A Novel Cell Culture Model to Study Ischemia-Reperfusion Injury in Lung Transplantation

Jonathan A. Cardella, B.Sc.

A thesis submitted in conformity with the requirements for the degree of Master of Science, The Institute of Medical Science, University of Toronto.

Division of Thoracic Surgery, University Health Network, Toronto General Hospital and The Institute of Medical Science, School of Graduate Studies, University of Toronto.

© Copyright by Jonathan A. Cardella 1999.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-46106-8
ABSTRACT

A Novel Cell Culture Model To Study Ischemia-Reperfusion in Lung Transplantation


Lung transplantation has emerged as a therapeutic modality for patients with end-stage lung disease. However ischemia reperfusion injury remains a significant clinical problem, occurring in 20% of patients. Many cell culture models have been developed to study ischemia-reperfusion (I/R) injury, however none are specific to the conditions of lung preservation and transplantation. We designed a cell culture model that mimics the conditions of clinical lung transplantation, where preservation is aerobic and hypothermic. Human pulmonary epithelial cells (A549 cell line), were preserved in 100% O₂ at 4°C for varying periods in Low Potassium Dextran Glucose solution, simulating ischemia, followed by the reintroduction of warm (37°C) Dulbecco’s Modified Eagle Medium plus 10% fetal bovine serum to simulate reperfusion. Cultures were assayed for cell attachment and viability. Sequential extension of ischemic times to 24 h showed a time-dependent loss of cells. There was a further decrease in cell number following simulated reperfusion. Cell detachment was due mainly to cell death, as determined by cell viability assays. The effects of chemical components in the preservation solution and various gas mixtures during ischemia were examined using this model system. Because this model closely mimics I/R injury in lung transplantation, it could be used to study cellular and molecular mechanisms related to this clinical situation.
ACKNOWLEDGEMENTS

The time I have spent in The Thoracic Surgery Laboratory have been some of the most enjoyable of my life. Dr. Mingyao Liu has been exceptional as a supervisor. Not only has he provided his knowledge, time, enthusiasm, advice and tutelage but has become a friend and mentor. His patience and honesty have allowed me to take advantage of the learning opportunities presented to me over the previous two years. Dr. Shaf Keshavjee has been particularly helpful to me in helping to direct my research, career and personal goals. He has also become a friend and mentor and, in my mind, sets an example of success in academic medicine. Thank you Mingyao and Shaf, for everything. I would also like to thank Dr. Art Slutsky who was instrumental in my research through timely and necessary critical evaluation as well as a constant source of career guidance. Dr. Martin Post has also been invaluable as a source of knowledge and critical appraisal.

The success of The Thoracic Surgery Laboratory is a credit to the fellows and technicians that work there. Ioan Mates is a superb technician and a great friend and I would like to thank him for his help, support and the positive example he sets. I also appreciate the friendship, camaraderie, advice and collaboration of Dr. Stephen Cassivi. Thank you also to Dr. Stefan Fischer who has provided me with much guidance and has been an estimable collaborator and friend. Laura Bartolamiol has provided excellent guidance and help usually at moments notice and for that I thank her. Xiao-Hui Bai, Xiao-Ming Zhang and Lu Cai have been excellent teachers, good friends and enjoyable people to work with. Drs. Eric Mourgeon, Noritaka Isowa, Michiharu Suga, Alexandre Xavier, Jose Matute Alexandra MacLean, Jorge Moreira Annette Boehler and David Hopkinson have all contributed and helped me throughout
the course of my research. The staff of the operating room has been excellent in helping me with any experiments I have performed there. Finally I would like to thank all my friends for their support over the years.
DEDICATION

This thesis is dedicated to my family. To my parents, Piera and Carl, you have always set the example and inspired me to do my best, instilled in me the importance of hard work, friends and family, picked me up when I was down, shared my victories and my defeats. To my brother David and sister Jennifer, you are a tough act to follow. You have succeeded in different career paths and have always motivated, challenged and set an example for me. To my family, this work represents your contributions that helped shape the person that I am and the work that I do. Thank-You.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>II. List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>III. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>(a) HISTORICAL</td>
<td>2</td>
</tr>
<tr>
<td>(b) EARLY LUNG PRESERVATION STRATEGIES</td>
<td>3</td>
</tr>
<tr>
<td>Maintenance of lung metabolism</td>
<td>3</td>
</tr>
<tr>
<td>Inhibition of lung metabolism</td>
<td>3</td>
</tr>
<tr>
<td>(c) CONDITIONS OF LUNG PRESERVATION</td>
<td>4</td>
</tr>
<tr>
<td>Pulmonary preservation solutions</td>
<td>7</td>
</tr>
<tr>
<td>Intracellular based preservation solutions</td>
<td>7</td>
</tr>
<tr>
<td>Extracellular based preservation solutions</td>
<td>8</td>
</tr>
<tr>
<td>Preservation gas</td>
<td>9</td>
</tr>
<tr>
<td>Preservation temperature</td>
<td>10</td>
</tr>
<tr>
<td>Current “gold standard” of lung preservation</td>
<td>11</td>
</tr>
<tr>
<td>(d) ISCHEMIA REPERFUSION (I/R) INJURY</td>
<td>13</td>
</tr>
<tr>
<td>Biochemical basis of I/R injury</td>
<td>14</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>14</td>
</tr>
<tr>
<td>Cytokines</td>
<td>15</td>
</tr>
<tr>
<td>Complement</td>
<td>16</td>
</tr>
<tr>
<td>Experimental approaches to I/R injury</td>
<td>17</td>
</tr>
<tr>
<td>Animal models of I/R injury</td>
<td>17</td>
</tr>
</tbody>
</table>
(e) THE IMPORTANCE OF MAINTAINING CELL VIABILITY 18

