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ORIGINAL ARTICLE

Flush at room temperature followed by storage on ice creates the best lung graft preservation in rats

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Summary

Current clinical lung preservation techniques have not eliminated ischaemia-reperfusion (I/R) injury, despite many improvements. The optimal combination of flush and storage temperatures remain unclear in lung preservation. This is the first study to investigate a range of temperatures with 24-h inflated storage using consistent state-of-the-art preservation techniques. A rat lung transplant model was used to investigate the optimal combination of flush and storage temperatures. In six groups, rat lungs were flushed at 4 °C, 10 °C or room temperature ($F_4/F_{10}/F_{Rt}$) with Perfadex and stored inflated for 24 h in Perfadex on melting ice or at 10 °C (S_{ice}/S_{10}). Left donor lungs were transplanted for analysis. During 2-h reperfusion, the lung graft function was measured (blood gases, maximum ventilation pressure and static compliance) and lung graft injury was also assessed (W/D ratio, total lung protein, Tryptase, Myeloperoxidase). Right donor lungs were assessed for W/D ratio only after flush and storage. For baseline measurements, left lungs without intervention were used. The combination of F_{Rt}–S_{ice} showed a significantly higher pO_2, lower P_{max}, low W/D ratios and total protein levels of left lungs after reperfusion when compared with F_4–S_{ice} and baseline. Storage at 10 °C did not improve preservation. We conclude that F_{Rt}–S_{ice} creates the best lung graft preservation.

Introduction

Lung transplantation is considered to be the most effective therapy in end-stage pulmonary disease, but its application is limited by severe donor organ shortage. However, if donor lungs meet the selection criteria for transplantation, these lungs are still at risk for development of ischaemia/reperfusion (I/R) injury. I/R injury is a major cause for early graft dysfunction after transplantation [1]. One strategy to reduce I/R injury, and injury extent, is improvement of the preservation method [2].

Experimental lung preservation studies, in both animals and humans, have shown that several preservation procedures are beneficial in the preservation and outcome of lungs, and reduce I/R and subsequent graft injury after transplantation. These procedures involve widespread use of a Perfadex preservation solution [1,3,4], combined antegrade and/or retrograde flush perfusion [5,6], inflated lung storage [1], and slow reperfusion after implantation [7].

The above procedures gradually became clinical standard practice and have led to the current lung preservation techniques [2]. Nonetheless, I/R and graft injury still remain.

To further optimize donor lung graft preservation, we think that both flush and storage temperatures need to be re-examined. If optimized, these would certainly lead to improved graft function and less injury. Many experimental studies have investigated preservation temperatures and their influence on graft quality with or without reperfusion.

Currently, the most common clinical preservation temperatures are 4 °C flush (F_4) with storage on melting ice (ice). Some studies advocate higher flush temperatures from 4 to 25 °C [8–13], while others suggest 10 °C lung storage [8,14–18]. However, as most studies use different preservation procedures, it is extremely difficult to form a consensus concerning the best preservation temperatures.

Therefore, we investigated the three most common experimental lung preservation temperatures using a rat lung transplant I/R model. We applied state-of-the-art improvements in the field of lung preservation with a prolonged 24 h storage, namely, a Perfadex flush solution [19,20], nitroglycerin supplementation [21], antegrade and retrograde flushing [5,6,22–24], inflated lung storage, slow alveolar recruitment [25], and slow reperfusion [7,26]. The primary aim of this study was to investigate whether or not a flush temperature of 10 °C or room temperature (Rt) combined with storage on melting ice could improve lung preservation compared with the current clinical standard, a 4 °C flush and storage on melting ice (F_4–S_{ice}). Second, we investigated the effect of increasing storage temperatures from ice to 10 °C in combination with the above flush temperatures (4 °C, 10 °C and Rt). After preservation, the lungs were transplanted and tested for function and injury during a 2-h reperfusion period.

Table 1. Experimental groups plus baseline.

Group	Antegrade and retrograde flush (°C)	Storage (°C)
F_4–S_{ice}	4	Ice
F_{10}–S_{ice}	10	Ice
F_{Rt}–S_{ice}	Rt	Ice
F_4–S_{10}	4	10
F_{10}–S_{10}	10	10
F_{Rt}–S_{10}	Rt	10
Baseline	No	No

F, flush; S, storage; Rt, room temperature; ice, melting ice.

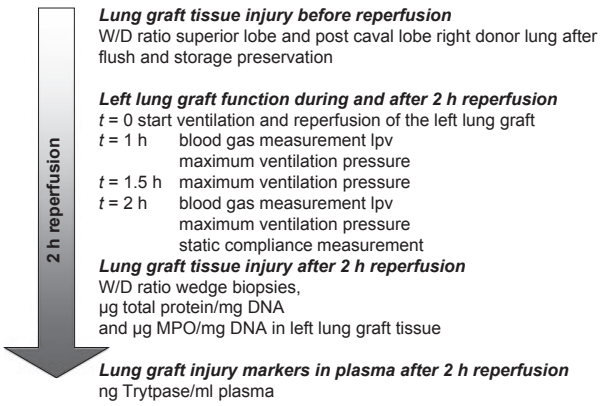

Figure 1 Assessment of the left lung graft in time.

