in RF. The difference between the 3 groups was not significant (Figure 1.2D).

Wet-to-dry weight ratio

The W/D ratio in the non-perfused right lung and in the left lung after reperfusion is shown in Figure 1.3. There was no significant difference between the 3 groups prior (p = 0.12) and after (p = 0.27) reperfusion. W/D ratio in RF and AF, however, was significantly lower after reperfusion (left lung) than before reperfusion (right lung) (4.9 ± 0.1 versus 5.7 ± 0.1; p = 0.0011 and 5.0 ±0.1 versus 5.6 ± 0.1; p = 0.0007, respectively). There was no significant difference in NF (5.2 ± 0.1 versus 5.5 ± 0.1; p = 0.13, respectively).

Hemoglobin concentration

Hemoglobin concentration (g/dl) in the outflowing flush solution over time in RF and AF is depicted in Figure 1.4. At time point 0 the concentration was higher in RF compared to AF (3.4 ± 1.1 versus 0.6 ± 0.1; p = 0.04) but decreased quickly during the flush.

IL-1β and TNF-α

Concentrations of IL-1β and TNF-α in the right lung after explantation and in the left lung after reperfusion are shown in Table 1.3. There were no significant differences for both cytokines amongst the 3 groups.

Table 1.3: IL-1β and TNF-α protein levels in bronchial lavage fluid from the right and the left lung in all study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right lung</td>
<td>Left lung</td>
</tr>
<tr>
<td>NF</td>
<td>80 ± 4</td>
<td>63 ± 6</td>
</tr>
<tr>
<td>AF</td>
<td>69 ± 4</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>RF</td>
<td>78 ± 5</td>
<td>68 ± 12</td>
</tr>
<tr>
<td>p-value</td>
<td>0.20</td>
<td>0.74</td>
</tr>
</tbody>
</table>

NF: no flush; AF: anterograde flush; RF: retrograde flush

Values are expressed as mean ± SEM.
Retrograde flush following topical cooling is superior to preserve the non-heart-beating donor lung

Chapter 1

Histology

Histological examination of biopsies from the right, non-perfused lung showed capillaries occluded with microthrombi in 6/6 NF and in 6/6 AF versus only 1/6 RF (Figures 1.5A - C). One biopsy from the left lung in NF was excluded from the analysis for technical reason. After reperfusion only a small amount of microthrombi were left (2/5 NF, 1/6 AF and 0/6 RF).

DISCUSSION

In this study, we compared graft performance in NHBD lungs after retrograde flush versus anterograde flush and no flush. We found that pulmonary vascular resistance was significantly lower after retrograde flush. Hemoglobin concentration in the outflowing flush solution was also significantly higher in RF versus AF at the start of the flush. PTAH staining of lung biopsies revealed less microthrombi in the non-perfused lung after retrograde flush. We assume that these findings were directly related to each other and that a better washout of microthrombi resulted in a lower PVR upon reperfusion.

Lung transplantation, nowadays, is limited by a scarcity of suitable organ donors. Clinical series have recently suggested that the use of pulmonary grafts from NHBD’s may be a valuable option to alleviate this shortage. In order to safely use the lung from a NHBD, the length of warm ischemic period can be reduced by insertion of thoracic drains for topical cooling [5,8]. Microthrombi formed in NHBD lungs during the pre-flush ischemic interval are considered to represent a risk for graft dysfunction following transplantation [2]. Administration of agents like heparin or fibrinolytic agents may help to better preserve organ function [2,12]. Flushing the lungs during procurement may be a strategy to remove residual microthrombi thereby improving graft performance following transplantation. All these protective strategies in the NHBD, however, may raise ethical questions [10]. Furthermore, permission to intervene after death depends on the legislation for organ donation and harvesting (presumed consent versus explicit consent) and differs from country to country. Several groups have previously investigated the preferred route of pulmonary flush after warm ischemia in the NHBD [3, 11-15]. Most of these reports, however, failed to compare retrograde pulmonary flush, anterograde pulmonary flush or no flush in non-heparinized animals in one experimental set up. We recently already demonstrated that retrograde flush of the pulmonary graft immediately after 1 hour of warm ischemia resulted in a lower PVR during reperfusion [9]. The objective of the present study was to create a situation resembling an NHBD category I according to the Maastricht classification [16]. Therefore the animals were sacrificed by ventricular fibrillation. There was no attempt for cardio-pulmonary resuscitation and animals were not heparinized. After 1 hour of warm ischemia preservation was started by means of topical cooling. The lungs were then harvested after a retrograde, anterograde or no flush and evaluated in the ex vivo model. During the flush ventilation was restarted in a controlled way with a low tidal volume and a low

Figure 1.4: Hemoglobin concentration (g/dl) in the outflowing flush solution. There was a significant difference between RF and AF at time point 0 (* p = 0.04). AF: anterograde flush; RF: retrograde flush

Figure 1.5: Histology of lung specimens obtained from the right lung (phosphotungstic acid hematoxylin stain; original magnification x 200). Residual microthrombi (arrow) were observed more frequently in the capillaries after NF (A) and AF (B) compared to RF (C).
frequency to avoid cold lung injury related to mechanical stress. To our knowledge this study is the first to investigate the best route of pulmonary flush after additional topical cooling in a large animal model. In the early days of lung transplantation prior to cold flush preservation, the lungs were cooled and stored by immersion in 4°C Collins solution as initiated by the Toronto Lung Transplant Group [17].

The effect of topical cooling on NHBD graft performance has also been investigated in several animal studies. Steen and colleagues demonstrated in a pig transplant model that topical cooling is an excellent method to preserve the graft inside the cadaver [18]. Animals were heparinized and no pulmonary flush was performed. In a previous study, our group compared post-mortem ventilation with topical cooling as a method to protect the graft during the warm ischemic period. We found that topical cooling was superior to preserve the graft inside the warm cadaver [5] and that this can safely be extended up to 6 hours [19]. Kutschka and coworkers reported a study comparing anterograde flush in the heart-beating donor (HBD) versus topical cooling for 30 minutes in NHBD in a unilateral porcine lung transplant model [20]. In both groups, the lungs were stored for 24 hours at 8°C in a low potassium dextran solution. Surprisingly, hemodynamic function and animal survival time were superior in the topical cooling group compared to the flush group.

Only one recent study focused on the use of pulmonary flush following topical cooling, Snell and colleagues reported that anterograde flush of lungs in a dog model after 120 minutes of topical cooling preceded by 120 minutes of warm ischemia is feasible [21]. Outcome was comparable with other strategies of NHBD lung preservation and evaluation. These authors also stated that a blood flush evaluation preceding flush cooling might have a role in the assessment of the allograft from the NHBD. A limitation of that study, however, was the limited number of experiments performed.

In all these previous studies, animals were heparinized. We did not administer heparin prior or after cessation of circulation since our first interest was to investigate solely the benefit of pulmonary flush.

Erasmus et al. compared AF followed by RF after a short warm ischemic period versus topical cooling after 1 hour of warm ischemia in a pig model and concluded that lung function was impaired after topical cooling [22]. This was characterized by a large increase in the alveolar-arterial oxygen gradient, lung edema and an increased maximum ventilation pressure. In contrast to the previously discussed studies, cardiac death was induced by ventilator switch off instead of cardiac fibrillation. These authors hypothesized that the hypertensive period preceding cardiac arrest might have caused endothelial damage and a release of pro-inflammatory cytokines. This in combination with 1 hour of warm ischemia might have been the cause of impaired lung function in the topical cooling group.

In this study all our animals were sacrificed by ventricular fibrillation since our first interest was to evaluate the effect of pulmonary flush after additional topical cooling preceded by 1 hour of warm ischemia. We acknowledge that our animals were only exposed to a short agonal phase that may explain the better outcome in our study.

The group from Varela in Madrid reported the first large series of lung transplantation from out-of-hospital NHBD [8,23]. After a warm ischemic interval of maximum 120 minutes, donors are heparinized and lungs are preserved by means of topical cooling via chest drains. During harvesting, an anterograde flush with Perfadex® through the pulmonary artery is performed followed by a flush with blood for graft evaluation. The procedure is completed with a retrograde flush for further preservation. Lung transplantation was performed successfully in 16 patients and all were oxygen independent at discharge [23]. This group provided clinical evidence that pulmonary flush after topical cooling is feasible and results in good outcome.

Assessment of the graft was performed using our well established model of isolated ex vivo reperfusion. In this model, reperfusion is performed in a controlled setting with a maximum inflow pressure of 15 mmHg. The maximum flow through the lung is therefore determined by a decrease in PVR. We noticed a significantly higher flow in RF compared to AF and NF resulting from lower pulmonary vascular resistance. Oxygenation capacity tended to be lower in RF at the start of the assessment. The reaction time of oxygen to bind to hemoglobin and the time required for oxygen to diffuse through the alveolo-capillary membrane are considered to be important in blood oxygenation. Other investigators have demonstrated that pulmonary capillary transit time is decreased during a state of increased cardiac output reflected by a deficit in oxygen transport [24]. Wedging of microthrombi in the capillary vessels may well explain the lower flow in AF and NF and subsequent somewhat better oxygenation. However, it is likely that after transplantation the high PVR in AF and NF will persist resulting in pulmonary hypertension, hydrostatic edema, impaired oxygenation and finally graft failure. These data are consistent with the findings at histological examination of lung biopsies showing more microthrombi in the right
Retrograde flush following topical cooling is superior to preserve the non-heart-beating donor lung in NF and AF compared to RF. This may also explain the longer time that was needed to warm up the lung in NF (p < 0.05) and AF (NS) compared to RF (Table 1.2). These findings need to be further confirmed in a transplant model. We speculate that the difference in graft performance between groups would have become more evident in a transplant model compared to our pressure-controlled reperfusion model.

The role of IL-1ß and TNF-α in ischemia-reperfusion injury was elucidated in previous studies [25-27]. NHBD graft function deteriorates with increasing warm ischemic intervals and this is reflected by an increase in IL-1ß protein levels in the bronchial lavage before [26] and after reperfusion [27]. In the present study, we could not observe any significant difference in IL-1ß and TNF-α protein levels between the 3 groups before (non–perfused right lung) and also not after reperfusion (left lung). We think this may be related to the short warm ischemic period. The results in the present study confirm once more that a warm ischemic period limited to 60 minutes followed by topical cooling is not detrimental for the graft and demonstrate that no additional inflammatory injury is provoked by performing a pulmonary flush under controlled ventilatory settings.

In summary, we demonstrated that retrograde flush following topical cooling resulted in a better washout of blood and microthrombi and subsequent reduced pulmonary vascular resistance in our isolated lung reperfusion model. Based on these findings we would recommend to flush the NHBD lung in a retrograde manner.

REFERENCES

CHAPTER 2

Retrograde flush following warm ischemia in the non-heart-beating donor results in superior graft performance at reperfusion

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ABSTRACT

Objective
The use of non-heart-beating donors (NHBD) has been propagated as an alternative to overcome the scarcity of pulmonary grafts. The presence of postmortem thrombi however, is a concern for the development of primary graft dysfunction. In this isolated lung reperfusion study we looked at the need and the best route of preharvest pulmonary flush.

Methods
Domestic pigs were sacrificed by ventricular fibrillation and divided in 3 groups (n = 6/group). After 1 hour of in situ warm ischemia, lungs in group I were retrieved unflushed [NF]. In group II, lungs were explanted after an anterograde flush [AF] through the pulmonary artery. Finally, in group III lungs were explanted after a retrograde flush [RF] via the left atrium. After 3 hours of cold storage, the left lung was assessed during 60 minutes in our ex vivo reperfusion model. Wet-to-dry weight ratio (W/D) was calculated after reperfusion.

Results
Pulmonary vascular resistance (dynes x sec x cm⁻⁵) was 1145 ± 56 [RF] versus 1560 ± 123 [AF] and 1435 ± 95 [NF] at 60 minutes of reperfusion (p < 0.05). Oxygenation and compliance were higher and plateau airway pressure was lower in RF versus AF and NF although the difference did not reach statistical significance. No differences in W/D were observed between groups after reperfusion. Histological examination revealed fewer microthrombi in the left lung in RF compared to AF and NF.

Conclusion
Retrograde flush of lungs from NHBD improves graft function by elimination of microthrombi from the pulmonary vasculature resulting in lower PVR upon reperfusion.

INTRODUCTION

In 1963, James Hardy performed the first human lung transplantation with a graft from a non-heart-beating donor (NHBD) [1]. Since the introduction and the acceptance of brain death criteria in 1968, transplantation with lungs from heart-beating donors (HBD) became the mainstay therapy for selected patients with end-stage pulmonary disease refractory to medical therapy. This treatment has enjoyed increasing success with better early and late survival [2]. However, donor organ shortage is the main limiting factor to this lifesaving treatment. Only 15-30% of HBD have lungs that are suitable for transplantation [3,4]. During the last decade, the number of lung transplantations but even more the number of patients on the waiting list has increased steadily. As a result of the disparity between the growth in demand and the inadequate organ supply, there is currently a renewed interest in the use of NHBD [5].

There is growing experimental [6,7] and clinical [8-11] evidence that one hour of warm ischemia does not compromise the performance of the pulmonary graft from the NHBD. However, formation of microthrombi after circulatory arrest is still a concern for the development of ischemia-reperfusion injury. Flushing the lungs during procurement may be a strategy to remove the microthrombi thereby improving graft performance. Previous studies in HBDs have shown that the quality of the pulmonary graft can be improved using a combined technique with an anterograde flush through the pulmonary artery followed by a retrograde flush through each of the pulmonary veins [12]. In a previous publication mimicking the clinical scenario in the uncontrolled NHBD (Maastricht Categories I-II) [13], we have shown that a retrograde flush after additional topical cooling inside the cadaver is superior compared to anterograde flush or no flush [14]. Studies looking at the best route of pulmonary flush in the controlled NHBD (Maastricht Categories III-IV) [13] immediately after the warm ischemic period prior to cold storage have not been performed so far.

The aim of this NHBD isolated pig lung reperfusion study, therefore, was to compare anterograde pulmonary flush versus retrograde flush versus no flush followed by cold storage on graft function and on residual microthrombi.
Chapter 2

MATERIAL AND METHODS

Experimental Groups

Eighteen domestic pigs were randomly divided in 3 groups (n = 6 per group; weight: 31.7 ± 0.8 kg). In all 3 groups, pigs were sacrificed by ventricular fibrillation and left untouched for 1 hour. In the first group lungs were retrieved unflushed (NF). The lungs in the second group were flushed in an anterograde way (AF). Finally, in the third group a retrograde flush (RF) was performed. After explantation the heart-lung block in all groups was stored on ice for 3 hours (4°C).

Animal Preparation

Domestic pigs were premedicated with an intramuscular injection of Xylazine (5 ml Xyl-M® 2%, V.M.D. nv/sa, Arendonk, Belgium) and Zolazepam/Tiletamine (3 ml Zoletil® 100, Virbac s.a., Carros, France). The animals were installed in a supine position and intubated with an endotracheal tube 7.5 (Portex Tracheal Tube, SIMS Portex, Ltd. Hythe, Kent, UK) and ventilated with a volume-controlled ventilator (Titus®, Dräger, Lübeck, Germany) with an inspiratory oxygen fraction (FiO₂) of 0.5, a tidal volume of 10 ml/kg body weight and a frequency of 20 breaths/minute. Positive end-expiratory pressure was set to 5 cmH₂O. Anaesthesia was maintained with isoflurane 0.8 – 1% (Isoba® Vet, Schering – Plough Animal Health, Harefield, Uxbridge, England) and muscle relaxation with intermittent boli of pancuronium bromide (Pavulon 2 mg/ml, Organon, Teknika, Boxtel, The Netherlands). A catheter (Secalon T, 14G/2.0x160 mm, Becton Dickinson, Singapore) was placed in the right common carotid artery for measurement of the systemic arterial pressure (SAP) and sampling of arterial blood. A Swan-Ganz thermodilution catheter (Baxter Healthcare Corp., Irvine, CA, USA) was inserted through the right external jugular vein into the pulmonary artery. With this catheter, hemodynamic parameters including pulmonary artery pressure (PAP) and pulmonary capillary wedge pressure (PCWP) were monitored. Hemodynamic parameters (SAP, PAP and PCWP) and aerodynamic parameters (Plateau airway pressure and Compliance) were continuously recorded and stored.

The pigs were sacrificed by inducing ventricular fibrillation with a subxyphoidal needle puncture using a square-pulse generator (amplitude range +15 to -15 V, current < 300 mA, frequency 50 Hz). After cardiac arrest, the endotracheal tube was disconnected from the ventilator and left open to the air. The cadavers were left untouched for 1 hour at room temperature. Temperature of the lung was measured via a probe in the endotracheal tube and rectal temperature was monitored. All animals received human care in compliance with Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (NIH Publication No. 85-23, Revised 1996). The study was approved by the institutional review board on animal research at the Katholieke Universiteit Leuven.

