Pentoxifylline is as effective as leukocyte depletion for modulating pulmonary reperfusion injury

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Objective: Previous studies have suggested the amelioration of lung reperfusion injury when initial reperfusion is undertaken with leukocyte-depleted blood. Pharmacologic agents, such as pentoxifylline, are also effective, but no previous studies have demonstrated which is superior. We investigated these agents in a porcine model of left single-lung transplantation.

Methods: Donor lungs were preserved with modified Euro-Collins solution for a mean ischemic time of 18.6 hours. Gas exchange, pulmonary vascular resistance, neutrophil elastase level, and free radical release (measured on the basis of malonaldehyde levels) were assessed over a 12-hour period. Group A (n = 5) was a control group with no interventions added. Group B was reperfused through an extracorporeal circuit incorporating a leukocyte-depleting filter for 30 minutes before conventional blood flow was restored. Group C was reperfused with the addition of intravenous pentoxifylline ($2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$).

Results: Groups B and C were similar in terms of oxygenation, pulmonary vascular resistance, and free radical release. Group B displayed increased levels of neutrophil elastase. Both groups were superior with regard to these outcome measures compared with control group A.

Conclusions: Pentoxifylline, when administered to recipient animals, attenuates reperfusion injury to a degree similar to that seen with leukocyte-depleted reperfusion. This technique is simple, safe, and as effective as using a more complex extracorporeal circuit incorporating a leukocyte-depleting filter to ameliorate acute lung injury.

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Despite advances in lung preservation technology, early graft dysfunction remains a significant problem. The pathophysiology of lung graft ischemia-reperfusion injury is complex but predominantly involves the sequestration of leukocytes in the lung, with subsequent release of toxic granular enzymes and oxygen free radicals.¹

Several interventions are thought to modulate reperfusion injury but have seldom been directly compared in a single standardized model. Some of the more promising interventions include the administration of intravenous pentoxifylline and, alterna-

tively, the perfusion of the graft lung with leukocyte-depleted blood obtained by using a suitable filter contained within an extracorporeal circuit.

Pentoxifylline administration has been successful in ameliorating reperfusion injury after skeletal muscle ischemia and experimental lung and liver transplantation. It acts through a variety of mechanisms, including inhibition of leukocyte-endothelial interactions and oxygen free radical scavenging. There might also be inhibitory effects on cytokines.

Leukocyte-depleting filters have been used in heart-lung transplant models when incorporated into the cardiopulmonary bypass circuit and in isolated rat lung grafts.^{2,3} Significantly improved early graft function has been achieved compared with that seen in control animals. Recent experience with the reperfusion of human lung grafts indicates that this is a potentially useful intervention to ameliorate early graft dysfunction.⁴

We investigated the comparative benefits of these interventions and were uniquely able to directly compare their effects in a porcine model of single-lung transplantation with follow-up for 12 hours. This enabled us to assess which intervention might be superior for subsequent use in clinical practice.

Methods

Fifteen female Landrace pigs (mean weight, 45.6 kg) were divided randomly into 3 groups ($n = 5$ in each). A similar number were size and weight matched and acted as donor animals. All grafts were reperfused at a mean pulmonary artery pressure of 20 mm Hg.

Group A was a control group with no intervention administered. Group B was perfused through a leukocyte-depleting filter for the first 30 minutes of reperfusion and then perfused ordinarily through the pulmonary artery. Group C was reperfused with the addition of intravenous pentoxifylline (20 mg/kg loading dose, followed by $2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Pentoxifylline was administered to the recipient animal only and commenced 5 minutes before reperfusion of the graft.

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 National Academy of Science and published by the National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1985). All conditions associated with the United Kingdom Animals (Scientific Procedures) Act of 1986 were also met.

Donor Operation

Our model of single-lung transplantation has been described previously.⁵ In summary, animals were anesthetized with initial intramuscular premedication by using diazepam (2 mg/kg) and ketamine (15 mg/kg). Subsequent anesthesia was induced with propofol (20 mg/kg) and maintained with isoflurane and intravenous alfentanil. Animals were intubated with an endotracheal tube

(outside diameter, 9.5 mm) and ventilated at a tidal volume of 15 mL/kg at an inspired oxygen concentration of 100%.

Heart-lung blocks were retrieved in a standard manner, and lungs were preserved by means of flush perfusion with 60 mL/kg modified Euro-Collins solution administered through the main pulmonary artery.