Materials and methods

Experimental groups and baseline

The effect of preservation, specifically flush and storage temperatures, on lung function and lung graft injury were assessed after orthotopic unilateral left lung transplantations and 2-h reperfusion. Six experimental groups (n = 72) and a baseline group (n = 6), as defined in Table 1, were used. For each experimental group, six donor and six recipient rats were used. All donor lungs were antegrade plus retrograde flushed with an enriched Perfadex solution of 4 °C, 10 °C or Rt using equal volumes per kg bodyweight (BW). Donor lungs were inflated and submersed in this solution and stored for a period of 24 h on ice or at 10 °C. All preserved right donor lungs were assessed for preservation injury (n = 36). All preserved left donor lungs were transplanted and reperfused for 2 h and then assessed for function and injury (n = 36); see Fig. 1 for experimental timeline. Immediately harvested noninjured baseline donor lungs without preservation or reperfusion were used as baseline measurements for compliance (left lung) and injury both in tissue (right lung) and plasma (n = 6).

Animals and animal care

Inbred Lewis rats (250–350 g) were obtained from Harlan (Horst, the Netherlands). Animal experiments were performed after receiving the approval of the Institutional Animal Care and Use Committee of the University of Groningen. All animals received humane care in compliance with the Dutch law on experimental animal care.

Donor procedure; flush and storage

All donor rats were anaesthetized by intramuscular administrations of Ketamine (Alfasan International BV, Woerden, the Netherlands) and Dexdomitor (Orion Pharma, Espoo, Finland), and subcutaneous injections of Atropine Sulfate (Centrafarm, Etten-Leur, the Netherlands) were given to control salivation. After endotracheal intubation with a 14-G angiocatheter (Becton Dickinson BV, Breda, the Netherlands), the donor was ventilated with 50% oxygen at 45 breaths/min. A positive end expiratory pressure (PEEP) of 2 cmH_2O and a maximum ventilation pressure (P_{max}) of 16 cmH_2O were set. After preparing the heart lung block for flushing, as described in detail by Prop *et al.* [27], a final lung recruitment manoeuvre was done to eliminate the atelectatic lung regions.

All ventilated lungs were flushed antegrade and retrograde with a perfusion solution composed of Perfadex® (Vitrolife, Göteborg, Sweden), buffered with 0.3 mmol/l THAM and 0.27 mmol/l $CaCl_2$ (Department of Hospital

and Clinical Pharmacy UMCG), and 100 ml/l Nitropohl (G. Pohl-Boskamp GmbH and Co., Hohenlockstedt, Germany). Antegrade flushing of 50 ml/kg BW was carried out at a constant pressure of 20 cmH_2O via a 16-G RVS-needle in the pulmonary artery. The subsequent retrograde flush of 50 ml/kg BW was performed at a pressure of 15 cmH_2O via a 14-G cannula in the left atrium. The flush flow index, which is an indicator of flush temperature-induced vasoconstriction, was calculated for all antegrade and retrograde flush temperatures. The amount of flushed perfusion solution per kg BW was calculated per minute (ml/kg BW/min). Flush temperature was measured and carefully maintained in the F_4 and F_{10} groups. The mean flush temperature for Rt groups was 22.5 ± 0.7 °C.

The heart lung block was excised and submersed in Perfadex® for inflated storage with 50% oxygen and a PEEP of 5 cmH_2O. The lungs were stored for 24 h on ice (0.4 °C) or in a digitally controlled thermo box (10 ± 0.1 °C). After flush and storage, the superior lobe and the postcaval lobe of right donor lungs were used for the W/D ratio measurement; see Fig. 1. The left donor lungs were prepared for transplantation and 2-h reperfusion.

Recipient procedure and orthotopic left lung transplantation

Each recipient was initially anaesthetized in a box flushed with 4% isoflurane (Pharmachemie BV; Teva Group, Haarlem, the Netherlands), and then 0.25 mg atropine was administered subcutaneously. Endotracheal intubation was carried out and ventilation was set at 100% oxygen and 2% isoflurane for anaesthesia maintenance. To prevent dehydration, an infusion with saline solution was given via the tail vein and sustained at a flow of 3 ml/h. Via a left thoracotomy with excision of the fourth and fifth rib, the left recipient lung was removed [28]. The left donor lung was transplanted into the recipient using the method of de Perrot [29]. This method enables separate left and right lung ventilation using separate tubes. After anastomosing the vessels, a thin silicon cannula was positioned in the left pulmonary vein (LPV) for blood sampling. The left implanted donor lung was ventilated with 100% oxygen, a tidal volume of 6 ml/kg BW, a PEEP of 4 cmH_2O and a frequency of 45 breaths/min. Before reperfusion, a vessel clamp was partially positioned on the left pulmonary artery and maintained for 2 min. This enabled a slow reperfusion after unclamping the pulmonary artery and vein; total reperfusion time was 2 h. Blood gases, the acid base and the electrolyte balance were checked in blood samples drawn from the left ventricle (LV) prior to and after 2 h of reperfusion. All experiments were terminated after 2-h reperfusion by drawing ample blood from the LV. This blood was collected in 1 mL tubes containing ethylenediamine tetraacetic acid, centrifuged at 3000 rpm for 3 min and the obtained plasma was stored at −80 °C awaiting further analysis.