Preservation of the Heart-Lung Block

After 1 hour of warm ischemia a sternotomy was performed. The thymic tissue was excised and the pericardium and pleural cavities were widely opened. The lungs were inspected. The pulmonary artery, ascending aorta and caval veins were encircled. Gross thrombi in the pulmonary artery and left atrium were removed as much as possible. In NF the lungs were explanted without flush. In AF the pulmonary artery was cannulated (DLP Inc, Grand Rapids, MI, USA) through the right ventricular outflow tract and secured with a purse-string. The caval veins were ligated and the ascending aorta was clamped. The pulmonary artery was isolated from the right ventricle by a ligature around the tip of the catheter just distal to the pulmonary valve. The right and the left atrium were incised for venting of the heart. The lungs were flushed in an anterograde way with 50 ml/kg Perfadex® (Vitrolife, Göteborg, Sweden) at room temperature (18°C) buffered with Trometamol (0.3 ml/l, 2 g/5ml, Addex-THAM) and CaCl₂ (0.6 ml/l, 11mEq). During the flush, ventilation was restarted with the same ventilatory settings. In RF the left atrium was cannulated (MOD Cannula 18 Fr, International Medical Products NV/SA, Brussels, Belgium) through a purse-string and the lungs were flushed under the same circumstances but in a retrograde manner. The anterior aspect of the main pulmonary artery was incised for drainage of the flush solution. The pressure during AF and RF was maintained at 15 mmHg by adjusting the height of the perfusion bag. After excision of the heart-lung block, the lungs were collapsed and immersed in cold (4°C) Perfadex® and stored on ice for 3 hours.

Preparation of the Heart-Lung Block

The lungs in all 3 groups were prepared in the same way for ex vivo evaluation in the isolated reperfusion system after the cold storage. The right lung was separated from the heart-lung block and used as a control. The pulmonary artery was cannulated through the right ventricular outflow tract using a 36 Fr cannula and isolated with
a ligature around the catheter distal to the pulmonary valve. A small catheter was placed in the pulmonary artery for measurement of PAP. The ascending aorta was clamped. The left atrium was cannulated through the apex of the left ventricle with a second 36 Fr cannula and secured with a purse-string. Finally, an endotracheal tube nr. 8 was placed in the trachea for ventilation of the pulmonary graft.

**Preparation of the Perfusate**
Autologous blood (1200 ml) was rapidly withdrawn from each animal at the moment of sacrifice via the catheter in the right external jugular vein and collected in a sterile bag containing 5000 IU of heparin (Natrium Heparine B. Braun, 25000 IU/5ml, B. Braun Medical SA, Jaén, Spain). This whole blood was centrifuged with a Cell Saver (Sequestra 1000, Medtronic Inc, Parker, CO, USA) and washed with saline for 12 minutes at 5600 rpm. Leukocytes were sequestered using a leukocyte filter (Imugard III-RC, Terumo Europe N.V., Haasrode, Belgium). The remaining red blood cells (350 ml) were then diluted to a hematocrit of 15% with a low potassium dextran solution (Perfadex®) and human albumin (final concentration: 8%, CAF-DCF, Brussels, Belgium). The perfusate was finalized by adding CaCl₂ (2.4 ml/l, 100 mg/ml), heparin (10000 IU/l) and sodium bicarbonate (45 ml/l, 16.8g/250ml Baxter, Lessines, Belgium). The total volume of the perfusate was 1400 ml.

**Isolated Reperfusion Circuit**
The ex vivo reperfusion system consisted of a hardshell reservoir (Minimax Hardshell reservoir, Medtronic), a centrifugal pump (Bio-medicus, Medtronic, Minneapolis, MN, USA), a heater/cooler system (Bio-Cal, Heater Cooler Model 370, Medtronic, Minneapolis, MN, USA) and a hollow fiber oxygenator (Capiox®SX, Terumo, MI, USA) with integrated heat exchanger. The heating element of the gas exchanger was connected to the heater/cooler system. The left lung and the heart were then placed in a specially designed evaluation box and mounted in the reperfusion system. The cannula in the pulmonary artery was connected to the inflow tubing and the outflow tubing was connected to the cannula in the left atrium.

**Technique of controlled reperfusion and ventilation**
Reperfusion of the left lung was started after de-airing of the inflow tubing with normothermic (37°C) oxygenated perfusate (O₂: 0.4 l/min). Pulmonary artery pressure was gradually increased to a maximum of 15 mmHg and the left atrial pressure on the outflow was kept at 0 mmHg by adjusting the height of the blood reservoir. This resulted in warming up of the lung and a gradual increase in pulmonary artery flow. Ventilation with a FiO₂ 0.5 was started when the temperature of the outflowing perfusate reached 34°C and was slowly increased to a tidal volume of 140 ml, a frequency of 14 breaths/min and a PEEP of 5 cmH₂O. At that moment, the initially oxygenated perfusate was partially deoxygenated with a gas mixture of CO₂ (8%), O₂ (6%) and N₂ (86%).

**Assessment of the Graft**
Thirty-five minutes after the onset of reperfusion the temperature of the lung parenchyma reached 37.5 °C. At this moment functional graft parameters were recorded up to one hour. Pulmonary artery pressure (PAP) (mmHg) was measured via an 18 Gauge catheter in the main pulmonary artery. The pressure in the left atrium (LAP) was measured on the outflow. An electromagnetic flow probe (FF 100T 10 mm probe, Nihon Kohden, Tokyo, Japan) was inserted in the tubing on the inflow for continuous measurement of the pulmonary artery flow (PAF) (l/min). Pulmonary vascular resistance (PVR) was calculated using the formula: PVR = [PAP – LAP] x 80/PAF and expressed in dynes x sec x cm⁻⁵. Dynamic lung compliance (Compl) (ml/cmH₂O) and plateau airway pressure (Plat AwP) (cmH₂O) were recorded. PO₂ and PCO₂ were continuously measured in the perfusate via probes (Terumo CDI™, 500 shunt sensor, Leuven, Belgium) on the outflow tubing using an inline blood gas analyzer (CDITM 500, Terumo, Borken, Germany). Oxygenation capacity was calculated using the formula PO₂/FiO₂ (mmHg).

Temperature (°C) of the inflowing and outflowing perfusate was continuously measured, the last being considered as the graft temperature. All data were recorded online and stored on a central server (Datex AS/3 and S5 collect 3.0 Software respectively, Datex-Ohmedia, Helsinki, Finland).

At the end of the reperfusion, both right and left lung were dried in an oven at 80°C for 48 hours to a constant weight and their wet-to-dry ratio (W/D) was calculated and used as a parameter of pulmonary oedema.

**Histology**
At the end of the experiment, tissue samples were obtained from the right and the left lung. Specimens were fixed in 6% formaldehyde, dehydrated and stained with phosphotungstic acid hematoxylin (PTAH) to detect fibrin deposits. Histological analysis was performed by one experienced pathologist (EV) who was blinded to the experimental set-up.
Data analysis was performed using GraphPad Prism 4 for Windows (GraphPad Software, San Diego, California, USA). Results were expressed as mean ± standard error of the mean (SEM). Parameters between study groups were compared using a one-way analysis of variance with multiple comparisons. An unpaired t-test was used to test for significant difference for flush time between AF and RF. A chi-square test was used to test for significant difference in fibrin deposits. A p-value of < 0.05 was considered significant.

RESULTS

Study groups

Baseline parameters in the 3 groups prior to sacrifice are listed in Table 2.1. There were no significant differences in animal weight, PAP, Plat AwP, Compl and PaO₂/FiO₂ between the 3 groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal Weight (kg)</th>
<th>PAP (mmHg)</th>
<th>Plat AwP (cmH₂O)</th>
<th>Compl (ml/cmH₂O)</th>
<th>PaO₂/FiO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>30 ± 1</td>
<td>12 ± 1</td>
<td>13 ± 0</td>
<td>34 ± 1</td>
<td>626 ± 25</td>
</tr>
<tr>
<td>AF</td>
<td>32 ± 1</td>
<td>12 ± 1</td>
<td>15 ± 1</td>
<td>32 ± 1</td>
<td>599 ± 29</td>
</tr>
<tr>
<td>RF</td>
<td>33 ± 1</td>
<td>12 ± 1</td>
<td>14 ± 0</td>
<td>34 ± 3</td>
<td>564 ± 22</td>
</tr>
</tbody>
</table>

| p-value     | 0.52               | 0.23       | 0.61            | 0.25             |

There were no significant differences between no flush (NF), anterograde flush (AF) and retrograde flush (RF) for animal weight, pulmonary artery pressure (PAP), plateau airway pressure (Plat AwP), compliance (Compl) and oxygenation capacity (PaO₂/FiO₂). Data are presented as mean ± SEM (n=6/group).

Graft characteristics in the three study groups are compared in Table 2.2. There were no statistically significant differences among the 3 groups regarding endobronchial temperature at the end of the warm ischemic period, warm and cold ischemic intervals and graft temperature at the end of the cold ischemic period. Flush time was significantly shorter in AF compared to RF (p < 0.05). During RF, continuous washout of small blood clots was visible. The time to warm up the lung to 34°C and start ventilation in the ex vivo circuit was significantly longer in NF compared to RF and AF (p < 0.01).

<table>
<thead>
<tr>
<th>Group</th>
<th>Endobronchial temperature (°C)</th>
<th>Flush time (sec)</th>
<th>WIT (min)</th>
<th>CIT (min)</th>
<th>Graft temperature (°C)</th>
<th>Warming up (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF</td>
<td>35 ± 0</td>
<td>-</td>
<td>62 ± 2</td>
<td>186 ± 2</td>
<td>4 ± 0</td>
<td>27 ± 0</td>
</tr>
<tr>
<td>AF</td>
<td>36 ± 1</td>
<td>395 ± 10*</td>
<td>63 ± 1</td>
<td>184 ± 1</td>
<td>4 ± 1</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>RF</td>
<td>35 ± 1</td>
<td>561 ± 28</td>
<td>59 ± 2</td>
<td>189 ± 2</td>
<td>4 ± 0</td>
<td>22 ± 1</td>
</tr>
</tbody>
</table>

| p-value     | 0.72                           | 0.0003          | 0.24      | 0.16      | 0.66                   | 0.0017          |

Flush time was significantly shorter in AF compared to RF (p = 0.0003). The warming up time before the start of ex vivo assessment was significantly longer in NF versus RF and AF (p = 0.0017). There were no significant differences for the other characteristics, except for end of warm ischemia, end of cold ischemia, NF: no flush, AF: anterograde flush, RF: retrograde flush, WIT: warm ischemic time, CIT: cold ischemic time.

Graft function

Pulmonary vascular resistance

PVR (dynes x sec x cm⁻⁵) on the ex vivo circuit was significantly lower in RF compared to AF from 45 minutes (1225 ± 54 versus 1581 ±111; p < 0.05) until the end of the reperfusion (1145 ± 56 versus 1560 ± 123; p < 0.05) and also when compared to NF, starting at 55 minutes (1186 ± 69 versus 1448 ± 82; p < 0.05). There was no significant difference in PVR between AF and NF (Figure 2.1A).

Dynamic lung compliance

During the assessment period, there was a trend towards a higher dynamic lung compliance (ml/cmH₂O) in RF (20.7 ± 3.9) compared to AF (16.2 ± 1.3) and NF (17.6 ± 1.8) at 35 minutes (p = 0.48) and 23.7 ± 2.7 versus 18.1 ± 1.4 and 17.6 ± 1.8, respectively, at 60 minutes (p = 0.09) (Figure 2.1B).

Plateau airway pressure

There was no significant difference in plateau airway pressure between the three groups. However, Plat AwP (cmH₂O) was lower in RF compared to AF and NF, from 45 minutes (12.9 ± 1.3 versus 14.4 ± 0.6 and 13.6 ± 0.9, respectively; p = 0.58) until the end of reperfusion (12.6 ± 1.1 versus 13.6 ± 0.5 and 13.9 ± 0.8, respectively; p = 0.56) (Figure 2.1C).

Oxygenation capacity

During assessment there was a trend towards better oxygenation (mmHg) in RF compared to AF and NF. The values were 643.2 ± 41.7 versus 630.5 ± 29.9 and 635.7 ± 14.4, respectively, at 35 minutes (p = 0.24) and 673.2 ± 41.7 versus 636.3 ± 35.5 and 621.0 ± 24.7, respectively, at 60 minutes (p = 0.56) (Figure 2.1D).
Retrograde flush following warm ischemia in the non-heart-beating donor results in superior graft function.

**Wet-to-dry weight ratio**

The W/D ratio of both lungs is shown in Figure 2.2. There was no significant difference between the 3 groups before (p = 0.17) and also not after reperfusion (p = 0.90). W/D ratio in RF, AF and NF, however, was significantly lower after reperfusion (left lung) than before reperfusion (right lung) (4.8 ± 0.06 versus 5.4 ± 0.08 (p < 0.0001); 4.7 ± 0.06 versus 5.4 ± 0.09 (p = 0.0001) and 4.7 ± 0.09 versus 5.2 ± 0.09 (p = 0.003), respectively).

**Gross Appearance**

There was a better whitening of the lungs resulting from a more homogeneous washout of residual blood in RF compared to AF.

![Figure 2.2: Wet-to-dry weight (W/D) ratio before (right lung, closed squares) and after reperfusion (left lung, open squares). There was no significant difference between the 3 groups. NF: no flush; AF: anterograde flush; RF: retrograde flush](image)

**Figure 2.2**

- A: Pulmonary vascular resistance: * p < 0.05: RF versus AF, † p < 0.05: RF versus NF;
- B: Dynamic lung compliance: NS;
- C: Plateau airway pressure: NS;
- D: Oxygenation capacity (PO$_2$/FiO$_2$): NS

**Figure 2.1:** Pulmonary graft function (mean ± SEM) from 35 minutes until 60 minutes of ex vivo reperfusion. NF: no flush, AF: anterograde flush and RF: retrograde flush.

**Figure 2.3:** Histology of lung specimens obtained after reperfusion of the left lung (phosphotungstic acid hematoxylin stain; original magnification x 100). Residual microthrombi (arrows) were observed more often in the capillaries after NF (A) and AF (B) compared to RF (C).
Retrograde flush following warm ischemia in the non-heart-beating donor results in superior graft survival after lung transplantation in recent years. As a result of its own success, immunosuppression and postoperative care have all contributed to improved survival after lung transplantation in recent years. As a result of its own success, there is now an important discrepancy between the number of suitable donor lungs and the number of patients on the waiting list. A valuable option to increase the donor pool may be the use of lungs from non-heart-beating donors [5]. The lung is unique amongst other solid organs since pulmonary tissue may remain viable by using the oxygen in the alveoli via diffusion, even after cessation of circulation [15,16]. However, diffuse microthrombi formation that can occur during the warm ischemic interval is a major concern in lung transplantation from non-heart beating donors.

Numerous studies have been reported in the literature comparing the effect of different preservation routes in the heart-beating donor. Anterograde flush is the technique most frequently applied clinically. It improves the pulmonary microcirculation and preserves the endothelial-epithelial barrier. Retrograde pulmoplegia, via the left atrium into the pulmonary venous system using the pulmonary artery for outflow, is characterized by a low vascular resistance and high volume capacity resulting in a more uniform distribution of the preservation solution [17,18]. There is an advantage of flushing both the pulmonary and bronchial vessels and of limiting the effect of pulmonary arterial hypothermic vasoconstriction. Furthermore, retrograde flush can washout residual blood, possible microthrombi and other tissue emboli that may obstruct the pulmonary vessels [12]. Experimental [19-23] and clinical [12,17,24] reports have shown that retrograde flush is not detrimental and improves graft performance with less edema and improved oxygenation. Ventilation during perfusion of the preservation solution results in better distribution regardless of the route of delivery [18]. Many transplant groups are now using a combined technique starting with anterograde flush via the pulmonary artery followed by a retrograde flush via the pulmonary veins after the heart has been excised and with the lungs still ventilated in the donor.

Ware et al. recently reported that on pathological examination of 17 donor lungs rejected for transplantation, gross or microscopic evidence of either pulmonary arterial thrombosis and/or pulmonary infarction was present in 35% of donor lungs [25]. These thrombi may well contribute to primary lung graft dysfunction. A retrograde flush may be a strategy to reduce the amount of pulmonary arterial thrombi. In a first publication [14] mimicking the situation in the uncontrolled NHBD [13] we investigated the best route for pulmonary flush following warm ischemia and additional in situ topical cooling. In that study we already demonstrated that retrograde flush is superior compared to anterograde flush or no flush in this type of donors [13]. The current study looked at the situation in the controlled NHBD where the lungs are explanted after a short period of warm ischemia followed by cold storage outside the cadaver. This has lead to identical findings with superior graft function after retrograde flush as a result of less residual microthrombi at the onset of reperfusion.

An ex vivo lung perfusion study comparing anterograde flush versus retrograde flush versus no flush in non-heparinized animals after a warm ischemic period of 1 hour prior to cold storage, has never been performed so far (Table 2.3). In a dog model of left lung transplantation Hayama et al. showed that an additional retrograde flush after an anterograde flush improved gas exchange and resulted in less edema. This was due to a better washout of blood cells and residual pulmonary...
Retrograde flush following warm ischemia in the non-heart-beating donor results in superior graft performance. In the present study, animals were sacrificed by ventricular fibrillation after baseline measurement. The objective of this study was to mimic the clinical scenario in a controlled NHBD with lungs flushed immediately after a short period of warm ischemia. No drugs were administered prior to circulatory arrest and animals were also not heparinized after cessation of circulation. In contrast with other studies lungs in the present study were flushed with Perfadex® at room temperature (18°C). This protocol was based on the study of Wang et al. demonstrating that lungs flushed with a low potassium dextran solution at 23°C performed better than those flushed at 10°C. A uniform and clean washout of the pulmonary vascular bed was obtained while the temperature of the flush solution was 23°C [34]. A potential drawback of perfusing lungs at room temperature is a prolongation of the warm ischemic period with 3-5 minutes. Steen et al. previously demonstrated that surface cooling is quicker when the lungs are in a collapsed state [35]. The lungs in the present study, therefore, were not inflated after the flush and remained atelectatic during the cold ischemic period. This also made comparison between the 3 groups possible.