Endothelial cells 18
Epithelial cells 19

(f) CELL CULTURE MODELS OF I/R INJURY 22

General I/R cell culture models 22
Lung transplantation specific cell culture models of I/R injury 23

(f) OBJECTIVES 25

IV. MATERIALS AND METHODS 26

Rationale for the cell culture model design to mimic I/R injury in lung transplantation 27
Cell culture 28
A simulated I/R injury model system 30
Evaluation of cell attachment 30
Fluorescein Diacetate – Propidium Iodide Staining 30
Preservation Solutions 32
Preservation Gases 34
Measurement of pH 34
Statistical Analysis 34

V. RESULTS 36

Aerobic hypothermic ischemia and reperfusion induce cell detachment 37
Cell attachment provides an indirect quantification of cell viability 40
Effects of chemical components on cell viability 40
Preservation gas mixture has a marked effect on cell viability 46

VI. DISCUSSION 50
(a) GENERAL DISCUSSION 51
Critique of the model 51
Validation of the model 54
(b) EFFECTS OF CHEMICAL COMPONENTS 54
Effect of glucose 55
Effect of dextran 40 55
Effect of calcium 55
(c) EFFECT OF pH 58
(d) EFFECT OF PRESERVATION GAS 62
(d) SUMMARY 63
Future directions 64

VII. REFERENCES 66
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Organ Preservation Strategies</td>
<td>6</td>
</tr>
<tr>
<td>Table 2</td>
<td>Primary Functions of Type II Cells</td>
<td>21</td>
</tr>
<tr>
<td>Table 3</td>
<td>Composition of Preservation Solutions</td>
<td>33</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Procedure of human lung preservation and transplantation</td>
<td>12</td>
</tr>
<tr>
<td>Figure 2</td>
<td>A cell culture model that mimics I/R in lung transplantation</td>
<td>29</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Timeline and sampling points of experimental groups</td>
<td>31</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Aerobic hypothermic ischemia and reperfusion</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>decreased cell attachment in a time dependant manner</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a) Post ischemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) Post reperfusion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(c) I+R control</td>
<td></td>
</tr>
<tr>
<td>Figure 5</td>
<td>Cell Viability as determined by Fluoroscein-Diacetate Propidium Iodide staining</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>(a) Live cells fluoresce green</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) Dead cells fluoresce red</td>
<td></td>
</tr>
<tr>
<td>Figure 6</td>
<td>Cell attachment provides an indirect representation of cell viability</td>
<td>43</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Dextran did not confer better cellular preservation when added to D10 while adding Ca^{++} in LPDG significantly increased cell viability after reperfusion</td>
<td>44</td>
</tr>
<tr>
<td>Figure 8</td>
<td>The removal of glucose or dextran from LPDG solution did not significantly affect cell viability</td>
<td>45</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>Figure 9</td>
<td>The presence of 5% CO₂ during ischemia for cells preserved in D10 or DMEM significantly increased cell attachment after ischemia and reperfusion</td>
<td></td>
</tr>
<tr>
<td>Figure 10</td>
<td>Varying concentrations of O₂ and CO₂ in the preservation gas provided equivalent cellular preservation after 24 h at 4°C in LPDG solution</td>
<td></td>
</tr>
<tr>
<td>Figure 11</td>
<td>The calcium paradox</td>
<td></td>
</tr>
<tr>
<td>Figure 12</td>
<td>The cytotoxic effects of alkalosis</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION
INTRODUCTION

HISTORICAL
The first successful lung homograft procedure was performed in 1947 by the Russian physiologist, Demikhov. Using a canine model he was able to achieve allograft survival of up to 8 days at which time rejection was noted (1). It was not until 1963 that Hardy, following extensive work with dogs, performed the first successful single lung transplant in man (2). The patient survived for 18 days eventually succumbing to renal failure and malnutrition. In the next seventeen years 38 human lung transplantation procedures were performed with two patients surviving for 6 and 10 months respectively while experiencing significant morbidity. Poor healing of the bronchus at the anastomotic site, rejection of the transplanted lung as well as inadequate lung preservation techniques hindered the early attempts at lung transplantation (3, 4). In 1983, bolstered by the development of the immunosuppressant cyclosporine to treat rejection, the Toronto Lung Transplant Program performed what is considered to be the first successful single-lung transplant in a human with long term survival (5). The patient survived six and a half years. Using an omental wrap to supply blood to the transplanted bronchus helped to ameliorate the problem of interruption of the bronchial blood supply (6). These two advances helped make transplantation a viable therapeutic modality for selected patients with end stage lung disease.
EARLY LUNG PRESERVATION STRATEGIES

At the time the Toronto group performed its first successful lung transplant little was known about organ preservation, in particular lung preservation. Furthermore the lung must be able to function at near optimal levels immediately following transplantation for life to be sustained (7). The combination of these two factors made initial attempts at lung transplantation difficult as long ischemic times were intolerable, therefore donors and recipients had to be located at the same hospital. The early investigations in lung preservation took 2 distinct approaches: 1) maintenance of metabolic rates or, 2) inhibition of metabolic rates (8).