After separation of the left lung from the block, a pulmonary artery pressure monitoring line (Cavafix Certo 18G; Braun, Melsungen, Germany) was inserted through a purse-string suture into the distal left pulmonary artery. A pulmonary venous sampling line (Flocare; Nutricia, Madrid, Spain) was similarly placed through the left atrial cuff directed into a distal pulmonary vein, allowing for later sampling of venous blood from the graft to avoid the mixing of blood from the contralateral native lung. Lungs were stored inflated at a temperature of 4°C for a mean ischemic time of 18.6 hours.

Recipient Operation

Recipient animals were premedicated with intramuscular azaperone (8 mg/kg) and diazepam (2 mg/kg). After induction with intravenous propofol, animals were maintained on intravenous pentobarbitone ($30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and alfentanil. Venous and arterial pressure monitoring lines were inserted as in donor animals.

Two endotracheal tubes were inserted through a tracheostomy. A 9.5-mm outside diameter tube was placed into the trachea to ventilate both lungs initially and, subsequently the native lung alone. The second 6.5-mm tube was advanced through the left bronchial anastomosis after its completion to ventilate the graft lung independently. Each endotracheal tube was connected to a separate ventilator to permit individual lung ventilation after transplantation, with a tidal volume of 15 mL/kg at 12 breaths/min for each lung. The fraction of inspired oxygen was 100%.

A left thoracotomy was performed, followed by a left pneumonectomy. Implantation of the donor lung proceeded in an established fashion, constructing anastomoses of the left atrium, bronchus, and left pulmonary artery in order. The contralateral pulmonary artery was encircled by a tape and snigger such that the left pulmonary artery pressure could be manipulated. A pressure monitoring line was placed in the recipient left atrium, and a dedicated sampling line was inserted into the proximal left pulmonary artery.

Pulmonary artery flow was measured by using a 10-mm Transonic A-Series flow probe (Linton Instruments, Norfolk, United Kingdom) placed around the left pulmonary artery distal to the anastomotic line. A similar 12-mm probe was placed around the descending aorta to provide a guide to the cardiac output. Both flow probes were connected to a dual-channel HT207 Medical volume flowmeter (Transonic Systems Inc, Ithaca, NY).

All data sources were routed through a CED 1401 32-channel digital to analogue converter (Cambridge Electronic Design Ltd, Cambs, United Kingdom) and acquired on a microcomputer by using Spike 2 (Version 4.0) data acquisition software (Cambridge Electronic Design Ltd). Data were collected continually over the 12-hour postoperative period and stored on hard disk for subsequent analysis.

Animals in group A received no intervention to modulate reperfusion injury and acted as a control group apart from systemic

heparinization (300 U/kg). Because the pulmonary artery pressure at reperfusion might influence subsequent graft function, the pressure was kept at 20 mm Hg by constricting or releasing the tourniquet on the contralateral pulmonary artery as required. The same principle was applied to the other groups to ensure that reperfusion pressure was constant in all cases.

In animals randomized to group B, a simple circuit was used to allow preliminary leukocyte-depleted perfusion of the graft lung. Blood was drawn from the external jugular vein through a large-bore cannula (Polystan Venous Paediatric Catheter 145016 16FG; Polystan A/S, Walgerholm, Denmark) and passed through 3-in tubing through a calibrated roller pump, distal to which a Pall BC1-B leukocyte-depleting cardioplegia filter (Pall Biomedical, Portsmouth, United Kingdom) was placed. This filter combines leukocyte filtration technology with a conventional gaseous and particulate microemboli-removing 40- μ m screen. The filter has been designed to allow flow rates of up to 500 mL/min while retaining the ability to efficiently filter leukocytes.

Beyond this, a cannula (Polystan Bent Hard Tip 18FG 100184, Polystan A/S) returned filtered blood to the left pulmonary artery. The cannula was inserted into the artery through a purse-string suture proximal to the anastomotic suture line and then advanced past the anastomosis such that the tip lay just proximal to the left upper lobe artery branch. With the left pulmonary artery occluded proximally, the lung could be reperfused by blood from the filter circuit for 30 minutes at a pressure of 20 mm Hg, after which the vessel was decannulated, allowing flow from the recipient pulmonary artery to enter the pulmonary vascular bed. Three hundred units per kilogram of heparin was administered as a bolus to the animal before cannulation of the pulmonary artery. The experiment was then executed as usual. Blood remaining in the circuit (priming volume, 500 mL) was returned slowly to the animal over a 2-hour period through the remaining external jugular venous cannula.