Assessment of the graft was performed using our well established isolated reperfusion model. In this ex vivo circuit, the left lung is reperfused in a controlled setting with an inflow pressure limited to 15 mmHg. The maximum flow through the lung is therefore determined by a decrease in PVR. In this study we demonstrated that PVR was significantly lower in RF compared to AF and NF. We speculate that microvascular obstruction may be responsible for the higher PVR in NF and AF. W/D ratio was significant lower after reperfusion (left lung) with a perfusate based on a LFPGD solution and albumin than before reperfusion (right lung). This may open the perspective to dry out edematous lungs prior to transplantation using an ex vivo reperfusion circuit.

A limitation of our study is that we did not perform a combined anterograde plus retrograde flush. We were, however, interested in the effect of each specific flush route. We hypothesize that retrograde flush followed by anterograde flush may further improve graft performance and may become the gold standard in NHBD.

Another limitation of our experimental set-up is the short evaluation period of graft performance. The present findings, therefore, need to be further confirmed in a transplant model.

In conclusion, we demonstrated that retrograde flush of the controlled NHBD lung at room temperature via the left atrium results in superior graft performance in an ex vivo reperfusion circuit. This study confirms once more that 1 hour of warm ischemia followed by warm flush preservation and further cold storage in an atelectatic state is not detrimental for lungs.
Table 2.3: Comparison of previously published studies using flush to preserve lungs from non-heart-beating donor

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Reference</th>
<th>WIT (h)</th>
<th>CIT (h)</th>
<th>Model</th>
<th>Species</th>
<th>Solution</th>
<th>Heparin Flush</th>
<th>Notes</th>
</tr>
</thead>
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<tr>
<td>1991</td>
<td>Egan</td>
<td>(6)</td>
<td>1-2</td>
<td>4</td>
<td>NY</td>
<td>Dog</td>
<td>AF/LPG</td>
<td>Yes</td>
<td></td>
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<tr>
<td>1993</td>
<td>Ulicny</td>
<td>(28)</td>
<td>4</td>
<td></td>
<td>YY</td>
<td>Dog</td>
<td>AF</td>
<td>Yes</td>
<td></td>
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<tr>
<td>1995</td>
<td>Umemori</td>
<td>(32)</td>
<td>1-2</td>
<td>3</td>
<td>NNN</td>
<td>Dog</td>
<td>LPG</td>
<td>No, AF</td>
<td></td>
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<tr>
<td>2000</td>
<td>Luh</td>
<td>(27)</td>
<td>1.5</td>
<td>3</td>
<td>YY</td>
<td>Pig</td>
<td>AF/RF</td>
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<tr>
<td>2003</td>
<td>Hayama</td>
<td>(26)</td>
<td>2</td>
<td></td>
<td>YY</td>
<td>Dog</td>
<td>AF/RF</td>
<td>No, LPG</td>
<td></td>
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<tr>
<td>2004</td>
<td>Wittwer</td>
<td>(29)</td>
<td>1.5</td>
<td>3-5</td>
<td>YY</td>
<td>Pig</td>
<td>RFLPDG</td>
<td>Yes</td>
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<td>2004</td>
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<td>1.5-3</td>
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<td>YY</td>
<td>Pig</td>
<td>RFLPDG</td>
<td>Yes</td>
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<td>2005</td>
<td>Inokawa</td>
<td>(31)</td>
<td>2</td>
<td></td>
<td>NY/NA</td>
<td>Dog</td>
<td>LPG</td>
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REFERENCES


CHAPTER 3

Retrograde flush is more protective than heparin in the uncontrolled DCD lung donor

Caroline Van De Wauwer, Arne P. Neyrinck, Filip R. Rega, Erik Verbeken, Dirk E.M. Van Raemdonck
ABSTRACT

Objective
Formation of microthrombi after circulatory arrest is a concern for the development of reperfusion injury in lung recipients from donation after circulatory death (DCD) donors. In this isolated lung reperfusion study we compared the effect of postmortem heparinization versus preharvest retrograde pulmonary flush or both.

Methods
Domestic pigs (n=6 per group) were sacrificed by ventricular fibrillation and left at room temperature for 1 hour. This was followed by 2.5 hours of topical cooling. In control group [C], no heparin and no pulmonary flush were administered. In group [R] lungs were flushed with Perfadex® in a retrograde way prior to explantation. In group [H], heparin (300IU/kg) was administered 10 minutes after cardiac arrest followed by closed chest massage during 2 minutes. In the combined group animals were heparinized and lungs were explanted after retrograde flush [HR]. The left lung was assessed during 60 minutes in an ex vivo reperfusion model.

Results
Pulmonary vascular resistance at 50 minutes and at 55 minutes was significantly lower in [R] and [HR] compared to [C] and [H] (p < 0.01 and p < 0.001) and at 60 minutes in [R], [H] and [HR] compared to [C] (p < 0.001). Oxygenation, compliance and plateau airway pressure were more stable in [R] and [HR]. Plateau airway pressure was significantly lower in [R] compared to [H] at 60 minutes (p < 0.05). No differences in wet/dry weight ratio were observed between groups.

Conclusion
This study suggests that preharvest retrograde flush is more protective than postmortem heparinization to prevent reperfusion injury in lungs recovered from DCD donors.

INTRODUCTION

The first human lung transplantation in 1963 was performed with a left lung recovered from an uncontrolled donation after circulatory death (DCD) [1]. After failed resuscitation heparin was injected in the heart of the deceased patient and closed cardiac massage and ventilation were continued until explantation. The left lung was flushed with a cold heparinized glucose solution and rhythmically inflated with pure oxygen until the moment of implantation. The following milestone in uncontrolled DCD was the transplantation of lungs after ex vivo evaluation in 2001 [2]. In this case 50000 IU of heparin was administered through a central venous line 10 minutes after declaration of death, followed by 20 chest compressions. The lungs were preserved by topical cooling, retrieved without flush and evaluated ex vivo.

Intra-pulmonary thrombi formation after circulatory arrest is a concern in DCD. In controlled DCD, pretreatment (i.e. heparin or phentolamine) can be given before death [3-5] or after the 5-min no-touch interval [6]. In some centers donors are optimized but no heparin is given [7]. In uncontrolled donation, there is evidence that retrograde flush following topical cooling results in a better washout of blood and microthrombi and reduces pulmonary vascular resistance [8]. Heparinization of the uncontrolled DCD followed by chest compression can potentially cause lung contusions and subsequent pulmonary hematomas. There is concern of dispersing microthrombi trough the lung.

The aim of this study was to compare the possible benefit of postmortem heparinization versus a preharvest retrograde flush or both in uncontrolled lung donors.

MATERIAL AND METHODS

Experimental Groups
Domestic pigs were randomly divided into four groups (n = 6/group). In all four groups lungs were subjected to 1 hour of warm ischemia and 2.5 hours of topical cooling. Pulmonary grafts were recovered without heparinization or flush in the control group [C]. A retrograde flush with Perfadex® was performed after topical cooling in the second group [R]. In the third group [H] animals were given heparin (300IU/kg) via the central venous line 10 minutes after cardiac arrest, and then closed.
Retrograde flush is more protective than heparin in the uncontrolled DCD lung donor.

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Positive end-expiratory pressure was set to 5 cmH\textsubscript{2}O.

Animals were premedicated with an intramuscular injection of Xylazine (5 ml Xyl-M\textsuperscript{®} 2%, V.M.D. nv/sa, Arendonk, Belgium) and Zolazepam/Tiletamine (3 ml Zoletil\textsuperscript{®} 100, Virbac s.a., Carros, France), installed in a supine position, intubated with an endotracheal tube 7.5 (Portex Tracheal Tube, SIMS Portex, Ltd. Hythe, Kent, UK), and ventilated with a volume-controlled ventilator (Titus\textsuperscript{®}, Dräger, Lübeck, Germany) with an inspiratory oxygen fraction (FiO\textsubscript{2}) of 0.5 and a tidal volume of 10 ml/kg body weight. Respiratory rate was adjusted to achieve an end-tidal CO\textsubscript{2} of 40 mmHg. Positive end-expiratory pressure was set to 5 cmH\textsubscript{2}O. Anaesthesia was maintained with isoflurane 0.8 – 1% (Isoba\textsuperscript{®} Vet, Schering – Plough Animal Health, Harefield, Uxbridge, UK) and muscle relaxation with intermittent boli of pancuronium bromide (Pavulon 2 mg/ml, Organon, Teknika, Boxtel, The Netherlands). A 14 G catheter (Secalon\textsuperscript{®} T, Becton Dickinson Ltd., Singapore) was placed in the right common carotid artery for measurement of the systemic arterial pressure (SAP) and sampling of arterial blood. A Swan-Ganz catheter was inserted through the internal jugular vein into the main pulmonary artery to measure pulmonary artery pressures (PAP) and pulmonary capillary wedge pressure (PCWP). Hemodynamic parameters (SAP, PAP and PCWP) and ventilatory parameters (plateau airway pressure and compliance) were continuously monitored and stored on a computer.

After a stabilization period, pigs were sacrificed by inducing ventricular fibrillation with a subxyphoidal needle puncture using a square-pulse generator (amplitude range +15 to -15 V, current < 300 mA, frequency 50 Hz). After cardiac arrest, the endotracheal tube was disconnected from the ventilator and left open to the air. According to the study protocol pigs were heparinized or not.

The cadavers were left untouched for 1 hour at room temperature. Lung temperature was measured via a probe in the endotracheal tube and rectal temperature was monitored.

All animals received humane care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (NIH Publication No. 85-23, Revised 1996). The study was approved by the institutional review board on animal research at the KU Leuven.

Preservation of the Heart-Lung Block

Two chest drains were inserted in each pleural cavity after 1 hour of warm ischemia. Lungs were then cooled with cold saline in a closed circuit. Via the deep drains, cold saline solution was infused and continuous recirculated with a roller-pump from a reservoir placed in an ice basket. The system was filled with approximately 6 litres of cold saline. To ensure that the lungs were well immersed in the fluid, the superficial drains were connected to an overflow system of 5 cmH\textsubscript{2}O. Temperature of the lung was measured via a probe in the endotracheal tube and rectal temperature was monitored. After 2.5 hours of topical cooling, sternotomy was performed. The thymic tissue was excised, the pericardium and pleural cavities were widely opened and lungs were inspected. The pulmonary artery, ascending aorta and caval veins were encircled. Gross microthrombi in the pulmonary artery and left atrium were evacuated as much as possible. In [C] and [H] lungs were explanted without flush. In [R] and [HR] the left atrium was cannulated (MOD Cannula 18 Fr, International Medical Products NV/SA, Brussels, Belgium) through a purse-string and lungs were flushed in retrograde manner with 50 ml/kg Perfadex\textsuperscript{®} (Vitrolife, Göteborg, Sweden) at room temperature (18°C) buffered with Trometamol (0.3 ml/l, 2 g/5ml, Addex-THAM) and CaCl\textsubscript{2} (0.6 ml/l, 11mEq). The caval veins were ligated and the ascending aorta was clamped. The inferior caval vein was incised for venting of the heart. During the flush, ventilation was restarted with a low tidal volume and a low frequency to avoid cold lung injury related to mechanical stress. Lungs were kept on ice during the 30 minutes of preparation.

Preparation of the Heart-Lung Block

Lungs in all groups were prepared in the same way for ex vivo evaluation. The right lung was separated from the heart-lung block and used as a control. The pulmonary artery was cannulated through the right ventricular outflow tract using a 36 Fr cannula and isolated with a ligature around the catheter distal to the pulmonary valve. A small catheter was placed in the pulmonary artery for measurement of PAP. The ascending aorta was clamped. The left atrium was cannulated through the apex of the left ventricle with a second 36 Fr cannula and secured with a purse-string. Finally, an endotracheal tube nr. 8 was placed in the trachea for ventilation of the pulmonary graft.
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Preparation of the Perfusate

Autologous blood (1200 ml) was withdrawn from each animal after circulatory arrest via a catheter in the right external jugular vein and collected in a sterile bag containing 5000 IU of heparin (Natrium Heparin B. Braun, 25000 IU/5 ml, B. Braun Medical SA, Jaén, Spain). This whole blood was centrifuged with a Cell Saver (Sequestra 1000, Medtronic Inc, Parker, CO, USA) and washed with saline for 12 minutes at 5600 rpm. Leukocytes were sequestered using a leukocyte filter (Imugard III-RC, Terumo Europe N.V., Haasrode, Belgium). The remaining red blood cells (350 ml) were then diluted to a hematocrit of 15% with a low potassium dextran solution (Perfadex®, Vitrolife, Göteborg, Sweden) and human albumin (final concentration: 8%, CAF-DCF, Brussels, Belgium). The perfusate was finalized by adding CaCl\(_2\) (2.4 ml/l, 100 mg/ml), heparin (10000 IU/l) and sodium bicarbonate (45 ml/l, 16.8 g/250 ml Baxter, Lessines, Belgium). The total volume of the perfusate was 1400 ml.

Isolated Reperfusion Circuit

The ex vivo reperfusion system consisted of a hardshell reservoir (Minimax® Hardshell reservoir, Medtronic, Minneapolis, MN, USA), a centrifugal pump (Biomedicus, Medtronic), a heater/cooler system (Bio-Cal, Heater Cooler Model 370, Medtronic, Minneapolis, MN, USA), and a hollow fiber oxygenator (Capiox®SX, Terumo, MI, USA) with integrated heat exchanger. The heating element of the gas exchanger was connected to the heater/cooler system. The left lung and the heart were then placed in a specially designed evaluation box and mounted in the reperfusion system. The cannula in the pulmonary artery was connected to the inflow tubing and the outflow tubing was connected to the cannula in the left atrium.

Technique of controlled reperfusion and ventilation

Reperfusion of the left lung was started with normothermic (37°C) oxygenated perfusate (O\(_2\): 0.4 l/min) after de-airing of the inflow tubing. Pulmonary artery pressure was gradually increased to a maximum of 15 mmHg and the left atrial pressure on the outflow was kept at 0 mmHg by adjusting the height of the blood reservoir. This resulted in warming up of the lung and a gradual increase in pulmonary artery flow. Ventilation with a FiO\(_2\) 0.5 was started when the temperature of the outflowing perfusate reached 34°C and slowly increased to a tidal volume of 140 ml, a frequency of 14 breaths/min and PEEP of 5 cmH\(_2\)O. At that moment, the perfusate was partially deoxygenated to a PO\(_2\) of 50 – 60 mmHg with a gas mixture of CO\(_2\) (8%), O\(_2\) (6%) and N\(_2\) (86%).

Assessment of the Graft

Forty minutes after the onset of reperfusion, the temperature of the lung parenchyma reached 37.5°C. At this moment functional graft parameters were recorded up to one hour. Pulmonary artery pressure (PAP) (mmHg) was measured via an 18 Gauge catheter inserted in the main pulmonary artery. The pressure in the left atrium (LAP) (mmHg) was measured on the outflow line. An electromagnetic flow probe (FF 100T 10 mm probe, Nihon Kohden, Tokyo, Japan) was inserted in the tubing on the inflow line for continuous measurement of the pulmonary artery flow (PAF) (l/min). Pulmonary vascular resistance (PVR) was calculated using the formula: PVR = [PAP – LAP] x 80/PAF and expressed in dynes x sec x cm\(^{-5}\). Dynamic lung compliance (Compl) (ml/cmH\(_2\)O) and plateau airway pressure (Plat AwP) (cmH\(_2\)O) were recorded. PO\(_2\) and PCO\(_2\) were continuously measured in the perfusate via probes (Terumo CDITM, 500 shunt sensor, Leuven, Belgium) on the outflow tubing using an inline blood gas analyzer (CDITM 500, Terumo, Borken, Germany). Oxygenation capacity was calculated using the PO\(_2\)/FiO\(_2\) ratio (mmHg).

Temperature (°C) of the inflowing and outflowing perfusate was continuously measured, the last being considered as the graft temperature. All data were recorded online and stored on a central server (Datex AS/3 and S5 collect 3.0 Software respectively, Datex-Ohmeda, Helsinki, Finland).

At the end of the reperfusion, both right and left lung were dried in an oven at 80°C for 48 hours to a constant weight and their wet-to-dry weight ratio (W/D) was calculated and used as an indicator of pulmonary edema.