**Maintenance of lung metabolism**

Preservation of the lung while maintaining its metabolic rate was attempted using normothermic ischemia. A deflated lung is only able to withstand 30 min of normothermia before lung function is significantly compromised (8). However, normothermia combined with inflation or ventilation with O2 of an ischemic lung in baboons and dogs prolonged adequate preservation from 3 h to 10 h (9). It became clear that complete deflation of the lung is injurious during ischemia and Stevens reasoned that the surfactant system is damaged after the lung is collapsed and this results in a depression of pulmonary function when blood perfusion is restored (10).

**Inhibition of lung metabolism**

Metabolic inhibition of the lung during ischemia has been achieved primarily by the use of hypothermia (8). Preservation of the lung with fluorocarbon or subzero
temperatures was quickly abandoned as a method of preservation because of the massive morphological and functional damage associated with it (11, 12). Conaughton and colleagues employed lung preservation using atelectatic immersion at 10°C. This model provided adequate preservation for 6 h (13). This was the method used by the Toronto group in 1983 (6). This work was improved by Blumenstock who replaced the pulmonary blood with plasma as perfusate, ventilated the lungs with room air and used a preservation temperature of 4°C. This method provided adequate lung function and preservation of histological characteristics for 18 h to 28 h (14). However, the most consistent long-term results were produced by Noirclerc and co-workers who maintained consistent 24 h lung preservation in 43 of 48 dogs. The authors employed the following technique: 1) heparinization of the donor; 2) a pulmonary flush with Collins III solution; 3) ventilation of the lung, with oxygen, during flushing; 4) preservation at 4°C with the lung submerged in Collins III solution (8). This is similar to the method employed by Hardy’s group in 1963.

Since the early stages of research, lung transplantation and preservation have made many advances. Research has now begun to focus on the specific requirements for optimal lung preservation.

CONDITIONS OF LUNG PRESERVATION

Organ transplantation presents a unique physiological stress to the organ being transplanted. Typically, in organs other than the lung, ischemia for transplantation is hypothermic and anaerobic (15). Hypothermia is essential in organ preservation for transplantation for a number of reasons. Firstly, hypothermia significantly decreases the
rate at which intracellular enzymes degrade (15). Secondly, hypothermia at 4°C slows cellular metabolism to 5 percent of the normal rate (16). Presumably, this slowed reaction rate will allow for conservation of metabolic substrate, a decrease in cytotoxic metabolite production and a decrease in cell death. A unique feature for lungs, which are preserved for transplantation, is that they are inflated with O₂; this has been shown to be superior to inflation with nitrogen (17). During this period, the cells of the lung are able to consume O₂ to maintain aerobic respiration during preservation (18, 19). Therefore, optimal lung preservation for transplantation is unique in that the cells of the lung are able to maintain aerobic metabolism during the hypothermic preservation period (Table 1).
### Table 1: Organ Preservation Strategies

<table>
<thead>
<tr>
<th>LUNG</th>
<th>OTHER ORGANS</th>
</tr>
</thead>
<tbody>
<tr>
<td>➡ Ischemic (i.e. no blood flow)</td>
<td>➡ Ischemic</td>
</tr>
<tr>
<td>➡ Hypothermic (4°C)</td>
<td>➡ Hypothermic</td>
</tr>
<tr>
<td>➡ Aerobic</td>
<td>➡ Anaerobic</td>
</tr>
</tbody>
</table>

Comparison between the organ preservation strategies of the lung and other transplantable organs (i.e. kidney, liver, heart and pancreas)
**Pulmonary preservation solutions**

In addition to hypothermic and aerobic conditions during ischemia, lungs are generally preserved using an electrolyte solution. The electrolyte solution is introduced into the vasculature of the lung through a single pulmonary artery flush. While topical cooling, donor core cooling and autoperfusion of the heart lung block have been used, however, they are primarily assigned to laboratory investigations and are rarely used clinically (20, 21). The delicate alveolar capillary network of the lung makes it particularly susceptible to I/R injury (22). As a result a great deal of investigation has been performed to determine the ideal preservation solution that will maintain the integrity of this network. Principally, the types of preservation solutions can be divided into two groups: “intracellular” and “extracellular” solutions.

**Intracellular based electrolyte preservation solutions**

The introduction of Collins solution (its electrolyte composition is similar to intracellular fluid) for organ preservation revolutionized kidney preservation by allowing ischemic times greater than 24 h. Collins solution has a high “intracellular like” potassium concentration of 115 mM. The theory behind the use of a high potassium concentration is that it would directly prevent cell swelling as well as limit potassium ion leakage from preserved cells (23). Euro-Collins solution modified with the addition of glucose (Modified Euro-Collins) has been used with some success in lung transplantation and clearly confirmed the superiority of pulmonary artery flushing versus topical cooling for preservation (24-27). The tolerable ischemic time when using modified Euro-Collins solution is approximately 6 h provided that prostacyclin is administered during the donor
flush (28). The reason that the vasodilator prostacyclin is required lies in the hyperkalemic nature of modified Euro-Collins solution. Potassium is a potent vasoconstrictor. Without the use of a vasodilator in conjunction with modified Euro-Collins an inadequate distribution of flush solution is inevitable resulting in non-uniform cooling and poor preservation of regions of the lung (22, 29).