Assessment of left lung graft function after reperfusion

Blood gas measurement

During reperfusion, blood gas measurements, namely, specific oxygenation capacity of left lung grafts were measured by drawing blood samples from the LPV at 1 and 2 h intervals; see Fig. 1. Blood gas measurements, pH, partial oxygen tension (pO_2), partial carbon dioxide tension (pCO_2) and oxygen saturation (sO_2), were performed using the i-STAT® System (Princeton, NJ, USA).

Maximum ventilation pressure

During reperfusion, 5 min before the 1-, 1.5- and 2-h reperfusion intervals, P_{max} was assessed. These intervals are further referred to in this paper as 1-, 1.5- and 2-h reperfusion intervals.

Static compliance measurement

After termination, an in situ static pressure–volume measurement was performed at increasing pressures from 5 to 35 cmH_2O with a 1-min stabilization interval [30]. At a pressure of 35 cmH_2O, compliance was defined as the maximum volume of air per kg BW (ml/kg BW).

Assessment of lung graft tissue injury

W/D ratio measurement before and after 2-h reperfusion

W/D ratio, an indicator of pulmonary oedema, was assessed twice, first after preservation and then after transplantation following the static compliance measurement (Fig. 1). Preservation injury by W/D ratio was only assessed in the superior and the postcaval lobe of the right donor lung. I/R-induced tissue injury after 2-h reperfusion was investigated in the superior and inferior wedge biopsies of the left lung grafts. The wedge biopsies of the lobes of the right donor lungs and the left lung grafts were placed in a block heater at 70 °C to dry for 3 days. The W/D ratio was calculated by subtracting the dry lung weight from the wet lung weight and this was divided by the dry lung weight.

Total protein/DNA ratio after reperfusion

The ratio of total protein per amount tissue DNA is an indicator for vascular permeability. This ratio was determined in left lung graft tissue after 2 h of reperfusion. First, the lung tissue had to be lysed, as described by the Myeloperoxidase (MPO) Elisa kit for rats (Hycult Biotech, Uden, the Netherlands). Second, total protein per mL lysate was determined using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). A sample of 100 µl lysate and 2 µg Hoechst 33 258 per 100 µl TNE buffer solution was

incubated for 15 min. To determine total DNA per ml lysate, all samples were measured using a fluorometer (excitation at 360 nm, emission at 460 nm). All materials were obtained from Sigma Chemical Co., St Louis, MO, USA. The samples were evaluated using a standard, ranging from 0.1 to 1.0 μg/μl DNA (Fluka BioChemica 31149) in 0.1% Triton-X100. To exclude the confounding effect of oedema formation in lung tissue, total DNA instead of lung tissue weight was used to determine ug total protein/mg DNA ratio.

MPO/DNA ratio after reperfusion

Myeloperoxidase levels reflect the inflammatory state and oxidative stress of tissue [31]. The enzymatic activity of MPO was measured in lysate of left lung tissue after 2-h reperfusion using an Elisa kit for rats (Hycult Biotech, Uden, the Netherlands) where the MPO concentration was assessed. The previously measured total DNA was used to determine μg MPO/mg DNA ratio.

Assessment of lung graft injury markers in plasma

Local lung inflammation activates mast cells [32] which subsequently release tryptase [33]. The tryptase was measured after 2-h reperfusion in plasma by quantitative analysis using a standardized protocol developed by HaemoScan (Groningen, the Netherlands) (Fig. 1). The conversion of the substrate N-p-Tosyl-Gly-Pro-Arg-p-Nitroanilide was a measure for the tryptase activity in plasma. The final measurement, including a standard reference, was performed using spectrophotometry (405 nm).

Statistical analysis

All statistical analyses were performed using SPSS (v19.0, SPSS Inc., Chicago, IL, USA). This study analysed the effect of the combination of flush and storage temperatures on lung graft function and injury. To evaluate the overall main effect of flush and storage temperatures on the assessed lung function and injury parameters, analysis of variances (ANOVA) with and without repeated measures was performed, applying the general linear models (GLM) procedure. To analyse the main effect of flush and storage temperature on repeated measurements, flush and storage were used as fixed factors (between-subject factor group), time was designated as the within factor, and the assessed parameter at the chosen time intervals was assigned as the dependent variable. For all single measurements, flush and storage were used as two fixed factors (between-subject factor group) and the assessed parameter was assigned as the dependent variable.

A one-way ANOVA was performed to evaluate the differences among mean values between experimental groups and baseline. Therefore, the experimental group was the fixed factor and the investigated parameter was designated as the variable. When the ANOVA test showed statistical significance, the Fisher's least significant difference (LSD) post-hoc test was applied. Data are presented as mean ± standard deviation (SD). If the ANOVA criteria were not met, the data were transformed into the natural logarithm to achieve homogeneity of variances. Flush flow data could not be transformed and therefore a Kruskal–Wallis test followed by a Mann–Whitney test with Bonferroni correction was applied and presented as median (25–75 percentile). For all analyses, a *P*-value of less than 0.05 was considered statistically significant.