Histology

At the end of the experiment, tissue samples were obtained from the right and the left lung. Specimens were fixed in 6% formaldehyde, dehydrated and stained with phosphotungstic acid hematoxylin (PTAH) to detect fibrin deposits. Histological analysis was performed by one experienced pathologist (EV) who was blinded to the experimental set-up.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 5 (San Diego, CA, USA). Graft parameters between study groups were compared using a one-way analysis of variance with a Newman-Keuls multiple comparison test. A paired t test was used to test for
significant difference for wet-to-dry weight between the left perfused and the right non-perfused lung. Data regarding pulmonary graft function are expressed as mean ± standard error of the mean (SEM). All other data are expressed as mean ± standard deviation (SD). A p-value < 0.05 was considered as significant.

RESULTS

Study groups

There were no significant differences for animal weight, plateau airway pressure, compliance and oxygenation capacity (Table 3.1). Pulmonary graft characteristics were comparable for warm ischemic time, cold ischemic time and graft temperature. However, there was a significant difference in the time necessary to warm the lung up to 34°C at the time of reperfusion (Table 3.2). The time (min) to warm up was shorter in [HR] compared to [C] and [H] (17 ± 2 versus 30 ± 6 and 34 ± 8; p < 0.01 and p < 0.001 respectively) and between [R] and [C], [H] (20 ± 4 versus 31 ± 6 and 34 ± 8; p < 0.01) respectively. There was also significant difference between [H] and [C] at 55 minutes (1281 ± 75 versus 1517 ± 82; p < 0.05) and at 60 minutes of reperfusion (1224 ± 64 versus 1576 ± 88; p < 0.01) (Figure 3.1A).

Table 3.1: Baseline parameters prior to circulatory arrest in the 4 groups

<table>
<thead>
<tr>
<th>Group (n = 6 per group)</th>
<th>Animal weight (kg)</th>
<th>Compl (ml/cmH₂O)</th>
<th>Plat AwP (cmH₂O)</th>
<th>pO₂/FiO₂ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C]</td>
<td>35 ± 4</td>
<td>31 ± 5</td>
<td>15 ± 2</td>
<td>626 ± 81</td>
</tr>
<tr>
<td>[R]</td>
<td>40 ± 5</td>
<td>36 ± 8</td>
<td>16 ± 2</td>
<td>623 ± 97</td>
</tr>
<tr>
<td>[H]</td>
<td>38 ± 6</td>
<td>35 ± 7</td>
<td>15 ± 2</td>
<td>635 ± 39</td>
</tr>
<tr>
<td>[HR]</td>
<td>36 ± 4</td>
<td>34 ± 2</td>
<td>16 ± 1</td>
<td>617 ± 76</td>
</tr>
<tr>
<td>p - value</td>
<td>0.25</td>
<td>0.46</td>
<td>0.85</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Compl: compliance; Plat AwP: plateau airway pressure. Values are expressed as mean ± SD. [C]: no flush, [R]: retrograde flush, [H]: heparinization, [HR]: heparinization and retrograde flush

Table 3.2: Pulmonary graft characteristics before reperfusion in the 4 groups

<table>
<thead>
<tr>
<th>Group (n = 6 per group)</th>
<th>WIT (min)</th>
<th>CIT (min)</th>
<th>Graft temperature (°C)</th>
<th>Warming up (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C]</td>
<td>60.0 ± 0</td>
<td>180.2 ± 0.4</td>
<td>7.1 ± 1.0</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>[R]</td>
<td>60.2 ± 0.4</td>
<td>180.0 ± 0.0</td>
<td>6.5 ± 1.5</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>[H]</td>
<td>61.8 ± 2.0</td>
<td>180.0 ± 0.0</td>
<td>6.4 ± 1.0</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>[HR]</td>
<td>61.2 ± 2.0</td>
<td>180.0 ± 0.0</td>
<td>5.5 ± 0.7</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>p - value</td>
<td>0.13</td>
<td>0.41</td>
<td>0.14</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

WIT: warm ischemic time; CIT: cold ischemic time. Values are expressed as mean ± SD. [C]: no flush, [R]: retrograde flush, [H]: heparinization, [HR]: heparinization and retrograde flush

*p < 0.01 [H] versus [C] and [R] versus [H] and [C]

Figure 3.1: Pulmonary graft function and flow during the evaluation period.

A: Pulmonary vascular resistance: † p < 0.05 [R] versus [C], *p < 0.05 [R] versus [H]. † p < 0.05 [HR] versus [C]. ♠ p < 0.05 [HR] versus [H] and *p < 0.05 [H] versus [C].

B: Flow: + p < 0.05 [C] versus [HR], • p < 0.05 [H] versus [HR], ♠ p 0.05 [C] versus [HR] and [C] versus [R]. ♠ [H] versus [HR] and [H] versus [R], ♠ p 0.05 [C] versus [R], [C] versus [HR] and [C] versus [H].

[C]: control group, [R]: retrograde flush, [H]: heparinization, [HR]: heparinization and retrograde flush. Values are expressed as mean ± SEM.
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Flow
There was a significantly higher flow (l/min) in [R] and [HR] compared to [C] and [H] from 15 minutes until the end of the reperfusion (p < 0.01). At the end of the reperfusion, the flow in [H] was higher than the flow in [C] (p < 0.05) (Figure 3.1B).

Dynamic lung compliance
Compliance (ml/cmH₂O) was higher in [R] and [HR] during the assessment (Figure 3.2A). At 60 minutes there was a significant difference between [R] and [C] (22 ± 3 versus 14 ± 1; p < 0.05).

Plateau airway pressure
Plateau airway pressure (cmH₂O) was significantly lower in [R] compared to [H] at 45 minutes (12 ± 1 versus 18 ± 2; p < 0.05) and at 60 minutes of reperfusion (11 ± 1 versus 15 ± 1; p < 0.05) (Figure 3.2B). There was no significant difference between the other three groups, although plateau airway pressure was lower in [R] during the whole assessment.

Gas Exchange
There was no significant difference in oxygenation capacity (PO₂/FiO₂ (mmHg)) (Figure 3.3A). In [C] oxygenation capacity decreased during the reperfusion. PCO₂ in the outflow was lower in [H] compared to [C], [R] and [HR] at 45 minutes (27 ± 5 versus 42 ± 3, 46 ± 3 and 41 ± 3; p < 0.05) and compared to [C] at 50 minutes of reperfusion (35 ± 5 versus 47 ± 3; p < 0.05) (Figure 3.3B).

Wet-to-dry weight ratio
The W/D ratio for both lungs is shown in Table 3.3. There was no significant difference between the four groups before (p = 0.08) and also not after reperfusion (p = 0.19). W/D ratio was significantly lower after reperfusion (left lung) than before reperfusion (right lung) in [R], [H] and [HR].
### Table 3.3: W/D ratio in the right (non-perfused) and left (perfused) lung in all 4 groups.

<table>
<thead>
<tr>
<th></th>
<th>Right Lung</th>
<th>Left Lung</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C]</td>
<td>5.5 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>[R]</td>
<td>5.7 ± 0.4</td>
<td>5.0 ± 0.2</td>
<td>0.0018</td>
</tr>
<tr>
<td>[H]</td>
<td>5.3 ± 0.1</td>
<td>4.9 ± 0.3</td>
<td>0.032</td>
</tr>
<tr>
<td>[HR]</td>
<td>5.9 ± 0.6</td>
<td>4.9 ± 0.2</td>
<td>0.0075</td>
</tr>
</tbody>
</table>

*p*-value 0.08 0.19

Values are expressed as mean ± SD. [C]: no flush, [R]: retrograde flush, [H]: heparinization, [HR]: heparinization and retrograde flush.

### Histology

Histological examination of the left lung showed that capillaries and small arteries were more often occluded with microthrombi in [C] (2/6 specimens) and [H] (2/6 specimens) compared to [R] (0/6 specimens) and [HR] (0/6 specimens) (Figure 3.4 A-D). For the right lung, there were more microthrombi in [C] (6/6 specimens) and [H] (6/6 specimens) compared with [R] (1/6 specimens) and [HR] (0/6 specimens).

### DISCUSSION

The results of our study demonstrate that heparin alone or in combination with a retrograde flush has no additional benefit in uncontrolled DCD. There was no difference between [R] and [HR] in the time to warm up lungs. The time in [C] and [H] is comparable and significantly longer than in [R] and [HR]. PVR and flow were almost equal in [R] and [HR] but different from [C] and [H]. This trend was also seen in compliance. Plat AwP was worse in [H]. There was no difference between groups in oxygenation capacity or W/D weight ratio for the left lung.

Inokowa et al. reported that post-mortem heparinization followed by cardiac massage is possible and results in a lower pulmonary vascular resistance and a better oxygenation [9]. In a further study, the authors suggest that the optimal time of postmortem heparinization is within 30 minutes after cardiac arrest [10]. In both studies, animals were left at room temperature for 2 hours; the lungs were flushed in an anterograde manner and were then preserved cold for 60 minutes. This was followed by left lung transplantation. The animals received high doses of heparin (1000 IU/kg) followed by closed chest cardiac massage for 2 minutes.

Several studies routinely used heparin in controlled DCD with results comparable to donors after brain death (DBD) [11-13]. However, it is difficult to compare those studies since there is a difference in animals, amount of heparin, warm ischemic time, and ventilation during warm ischemic time, cold ischemic time, flush solution and type of flush. Nowadays, heparin is given before or after cardiac arrest in the controlled DCD donor in some transplant centres [3-6].

In the uncontrolled DCD studies, heparin was used pre-arrest [14,15] or post-arrest [16-18] as a part of the study protocol. Hodyc et al investigated the need of pre-arrest heparinization and ventilation during warm ischemia [19]. Rats received 600 IU of heparin intrahepatically. After euthanasia with sodium thiopental lungs were subjected to 60 min warm ischemia and 90 min cold ischemia. Ventilation during warm ischemia resulted in massive pulmonary edema. Better oxygenation was observed in heparinized animals compared to the non-heparinized animals.

In a recently published study in an uncontrolled DCD setting, animals received heparin (300 IU/kg) after a hands-off period of 10 minutes. This was followed by 2 minutes of cardiac massage. After 1 hour of warm ischemia and 2 hours of topical...
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Before change in PaO2, evidence that, during ex vivo evaluation, physiologic parameters can deteriorate received heparin regarding PaO2. There was no significant difference between the control group and the group who received heparin regarding PaO2, PVR, W/D ratio and histology [20]. There is evidence that, during ex vivo evaluation, physiologic parameters can deteriorate before change in PaO2 occurs [21]. Unfortunately, compliance and airway pressure were not mentioned in this study [20]. In a previous study, we demonstrated that retrograde flush is superior to anterograde flush or no flush in the preservation of DCD donor lungs [8]. Therefore, in the present study we decided to compare the use of heparin with retrograde flush or no flush.

Heparin is known for its anticoagulant activity but it’s also reported that it prevents postischemic endothelial cell dysfunction [22] independently from its anticoagulant, antiplatelet or anticompliment activities. There were marked differences in RBC-reperfusion of alveolar capillaries in a study comparing heparin and the nonanticoagulant N-acetyl (NA) heparin with a control group [23]. In the control group there was no capillary reperfusion. Pulmonary vascular resistance was higher and the blood flow was lower in the control group. The absence of a difference between the heparin and NA heparin group may confirm the effect reported by Sternbergh. However, in our study there was no difference between the animals treated with or without heparin.

Pulmonary vascular endothelium maintains its function when it is well preserved but it promotes platelet aggregation and coagulation if injured [24]. Fibrinolytic agents such as urokinase or tissue plasminogen activator convert plasminogen in plasmin. Plasmin breaks down fibrin resulting in fibrin degradation products and thus resolves the formed clot. When added to the flush solution or to the reperfusion solution they can dissolve the formed fibrin and microthrombi. In a left lung transplantation model, urokinase was injected in the main pulmonary artery after anterograde flush with a low-potassium-dextrose-glucose (LPDG) solution. This resulted in a better outcome after 2 hours of warm ischemia compared with a group without added urokinase [25]. Histology in the non-urokinase group showed thrombus formation and perivascular haemorrhage. In a study hereafter the effect of a longer cold ischemic time (12, 18 or 24 hours) in combination with inflation of the lung and flush with a low potassium dextran glucose solution containing EPC-K, was evaluated [26]. Lungs subjected to 12 or 18 hours of cold ischemia showed excellent graft performance during the 6 hours after transplantation. In contrast lungs subjected to 24 hours of cold ischemia, showed a lower oxygenation and a higher level of extravascular lung water reflecting lung oedema. Urokinase administered 60 minutes after cardiac arrest in addition to heparin resulted in better oxygenation, lower pulmonary vascular resistance, lower airway pressure and lower wet/dry weight ratio compared to heparin alone [27]. The use of urokinase during EVLP resulted in a lower PVR and increased oxygenation after 2 hours of warm ischemia [28]. Administration of recombinant tissue-type plasminogen activator (rt-PA) during reperfusion resulted in improved early lung function in canine lung transplantation model with 2 hours of warm ischemia and cold storage for 3 hours [29].

Histological examination of the lung biopsies showed more microthrombi in [C] and [H] compared to [R] and [HR]. This is consistent with the longer time that was necessary to warm up the lung in [C] and [H]. Microscopic evaluation of lungs harvested in a heparin-free donation scenario revealed no microvascular thrombi in the alveolar capillaries or in the pulmonary vasculature [30]. Although, these were controlled DCD lungs flushed retrograde and anterograde with Perfadex enriched with 50 000 IU heparin. This finding can confirm the results of our experimental set-up.

We acknowledge the limitations of our study. First, the period to evaluate the graft was limited in time. Therefore the findings need to be confirmed during a longer evaluation period in a transplant model or during a longer ex vivo evaluation. Second, we only compared the use of heparin in combination with a retrograde flush. However, in clinical practice, lungs are flushed first anterograde followed by a retrograde flush on the backtable.

The objective of the study was to mimic a DCD category I - II situation. There is experimental and clinical evidence that a limited period of warm ischemia (60 – 90 minutes) does not compromise the pulmonary graft from the DCD donor. Therefore the maximum warm ischemia time was limited to 1 hour after which preservation was started by means of topical cooling during 2.5 hours. In the clinical situation this gives the transplant coordinator the opportunity and the time to contact the next of kin and to start the legal procedures regarding organ retrieval.

In conclusion, we have demonstrated that the administration of heparin after cardiac arrest has no additional benefit in the uncontrolled DCD. A well performed retrograde flush is more effective to prevent from lung injury. EVLP is an appropriate and necessary tool to evaluate lungs from uncontrolled DCD. Addition of fibrinolytic agents to the reperfusion solution may have added beneficial effect.
REFERENCES


CHAPTER 4

The mode of death in the non-heart-beating donor has an impact on lung graft quality

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ABSTRACT

Objective
We hypothesized that the agonal phase prior to cardiac death may negatively influence the quality of the pulmonary graft recovered from non-heart-beating donors (NHBD). Different modes of death were compared in an experimental model.

Methods
Non-heparinised pigs were divided in 3 groups (n=6/group). Animals in group I [FIB] were sacrificed by ventricular fibrillation resulting in immediate circulatory arrest. In group II [EXS], animals were exsanguinated (45 ± 11 minutes). In group III [HYP], hypoxic cardiac arrest (13 ± 3 minutes) was induced by disconnecting the animal from the ventilator. Blood samples were taken premortem in HYP and EXS for measurement of catecholamine levels. After 1 hour of in situ warm ischemia, unflushed lungs were explanted and stored for 3 hours (4°C). Left lung performance was then tested during 60 minutes in our ex vivo reperfusion model. Total protein concentration in bronchial lavage fluid was measured at the end of reperfusion.

Results
Premortem noradrenalin (mcg/l) concentration (baseline: 0.03 ± 0) increased to a higher level in HYP (50 ± 8) versus EXS (15 ± 3); p = 0.0074. PO2 (mmHg) at 60 minutes of reperfusion was significantly worse in HYP compared to FIB (445 ± 64 versus 621 ± 25; p < 0.05), but not to EXS (563 ± 51). Pulmonary vascular resistance (dynes x sec x cm-5) was initially higher in EXS (p < 0.001) and HYP (NS) versus FIB (15824 ± 5052 and 8937 ± 4933 versus 1482 ± 61, respectively) but normalized thereafter. Wet-to-dry weight ratio was higher in HYP compared to FIB (5.2 ± 0.3 versus 4.7 ± 0.2, p = 0.041), but not to EXS (4.9 ± 0.2). Total protein (g/l) concentration was higher, although not significant in HYP and EXS versus FIB (18 ± 6 and 13 ± 4 versus 4.5 ± 1.3, respectively).

Conclusion
Premortem agonal phase in the NHBD induces a sympathetic storm leading to capillary leak with pulmonary oedema and reduced oxygenation upon reperfusion. Graft quality appears inferior in NHBD lungs when recovered in controlled (HYP) versus uncontrolled (EXS and FIB) setting.