The limitations of using an intracellular solution clearly lie in the hyperkalemia associated with their use. As a result laboratory investigation moved to the development of "extracellular" preservation solutions for extending preservation time.

**Extracellular based electrolyte preservation solutions**

The initial experience with extracellular solutions for preservation gave poor results. Preservation with Ringer's lactate solution, serum and plasma fractions of blood as well as tissue culture media, such as RMPI, all gave poor results in I/R injury models (7, 30, 31). In 1989 Keshavjee et. al. reported that the use of a low potassium dextran (LPD) solution gave superior lung preservation than did modified Euro-Collins solution after 12 h of ischemia and reperfusion (32). Further investigations revealed that the method by which LPD conferred protection was through its low potassium content and the presence of dextran 40 (33). The potassium content of LPD is 4 mM. Presumably the mechanism of protection in comparison to modified Euro-Collins solution is the decrease in pulmonary vasoconstriction as mentioned earlier. Furthermore, the role of dextran 40 is to aid in microcirculation through its antithrombogenic effects as well as its positive contribution to the deformability of erythrocytes allowing for more efficient reperfusion (33, 34).
The unique ability of the lung to maintain aerobic metabolism prompted modifications to the LPD solution. The cells of the lung are able to maintain metabolism exclusively deriving energy from carbohydrate (35). One-percent glucose was introduced as an adjunct to LPD making the solution LPDG thus allowing glucose to be used as a metabolic substrate for the lung during ischemia (19). LPDG solution provided significantly better lung function after 24 h preservation at 10°C compared to LPD solution. Furthermore the metabolic intermediates of glucose and the citric acid cycle were maintained throughout the storage period (19).

Further study into the role of potassium using University of Wisconsin (UW) solution, another “extracellular” solution, designed for liver preservation by Belzer (36). Lowering the potassium level in UW solution to 20 mM or 4 mM significantly improved lung function following 30 h of ischemia and reperfusion (37, 38).

The body of evidence declaring extracellular solutions as being superior to intracellular solutions is overwhelming (29, 32, 39-42). Furthermore, in many laboratory investigations, an extracellular dextran 40 based perfusate has been used as a baseline solution to which various agents have been added (43-46). Current evidence overwhelmingly favors the use of extracellular solutions for lung preservation.

**Preservation gas**

The issue of inflation in lung preservation was initially explored in the 1970’s and it was generally accepted, as mentioned earlier, that inflation of the lung during storage was beneficial (47). It was postulated that the collapse of the lung during preservation injured the surfactant and therefore decreased pulmonary function after the
reestablishment of flow during reperfusion (8). The issue of gas concentration in the inflation preservation gas is controversial. Koyama and coworkers showed that lungs inflated with nitrogen ventilation during preservation were better preserved compared to ventilation with 100% O₂ or room air, under conditions of warm ischemia (48). However, Weder et al. showed the opposite result where nitrogen inflation was injurious and that optimal lung preservation would utilize O₂ for aerobic metabolism during cold preservation (17). Clearly, inflation of the preserved lung is required for optimal function and if optimal lung preservation relies on aerobic metabolism then O₂ must be present in the preservation gas.

**Preservation Temperature**

It is evident from earlier studies that the tolerable warm ischemic time for a lung is 2 h (8). Therefore the necessity of hypothermic preservation becomes evident. The optimal preservation temperature for lung appears to be 10°C, compared to preservation at 1°C, 4°C, 22°C or 38°C over a variety of preservation time periods (49-51). In clinical settings, lungs preserved for transplantation are maintained in an ice slush bath that is approximately at 4°C that is conveniently maintained in a cooler by the slush surrounding the organ.
Current "gold standard" of lung preservation

The optimal preservation condition for lungs is not obvious, however, based on laboratory evidence the use of an extracellular solution, with O₂ in the preservation gas under hypothermia seems to provide the "ideal" preservation conditions that maximizes pulmonary function at the time of reperfusion (Fig. 1). Therefore, it is these conditions that are simulated in the current study.
1. **FLUSH**
   Low Potassium Dextran Glucose (LPDG) and Inflated with 100% O₂

2. **ISCHEMIA**
   Preservation at 4°C
   6-8 hours

**REPERFUSION**
Lungs are transplanted and 100% O₂ ventilation is maintained

---

**Figure 1:** Current procedure of human lung preservation and transplantation: 1) Lungs are flushed with pulmonary preservation solution and filled with 100% O₂. 2) They are placed in ice slush for preservation, the current clinical tolerance is 6-8 h. 3) The lungs are then reimplanted, reperfused and the patient is maintained on 100 O₂ ventilation.
ISCHEMIA-REPERFUSION (I/R) INJURY

Since the 1980's lung transplantation has emerged as a therapeutic modality for the treatment of patients with end-stage pulmonary disease (26). Lung transplantation, as other forms of transplantation, is severely limited by the shortage of donor organs. Currently, there are three potential alternatives to conventional brain death organ donation. They are: living related lobar transplantation, xenotransplantation and the use of non-heartbeating donors. Living related lobar lung transplants are being used but only to a limited degree in a few centres (52). Furthermore, while no mortality has been reported in the donor procedure there is morbidity associated with it (53). The problem of hyperacute rejection continues to make xenotransplantation a non-viable option as a source of donor lungs at the present time (54, 55). Finally, non-heartbeating donors could potentially provide a source of additional donor organs, however, they have provided less than adequate post transplant lung function (56, 57).