Results

Donor procedure; flush and storage

Antegrade and retrograde flush flow indices for all Rt flushed groups were significantly higher compared with the 4 °C flushed groups ($P < 0.016$). The median antegrade Rt flush flow index was 57.0(46.2–67.1) and the median retrograde Rt flush flow index was 90.1(73.7–103.8) ml/kg BW/min, while the median antegrade and retrograde 4 °C flush flow indices were significantly lower, being 38.4(37.6–39.4) and 39.6(31.1–58.1) ml/kg BW/min, respectively,. Mean storage time for all experimental groups was 23.5 ± 0.6 h and total warm ischaemia time, consisting of preparation of heart lung blocks and implantation of left lungs, was 1.23 ± 0.2 h.

Recipient procedure; stability and survival after reperfusion

All rats with the removal of a left recipient lung survived the single right lung ventilation, the subsequent implantation of the left donor lung, and the 2-h reperfusion period. Equal blood gases were measured in the left ventricle prior to reperfusion. Mean pH was 7.51 ± 0.04, the pO_2 was 74.1 ± 9.3 kPa, the pCO_2 was 4.6 ± 0.84 kPa and the sO_2 was 100%. These blood gases remained similar between all experimental groups after 2-h reperfusion, with the exception of pCO_2 (3.9 ± 0.8 kPa).

Lung graft function after reperfusion

Blood gas measurement

Increasing flush temperature from 4 °C to Rt resulted in a significant positive main effect on pO_2 ($P = 0.024$). The blood pO_2 values measured from the LPV at 1- and 2-h reperfusion intervals were significantly higher in both F_{10}–S_{ice} and F_{Rt}–S_{ice} compared with F_4–S_{ice} (Fig. 2). However, when increasing the storage temperature to 10 °C, a substantial decrease in pO_2 was seen especially in the F_4 lungs.

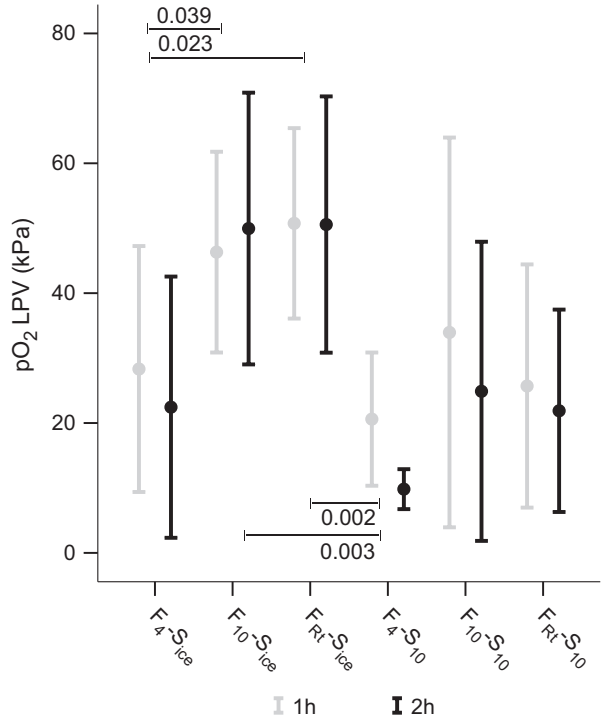

Figure 2 pO_2 measured in blood from the LPV of the left donor lung at 1- and 2-h reperfusion intervals. Within all groups, pO_2 did not change over time ($P = 0.058$). ANOVA, further refined by GLM repeated measures, revealed differences between groups ($P = 0.009$). Between groups, both flush and storage temperatures showed a significant main effect concerning pO_2 ($P = 0.033$ and $P = 0.005$, respectively) resulting in a higher pO_2 when applying F_{Rt} and S_{ice} temperatures. Both F_{10}–S_{ice} and F_{Rt}–S_{ice} showed a significantly higher pO_2 when compared with F_4–S_{ice}. Increasing storage temperatures did not further improve pO_2. Data presented in the figure are mean ± SD. F, flush; S, storage.