Group C animals received intravenous pentoxifylline, which was commenced 5 minutes before reperfusion of the lung graft. A loading dose bolus of 20 mg/kg was administered, followed by an infusion of 2 mg $\cdot$ kg⁻¹ $\cdot$ h⁻¹ for the duration of the experiment. Animals in group C also received 300 U/kg heparin to match the pharmacologic conditions of Group B. No untoward effects of drug administration were noted in any animal.

In all animals the pulmonary venous oxygen partial pressure (in millimeters of mercury) was obtained from pulmonary venous sampling line samples analyzed immediately on a blood gas analyzer (Nova Biomedical Stat Profile 5, Waltham, Mass).

Pulmonary vascular resistance (PVR; in millimeters of mercury per liter per minute) was calculated on the basis of the following formula:

$$\frac{(\text{Mean pulmonary artery pressure [mm Hg]} - \text{Left atrial pressure [mm Hg]})}{\text{Left pulmonary flow [L/min]}}$$

Malonaldehyde (MDA), an important decomposition product of lipid peroxides, is an indirect measure of free radical activity. A spectrophotometric assay using an LPO-586 method (Calbiochem-Novabiochem International, San Diego, Calif) was used to quantify MDA in pulmonary venous blood.

Five-milliliter samples of whole blood were collected in 48 μ L of potassium ethylenediamine tetraacetic acid, 0.17 mol/L. After centrifugation at 2500g for 10 minutes at 4°C, 200 μ L of the supernatant was collected in duplicate for use in the assay. The sample was incubated for 40 minutes at 45°C with the chromogenic substance and then cooled on ice, and absorbance was measured with a spectrophotometer at 586 nm. The sensitivity of this method was determined as 0.5 μ mol/L. In whole blood samples of 200 μ L, the lower limit of measurable MDA was 2.5 μ mol/L. Reproducibility was determined by using assays performed over 10 days under identical experimental conditions. By using standard concentrations from 0 to 20 μ mol/L, the SEM was less than 5%.

For the assay of neutrophil elastase, blood (5 mL) was collected in ethylenediamine tetraacetic acid and centrifuged within 2 hours of collection at 3000 rpm (1500g) for 10 minutes. Fifty-microliter specimens of plasma or control samples were added to 2500 μ L of phosphate-buffered saline. All samples were duplicated at this stage. One thousand microliters of distilled water was added before incubation for 60 minutes at 25°C. Five hundred microliters of antibody-enzyme solution was then added, and after a further incubation of 30 minutes (25°C), the sample was washed 3 times with distilled water, and 500 μ L of substrate solution was added (20 μ mol of 4-nitrophenyl phosphate/10 mL of diethanolamine [pH 9.8] 1 mol/L, magnesium chloride 0.5 mmol/L). The reaction was terminated with 100 μ L of sodium hydroxide (2 mol/L) after a further 30 minutes of incubation protected from light.

Five hundred microliters of the sample with 100 μ L of sodium hydroxide was then used to measure absorbance at 405 nm. The mean absorbance from the duplicate determinations was calculated. The concentration of elastase from the absorbance of each sample was then determined from the calibration curve. Results were thus received in micrograms per liter of neutrophil elastase. Free hemoglobin did not affect the assay, which was of importance in leukocyte filter experiments in which free hemoglobin levels at the upper limit of the normal range were observed.

Statistical Methods

A misleading impression might be gained of trends in data because graphically a line joining mean values together might not describe individual subjects within a study group adequately. Furthermore, no account is taken of the fact that measurements at different time points are from the same subjects with successive observations of an individual subject likely to be correlated.⁶ Statistical examination using the individual, rather than the mean of the group at each time point, as the basic unit for analysis can be performed to overcome these potential problems. A single number to summarize an individual subject's response curve with time must be sought (ie, a summary measure). This reduces a large number of dependent observations to a smaller number of summary measures.

In our study the summary measure of area under the curve was used to describe the behavior of individual animals. Groups of animals were then compared by using Scheffe analysis of variance.