Although options exist for increasing the donor pool they are limited and currently the majority of lungs for transplantation come from brain dead donors. Of the potential donor pool, it is estimated that organs are retrieved from only 20 to 30% of these patients due to religious, social or other reasons. This small pool is further decreased because only 5 to 10% of organ donors have lungs that are suitable for transplantation (1).

One of the major problems associated with lung transplantation is ischemia-reperfusion (I/R) injury. Organs for transplantation endure two main insults, ischemia during preservation followed by blood reperfusion after implantation (58). Both of these insults inflict distinct and separate injuries on the transplanted lung (1). Patients
undergoing I/R injury suffer primary lung dysfunction manifested as hypoxemia, decreased pulmonary compliance, pulmonary edema, and diffuse alveolar damage (59). Approximately 20% of patients undergoing lung transplantation experience significant I/R injury (60). Moreover, the current clinical tolerance of cold ischemia in lungs is 6 h to 8 h (58). These two problems result in transplantation being performed on an emergent basis with the selection of only "perfect" donor lungs for transplantation. If tolerable ischemic times could be extended and I/R injury ameliorated this would permit: 1) an expansion of the donor pool by allowing distant organ procurement and the use of "marginal" donor lungs and 2) lung transplantation to be performed on a semi-elective rather than emergent basis. Furthermore, an amelioration of I/R injury might result in fewer rejection episodes, as class II major histocompatibility antigens are upregulated during I/R injury (61).

Biochemical basis of I/R injury

In the more recent lung transplantation work, some of the cellular and molecular events that precipitate I/R injury have been elucidated. While not an exhaustive list, for summary purposes these can be grouped into the contributions of: 1) leukocytes 2) cytokines and 3) the complement system (58, 59). These all contribute to mediating the inflammatory response that is seen during I/R injury.

Leukocytes

The movement of leukocytes out of the pulmonary capillaries at the site of injury is an important factor for inflammation in I/R injury. The leukocytes tend to move
between the endothelial cells and out of the capillary in a multi-step process (59). The first step involves weak interactions between the glycoprotein moieties on the leukocyte and a group of molecules expressed on the endothelial cells known as selectins (62). Both E-Selectin and P-selectin are induced during reperfusion and their weak interaction with the leukocyte causes it to roll along the endothelial surface allowing stronger interactions to occur in later steps (59). The second step occurs when the integrins on the surface of the leukocyte interact with intercellular adhesion molecule 1 (ICAM-1) on the endothelial cell surface. This halts the leukocyte rolling and permits the extravasation of the leukocyte across the endothelium. The final step is the release of molecular mediators by the leukocytes that will further injure endothelial and epithelial cells.

Free radicals are one of these mediators and are produced by a class of leukocytes called neutrophils. Free radicals are capable of eliciting a variety of injuries to lipids, DNA and protein (63). Furthermore, activated neutrophils are stiffer than non-activated cells, resist deformation and therefore, more likely to impair microcirculation. Moreover, degraded neutrophils are able to release elastase and protease, which can further damage cells (64). Finally, neutrophils are able to produce large amounts of inflammatory cytokines (65-67).

Cytokines

Cytokines are a class of chemotactic agents that act as extracellular signaling proteins. The upregulation of pro-inflammatory cytokines such as interleukin-2 (IL-2), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) has been shown in lung transplant I/R injury models (68). These pro-inflammatory cytokines can activate and/or
mediate acute inflammation reactions. For example, TNF-α causes endothelial cells to
become increasingly adhesive to leukocytes such as neutrophils (69). Furthermore TNF-α
results in increased neutrophil phagocytosis and degranulation which can injure the
pulmonary endothelium (70).

Complement

The complement system consists of about 20 interacting proteins that are
produced by the liver and circulate in the blood. Their role is to mediate the immune
system and inflammatory reactions (62). There are two pathways that can lead to
activation of the complement cascade. They are the classical and alternative pathways.
Both pathways are regulated through C3 and C5 proteins. Activation of these proteins by
their respective enzymes (C3 and C5 convertase) will result in the activation of the
complement cascade. Subsequently, increases in vascular permeability and leukocyte
adhesion will follow for C3 activation and direct cell lysis will occur if both C3 and C5
are activated (71). Complement is activated following ischemia and reperfusion of rabbit
lungs (72). Furthermore, soluble complement receptor 1 (sCR1) has been shown to be
successful in ameliorating I/R injury in a pig lung transplant model by blocking the
activation of C3 and C5 proteins (73).

These represent some of the molecular and cellular mechanisms that are involved
in I/R induced lung injury. They have highlighted the importance of studying the
molecular mediation of the extremely complex process of I/R injury, and, have shed
some light into potential remedial treatments.
Experimental approaches to studying I/R injury

As stated the early attempts at lung transplantation were hindered by failure in the surgical technique. With the improvement in technique, the focus for lung transplantation research changed to optimizing whole organ preservation and ameliorating reperfusion injury. Clinical studies are clearly the “Gold Standard” in solving a clinically based problem such as I/R injury. These studies have allowed us to define the physiological hallmarks of I/R injury mentioned earlier. However, ethical considerations make experimentation in patient populations difficult. Furthermore, patient populations are heterogeneous in nature. Their indications for transplantation, genetic makeup, lifestyle etc. make controlling these studies difficult. Therefore, investigations in lung transplantation moved to the optimization of whole organ function using animal models.