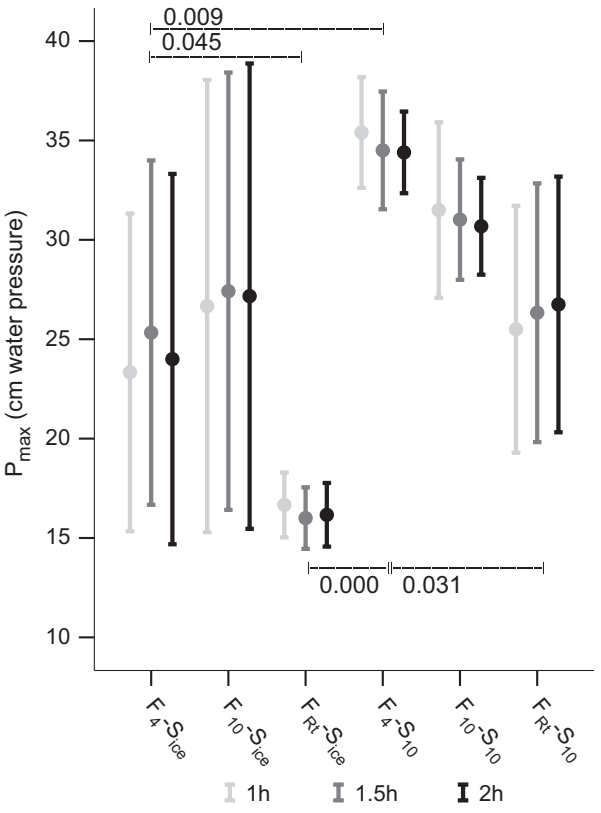

Figure 3 This figure shows the P_{max} at 1-, 1.5- and 2-h reperfusion intervals of left donor lungs. No significant changes within groups over time were observed ($P = 0.423$). ANOVA, refined by GLM repeated measures, showed significant differences between groups ($P = 0.001$). Only the F_{Rt}-S_{ice} group showed a significant low P_{max} during reperfusion. Increasing the storage temperature did not further improve P_{max}. Flush and storage temperatures showed a significant main effect for P_{max}, $P = 0.008$ and $P = 0.001$, respectively. The main effect for Rt flush resulted in a lower P_{max} ($P = 0.005$ vs. F_4). For storage, the main effect showed S_{ice} led to a reduction in P_{max} ($P = 0.001$). Data presented in this figure are mean ± SD. F, flush; S, storage.

In all groups, pH, pCO_2 and sO_2 did not significantly differ throughout 2-h reperfusion.

Maximum ventilation pressure

Only the F_{Rt}–S_{ice} group showed a significantly steady low P_{max} at all reperfusion intervals ($P = 0.045$). Despite the high pO_2, the transplanted lungs in the F_{10}–S_{ice} group were unable to achieve a steady low P_{max} (Fig. 3). Both of the above results are supported by statistical analysis which demonstrated that Rt flush alone has a main effect in lowering P_{max} compared with F_4 ($P = 0.004$) and F_{10} ($P = 0.007$). Raising storage temperatures to 10 °C did not show further improvement. On the contrary, F_4–S_{10} showed a significant deterioration in P_{max} when compared with F_4–S_{ice} ($P = 0.009$).

Static compliance measurement

Comparing the 4 °C (F_4–S_{ice}) flush temperature to Rt in the F_{Rt}–S_{ice} group did not significantly increase the static compliance. All groups were statistically lower in compliance than the baseline group ($P < 0.000$). In groups stored at 10 °C, the combination F_4–S_{10} resulted in a significantly lower compliance (Fig. 4) ($P = 0.027$).

Lung graft tissue injury before reperfusion

W/D ratio after preservation

Right donor lungs stored on ice showed comparable W/D ratios after flush and storage (Fig. 5a). Interestingly enough, increasing storage temperatures from ice to 10 °C demonstrated a significantly lower W/D ratio in right donor lungs ($P < 0.000$) for both F_4–S_{10} and F_{10}–S_{10}, compared with F_4–S_{ice} and baseline group. Statistical analysis showed that only storage had a significant main effect on W/D ratios ($P < 0.001$), with a reducing effect of S_{10} on W/D ratios.

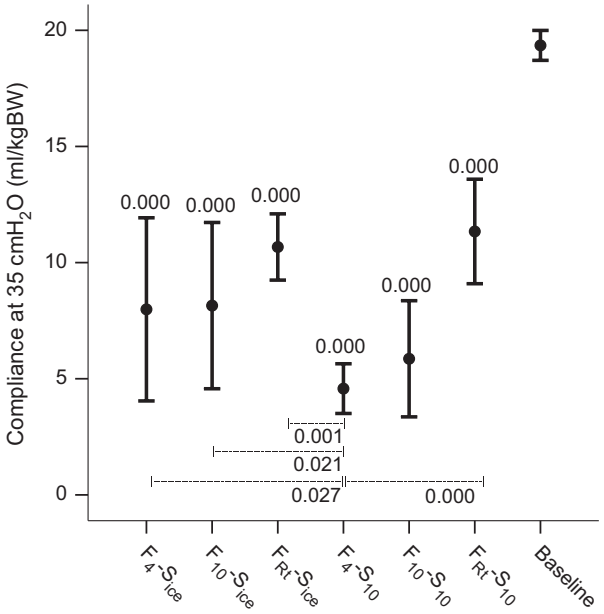

Figure 4 A static compliance measurement was performed after 2-h reperfusion on all left donor lungs. At a pressure of 35 cmH_2O, the maximum volume of air per kg of bodyweight (ml/kg BW) was calculated. One-way ANOVA showed significant differences between groups ($P < 0.001$). Compared to the baseline and the current clinical standard (F_4–S_{ice}), F_4–S_{10} showed the worst compliance. A significant main effect of flush temperature on compliance was found ($P = 0.002$), showing an improved compliance for F_{Rt} ($P = 0.001$ vs. F_4). Values are presented as mean ± SD. *P*-values above the error bars are compared to the baseline and those under the errors bars represent significant differences between the experimental groups. F, flush; S, storage.