Results

The perfusion of lungs at 20 mm Hg with leukocyte-depleted blood for 30 minutes before normal pressure reperfusion conferred significant benefits in terms of gas ex-

Figure 1. Pulmonary venous oxygen partial pressure (*PVO₂*, in millimeters of mercury) with time after reperfusion. Results are shown as the mean value for 5 animals in each intervention group. Error bars indicate the 95% confidence interval.

change. The pulmonary venous oxygen partial pressure, as assessed by the summary measure of area under the curve, was significantly higher than that in control animals subjected to similar pressure control at the time of reperfusion ($P < .001$).

In comparison with lungs reperfused with the addition of the pharmacologic intervention, a similar pulmonary venous oxygen partial pressure was observed in pentoxifylline-treated animals. No significant difference in oxygenation was seen when comparing leukocyte filtration and pentoxifylline treatment ($P = .414$). No difference in partial pressure of carbon dioxide was noted in either treatment group compared with that in the control animals ($P > .05$, Figure 1).

MDA levels (a measure of oxygen free radical activity) were significantly less in groups B and C than in group A ($P < .05$). Groups B and C were similar, however ($P > .05$, Figure 2).

Analysis of neutrophil elastase indicated that higher levels were observed in lungs reperfused through the filter. Elastase levels after filtration were significantly higher than in control lungs ($P = .002$) or lungs treated with pentoxifylline ($P \leq .001$). Elastase levels were, however, significantly less in animals treated with pentoxifylline compared with those in control animals ($P < .001$). The high levels of elastase in group B were probably a result of the degree of neutrophil activation during circulation through the extracorporeal circuit and hemolysis (Figure 3).

PVR for filtered lungs was significantly less than that seen in control animals ($P < .0001$), but PVR was similar when compared with that in lungs reperfused with the addition of pentoxifylline ($P = .95$, Figure 4).

Figure 2. MDA levels with time after reperfusion. Results are shown as the mean value for 5 animals in each intervention group. Error bars indicate the 95% confidence interval. Preperfusion value is shown at time zero.

Figure 3. Elastase levels with time after reperfusion. Results are shown as the mean value for 5 animals in each intervention group. Error bars indicate the 95% confidence interval. Preperfusion value is shown at time zero.

Left atrial pressures and right ventricular pressures were no different in filter group animals compared with pressures in any other group ($P > .05$).

Repeated sampling did not significantly affect the hematocrit level of animals in any group over the time course of the experiment.

Figure 4. PVR with time after reperfusion. Results are shown as the mean value for 5 animals in each intervention group. Error bars indicate the 95% confidence interval. Prereperfusion value is shown at time zero.

Discussion

In our experiments groups B and C were similar in terms of oxygenation, PVR, and free radical release. Group B, however, displayed increased levels of neutrophil elastase, which is likely to be a reflection of hemolysis and neutrophil activation in an extracorporeal circuit.

Both groups were superior with regard to these outcome measures compared with control group A. Pentoxifylline, when administered to recipient animals, attenuates reperfusion injury to a degree similar to that seen with leukocyte-depleted reperfusion. We believe that the simple infusion of a pharmacologic agent is simple, safe, and as effective as using a more complex extracorporeal circuit incorporating a leukocyte-depleting filter to ameliorate acute lung injury.

Our model of left single-lung transplantation provided a stable and reproducible apparatus, permitting control of ventilation and perfusion of the lung graft while the animal was supported by the contralateral lung, irrespective of the function in the newly implanted graft. The rigorous separation of the lungs in terms of sample collection ensures that there is no mixing of blood from the contralateral side.

Importantly, this study enables direct comparisons to be made between various interventions thought to be of benefit in attenuating reperfusion injury. The removal of leukocytes from blood reperfusing the transplanted lung is surely the gold standard against which other interventions must be measured. The disadvantages of neutrophil activation by an extracorporeal circuit, however, are a well-recognized problem with such systems.

Pentoxifylline appears to be a promising agent in combating reperfusion injury in a variety of organs and has certainly been shown to be beneficial in lung injury.⁷⁻¹⁰ Until now, no comparison has ever been made with leukocyte depletion under consistent conditions in the same model.

The concept of leukocyte depletion is not new. In 1928, Fleming used cotton wool filters to extract white cells from whole blood, and the filtration of whole blood to deplete it of leukocytes has been practiced since 1962. Modern specific leukocyte filters are formed from tightly packed fibers of combed cotton, nylon, or cellulose acetate. The mode of action is not completely understood because the filters not only retain activated and adherent neutrophils but also lymphocytes, monocytes, and some platelets.