Animal models of I/R injury

Because the main function of the lung is gas exchange the primary indicator of lung function is oxygenation (21). Therefore, most animal models employ this parameter as the primary endpoint. Large animal lung transplant models such as porcine, canine and primate models have been used extensively in experimental lung transplantation (12, 32, 40, 74). Furthermore, small animal models of lung transplantation have also been employed with some success. These can be divided into rat transplant models (75) or extracorporeal models using rats (76) or rabbits (29, 50, 77). While allowing for good evaluation, animal models are difficult to use to study the cellular and molecular mechanisms of I/R injury.
Investigations are emerging that explore cellular injury and death in ischemia and reperfusion (78, 79). These studies will provide insight into the mechanisms of cell injury and cell death underlying I/R injury and potential remedial treatments to ameliorate it.

THE IMPORTANCE OF MAINTAINING CELL VIABILITY

Recent studies from our laboratory indicate that a decrease in lung function post reperfusion correlates with a decrease in cell viability after ischemia (79). Therefore it is imperative to maintain cell viability to ensure proper lung function. Research into lung transplantation has now begun to shift its focus from whole organ physiological determinants of I/R injury to focusing on cellular and molecular mediators of I/R injury as well as types and modes of cell death.

Endothelial cells

The first site of contact of pulmonary preservation fluid during lung preservation, and blood during reperfusion, are the endothelial cells of the donor lung. As a result, these cells are susceptible to injury (59). There are three main physical stresses that act on the pulmonary capillary: 1) circumferential wall tension caused by transmural pressure, 2) surface tension of the alveolar lining layer, and 3) tension of the tissue elements in the alveolar wall (80). It is possible that during hypothermic storage of lungs the threshold of the lung to withstand these stresses is weakened (81). The results of these stresses are seen at the time of reperfusion. There is a weakening of the endothelial cell attachment to the basement membrane, as well as a corresponding thinning and an increase in surface to
volume ratio (82). Breaks in the endothelial cell to cell tight junctions are rarely seen (81, 82).

**Epithelial cells**

Due to the relatively high permeability of the endothelial cell junction, during preservation the epithelial cells are also exposed to pulmonary preservation fluid and furthermore are susceptible to cellular injury. The functions of Type II alveolar epithelial cells are four-fold: 1) they synthesis and secrete surfactant 2) they maintain the alveolar epithelium by differentiating into Type I cells 3) they are involved in ion transport from the apical to the basolateral surface to minimize alveolar fluid 4) they can participate in host defense and inflammatory responses by producing cytokines and chemokines (Table 2) (83). Alterations in surfactant are known to contribute to I/R injury (84-87). This in combination with the fact that Type II cells differentiate into Type I cells make the maintenance of their integrity during lung preservation and reperfusion crucial to upholding optimal lung function. During lung preservation, epithelial cells show cytoplasmic vacuolation, chromatin clumping and cellular edema (88). Furthermore, mitochondrial swelling is also evident (89). Upon reperfusion, epithelium cell blebbing is evident as edema fluid lifts the cells from their attachment to the basement membrane. This is likely due to the extremely tight cell to cell junctions between epithelial cells. Rather than letting fluid move between intracellular junctions, the fluid is forced to distend the cells resulting in epithelial blebs (81). The rupturing of these epithelial blebs accounts for the alveolar edema seen in injured lungs at the time of reperfusion.
Clearly, the maintenance of cell viability during ischemia and reperfusion is crucial to maintaining the overall health of the lung to ensure optimal gas exchange during the early stages of reperfusion.
Table 2: Primary Functions of Type II Cells

- Synthesis of surfactant
- Maintain alveolar epithelium by differentiating into type I cells
- Transepithelial sodium transport to minimize alveolar fluid
- Host defense: production of cytokines and chemokines
CELL CULTURE MODELS OF ISCHEMIA-REPERFUSION INJURY

Cell culture models can provide a powerful tool to study the molecular and cellular mechanisms associated with I/R injury. The complexities of animal models in lung transplantation make the delineation of contributing factors difficult. Immune mediated factors, physical factors and chemical factors all play integral roles in the pathogenesis of I/R injury (22, 59). Cell culture models allow the control of confounding factors and permits study on a particular aspect of I/R that is not possible in animal models. While many models exist to study I/R injury none embrace the tenets of lung preservation, which are aerobic hypothermic preservation followed by reperfusion. Furthermore, many of the I/R models in use do not employ lung specific cells.

General I/R Cell Culture Models

Ischemia in all organs for transplantation, except the lung, is hypothermic and anaerobic (15). Usually ischemic injury of an organ, such as myocardial infarction, is normothermic and anaerobic. There are many cell culture models that are currently used to mimic this clinical situation. For example, there are models that employed cardiomyocytes in the setting of normothermic anaerobic ischemia induced by hypoxic Phosphate Buffered Saline Glucose (PBSG) and reperfusion using PBS at 5% CO₂ and 95%. (90, 91). This varied only slightly from protocols that used different concentrations of O₂ and CO₂ at the time of reperfusion (92). Similar models are also employed where ischemia was induced on endothelial cells maintained under normothermia and hypoxia, which was maintained by 95% N₂/5%CO₂. In this system, reperfusion consisted of the
reintroduction of oxygenated culture medium at 37°C (93, 94). Furthermore, ischemia induced by Potassium Cyanide (KCN) and subsequent washout of KCN to simulate reperfusion has also been used for cell cultures modeling I/R injury in the liver transplant setting (95).