Lung graft tissue injury after reperfusion

W/D ratio

All transplanted left lungs showed increased W/D ratios after 2-h reperfusion compared with flushed and stored right donor lungs (Fig. 5a, b).

Statistical analysis showed a main effect for flush, as well as, storage on W/D ratios ($P < 0.018$). This finding is supported by the observation that an increase in flush temperature (4 °C to Rt) in both storage groups reduced W/D ratios. Despite the effect of a higher flush temperature, the

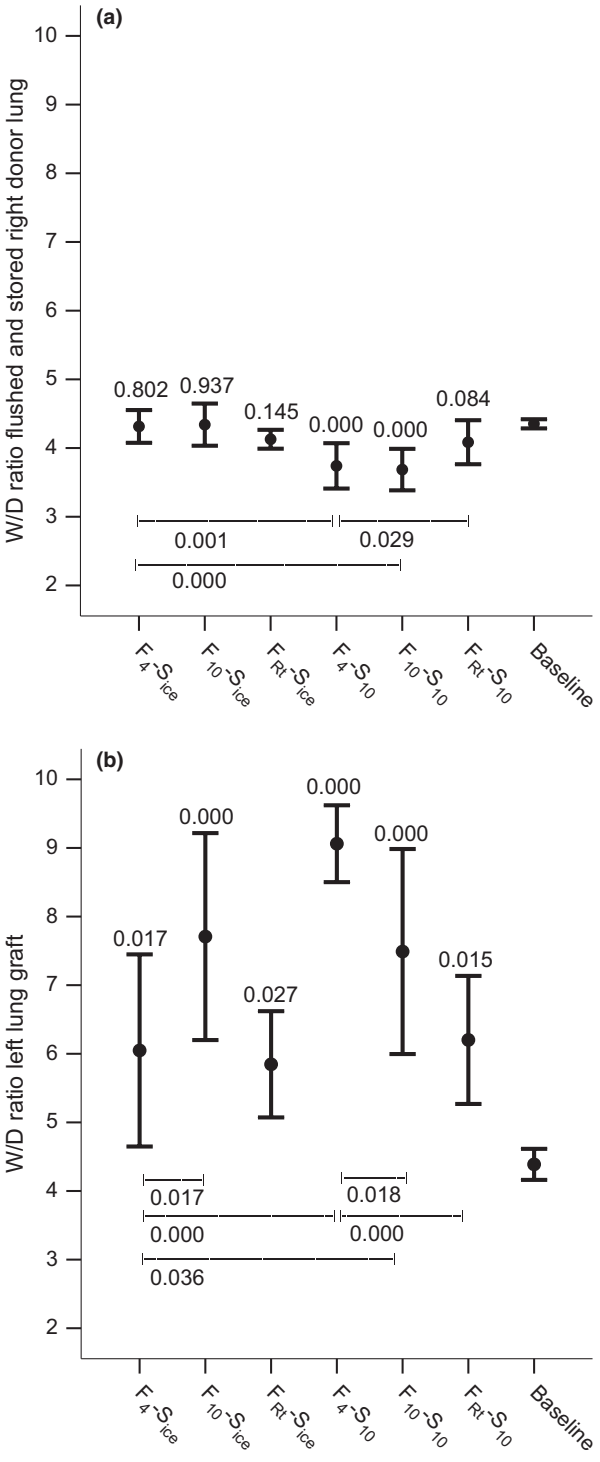

Figure 5 W/D ratio of preserved right donor lungs and left lung grafts after 2 h of reperfusion. The baseline left lungs were immediately harvested and not preserved or transplanted. (a) ANOVA showed significant differences between the right lung groups ($P < 0.001$). The F_4–S_{10} and F_{10}–S_{10} groups also had significant lower W/D ratios than the baseline and F_4–S_{ice}. Concerning storage, a main effect was shown: S_{10} reduced W/D ratio ($P < 0.001$). (b) The one-way ANOVA showed significant differences between groups ($P < 0.001$) for the left lung grafts. Especially in F_4–S_{10}, a worsened W/D ratio was observed. After reperfusion, both flush and storage showed a significant main effect on all transplanted left lungs: S_{ice} led to lower W/D ratios compared with S_{10} ($P = 0.018$). Furthermore, F_{Rt} also led to lower W/D ratio when compared with F_4 ($P = 0.003$) and F_{10} ($P = 0.004$). Values are presented as mean ± SD. *P*-values above the error bars are compared to the baseline and those under the errors bars represent significant differences between the experimental groups. F, flush; S, storage.