INTRODUCTION

Lung transplantation is the mainstay therapy for patients with end-stage pulmonary disease refractory to medical treatment. Donors that are declared dead by neurological criteria provide most of the lungs nowadays. However, only 15-30% of the brain-dead donors have lungs that are deemed transplantable [1]. The use of non-heart-beating donors (NHBD) may be an alternative solution for the persistent problem of organ shortage [2]. Recently, successful transplantation has been reported by several groups worldwide in several case reports or small clinical series from both uncontrolled [3,4] and controlled [5-8] NHBD. NHBD can be classified into 4 categories according to the Maastricht classification [9]. In category I (dead on arrival) and category II (failed resuscitation), cardiac death occurs unexpectedly outside the hospital and the situation for organ recovery is therefore “uncontrolled”. In category III (withdrawal of life support awaiting cardiac arrest) and category IV (cardiac arrest in brain-dead donor), circulatory arrest is anticipated and organs can be recovered under “controlled” circumstances. Exsanguination and myocardial infarction or fibrillation are common causes of death in the uncontrolled NHBD. This may lead to a period of hemodynamic instability prior to circulatory arrest and cardiac death. On the other hand, in patients with irreversible brain damage not fulfilling the brain death criteria the ventilatory support is withdrawn (controlled NHBD), hypoxia will also result in hemodynamic instability and circulatory stop. Little is known about the impact of premortem instability, the so called agonal phase, on the quality of the graft prior to retrieval and on its performance after transplantation. Previous experiments have shown that a period of hypotension followed by circulatory arrest impairs lung viability [10] and that prearrest hypoxic perfusion is less detrimental for the pulmonary allograft than for the cardiac allograft [11]. However, no study so far has compared different modes of cardiac death.

The purpose of this experimental porcine study was to investigate premortem hemodynamic disturbances during the agonal phase and to compare its influence on graft performance after preservation using an isolated lung reperfusion model between animals succumbing from different modes of death (hypoxia versus hypovolemic shock versus cardiogenic shock).
MATERIAL AND METHODS

Experimental Groups
Eighteen domestic pigs (n=6/group; weight: 37.2 ± 1.1 kg) were randomly divided in 3 groups. Pigs in the first group were sacrificed by inducing ventricular fibrillation (FIB) with a subxyphoidal needle puncture using a square-pulse generator (amplitude range +15 to -15 V, current < 300 mA, frequency 50 Hz) resulting in immediate cardiac arrest. After cardiac arrest, the endotracheal tube was disconnected from the ventilator and left open to the air. In the second group, the animals were exsanguinated (EXS) via a catheter in the external jugular vein. Finally in the third group, hypoxic arrest (HYP) was induced by disconnecting the animal from the ventilator. After 1 hour of warm ischemia, the heart-lung block was explanted and stored on ice for 3 hours (4°C).

Animal Preparation
Animals were premedicated with an intramuscular injection of Xylazine (5 ml Xyl-M® 2%, V.M.D. nv/sa, Arendonk, Belgium) and Zolazepam/Tiletamine (3 ml Zoletil® 100, Virbac s.a., Carros, France). The animals were installed in a supine position and intubated with an endotracheal tube 7.5 (Portex Tracheal Tube, SIMS Portex, Ltd. Hythe, Kent, UK) and ventilated with a volume-controlled ventilator (Titus®, Dräger, Lübeck, Germany) with an inspiratory oxygen fraction (FiO₂) of 0.5 and a tidal volume of 10 ml/kg body weight. Respiratory rate was adjusted to achieve an end-tidal CO₂ of 40 mmHg. Positive end-expiratory pressure was set to 5 cmH₂O. Anaesthesia was maintained with isoflurane 0.8 – 1% (Isoba® Vet, Schering – Plough Animal Health, Hereford, Uxbridge, UK) and muscle relaxation with intermittent bolus of pancuronium bromide (Pavulon 2 mg/ml, Organon, Teknika, Boxtel, The Netherlands). A 14 G catheter (Secalon® T, Becton Dickinson Ltd., Singapore) was placed in the right common carotid artery for measurement of the systemic arterial pressure (SAP) and sampling of arterial blood. A 7.5 F Swan-Ganz thermodilution catheter (Baxter Healthcare Corp., Irvine, CA, USA) was inserted through the right external jugular vein into the pulmonary artery. With this catheter, hemodynamic parameters including pulmonary artery pressure (PAP) and pulmonary capillary wedge pressure (PCWP) were monitored. Hemodynamic parameters (SAP, PAP and PCWP) and aerodynamic parameters (plateau airway pressure and compliance) were continuously monitored and stored on a computer.

After a stabilization period, the pigs were sacrificed according to the study protocol described above. No heparin was administered in any group. After cardiac arrest in EXS and FIB, the endotracheal tube was disconnected from the ventilator and left open to the air. At the end of the agonal phase, blood samples were taken in EXS and HYP to analyse catecholamine levels. All cadavers were left untouched for 1 hour at room temperature. Temperature of the lung was measured via a probe in the endotracheal tube and rectal temperature was monitored.

All animals received human care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (NIH Publication No. 85-23, Revised 1996). The study was approved by the institutional review board on animal research at the Katholieke Universiteit Leuven.

Preparation of the Heart-Lung Block
After 1 hour of warm ischemia a sternotomy was performed. The thymic tissue was excised and the pericardium and pleural cavities were widely opened. The lungs were inspected. The pulmonary artery, ascending aorta and caval veins were encircled. Gross thrombi in the pulmonary artery and left atrium were removed as much as possible and the lungs were explanted without flush. After excision of the heart-lung block, the lungs were collapsed and immersed in cold (4°C) Perfadex and stored on ice for 3 hours.

The lungs in all 3 groups were prepared in the same way for ex vivo evaluation in the isolated reperfusion system after the cold storage. The right lung was separated from the heart-lung block and used as a control. The pulmonary artery was cannulated through the right ventricular outflow tract using a 36 Fr cannula and isolated with a ligature around the catheter distal to the pulmonary valve. A small catheter was placed in the pulmonary artery for measurement of PAP. The ascending aorta was clamped. The left atrium was cannulated through the apex of the left ventricle with a second 36 Fr cannula and secured with a purse-string. Finally, an endotracheal tube nr. 8 was placed in the trachea for ventilation of the pulmonary graft.

Preparation of the Perfusate
Autologous blood (1200 ml) was withdrawn from each animal in HYP and FIB after circulatory arrest via the catheter in the right external jugular vein and collected in a...
The mode of death in the non-heart-beating donor has an impact on lung graft quality. In EXS, animals were sacrificed by exsanguination and this blood was collected for the preparation of the perfusate. This whole blood was centrifuged with a Cell Saver (Sequestra 1000, Medtronic Inc, Parker, CO, USA) and washed with saline for 12 minutes at 5600 rpm. Leukocytes were sequestered using a leukocyte filter (Imugard III-RC, Terumo Europe N.V., Haasrode, Belgium). The remaining red blood cells (350 ml) were then diluted to a hematocrit of 15% with a low potassium dextran solution (Perfadex®, Vitrolife, Göteborg, Sweden) and human albumin (final concentration: 8%, CAF-DCF, Brussels, Belgium). The perfusate was finalized by adding CaCl₂ (2.4 ml/l, 100 mg/mL), heparin (10000 IU/l) and sodium bicarbonate (45 ml/l, 16.8 g/250 mL Baxter, Lessines, Belgium). The total volume of the perfusate was 1400 ml.

**Isolated Reperfusion Circuit**

The ex vivo reperfusion system consisted of a hardshell reservoir (Minimax® Hardshell reservoir, Medtronic, Minneapolis, MN, USA), a centrifugal pump (Bio-medicus, Medtronic), a heater/cooler system (Bio-Cal, Heater Cooler Model 370, Medtronic, Minneapolis, MN, USA) and a hollow fibre oxygenator (Capiox®SX, Terumo, MI, USA) with integrated heat exchanger. The heating element of the gas exchanger was connected to the heater/cooler system. The left lung and the heart were then placed in a specially designed evaluation box and mounted in the reperfusion system. The cannula in the pulmonary artery was connected to the inflow tubing and the outflow tubing was connected to the cannula in the left atrium.

**Technique of controlled reperfusion and ventilation**

Reperfusion of the left lung was started with normothermic (37°C) oxygenated perfusate (O₂: 0.4 l/min) after de-airing of the inflow tubing. Pulmonary artery pressure was gradually increased to a maximum of 15 mmHg and the left atrial pressure on the outflow was kept at 0 mmHg by adjusting the height of the blood reservoir. This resulted in warming up of the lung and a gradual increase in pulmonary artery flow. Ventilation with a FiO₂ 0.5 was started when the temperature of the outflowing perfusate reached 34°C and slowly increased to a tidal volume of 140 ml, a frequency of 14 breaths/min and PEEP of 5 cmH₂O. At that moment, the perfusate was partially deoxygenated to a PO₂ of 50 – 60 mmHg with a gas mixture of CO₂ (8%), O₂ (6%) and N₂ (86%).

**Assessment of the Graft**

Forty minutes after the onset of reperfusion, the temperature of the lung parenchyma reached 37.5°C. At this moment functional graft parameters were recorded up to one hour. Pulmonary artery pressure (PAP) (mmHg) was measured via an 18 Gauge catheter inserted in the main pulmonary artery. The pressure in the left atrium (LAP) (mmHg) was measured on the outflow line. An electromagnetic flow probe (FF 100T 10 mm probe, Nihon Kohden, Tokyo, Japan) was inserted in the tubing on the inflow line for continuous measurement of the pulmonary artery flow (PAF) (l/min). Pulmonary vascular resistance (PVR) was calculated using the formula: PVR = [PAP – LAP] x 80/PAF and expressed in dynes x sec x cm⁻⁵. Dynamic lung compliance (Compl) (ml/cmH₂O) and plateau airway pressure (Plat AwP) (cmH₂O) were recorded. PO₂ and PCO₂ were continuously measured in the perfusate via probes (Terumo CDITM, 500 shunt sensor, Leuven, Belgium) on the outflow tubing using an inline blood gas analyzer (CDITM 500, Terumo, Borken, Germany). Oxygenation capacity was calculated using the PO₂/FiO₂ ratio (mmHg).

Temperature (°C) of the inflowing and outflowing perfusate was continuously measured, the last being considered as the graft temperature. All data were recorded online and stored on a central server (Datex AS/3 and S5 collect 3.0 Software respectively, Datex-Ohmedia, Helsinki, Finland).

At the end of the reperfusion, both right and left lung were dried in an oven at 80°C for 48 hours to a constant weight and their wet-to-dry ratio (W/D) was calculated and used as a parameter of pulmonary oedema.

**Biochemical analysis of plasma catecholamines**

The catecholamines were measured in plasma, using high performance liquid chromatography with electrochemical detection (HPLC-ED). Activated alumina was used to extract the catecholamines from plasma. After a wash step the analytes were eluted with an acetic acid solution and separated on a reversed phase column in ion pair chromography mode. Concentrations were determined using calibration curves covering a range from 0 to 2000 ng/l. The lower limit of the quantification was 0.05 ng/l.

**Biochemical analysis of total protein in bronchial lavage fluid**

Bronchial lavage (BL) was performed in the right lung after explantation and in the left lung immediately at the end of the reperfusion. Twenty-five ml of sterile saline at room temperature was instilled in the bronchus and aspirated with gentle suction.
after 1 minute in a standardized way. The returned fraction was centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatant was collected for further analysis and stored at -80°C. Total protein was measured in the bronchial lavage supernatant by means of the biuret method (Roche Diagnostics GmbH, Mannheim, Germany). Protein reacts in an alkaline solution with divalent copper to form a purple coloured biuret complex. The colour intensity is measured photometrically and is proportional to the protein concentration. The lower detection limit was 2 g/l with an upper limit of 150 g/l.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 4 (San Diego, CA, USA). Graft parameters between study groups were compared using a one-way analysis of variance with a Newman-Keuls multiple comparison test. A paired t test was used to test for significant difference for wet to dry weight between the left perfused and the right non-perfused lung. An unpaired t test was used to test for significant difference in catecholamine levels. A p-value < 0.05 was considered as significant. Data regarding pulmonary graft function and catecholamines are expressed as mean ± standard error of the mean (SEM). All other data are expressed as mean ± standard deviation (SD).

RESULTS

Study groups

Baseline parameters prior to sacrifice of the animals in these groups are depicted in Table 4.1. There was no significant difference for animal weight, PAP, Compl and PaO$_2$/FiO$_2$.

Table 4.1: Baseline parameters prior to circulatory arrest in the three animal groups.

<table>
<thead>
<tr>
<th>Group (n=6/group)</th>
<th>Animal Weight (kg)</th>
<th>PAP (mmHg)</th>
<th>Compl (ml/cmH$_2$O)</th>
<th>PaO$_2$/FiO$_2$ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIB</td>
<td>30 ± 3</td>
<td>12 ± 3</td>
<td>34 ± 3</td>
<td>626 ± 60</td>
</tr>
<tr>
<td>EXS</td>
<td>34 ± 2</td>
<td>11 ± 2</td>
<td>38 ± 5</td>
<td>637 ± 88</td>
</tr>
<tr>
<td>HYP</td>
<td>34 ± 1</td>
<td>12 ± 1</td>
<td>36 ± 3</td>
<td>594 ± 38</td>
</tr>
<tr>
<td>p-value</td>
<td>0.16</td>
<td>0.69</td>
<td>0.11</td>
<td>0.51</td>
</tr>
</tbody>
</table>

There was no significant difference for animal weight, pulmonary artery pressure (PAP), compliance (Compl) and oxygenation capacity (PaO$_2$/FiO$_2$). All data are expressed as mean ± SD. FIB: ventricular fibrillation, EXS: exsanguination, HYP: hypoxic cardiac arrest.

All animals in FIB were pulseless immediately after needle puncture as a result of myocardial fibrillation. Animals undergoing exsanguination (EXS) were pulseless after 45 ± 11 minutes. The agonal phase was characterized by changes in heart rate and in blood pressure. Initially there was a simultaneous decrease in heart rate and blood pressure (hypotensive bradycardia). In the next stage the heart rate increased but the blood pressure remained the same (hypotensive tachycardia). This was followed by a drop in the heart rate and a further decrease in blood pressure until pulselessness.

In the hypoxic arrest group (HYP) animals were pulseless after 13 ± 3 minutes. After disconnection of the endotracheal tube, there was an increase in the heart rate and in blood pressure (hypertensive tachycardia). Thereafter, both heart rate and blood pressure dropped continuously until loss of electrical activity. The hemodynamic changes during the agonal phase in one animal for EXS and HYP are demonstrated in Figure 4.1A and Figure 4.1B, respectively.

Figure 4.1: Example of hemodynamic changes in heart rate (HR) and in arterial blood pressure (BP) during the agonal phase in 1 animal from each study group. A: Exsanguination; B: Hypoxic cardiac arrest.
Pulmonary graft characteristics before reperfusion in the 3 groups are compared in Table 4.2. There was no significant difference between the 3 groups for the warm ischemic interval, cold ischemic interval and the temperature of the graft at the end of the cold ischemic period. The time necessary to warm up the lung to 34°C at the time of reperfusion was longer in EXS and HYP compared to FIB (p = 0.017).

Table 4.2: Pulmonary graft characteristics before reperfusion in the 3 study groups.

<table>
<thead>
<tr>
<th>Group (n=6/group)</th>
<th>WIT (min)</th>
<th>CIT (min)</th>
<th>Graft temperature  (°C)</th>
<th>Warming up (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIB</td>
<td>62 ±5</td>
<td>186 ± 6</td>
<td>4 ± 0</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>EXS</td>
<td>61 ± 1</td>
<td>183 ± 3</td>
<td>4 ± 0</td>
<td>38 ± 5†</td>
</tr>
<tr>
<td>HYP</td>
<td>60 ± 0</td>
<td>185 ± 5</td>
<td>5 ± 1</td>
<td>36 ± 3Û</td>
</tr>
</tbody>
</table>

Pulmonary graft function

**Pulmonary vascular resistance**

Pulmonary vascular resistance (dynes x sec x cm⁻¹) was higher in EXS (p < 0.001) and HYP (NS) compared to FIB at 40 minutes of reperfusion (15824 ± 5052 and 8557 ± 4933 versus 1482 ± 61). This difference disappeared during longer reperfusion (1531 ± 174 and 1369 ± 181 versus 1435 ± 95, respectively, at 60 minutes; p > 0.05) (Figure 4.3A).

**Dynamic lung compliance**

There was no difference in Compl (ml/cmH₂O) between FIB, EXS and HYP during the reperfusion (Figure 4.3B), but this parameter appeared more stable in FIB.

**Plateau airway pressure**

Plat AwP (cmH₂O) was initially higher in EXS and HYP compared to FIB although the difference did not reach significance (18 ± 3 and 16 ± 2 versus 13 ± 1, respectively) this normalized later on (Figure 4.3C).

**Oxygenation capacity**

PO₂ (mmHg) at 60 minutes of reperfusion was significantly worse in HYP versus FIB (445 ± 64 versus 621 ± 25; p < 0.05) but not when compared to EXS (563 ± 51). There was also no significant difference between FIB and EXS (Figure 4.3D).

**Protein concentrations**

The total protein concentration (g/l) in the BL fluid of the left lung was higher, although not significant in HYP and EXS compared to FIB (18 ± 6 and 13 ± 4 versus 4 ± 1, respectively).