These models provide an excellent platform by which to study I/R injury in vitro. They have established important concepts such as the ability of endothelial cells to generate free radicals and the role of lipid peroxidation in I/R injury (93, 94). Furthermore it was shown that preconditioning of cardiomyocytes can potentially ameliorate infarct related injury of the heart (91). Also the cytoprotective effects of glucose and acidosis during simulated liver ischemia have been elucidated (95). These models have proven to be powerful tools in studying I/R related cellular changes, however, it is difficult to reconcile the use of these models to mimic I/R injury in lung transplantation. Firstly, these models do not employ lung specific cells (e.g. human or rat cardiomyocytes or endothelial cells from rabbit aorta) (90, 91, 93). Furthermore, ischemia during lung preservation is aerobic and none of these models employ aerobic ischemia. Essentially they are hypoxia-reoxygenation models in the presence or absence of serum containing culture medium at 37°C.

**Lung Transplantation Specific Cell Culture Models of I/R Injury**

Cell culture has also been used to study the preservation of various lung cell types in vitro. In two separate studies Spaggiari and coworkers investigated preservation of human lung fibroblasts. In both studies cells were preserved in various different pulmonary preservation solutions at 10°C. There was no mention of O₂ concentrations
during the preservation period, and one would assume ambient air was used. Cell viability was determined by total protein and by protein synthesis rate and these studies explored the efficacy of extracellular based solutions as well as the effects of different concentrations and molecular sizes of dextran as an additive to lung preservation solution (96, 97).

Extensive work has been performed using endothelial cells in culture. In these studies the source of cells varied from human saphenous vein (98), human umbilical vein (99), porcine pulmonary artery (100, 101), bovine aorta (102) human pulmonary artery cell lines (103) and murine cell lines (104). The experimental designs of these studies did not closely simulate the conditions of lung transplantation. Some of the studies employed only hypothermic preservation (4°C-10°C) with ambient air preservation without a phase of simulated reperfusion (102-104). The studies that did employ a reperfusion phase used preservation conditions that were not appropriate to lung preservation in that they used ambient air during the preservation period. Furthermore, some investigators used 5%CO₂ and 95% room air during the reperfusion phase (98, 100, 101). This is not synchronous with the clinical situation where patients are generally maintained on 100% O₂ ventilation during the reperfusion period (6).

Despite their limitations in design, these models have provided a number of excellent insights into I/R related cellular alterations. These include the loss of actin stress fibers and the depolymerization of microtubules during cold preservation and their recovery following simulated reperfusion (100, 101). These alterations would be extremely difficult to detect using tissues from animal models.
The use of epithelial cells in cell culture models of I/R injury has not been explored extensively. Hachida and coworkers studied the role of pulmonary preservation fluids on primary cultured rat type II alveolar cells. This study however used warm preservation with 7% CO₂ to simulate ischemia (105). Another study performed by Maccherini and coworkers also employed primary cultured rat type II cells. Cells were preserved hypothermically (5°C) however, the O₂ concentration in that study was ambient (106). Neither study employed a simulated reperfusion phase.

Cell culture is a powerful tool for the study of I/R induced cellular changes in the lung transplant setting. However, no models specifically mimic the lung preservation and reperfusion setting.

OBJECTIVES

The objective of this project was to design a cell culture model that mimics the aerobic and hypothermic nature of lung preservation, as well as simulated reperfusion. Furthermore, this model was validated to ensure that cellular changes in this setting are congruous with results that have been yielded in the clinical and experimental work performed in lung transplantation. Moreover, factors such as O₂ tensions and CO₂ tensions during preservation as well as chemical additives to pulmonary preservation solutions were evaluated.
MATERIALS AND METHODS
MATERIALS AND METHODS

Rationale for the cell culture model to mimic I/R injury in lung transplantation

Currently cell culture models for I/R injury do not closely mimic the process of lung preservation. The model that we developed in the present studies closely mimics the protocol of human lung transplantation (Fig. 1). Cells are maintained in standard culture medium supplemented with 10% fetal bovine serum. To simulate the pulmonary artery flush portion of the lung procurement process, culture medium is removed and replaced with cold (4°C) pulmonary flush solution, LPDG. Preservation is attained by using isobaric 100% O₂ in a closed atmosphere for varying periods of time. Reperfusion is simulated by the removal of LPDG and the reintroduction of warm (37°C) serum-containing culture medium while maintaining 100% O₂ in a flow through situation (Fig. 2).

In clinical lung transplantation, prolonged organ ischemia results in poor lung function. Recently in our laboratory we have shown that the amount of cell death during ischemia correlates with lung function post transplantation. That is, prolonged ischemic times have resulted in more cell death and decreased organ function, during the acute phase of reperfusion (79). Moreover, animal models have shown that reperfusion exacerbates cell death following reperfusion (78). Therefore, we will validate this novel model by showing an ischemic time dependant increase in cell death. We will further validate it by showing that reperfusion further exacerbates cell death.
Cell culture

A549 is a human pulmonary epithelial cell line of Type II origin. It was derived from a human alveolar carcinoma and was used a lung specific cell in these studies (American Type Culture Collection, Rockville, MD). Cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Life Technologies, Grand Island, NY), containing gentamicin and supplemented with 10% fetal bovine serum, which is referred to as D10. Cells were maintained in T75 flasks (Nunc, Naperville, IL) in a humidified atmosphere at 37°C and 5% CO2/95% room air. Cells were sub-cultured using enzymatic digestion with 0.25% trypsin (Gibco) and 1 mM EDTA (Sigma, St. Louis, MO) when cells were approximately 80% confluent.