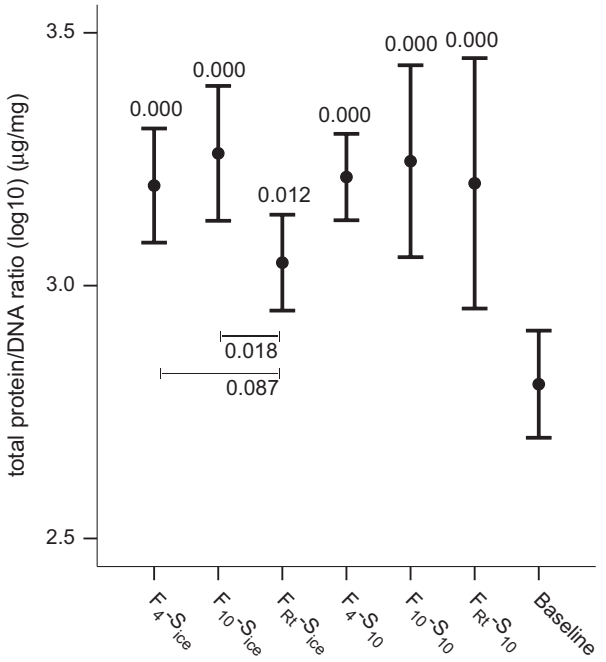

Figure 6 The total protein/DNA ratio was determined after 2-h reperfusion in left lung tissue. One-way ANOVA showed significant differences between groups ($P < 0.001$). Of all experimental groups, F_{Rt}–S_{ice} showed the lowest protein/DNA ratio. Data here are presented as mean ± SD. *P*-values above the error bars are compared to the baseline and those under the errors bars represent significant differences between the experimental groups. F, flush; S, storage.

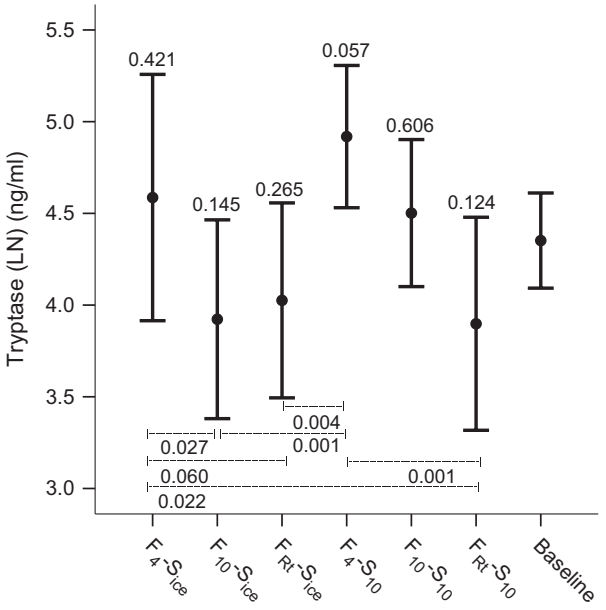

Figure 7 Tryptase activity was measured after 2-h perfusion in blood from the LV. ANOVA showed significant difference between groups ($P = 0.004$). Only flush showed a significant main effect on tryptase levels ($P = 0.003$). Both F_{10} and F_{Rt} showed a beneficial main effect on tryptase compared with F_4 ($P = 0.018$ and 0.001). Increasing flush temperature reduced tryptase levels in both S_{ice} and S_{10} stored lungs. Data here are presented as mean ± SD. *P-values* above the error bars are compared to the baseline and those under the errors bars represent significant differences between the experimental groups. F, flush; S, storage.

storage main effect showed that S_{10} worsened W/D ratios (Fig. 5b), in other words, F_4S_{10} and $F_{10}S_{10}$ showed a significantly higher W/D ratio ($P < 0.036$).

Total protein/DNA ratio

In comparison to noninjured tissue of the baseline group, a significant increase ($P < 0.012$) in total protein/DNA ratio in left lung tissue was seen in all experimental groups (Fig. 6). However, the F_{Rt}–S_{ice} group showed the lowest ratios of all experimental groups, especially when compared with both F_4–S_{ice} ($P = 0.087$) and F_{10}–S_{ice} ($P = 0.018$).

MPO/DNA ratio

Compared with the baseline MPO/DNA ratios (2.01 ± 0.52), all experimental groups showed significantly higher ($P < 0.009$) MPO levels in lung tissue. Between experimental groups, the MPO/DNA ratios did not show any significant differences; group ratios were 13.25 ± 5.07 (F_4–S_{ice}), 8.73 ± 2.22 (F_{10}–S_{ice}), 10.71 ± 2.15 (F_{Rt}–S_{ice}), 8.61 ± 5.81 (F_4–S_{10}), 11.96 ± 3.79 (F_{10}–S_{10}) and 9.02 ± 4.73 (F_{Rt}–S_{10}). The statistical analysis did not yield a main effect for flush or storage temperatures for MPO/DNA ratio in lung tissue.

Lung tryptase release in plasma after reperfusion

Increasing the flush temperature reduced tryptase levels significantly in both S_{ice} and S_{10} lungs (Fig. 7) ($P = 0.027$). Flush had a main effect on tryptase levels, resulting in reduced levels when applying a Rt flush ($P = 0.001$). Compared with noninjured lungs of the baseline group, only F_4–S_{10} showed the highest tryptase levels of all experimental groups ($P = 0.057$).

Discussion

The purpose of this study was to elucidate the optimal combination of flush and storage temperatures that would improve lung graft function and reduce graft injury. It was found that only Rt flush combined with storage on ice created the best lung graft preservation. To the best of our knowledge, this is the first study to incorporate the most commonly used experimental preservation temperatures in a prolonged inflated storage rat I/R lung transplant model with state-of-the-art preservation techniques. In addition, it is also distinguished from others by its assessment of the

contribution of flush and storage to lung preservation through a main effect analysis.