Catecholamine concentrations

Noradrenaline levels (mcg/l) taken immediately before cardiac arrest were significantly higher in HYP (50 ± 8) versus EXS (15 ± 3) (p = 0.0074) (Figure 4.2A). The premortem adrenaline levels were also higher in HYP compared to EXS although the difference did not reach significance (45 ± 15 and 14 ± 4, respectively) (Figure 4.2B). Noradrenaline and adrenaline levels in HYP and EXS were significantly higher premortem compared to the baseline values (0.03 ± 0) (p < 0.05) (Figure 4.2A-B).
Wet-to-dry weight ratio

The W/D ratio of the perfused left lung was significantly higher in HYP compared to FIB (5.2 ± 0.3 versus 4.7 ± 0.2, \( p = 0.041 \)) but not to EXS (4.9 ± 0.2). There was no significant difference in W/D ratio between the 3 groups for the non-perfused right lung. W/D ratio was significantly lower in FIB and in EXS in the left lung compared to the right lung. There was no difference for HYP (Table 4.3).

Table 4.3: W/D ratio in the right (non-perfused) and left (perfused) lung in all study groups.

<table>
<thead>
<tr>
<th></th>
<th>Right Lung</th>
<th>Left Lung</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIB</td>
<td>5.2 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>EXS</td>
<td>5.3 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td>0.011</td>
</tr>
<tr>
<td>HYP</td>
<td>5.5 ± 0.3</td>
<td>5.2 ± 0.3</td>
<td>0.041</td>
</tr>
</tbody>
</table>

\( p < 0.05 \): HYP versus FIB. All data are expressed as mean ± SD.

FIB: ventricular fibrillation, EXS: exsanguination, HYP: hypoxic cardiac arrest

DISCUSSION

The goal of this study was to investigate the impact of premortem instability and to compare different modes of death in the NHBD on graft performance. We therefore compared animals succumbing from cardiac arrest resulting from ventricular fibrillation, exsanguination or hypoxia. We demonstrated that lungs recovered from hypoxic animals were of inferior quality with significantly worse oxygenation at 60 minutes of reperfusion compared to lungs retrieved from animals with sudden death by myocardial fibrillation without agonal period. W/D ratio was also higher in HYP compared to FIB. Pulmonary vascular resistance was also higher in HYP (NS) and EXS (\( p < 0.001 \)) compared to FIB. As a result, the time necessary to warm the lung up in the ex vivo circuit was significantly longer in HYP and EXS versus FIB. Total protein concentration was higher although not significant in HYP and EXS versus FIB. Premortem noradrenaline concentration was significantly higher in HYP compared to EXS. These findings suggest that the premortem agonal phase during hypoxia induces a catecholamine storm leading to capillary leak with pulmonary edema and reduced oxygenation upon reperfusion much worse than during hypovolemia.

Cerebral herniation in the heart-beating donor is initially characterized by a hyperdynamic state with hypertension and tachycardia associated with changes in the circulating plasma catecholamines. This is followed by neurogenic hypotension. There is also evidence that systemic inflammatory pathways are activated during this process. The lung is susceptible to these changes resulting in neurogenic pulmonary edema and in an acute inflammatory lung injury [12,13].
Chapter 4

The use of lungs recovered from NHBD has recently been propagated as an alternative to overcome the critical organ shortage [14]. There are, however, still several concerns to the use of the lungs from NHBD [2]. The influence of the premortem instability is one of them. It is hypothesized that the injury to the graft in the premortem agonal period could be more noxious than the injury that occurs during the warm ischemic interval prior to cold preservation. The effect of premortem instability in the NHBD on the outcome has previously been addressed by some research groups [10,11]. However, no study compared the different modes of cardiac death. When our study was designed, we decided to compare the different situations that occur in patients dying from cardiac death qualifying as potential NHBD. We therefore looked at scenarios of sudden death following myocardial fibrillation as well as longer agonal periods prior to death resulting from hypoxia or hypovolemia. These scenarios reflect what can happen in the clinical situation for both uncontrolled (FIB and EXS) and controlled (HYP) donors after cardiac death.

Secher and colleagues described hemodynamic events during reversible hypovolaemic shock [15]. In the first stage, an increase in heart rate is associated with a normal or slightly increased blood pressure. This is followed by a decrease in heart rate and blood pressure. In the third stage blood pressure falls further and tachycardia is present. This stage can proceed to irreversible shock with cardiac arrest. We observed similar hemodynamic changes during the agonal phase in our animals that were exsanguinated.

After disconnection of the endotracheal tube in HYP, animals developed a marked increase in heart rate and blood pressure. This was paralleled with a significant increase in noradrenaline. Thereafter, both heart rate and blood pressure dropped until loss of electrical activity. This was also reported in a study by DeBehnke and colleagues were a canine model of asphyxial arrest with pulseless electrical activity was used [16]. Animals went through a characteristic pattern of tachycardia and hypertension followed by bradycardia and hypotension. Erasmus and colleagues also reported a period of central hypertension preceding cardiac arrest in NHBD pigs sacrificed after ventilator switch-off [17].

Tremblay and co-workers investigated in an isolated rat reperfusion model the influence of hemodynamic instability (mean blood pressure 30 – 40 mmHg) during 1 hour before death in combination with different lengths of warm ischemia (0, 2 or 3 hours) followed by cold flush preservation [10]. Haemorrhagic edema developed during reperfusion in lungs recovered from animals after 1 hour of haemorrhagic shock followed by 2 to 3 hours of warm ischemia. Lungs that were not subjected to an additional period of warm ischemia after circulatory arrest did better. This study suggests that the combination of 1 hour hypotension and 2 hours of warm ischemia is deleterious for the lung. Our group has previously demonstrated that the warm ischemia tolerance in the deflated lung is limited to 60-90 minutes [18,19].

In the present study, exsanguination was followed by 1 hour of warm ischemia. The lungs were harvested without flush and stored on ice for 3 hours. The left lung was then evaluated in an ex vivo reperfusion system. Gas exchange was slightly worse in EXS when compared to FIB but there was no significant difference. There was also no significant difference for compliance, pulmonary vascular resistance and plateau airway pressure.

In a rabbit model of isolated lung perfusion, Mauney et al. found that pulmonary allografts after hypoxic arrest and 20 minutes of warm ischemia showed no significant differences in pulmonary vascular resistance, oxygenation, airway resistance and baseline pulmonary artery pressure after 45 minutes of reperfusion [11]. Animals were heparinized before hypoxic arrest and lungs were not exposed to cold ischemia. In the present study no heparin was administered before hypoxic arrest and lungs were exposed to cold storage. Oxygenation was significantly worse after 60 minutes of reperfusion in HYP compared to FIB. The Groningen group also reported impaired lung function in an ex vivo pig lung perfusion study where animals were sacrificed by ventilator switch-off reflecting the clinical setting of controlled NHBD. This was followed by 1 hour of warm ischemia and topical cooling. Lungs were preserved for 6 hours using ex vivo lung perfusion. The authors hypothesized that the hypertensive period before cardiac arrest causes endothelial damage by mechanical stretching with release of pro-inflammatory cytokines [17].

We observed a higher pulmonary vascular resistance in HYP and EXS compared to FIB at the start of the perfusion. Hypoxic pulmonary vasoconstriction as in HYP is a response to a decreased alveolar oxygen tension which results in vasoconstriction of the small muscular pulmonary arteries increasing the pulmonary vascular resistance. Haemorrhagic shock as in EXS is characterized by hypovolaemia and hypoxia resulting in pulmonary vasoconstriction. We hypothesize that the longer duration of the agonal phase in EXS compared to HYP resulted in an initial higher PVR and that the increase of the catecholamines has less influence on PVR in the lungs. Reperfusion was performed in a controlled setting with a maximum inflow...
pressure of 15 mmHg. The high PVR in HYP and EXS resulted in a reduced flow through the lung and therefore a longer time necessary to warm up the lung.

Elevated catecholamines and mainly, norepinephrine cause an increase in total peripheral vascular resistance and in right ventricular systolic pressure leading to pulmonary congestion. It can also increase pulmonary venous tone with increased hydrostatic pressure in the pulmonary capillaries resulting in pulmonary edema. Norepinephrine stimulates proinflammatory cytokines resulting in increased permeability of the alveolar-capillary barrier and pulmonary edema. This was demonstrated experimentally in a study were rats received a continuous intravenous infusion of norepinephrine [20] and in clinical studies after lung transplantation [21].

Similar changes are described to explain pulmonary events in neurogenic pulmonary edema after brain death [22-24] and the inflammatory changes in patients with acute lung injury [25]. We also found a significantly higher level of catecholamines in the period preceding death in HYP compared to EXS. W/D ratio of the perfused left lung and protein levels were also higher in HYP. This suggests that the increase of catecholamines during the agonal phase after hypoxic cardiac arrest may have an important impact on graft performance.

As we were interested in the sole effect of the agonal phase on graft performance, no drugs were administered during the agonal phase or after circulatory arrest and the lungs were retrieved without pulmonary flush.

The present study suffers from several limitations to translate our conclusions to the clinical situation in NHBD. Firstly, the reperfusion model and therefore the period to evaluate graft performance are limited in time. The present findings, therefore, need to be confirmed in a transplant model. Secondly, animals with healthy lungs were used in this study. It is most likely that this is completely different in the clinical situation. Controlled NHBD dying from hypoxic cardiac arrest after ventilatory switch off may already have suffered some form of lung injury from barotrauma, aspiration or infection when ventilated in the hours after severe insult to the brain. Uncontrolled donors dying from hypovolaemic shock may also develop lung injury as a result of direct trauma or resuscitation manoeuvres with cardiac massage, administration of vasopressors and fluid resuscitation. Most patients dying from cardiogenic shock succumb from myocardial infarction. These patients do not always develop sudden cardiac arrest from myocardial fibrillation, as was the case in the present study. So the agonal period and thus inflammatory lung injury in the clinical situation may also contribute to the outcome.

In conclusion, in this experimental pig lung reperfusion study, we have demonstrated that the premortem agonal phase after switch-off procedure induces a catecholamine storm leading to capillary leak with pulmonary oedema and reduced oxygenation upon reperfusion. Pulmonary graft quality appears to be inferior when recovered from controlled (HYP) versus uncontrolled NHBD (EXS and FIB). Therefore, long periods of hemodynamic instability after ventilator switch-off should raise concerns in a clinical setting. Further studies are needed to identify an acceptable period whereby lungs can be safely transplanted in the setting of controlled donation.
The mode of death in the non-heart-beating donor has an impact on lung graft quality.
CHAPTER 5

In situ lung perfusion is a valuable tool to assess lungs from donation after circulatory death donors category I – II

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ABSTRACT

Donation after circulatory death (DCD) lung grafts are an alternative to extend the donor pool in lung transplantation. This study investigates the use of an in situ lung perfusion system (ISLP) in the donor to evaluate category I – II lungs. Pigs were sacrificed by ventricular fibrillation. All animals underwent 20 minutes of cardiopulmonary resuscitation and 5 minutes hands-off period after which heparin was administered. In group [WI-1], this was followed by 1 hour of warm ischemia (WI) and 2 hours of topical cooling (TC). In group [WI-2], 2 hours of WI was followed by 1 hour of TC. In group [WI-0], there was a minimal period of WI and no TC. In all 3 groups the lungs were then evaluated during 60 minutes with ISLP.

[WI-0] lungs showed a significantly higher compliance and Δ PO$_2$/FiO$_2$ compared to [WI-1] and [WI-2]. PCO$_2$ and lactate production were higher in [WI-2] versus [WI-0]. Wet/Dry weight ratio was significantly higher in [WI-2] compared to [WI-0] in two lung biopsy locations. A high W/D weight ratio was correlated with a lower compliance, higher lactate production and a higher PCO$_2$. ISLP is an effective way to assess the quality of lungs from category I – II DCD donors.

INTRODUCTION

Lung transplantation is a lifesaving treatment for well selected patients with benign end-stage pulmonary disease. One of the major limitations of this treatment is the donor organ shortage. Only 15% of lungs from multi organ donors are available for transplantation [1]. The use of lungs from donation after circulatory death (DCD) donors is one of the strategies to increase the donor pool. However, evaluating the lung function in uncontrolled DCD remains challenging.

Normothermic ex vivo lung perfusion (EVLP) was developed as a tool for assessing lung viability and for reconditioning of marginal and unacceptable donor lungs [2]. Recently, Cypel et al. reported in a prospective study that extended normothermic EVLP allows an assessment of DCD donor lungs and donation after brain death (DBD) donor lungs. Transplantation of these lungs led to results comparable with conventionally selected lungs [3]. Varella at al. initially evaluated uncontrolled DCD donor lungs using a pulmonary flush technique [4]. Recently, lungs were assessed using EVLP before implantation [5].

DCD is inevitable associated with a warm ischemia period. Warm ischemia is the ischemia of cells and tissues under normothermic conditions. It leads to endothelial dysfunction and to alveolar type II cell dysfunction resulting in pulmonary edema and graft dysfunction during reperfusion [6-9]. There is experimental and clinical evidence that 1 hour of warm ischemia does not compromise the function of the pulmonary graft [10]. However, reperfusion after 2 hours of warm ischemia results in deterioration of the pulmonary graft [6].

No study so far has evaluated the feasibility of an evaluation of lungs from uncontrolled DCD donors with a lung perfusion system in the donor. In in situ lung perfusion (ISLP) the lungs remain in the deceased body. The heart-lung block is connected to a reperfusion system and a bed site assessment is performed. At that time, there is no extra manipulation of the lungs by means of flush or harvest.

The primary endpoint of this study was to investigate the feasibility of an in situ lung perfusion system (ISLP) to make an assessment of the donor lung quality. The secondary endpoint was to determine predictors for lung injury in this model.
MATERIAL AND METHODS

Experimental groups
Twelve domestic pigs (n=4/group; average weight: 47.5 kg) were randomly divided into 3 equal groups. In all groups, the animals were sacrificed by inducing ventricularch fibrillation. This was followed by cardiopulmonary resuscitation for 20 minutes during which the systolic blood pressure was above 50 mmHg. During cardiopulmonary resuscitation, the animals were ventilated with an inspiratory oxygen fraction (FiO\textsubscript{2}) of 0.5, a tidal volume of 5 ml/kg and a respiratory rate of 5 breaths/minute. After a 5 minutes hands-off period, during which the endotracheal tube was disconnected from the ventilator and left open to the air, heparin (15000 IU intravenously) was given. The heparin was circulated by performing closed chest massage (20 times) and restarting of the ventilation. In group [WI-1], this was followed by 1 hour of warm ischemia, 2 hours of topical cooling and 1 hour of ISLP.

In group [WI-2], the warm ischemic period was extended up to 2 hours followed by 1 hour of topical cooling and 1 hour of ISLP. Finally, in group [WI-0] ISLP was performed after a short period of warm ischemia, without topical cooling.

Animal preparation
Animals were premedicated, positioned in a prone position and then intubated with an endotracheal tube. After re-positioning in supine position, the animals were ventilated with an inspiratory oxygen fraction (FiO\textsubscript{2}) of 0.5, a tidal volume of 10 ml/kg body weight, a respiratory rate of 10-12 breaths/minute and a positive end-expiratory pressure of 5 cmH\textsubscript{2}O.

Anaesthesia was maintained with sevoflurane 2-3% and muscle relaxation with a continuous infusion of pancuronium bromide (Pavulon 2 mg/ml, Organon, Teknika, Boxtel, and The Netherlands). Hemodynamic monitoring via a catheter placed in the right common carotid artery and blood gas analysis was performed in all animals. The right external jugular vein was used for infusion of fluids. Lung function was continuously monitored.

All animals received human care in compliance with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996 (NIH Publication No. 85-23, Revised 1996). The institutional animal care and use committee of the University of Groningen approved the study. The pigs were sacrificed according to the study protocol described above. In [WI-1] and [WI-2] the cadavers were left untouched at room temperature. Temperature of the lung was measured via a probe within the endotracheal tube. At the end of the warm ischemia, a sternotomy was performed and the lungs were inspected.

Topical cooling was started with a combination of cold saline and buffered Perfadex®. Endotracheal, chest cavity and rectal temperature was measured. Target was an endobronchial temperature of 12°C. At the end of the topical cooling, the pulmonary artery was cannulated through the right ventricular outflow tract and isolated with a ligature around the catheter distal to the pulmonary valve. A small catheter was placed in the pulmonary artery for measurement of pulmonary artery pressure (PAP). The left atrium was cannulated through the apex of the left ventricle with a second cannula and secured with a purse-string. Another small catheter was placed in the left atrium for measurement of the left atrium pressure (LAP).

After confirmation of asystole the sternotomy was performed in [WI-0]. The in situ lung perfusion was started after cannulation of the pulmonary artery and left atrium.

In Situ Lung Perfusion
The in situ lung perfusion evaluation was performed with the use of a Lung Assist. The Lung Assist (Organ Assist BV, Groningen, the Netherlands) is a combined heating/cooling and centrifugal pump system. A flow probe and temperature probes are also included. It is used in combination with a reservoir, an oxygenator and a ventilator. For the ISLP the system was primed with 1.5 l of Steen® solution. Steen solution is a buffered extracellular solution containing dextran and human serum albumin with an optimal colloid osmotic pressure (295 ± 20 mOsm/kg) preventing formation of lung oedema [11]. The ISLP was performed using an acellular perfusate with a hematocrit < 10%.