Using advanced 40- μ m polyester screen technology, manufacturers have developed biocompatible extracorporeal leukocyte-depletion filters capable of effective neutrophil removal that could be incorporated into the arterial return line of a cardiopulmonary bypass circuit. Gourlay and colleagues^{11,12} have indicated the efficacy of leukocyte depletion of the filter in both pulsatile and nonpulsatile bypass.

The role of white cell depletion in transplantation was previously investigated by using a bovine model.² After 12 hours of hypothermic preservation, heart-lung transplantations were performed with study animals undergoing reperfusion through a Pall RC100 filter in the return arm of the cardiopulmonary bypass system. During a 4-hour follow-up period, filtered animals had improved survival, better arterial oxygen tension, less extravascular lung water, and reduced systemic leukocyte counts. Combining superoxide dismutase administration with the use of leukocyte-depleted reperfusion further improved lung function after transplantation.

The ability of leukocyte-free reperfusion to prolong lung preservation times and retain satisfactory graft function was further confirmed by Schueler and associates.¹³ After core cooling during cardiopulmonary bypass, bovine lungs were preserved for 24 hours. After double-lung transplantation, reperfusion with a leukocyte-depleting filter in the extracorporeal circuit reduced the leukocyte count to 3% of its original value and improved systemic oxygenation, airway pressures, and lung water content and lowered PVR compared with that seen in control animals.

Our evaluation of the BC1B filter indicated that, from a purely practical standpoint, lungs could be reperfused from this extracorporeal circuit for 30 minutes before the 12-hour evaluation began, showing that a relatively easy method for extracorporeal perfusion of a unilateral lung graft can be effectively and safely accomplished.

In our experiments 30 minutes of leukocyte-depleted reperfusion protects the lung from subsequent injury. This could result from the downregulation of neutrophil-endo-

thelial adhesion molecules, which must occur during the initial phase of reperfusion and ameliorates neutrophil trapping in the lung graft. Recent studies on an isolated rabbit lung have also indicated that short periods of leukocyte-depleted reperfusion are beneficial, although the mechanism has yet to be investigated.¹⁴ A recent investigation in human lung transplant recipients used arterial blood collected and mixed to make a 4:1 solution of blood and modified Buckberg perfusate. This solution was passed through a leukocyte filter and into the transplanted pulmonary artery for 10 minutes at a controlled rate and pressure (<20 mm Hg) immediately before removal of the vascular clamp. All 5 patients in this clinical series demonstrated excellent post-reperfusion function in the lung grafts.⁴

Interestingly, neutrophil elastase levels were increased in those lungs reperfused by the filter and probably reflect a degree of hemolysis and neutrophil activation as blood passes through the filter and extracorporeal circuit. Pulmonary venous oxygenation was similar to that achieved with pentoxifylline but superior to that seen in the control group. PVR was comparable with that seen in the pharmacologic intervention group and was again superior to unmodified reperfusion in the control group.

Pentoxifylline has many functions, in particular inhibiting the adherence of neutrophils to endothelium and preventing leukocyte degranulation. It might also inhibit interleukin 1 and tumor necrosis factor, which are critical in the pathogenesis of ischemia-reperfusion injury. Pentoxifylline clearly improves and augments pulmonary function by reducing neutrophil trapping in the lung and subsequent free radical release.¹⁵⁻¹⁷

Previous studies in large-animal models in Europe and in North America have demonstrated improvements in functional outcome when the agent is incorporated into either the lung preservation flush solution or administered immediately before reperfusion.^{8,9,18} Our own studies comparing pentoxifylline with controlled pressure reperfusion and other pharmacologic interventions (eg, nitric oxide donors and phytic acid derivatives) have confirmed the effectiveness of this agent. Clearly pentoxifylline, when administered to the recipient only, confers significant advantages by attenuating reperfusion injury and permitting longer ischemic times to be tolerated. Little is known of function beyond 12 hours or the optimal dosing regimen, and this needs to be addressed in future studies.

Pentoxifylline, already licensed for use in human subjects with peripheral vascular disease, is an inexpensive and safe drug for use in lung transplant recipients and appears to be at least as effective as leukocyte-depleted blood reperfusion. It obviates the need for complex and expensive extracorporeal circuits and filters and the dangers with which they are inevitably associated in the operating room setting.

Adoption of pentoxifylline, a simple and cheap intervention, in human lung graft recipients might lead to considerable improvements in mortality and morbidity among lung transplant recipients, as well as shorter intensive care stays and costs.

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