This study shows that F_{Rt}–S_{ice} resulted in significantly better lung graft preservation than the most common clinically applied method, F_4–S_{ice}. The F_{Rt}–S_{ice} showed a higher pO2, a lower P_{max} and a higher compliance. Furthermore, lung injury assessment showed a reduction in total protein/DNA ratio and tryptase activity after 2-h reperfusion. Raising the storage temperature to 10 °C did not improve lung graft preservation. On the contrary, the F_4–S_{10} group resulted in a deteriorated graft function compared to the clinical standard, more specifically, a decline in pO_2, a high P_{max} during reperfusion, low compliance and significantly high W/D ratios and tryptase levels.

Two other major conclusions can be drawn from these results. First, that cold flushing (F_4) is harmful for lung vasculature [34–36], specifically the endothelial cells whereas F_{Rt} is protective for the endothelial cells. This conclusion is supported by the result that F_{Rt}–S_{ice} lung tissue had the lowest total protein/DNA ratio, indicating a reduced vascular permeability and therefore a better preservation of lung endothelium compared with the other groups. This endothelial protective mechanism at higher flush temperatures can possibly be explained by less vasoconstriction and a lower viscosity of the perfusion fluid, leading to a higher flush flow [6,10,22]. Our higher flush flow index at Rt may further prevent the endothelial cells from being exposed to toxic agents released from leucocytes, causing membrane leakage [9]. This protective effect of Rt flush has also been shown in other studies [8–13], but combined with shorter ischaemia times [9,10], deflated storage [37] or with different preservation solutions [11,12,14,27,37].

The second major conclusion that can be drawn is that S_{ice} during static inflated storage is not harmful for lung epithelial cells, but rather is necessary to prevent further metabolic deterioration. This is based on four results: lung preservation markers, length of warm ischaemia, pO_2 and the W/D ratio. First, it was found that F_{Rt}–S_{ice} gave the best lung preservation. Second, in F_{Rt}–S_{10}, we found that inflated lungs increased the storage solution temperature to 15 °C, resulting in a prolonged warm ischaemia period above 10 °C for 65 min (data not shown).

In S_{ice}, the storage solution warmed up to at most 8 °C and returned to S_{ice} within 25 min. The importance of effective cooling is supported by a pig lung transplant study applying topical cooling at 6–9 °C for 24 h, but than in combination with a deliberately chosen deflated storage to avoid the insulating effect of air. These pig lungs performed well after transplantation confirming the importance of effective cooling below 10 °C [38]. We hypothesize that the insulating effect of air in inflated lungs necessitates cooling to 4 °C.

Finally, the unexpected combination of high pO_2 and high W/D ratio after 2-h reperfusion in F_{10}–S_{ice}, also reported by others [9], is likely caused by intact epithelial cells. Although a higher W/D ratio reflects tissue injury, the epithelial cells that maintain proper pO_2 levels are preserved. As of yet, no structure–function correlation has been reported between gas exchange and epithelial pneumocyte type I injury when investigating preservation injury [39]. However, a sustained function of surfactant-producing type II pneumocytes [40] may very well be the additional cause of the good pO_2 levels in F_{Rt}–S_{ice} groups.

Flush flow and temperature, two interrelated factors, have a direct physical effect on the lung vasculature. In a vasodilatory state, the flush flow becomes a prominent factor to wash out blood and microthrombi [41]. Vasodilation contributes to a favourable diffusion of the preservation solution in the lung, which is optimal for prolonged lung preservation [41]. This is supported by the fact that the best preserved lungs in our study had the highest flush flow index of all groups, a finding also observed by Albes [10]. It follows then that if vasoconstrictive vasculature is not flushed properly, these cells ultimately depend on cooling, specifically topical cooling alone [41]. Therefore, our hypothesis is that lung function after reperfusion not only depends on the amount of flush-induced endothelial injury but also the maintenance of well-preserved pneumocytes. In support of this suggestion, a recent study shows that type II pneumocytes are more susceptible to I/R injury than most other cell types [42].

This study has two possible limitations. The first is that histology of the left lung graft was not performed because it interfered with static compliance measurements. Histology may have provided additional information about endothelial and epithelial structure and integrity. Second, because of the size and thermal insulating capacity differences of rat versus human lungs, the results of this study cannot be properly extrapolated to humans.

We conclude that flushing at Rt creates the best lung graft preservation in combination with inflated storage on ice. To extrapolate our findings in rats to humans, the next logical step would be assessment in a larger animal. Especially, thermal insulation after inflation of much larger lungs should be investigated as thermo insulating properties of air was already important in our small rat lung necessitating a rapid topical cooling after room temperature flush.

Authorship

AJM: Participated in research design, performance of the research, data analysis and writing the manuscript. GR, MEE: Participated in research design, data analysis and writing the manuscript. AHP: Participated in performance

of the research. WAO: Participated in data analysis and writing the manuscript. JP: Participated in research design and writing the manuscript.

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