Technique of ventilation and controlled reperfusion
Reperfusion of the heart-lung block was started, after retrograde flush of the tubing, with oxygenated Steen solution (FiO\textsubscript{2} 0.21) at room temperature (20°C). Pulmonary artery pressure was gradually increased to a maximum of 15 mmHg and the left atrial pressure was kept at 3-5 mmHg by adjusting the height of the blood reservoir. This resulted in warming up of the lung and a gradual increase in pulmonary artery flow. This up to maximum calculated flow of 40% of the total cardiac output (cardiac output = 100 ml/kg) or a mean PAP of 15 mmHg. When a temperature of 32°C was reached, ventilation was started with a FiO\textsubscript{2} 0.5 a tidal volume of 10ml/kg, a frequency of 12 breaths/min and PEEP of 5 cmH\textsubscript{2}O. At that moment, the perfusate was continuously deoxygenated to a PO\textsubscript{2} of 6.6 – 8 kPa with a gas mixture of CO\textsubscript{2} (8%), O\textsubscript{2} (6%) and N\textsubscript{2} (86%).
Assessment of the Graft
The functional graft parameters were recorded up to one hour. PAP (mmHg) and LAP (mmHg) were measured continuously. A flow probe on the inflow line measured the pulmonary artery flow (PAF) (l/min). Pulmonary vascular resistance (PVR) was calculated using the formula: \( PVR = \frac{PAP - LAP}{PAF} \) and expressed in Wood units. Dynamic lung compliance (Compl) and plateau airway pressure (Plat AwP) were recorded. \( PO_2 \), \( PCO_2 \) and lactate of the inflowing and outflowing perfusate were measured at 45 and 60 minutes. Oxygenation capacity was calculated using the \( \Delta PO_2/FiO_2 \) ratio (kPa) (\( \Delta PO_2 = \) perfusate left atrium \( PO_2 - \) perfusate pulmonary artery \( PO_2 \)). The lactate production during the last 15 minutes of reperfusion was calculated (outflow 60 minutes - outflow 45 minutes). The transpulmonary lactate gradient (\( \Delta \) lactate) was calculated as the difference between outflow lactate and inflow lactate at 0, 45 and 60 minutes.

Temperature (°C) of the inflowing and outflowing perfusate was continuously measured, the last being considered as the graft temperature.

At the end of the reperfusion, biopsies were taken from apical anterior part, the apical posterior part, the basal anterior part and the basal posterior part of the right lung in [WI-0] and from the left lung in [WI-1] and [WI-2]. W/D weight ratio was assessed as parameter for lung edema.

Histology
At the end of the experiment, tissue samples were obtained from the right lung in the [WI-0] and from the left lung in [WI-1] and [WI-2]. Specimens were fixed in 6% formaldehyde, dehydrated and stained with haematoxylin and eosin and examined for pathologic changes under a light microscope. Histological analysis was performed by one experienced pathologist who was blinded for the experimental set-up.

Correlations
The W/D weight ratios of all 3 groups were correlated with all the parameters to determine predictors for lung injury.

STATISTICAL ANALYSIS
Data were analyzed using GraphPad Prism 5 (San Diego, CA, USA). Graft parameters between 3 study groups were compared using a one way Anova test. Graft parameters between 2 study groups were compared using an unpaired t test.
In situ lung perfusion is a valuable tool to assess lungs from donation after circulatory death donors.

Chapter 5

Figure 5.1: Pulmonary graft function (mean ± SEM) from 45 minutes until 60 minutes after start of in situ reperfusion. A: Dynamic lung compliance: * p < 0.05 [WI-2] versus [WI-0] and ‡ p < 0.05 [WI-2] versus [WI-1]. B: Plateau airway pressure: NS between groups. C: Pulmonary vascular resistance: NS between groups.

Figure 5.2: Oxygenation capacity and PCO₂. A: Δ PO₂/FiO₂: * p < 0.05 [WI-2] versus [WI-0] and ‡ p < 0.05 [WI-2] versus [WI-1]. B: PCO₂: * p < 0.05 [WI-2] versus [WI-0].

PCO₂

There was more CO₂ in the outflowing perfusate in [WI-2] compared to [WI-0] at 45 minutes and 60 minutes of reperfusion (3.10 ± 0.15 versus 2.34 ± 0.10 (p = 0.0156) and 3.22 ± 0.15 versus 2.38 ± 0.05 (p = 0.0463); respectively) (Figure 5.2B). There was no significant difference in the PCO₂ of the inflowing perfusate.

Lactate

There was a continuous sharp increase of lactate in [WI-2]. In [WI-0] and [WI-1] the sharp increase was followed by a lesser increase of the lactate (p > 0.05) (Figure 5.3A). The lactate production (mmol/l) during the last 15 minutes of reperfusion is higher in [WI-2] compared to [WI-1] and [WI-0] (0.93 ± 0.23 versus 0.54 ± 0.11 and 0.3 ± 0.11; p = 0.0588) (Figure 5.3B).

In [WI-0] and [WI-1] there was an increase of Δ lactate during the first 45 minutes of reperfusion followed by a decrease during the last 15 minutes (p > 0.05) (Figure 5.3C). Instead in [WI-2] the Δ lactate continuously increased during reperfusion.
In situ lung perfusion is a valuable tool to assess lungs from donation after circulatory death donors.

Wet-to-dry weight ratio

W/D ratio was significantly lower in [WI-0] compared to [WI-1] and [WI-2] in the basal anterior lung biopsy. In the apical anterior lung biopsy W/D ratio was lower in [WI-0] compared to [WI-2] (Table 5.1).

Table 5.1: W/D weight ratio in all 3 study groups

<table>
<thead>
<tr>
<th></th>
<th>[WI-0]</th>
<th>[WI-1]</th>
<th>[WI-2]</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical anterior</td>
<td>4.55 ± 0.17</td>
<td>5.17 ± 0.13</td>
<td>5.87 ± 0.82</td>
<td>0.0132 [WI-0] versus [WI-2]</td>
</tr>
<tr>
<td>Apical posterior</td>
<td>4.92 ± 0.17</td>
<td>5.10 ± 0.15</td>
<td>5.87 ± 0.91</td>
<td>0.0736</td>
</tr>
<tr>
<td>Basal anterior</td>
<td>4.77 ± 0.32</td>
<td>7.02 ± 1.48</td>
<td>7.19 ± 1.37</td>
<td>0.0307 [WI-0] versus [WI-2]</td>
</tr>
<tr>
<td>Basal posterior</td>
<td>4.78 ± 0.17</td>
<td>6.14 ± 1.55</td>
<td>7.33 ± 1.62</td>
<td>0.0621 [WI-0] versus [WI-1]</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SD

Correlations

There was a negative correlation between compliance and the W/D weight ratios in the 4 biopsies and a positive correlation between PCO₂, lactate production and the W/D weight ratios in the 4 biopsies. For plateau airway pressure there was a positive correlation in 3 out of 4 biopsies and for ∆ lactate in 2 out of 4 biopsies (Table 5.2).

Gross Appearance

There was pulmonary oedema with oedema fluid in the endotracheal tube in [WI-2]. In [WI-0] and [WI-1] there was no pulmonary oedema.

Histology

Microscopically, lung injury was scored assessing alveolar congestion, haemorrhage, vascular thrombosis, infiltration or aggregation of neutrophils. There were no significant differences between the 3 groups.

Table 5.2: Correlations between W/D and other parameters

<table>
<thead>
<tr>
<th></th>
<th>Compliance</th>
<th>PCO₂</th>
<th>Plateau Airway Pressure</th>
<th>∆ Lactate</th>
<th>Lactate production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical Anterior</td>
<td>r = -0.81</td>
<td>0.74</td>
<td>0.87</td>
<td>0.51</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>p = 0.0007</td>
<td>0.003</td>
<td>&lt; 0.0001</td>
<td>0.045</td>
<td>0.011</td>
</tr>
<tr>
<td>Apical Posterior</td>
<td>r = -0.62</td>
<td>0.64</td>
<td>0.91</td>
<td>0.73</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>p = 0.016</td>
<td>0.012</td>
<td>&lt; 0.0001</td>
<td>0.034</td>
<td>0.047</td>
</tr>
<tr>
<td>Basal Anterior</td>
<td>r = -0.69</td>
<td>0.88</td>
<td>0.47</td>
<td>0.063</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>p = 0.0065</td>
<td>&lt; 0.0001</td>
<td>0.061</td>
<td>0.42</td>
<td>0.049</td>
</tr>
<tr>
<td>Basal Posterior</td>
<td>r = -0.65</td>
<td>0.94</td>
<td>0.63</td>
<td>0.22</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>p = 0.0112</td>
<td>&lt; 0.0001</td>
<td>0.013</td>
<td>0.24</td>
<td>0.028</td>
</tr>
</tbody>
</table>

Figure 5.3: A: Lactate levels during the reperfusion: NS between groups. B: Lactate production during the last 15 minutes of reperfusion: NS between groups. C: Transpulmonary lactate gradient (∆ lactate): NS between groups.
 DISCUSSION

Normothermic EVLP has proven to be a successful technique for assessment and reconditioning of marginal and unacceptable donor lungs from DBD donors and DCD donors in clinical and experimental work [11-13]. However, in DCD category I – II lung function is often unknown at the time of recovery. Therefore, we established an in situ lung perfusion system that can assess the lungs in the donor without preceding pulmonary flush preservation. The graft function at the end of ISLP in [WI-1] was comparable with the baseline parameters demonstrating that ISLP is a safe way to assess lungs from NHBD category I – II.

A key finding of our study is that resuscitation in combination with warm ischemia causes damage even in this ideal experimental setting. Another finding of this study is that pulmonary graft function of one animal in [WI-2] was equivalent to that of the lungs in [WI-1] animal. This observation however requires further study. A previous study of Egan et al. report that some animals survived an observation period after 2 hours of warm ischemia and after 5 hours of warm ischemia [14]. Also Hayama reports good results after 2 hours of warm ischemia in a study using dogs [15]. This may suggest that DCD category I – II donor lungs after both 1 hour and 2 hours of warm ischemia may be suitable for transplantation after evaluation and subsequent reconditioning with ISLP or EVLP.

Until today PO$_2$ is the gold standard for evaluation of donor lungs. However, there is recent evidence that during EVLP and subsequently during ISLP physiologic parameters are of greater importance than PO$_2$. Yeung et al. demonstrated that ex vivo PO$_2$ may not be the first indication of lung injury. In this study pulmonary edema was associated with a decrease in compliance and an increase in airway pressure before a decrease in PO$_2$ [16].

In our study there was a significant correlation between the compliance and W/D weight ratio. von Neergaard demonstrated that when the lung is completely filled with saline the lung compliance increases. However, in pulmonary edema not all alveoli are filled with fluid. A recent report shows that in pulmonary edema the liquid-filled edematous alveoli shrink resulting in an expansion of the air-filled neighbor alveoli. Subsequently, there is an over distension of the alveoli reducing the compliance of the air-filled alveoli and hence the overall lung compliance [17]. The correlation that we found between compliance and W/D ratio might well be explained by this mechanism.

The correlation that we found between compliance and W/D ratio might well be explained by this mechanism. We also found a relation between the PCO$_2$ and an increased W/D weight ratio. The gas exchange in the lung is determined by the balance between the capillary flow and the alveolar ventilation. In pulmonary edema this balance is disturbed causing an intrapulmonary shunt [18]. A decrease in PO$_2$ and an increase in PCO$_2$ is seen when the intrapulmonary shunt rises considerably. Under such conditions the PO$_2$ will be relatively independent of the changes in FiO$_2$. We hypothesize that in [WI-2] animals the alveolo-capillary membrane may suffer an injury resulting in macroscopic lung edema and an increased PCO$_2$.

Also an increased pulmonary lactate production was correlated with an increased W/D ratio indicating injury in our NHBD setting. The higher pulmonary lactate production in [WI-2] was associated with a higher W/D ratio.

In [WI-0] and [WI-1] the Δ lactate initially increased followed by a decrease. However in the more injured [WI-2] group there was a continuous increase. Since the ISLP circuit is closed with regard to non-volatile metabolites the rise of lactate must be the result of lactate production from pulmonary glucose degradation and of lactate release during lung injury [19]. Hypoxia is not necessarily the cause of pulmonary lactate production, since also well oxygenated lungs are known to be net lactate producers [20]. Moreover, hypoxemia due to pulmonary insufficiency in clinical studies did not correlate with a rise in lactate level or the lactate-pyruvate ratio (L/P ratio) [21,22]. Moreover, in the acute respiratory distress syndrome and sepsis, with lungs under aerobic conditions, the lungs were a primary contributor of lactate to the circulation [23,24]. Finally, a study on acute lung injury demonstrates the release of lactate by the lung by measuring the gradient over the lung [25].

A recent report evaluating declined lungs from DBD donors’ demonstrated two distinct patterns in L/P ratio. In some lungs the L/P ratio increased and then decreased suggesting the anaerobic use of glucose at the start of the EVLP followed by a more physiological lactate production. In the other group the L/P ratio remained high reflecting sustained anaerobic metabolism in more injured lungs [26]. This resulted in a higher peak airway pressure in the latter group. In the high L/P group EVLP was finished after 8 hours in 1 lung due to significant pulmonary edema. Our results support their theory.

The Madrid group recently updated their experience with 29 lung transplants from uncontrolled DCD donors and reported a high incidence of primary graft
dysfunction (PGD) [4,5]. Our results confirm that the DCD category I – II lungs are already injured at the time of harvesting. The notion that PCD led to higher mortality resulted in a more thorough evaluation of high-risk donors with EVLP after the initial evaluation in the donor. Based on our findings we believe evaluation and reconditioning by machine lung perfusion may be a very useful procedure. This might be either ISLP or EVLP. A major advantage of ISLP is that it provides a go / no-go at an earlier stage. With the ISLP there is the possibility to assess the lungs at the time of abdominal organ recovery. A disadvantage is that the lung perfusion system should be transported together with the donor team. When necessary re-assessment with EVLP can be performed in the recipient hospital.

In 2010 there were 15 DCD donors category I – II in the Netherlands resulting in 8 kidney donation procedures. There was no assessment or donation of lungs. In our own hospital in 2010 8 out of 61 patients could be eligible as DCD II lung donors [personal communication]. On a nation-wide basis such an extension would considerably enlarge the donor pool.

This study has several limitations. First, the use of a non-protective ventilation mode might have caused a modest lung injury in [WI-2]. Lung protective ventilation attenuates alveolar stretch and related injury during mechanical ventilation [27]. Second, the results of a study with a small sample size and with a limited evaluation period need to be confirmed in a model with a longer reperfusion time or a transplant model. Due to the short reperfusion period there were no histological differences. Finally, in the present study all animals developed sudden cardiac arrest from myocardial fibrillation. This is different from the clinical situation where exsanguination and myocardial infarction are the most common causes of death in the uncontrolled DCD donors. These patients do not always develop sudden cardiac arrest and the agonal phase and subsequently inflammatory lung injury may be more important.

In conclusion, we demonstrated that ISLP is an effective procedure to make a quality assessment of lungs from DCD category I – II in the donor. In addition, the reported data suggests that compliance, PCO₂ and lactate production may be predictors for lung injury.

**REFERENCES**

In situ lung perfusion is a valuable tool to assess lungs from donation after circulatory death donors.


CHAPTER 6

The use of non-heart-beating lung donors category III can increase the donor pool.

Caroline Van De Wauwer
Erik A.M. Verschuuren
Wim van der Bij
George D. Nossent
Michiel E. Erasmus
ABSTRACT

Objectives
The use of non-heart-beating (NHB) lung donors has been propagated as an alternative besides heart-beating (HB) lung donors to overcome organ shortage. We evaluated the effectiveness of NHB lung transplantation.

Methods
The donor and recipient data of all 35 NHB category III lung transplantations (LTx) between January 2005 and December 2009 were reviewed. For comparison, we collected recipient and donor data of a cohort of 77 HB lung transplantations. In both groups we assessed survival, primary graft dysfunction (PGD), FEV\textsubscript{1}, acute rejection and bronchiolitis obliterans syndrome (BOS).

Results
Thirty-five NHB lung transplantations were performed, five single LTx and 30 bilateral LTx in 12 male and 23 female patients. The donor oxygenation capacity was 61 kPa (IQR, 56 – 64). Warm ischemia time in the donor was 29 minutes (IQR, 24 – 30). Cold ischemic time of the last implanted lung was 458 minutes (IQR, 392 – 522). Cardiopulmonary bypass was used 13 times. PGD (1 -3) was observed in 45% of the patients at T0, in 42% at T24, in 53% at T48 and in 50% at T72. PGD 3 decreased from 24% at T0 to 6% at T72. The use of NO within 24 hours after transplantation was necessary in 3 patients with successful weaning in all. There was no significant difference for donor and recipient characteristics between NHB and HB lung transplantations. Survival, occurrence of PGD and acute rejection was equal to the HB cohort. The incidence of BOS was lower in the NHB group. The measured FEV\textsubscript{1} tended to be better in the NHB group.

Conclusion
Lungs from nonheparinized category III NHB donors are well suited for transplantation and can safely increase the donor pool.

INTRODUCTION

The first lung transplantation was performed by James Hardy in 1963 with a graft from a non-heart-beating donor [1]. Since the criteria for brain death were introduced and accepted in 1968, transplantation with lungs from heart-beating (HB) donors became the mainstay therapy for patients with end-stage lung disease refractory to medical therapy. Until today, donor organ shortage is the main limiting factor for this treatment. It is estimated that only 15 – 30% of all HB donors have lungs that are suitable for transplantation [2, 3]. This resulted in the reintroduction of the concept of lung transplantation from non-heart-beating (NHB) donors by Egan in 1991 [4]. In 1995, Love and co-workers performed the first clinical successful lung transplantation with lungs from a NHB donor [5]. The Maastricht classification drawn up in 1995 classifies the NHB donors as uncontrolled or controlled donors [6]. In category I (dead on arrival) and category II (failed resuscitation), cardiac death occurs unexpectedly and the situation for organ recovery is therefore “uncontrolled”.

In category III (withdrawal of life support, awaiting cardiac arrest) and category IV (cardiac arrest in brain-dead donor), circulatory arrest is anticipated and organs can be recovered under “controlled” circumstances. Steen et al. performed the first transplant from an uncontrolled donor in 2000 after \textit{ex vivo} evaluation of the lungs [7]. Subsequently de Antonio et al. reported lung transplantation using Maastricht category I and II donors [8]. The experience with the use of controlled donors, mainly Maastricht category III, is growing. This has been reported in recent articles [9-18]. The purpose of this study is to investigate whether the use of lungs from controlled NHB donors’ category III is a true alternative besides HB lung donors by comparing primary graft dysfunction, development of bronchiolitis obliterans syndrome, lung function and survival.

PATIENTS AND METHODS

Study Group
Between January 2005 and December 2009, a total of 145 lung transplantations were performed in our centre. Among these patients, 35 adults received lungs from category III non-heart-beating donors. For this retrospective study, the data of the 35 adult NHB recipients were assessed and compared with an existing cohort of 77 adult HB recipients that were transplanted during the same time period.
Donor Protocol

HB donors and NHB donors were selected according to the standard ISHLT criteria. When donation is considered, the Dutch National Donor Registry is checked to see whether the patient is registered positive as a donor. Blood group, gender, length, age, medical history, blood gas measurement and a recent chest x-ray are necessary for a first assessment. If judged suitable, a blood gas at 100% oxygen after at least 10 minutes of ventilation with 5 cmH2O positive end-expiratory pressure (PEEP) and a bronchoscopy are performed for final assessment of the suitability of the lungs for transplantation. After acceptance of a NHB donor, a time of withdrawal is agreed so all transplant teams can be present in the donor hospital in time. The technical procedure of withdrawal is up to the treating physicians and local protocols. No heparin is given before withdrawal of treatment. After circulatory arrest, a 5-minute no-touch period was respected. If necessary, the patient was reintubated and a bronchoscopy was performed. Lungs are preserved in our standard way with in situ anterograde flush and a retrograde flush on the back table both with Perfadex. The lungs are gently inflated and stored in Perfadex cooled by surrounding ice. The acceptable time between withdrawal of treatment and occurrence of circulatory arrest was 1 hour. The accepted warm ischemia time was 1 hour and was defined as the time between circulatory arrest and start of the anterograde flush. Cold ischemic time was defined as the interval between start of the anterograde flush in the donor and the reperfusion of the last implanted lung [14].

Recipient selection

Recipient selection and donor/recipient matching were performed using international guidelines. All recipients on the waiting list were candidates for both HB lungs and NHB lungs. Lung allocation was based on the waiting time on the waiting list or urgency status according to Eurotransplant allocation rules.

Primary Graft Dysfunction

Primary graft dysfunction (PGD) was graded according to the recommendations of the International Society for Heart and Lung Transplantation (ISHLT) considering the PaO2/FiO2 ratio and the findings on chest x-ray. The incidence was compared at different time points (T0 – within 6 hours of reperfusion, T24, T48 en T72). PGD was assessed and compared in all lung transplantations and separately in bilateral lung transplantations [19].

Acute Rejection and Bronchiolitis Obliterans Syndrome (BOS)

Bronchoscopy was performed at discharge from the hospital and thereafter when clinically indicated. Acute rejection was based on histological findings and was graded according to the recommendations of the ISHLT Lung Rejection Study Group [20].

All recipients received induction therapy with basiliximab and triple maintenance immunosuppression with corticosteroids, tacrolimus and azathioprine or mycophenolate.

BOS was a defined as a decrease in forced expiratory volume in 1 second (FEV1) compared to the baseline value and was classified following the recommendations of the ISHLT in BOS grade 0 – 3 [21].

Lung function

Lung function assessment was performed at every hospital visit. For this study, we report the FEV1 at 3 months, 6 months, 1 year and 2 years after transplantation. The FEV1 is expressed as a percentage of the predicted FEV1 of the recipient.

Variables

Data of 112 patients (35 NHB and 77 HB) were reviewed for donor variables and recipient variables. Age, gender, PaO2 after minimal 10 minutes of 100% oxygen and a PEEP of 5 cmH2O, days of mechanical ventilation, time until circulatory arrest, cause of brain damage, warm ischemic time and ischemic time of the last implanted lung were recorded as donor and preservation variables. Recipient variables collected were age, gender, LTx type, use of cardiopulmonary bypass, diagnosis, hospital stay, ICU stay and days of postoperative ventilation.

STATISTICAL ANALYSIS

Data analysis was performed using GraphPad Prism 5 (San Diego, CA, USA). All data are expressed as median (interquartile ranges) unless otherwise defined. Mann-Whitney test, the Fisher’s exact test and the chi-square test were used to test for significances between both groups. For PGD and BOS, a chi-square test was used to test for a significant difference between both groups at each time point. The Kaplan – Meier method was used to assess the patient survival. A p-value < 0.05 was considered significant.
RESULTS

Donors and Preservation

Between January 2005 and December 2009, a total of 61 lungs from NHB category III donors were offered to our institution. In 6 cases the lungs were considered but not accepted because of medical reasons. Logistic reasons, including no transplant capacity (n = 4), no suitable recipient (n = 2) and miscellaneous (n = 2) were other reasons to reject the lungs. Forty-seven NHB donor procedures were started resulting in 35 adult NHB lung donations and transplantations and 1 pediatric lung transplantation. Reasons for not using lungs after the donor procedure was started were absence of cardiac arrest within 1 hour (n = 7), emphysematous lung at inspection (n = 2), infection (n = 1) and lung edema after flush (n = 1).

Donor characteristics are listed in Table 6.1. In the NHB group, circulatory arrest occurred within 17 minutes (IQR, 10 – 39 minutes). The median warm ischemic time was 29 minutes (IQR, 24 - 30 minutes). The cold ischemic time for the last implanted lung was 458 minutes (IQR, 392 – 522) in NHB compared to 401 (IQR, 357 – 488) in HB (p = 0.18). There was no statistical significant difference between NHB an HB.

Recipient characteristics

Recipient characteristics are shown in Table 6.2. Thirty-five NHB lung transplantations were performed. Five single LTx and 30 bilateral LTx in 12 male and 23 female patients. Three patients underwent a re-transplantation for progressive BOS. Time to extubation, stay in the intensive care unit and hospital stay was comparable in both groups.

Table 6.1: Donor and preservation characteristics

<table>
<thead>
<tr>
<th></th>
<th>NHB (n = 35)</th>
<th>HB (n = 77)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47 (35 – 55)</td>
<td>46 (31 – 53)</td>
<td>NS</td>
</tr>
<tr>
<td>Female (%)</td>
<td>68.6</td>
<td>51.9</td>
<td>NS</td>
</tr>
<tr>
<td>pO2 at 100% (kPa)</td>
<td>61 (56 – 64)</td>
<td>61 (53 – 67)</td>
<td>NS</td>
</tr>
<tr>
<td>Time on ventilator (d)</td>
<td>2 (1 – 6)</td>
<td>1.5 (1 – 2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Brain damage (%)</td>
<td>IC 37.1</td>
<td>29.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAB 25.7</td>
<td>36.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brain anoxia</td>
<td>20</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Head trauma</td>
<td>11.4</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Miscellaneous</td>
<td>5.7</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>Circulatory arrest (min)</td>
<td>17 (10 – 39)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Warm ischemia (min)</td>
<td>29 (24 – 30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold ischemia (min)</td>
<td>458 (392 – 522)</td>
<td>401 (357 – 488)</td>
</tr>
</tbody>
</table>

ICB, intracranial bleeding; SAB, subarachnoidal bleeding; NHB, non-heart-beating; HB, heart-beating

Data are expressed as median (interquartile ranges) unless otherwise defined.

Primary Graft Dysfunction

PGD (grade 1-3) in the NHB group was observed in 45% of the patients at T0, in 42% at T24, in 53% at T48 and in 50% at T72. PGD grade 3 decreased from 24 % at T0 to 6% at T72. There was no significant difference in PGD between both groups at different time points, although the decrease in PGD 3 was less in the HB group compared to the NHB group (from 25% to 11% versus from 24% to 6%) (Figure 6.1A). When assessing and comparing only the bilateral lung transplantations, NHB lungs had less PGD at T0 and T24 (Figure 6.1B).

The use of inhaled nitric oxide (NO) within 24 hours after lung transplantation was necessary in 3 patients in the NHB group. There were no patients that required post-operative ECMO support. The use of NO was necessary in 3 patients in the HB group. One was successfully weaned, one died of PGD and one was successfully weaned after ECMO.

Acute Rejection and Bronchiolitis obliterans syndrome

In the NHB group, 2 patients (5.7%) developed acute rejection (A2) at 1 month and at 23 months after transplantation. A1 rejection was detected in 2 patients (2.6%) at 3 months and at 6 months in the HB group.

The incidence of BOS was lower in the NHB group compared to the HB group (Figure 6.2). At 1 year after transplantation, there was no BOS in the NHB group compared to the HB group (100% BOS 0 versus 85% BOS 0, p = 0.037).
The use of non-heart-beating lung donors category III can increase the donor pool.

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Figure 6.1: A: Primary graft dysfunction (PGD) after lung transplantation in non-heart-beating donors (NHB) and heart-beating donors (HB) at T0, T24, T48 and T72. B: PGD in NHB and HB after bilateral lung transplantation at different time points. Percentage of the recipient in each PGD grade at different time points.

Lung Function

Although there was no significant difference, lung function after transplantation assessed as a percentage of the predicted FEV₁ tended to be better in the NHB group compared to the HB group at 3 months, 6 months, 1 year and 2 years (Table 6.3).

Table 6.3: Transplant function as percentage of predicted forced expiratory volume in 1 second (FEV₁).

<table>
<thead>
<tr>
<th></th>
<th>3 months</th>
<th>6 months</th>
<th>1 year</th>
<th>2 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>32</td>
<td>32</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>% FEV₁</td>
<td>75 (54 – 90)</td>
<td>82 (57 -97)</td>
<td>87 (63 – 101)</td>
<td>85 (62 -112)</td>
</tr>
<tr>
<td>HB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>68</td>
<td>67</td>
<td>67</td>
<td>60</td>
</tr>
<tr>
<td>% FEV₁</td>
<td>69 (54 – 85)</td>
<td>74 (58 –92)</td>
<td>78 (56 –96)</td>
<td>79 (57 –97)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.40</td>
<td>0.50</td>
<td>0.19</td>
<td>0.31</td>
</tr>
</tbody>
</table>

NHB, non-heart-beating; HB, heart-beating. Data are expressed as median (interquartile ranges).

Survival

Recipient survival is shown in Figure 6.3. There was no significant difference between the NHB group and the HB group (p = 0.53). Five patients in the NHB group died during follow-up. Graft failure caused by BOS was the reason of death in 2 patients. In the HB group, 22 patients died during follow-up. Two patients died of PGD shortly after transplantation and 2 patients died due to graft failure.

Figure 6.2: The incidence of bronchiolitis obliterans (BOS) in the non-heart-beating (NHB) and the heart-beating donor (HB) at 6 months, 1 year and 2 years after transplantation. Percentage of the recipient in each BOS grade at different time points.

Figure 6.3: Patient survival. NHB: non-heart-beating donor; HB: heart-beating donor.
DISCUSSION

To our knowledge, this is the largest single-center study reporting the use of lungs from NHB donors’ category III. The presented study shows that lungs from category III NHB donors perform well compared to lungs from HB donors. Importantly there was no difference in survival at 1 year. Furthermore, postoperative ventilation, discharge from ICU and discharge from the hospital were comparable in both groups. Finally, in the NHB group there seemed to be less PGD 3 (3 – 24%), the FEV₁ was higher during follow-up and the incidence of BOS was lower compared to the HB group, although this was not significant.

The NHB donor program started in 2004 after the initiation of a renewed national protocol for NHB multiorgan donation by the Dutch Transplant Foundation (NTS). Since then, the number of NHB donors has increased from 4 in 2005 up to 12 in 2009. In 2008 and 2009, 40% and 37.5% respectively of our transplants were performed with NHB donors.

Our results with category III NHB donor lungs are comparable with the results of other NHB programs [9-13, 15-17]. In contrast, the Madrid group [8] with an uncontrolled NHB donor program reported a PGD 3 in 29% of their patients. This higher percentage PGD might be explained by the longer warm ischemic time (118 minutes) and the acute and less-controlled nature of the uncontrolled NHB donor procedure. Finally their lung function evaluation is less precise since there are no standard lung function data available in category I and category II NHB donors. Although their reported 1-year and 3-year survival after NHB donation is lower than in our study this lower survival was not different to their HB results.

The recent annual report of the ISHLT shows that acute rejection is detected in 36% of the patients in the first year after lung transplantation [22]. In our study, 2.8% in the NHB group developed A2 rejection and A1 rejection was detected in 2.6% of the patients in the HB group. This might be explained by tacrolimus-based immunosuppression regimen and by the use of induction therapy with an IL-2R antagonist. However, we only report the histological confirmed acute rejection. Our lower incidence is confirmed by the experience of other transplant groups [10,12,13,16,17]. BOS is present in more than 20% of the patients 2 years after transplantation and is one of the most common causes of death 1 year after transplantation [22]. Our study demonstrates 0% of BOS in the NHB group and 15% of BOS in the HB group 1 year after transplantation. After 2 years, the incidence between both groups is comparable. We hypothesize that a decrease of inflammatory lung injury before retrieval in the NHB donor as shown in animal experiments may be responsible for the lower BOS incidence at 6 months and 1 year after transplantation.

There are differences between category III NHB donation programs. Pretreatment (i.e., heparin, phentolamine) was given before death [10,12,13] or after the 5-minute interval [17] in other protocols. We only optimized the donor treatment before switch-off but added no treatment [14].

It is difficult to compare our warm ischemia with others. In our protocol warm ischemic time (WIT) was defined as the time between circulatory arrest and start of the antegrade flush, which is comparable with the report of De Vleeschauwer et al [13]. But it is different from the data reported by Snell et al where WIT was defined as the time between the absence of cardiac output and the start of cold flush preservation [18]. In other reports WIT is defined as part of the interval or the interval between withdrawal of life support and establishing perfusion of the donor lung with cold preservation solution [10,12] and in some studies WIT is not reported [15,17]. The most common factor in all reported series is the use of an antegrade flush followed by a retrograde flush through each of the pulmonary veins to remove any pulmonary microthrombi. We believe that the applied retrograde flush is essential for our good results in the Dutch situation where no heparin is used before withdrawal of treatment.

Although the number of NHB donors used is growing, there is still a potential pool of controlled NHB donors (category III) [23] and uncontrolled NHB donors (category I and II) that is not used. Evaluation of lungs in the uncontrolled donor remains challenging in the absence arterial blood gasses and previous medical history. The Madrid group initially evaluated the lungs using a pulmonary artery flush technique. At the time of organ procurement 300 ml of donor blood was taken. After an initial flush with Perfadex, the blood was flushed through the pulmonary artery. Subsequently, arterial blood gas analysis, corrected for temperature, was performed on the effluent from the left atrium.

Recently, lungs were assessed using an ex vivo lung perfusion (EVLP) system before implantation [24]. After assessing 3 lung blocks, two with a ΔPO₂ > 400 mmHg were
The use of non-heart-beating lung donors category III can increase the donor pool deemed acceptable for transplantation.

The first successful lung transplantation after ex vivo lung perfusion was performed by Steen et al in 2001 [7]. After 65 minutes of warm ischemia, 3 hours of topical, an ex vivo functional assessment at 37°C and further 8 hours of cold storage successful right lung transplantation was performed. Since then, EVLP is investigated extensively as a method to assess donor lungs but also as a tool to preserve and resuscitate donor lungs for a longer period of time [3, 25]. Currently, all the lungs with a donor arrest time longer than 30 minutes are assessed with EVLP in Toronto [10]. Other groups also have successful transplanted lungs from controlled NHB donors after EVLP. The use of EVLP opens the perspective for re-assessment of rejected NHB donor lungs, for assessment NHB donor lungs after a prolonged period of warm ischemia or after a period of cardiopulmonary instability during the agonal phase and for assessment of lungs from uncontrolled NHB donors, leading to the expansion of the NHB donor pool.

The present study suffers from some limitations. First, the study was retrospective and the experience increased with the amount of retrievals and transplantations performed. Secondly, the patients were not randomly assigned to a specific type of donor. Thirdly, there were more patients with bronchiolitis obliterans in the NHB group compared to the HB group. Therefore the findings need to be confirmed with a prospective study.

In conclusion, this study demonstrates comparable outcome between NHB donors and HB donors, thereby confirming that lungs from NHB donors may be a safe alternative to increase the donor pool.

REFERENCES

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