A simulated I/R injury model system

A549 cells (5x10^5 cells/well) were plated in 24-well plates (Corning Costar, Cambridge, MA) and maintained in 1 ml of D10 in a humidified atmosphere at 5% CO2/95% room air and 37°C for 18 h to allow cell attachment to the culture plate. Simulated cellular ischemia (referred to as ischemia hereafter) was achieved by the removal of D10 and introduction of 1 ml of cold (4°C) preservation solution. The cells were then placed into a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA) which was filled with 100% O2, or other gases as specified in the particular study group, and sealed. The chamber was then stored at 4°C for ischemic times of 6, 12, 18 and 24 h. Simulated reperfusion (referred to as reperfusion hereafter) was performed by the removal of the preservation solution and the re-introduction of D10 at 37°C. The cells were then constantly bathed with 100% O2 at 37°C for a period of 2 h (Fig. 2).
Figure 2: A cell culture model that mimics ischemia-reperfusion in lung transplantation. (a) Human lung epithelial cells (A549) were seeded and allowed to acclimate to standard culture conditions for 18 h at 37°C, 5% CO₂ in D10. (b) The D10 was removed and replaced with cold (4°C) LPDG lung preservation solution. (c) The cells were then placed into a modified incubator that was filled with 100% O₂ which was placed at 4°C for various periods of time simulating ischemia. (d) The LPDG was replaced with D10, placed in a 37°C incubator with 100% O₂ constant flow for 2 h to simulate reperfusion.
Cells were examined either 1) before hypothermic preservation as time = 0 controls, 2) after the simulated ischemia period, 3) after the 2 h simulated reperfusion period, or, 4) after a period of time that equals the ischemic time plus the reperfusion time while cells were maintained at 5% CO₂/95% room air and 37°C.

**Evaluation of cell attachment**

Cell attachment was quantified using a Coulter counter (Coulter Electronics, Hialeah, FA). This serves as an indirect assessment of cell viability because while in culture, when A549 cells die they detach from the culture plate. At each evaluation point, the solution covering the cells was removed and the cells in each well were gently washed once with 1 ml of PBS. The PBS was then removed and 1 ml PBS with 0.25% trypsin and 1 mM EDTA was added to each well. The cells were then incubated for 5 min at 37°C to detach the cells. An aliquot of cell suspension was added to a Dilu-Vial (VWR Scientific, Mississauga, ON) containing 10 ml of PBS. The cell number was then quantified using a Coulter Counter. This was then converted to a total cell number per well. Each well was sampled twice and each vial was quantified in duplicate. Each experiment was performed at least in triplicate, with four cell wells used for each treatment in the experiment.

**Fluorescein Diacetate - Propidium Iodide staining**

In order to determine the viability of attached and detached cells, Fluorescein Diacetate - Propidium Iodide (FDA-PI) cell viability staining was used. Following each evaluation point, the solutions covering the cells from four wells under identical treatment were pooled in a 15 ml tube and centrifuged at 2,000 rpm for 5 min. The
Figure 3: Timeline and sampling points of experimental groups. A549 cells were plated and allowed to acclimate to standard culture conditions for 16 h. (A) Sample taken as Time=0 control. Cells were then exposed to ischemia for 6, 12, 18 and 24 h under various conditions. (B) Sample is taken as end ischemia. Reperfusion is then initiated for a 2 h period using D10 and 100% O2. (C) Sample is taken as end reperfusion. (D) Sample taken as the post I/R control that represents cells maintained at standard culture conditions, (37°C, 5%CO2/95% Room air) in their respective preservation solutions for a period of time that equals their ischemic plus 2 h to account for the reperfusion. For example for an ischemic of 24 h, the post I/R control would be 24h + 2h = 26h.
supernatant was aspirated and discarded, and the pellet was resuspended in 100 µl of Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco). Stock solutions of FDA (5 mg/ml in acetone) and PI (0.02 mg/ml in DPBS) were stored at 4°C in the dark. Staining was achieved by the addition of a final solution containing 2 µg of FDA and 0.6 µg of PI to the cell suspension. The solution was then gently mixed and allowed to stand for 3 min. The stained suspension was then mounted on a hemocytometer and 4 fields were quantified under 100x magnification for a percentage of living cells. The viability of cells was examined with a fluorescent microscope using 520 nm and 590 nm filters. Viable cells fluoresced green, while non-viable cells were red. Attached cells were assessed in a similar way except the quantification was performed in situ with the cells still attached to the culture plate. Four microscope fields were assessed and a percentage of living cells were ascertained using the formula: # of living cells/ total number of cells. Each experiment was repeated in triplicate.

**Preservation solutions**

In the initial studies, Low Potassium Dextran Glucose (LPDG) (Biophausia, Uppsala, Sweden), was used as the preservation solution to be tested. Regular cell culture medium DMEM and D10 were also used for comparison. In subsequent experiments, to determine the effects of specific components, namely glucose, dextran 40 and Ca²⁺ in these solutions, several preparations were made with varying chemical compositions. The following modifications were made to the preservation solutions that were tested: D10 was supplemented with 20 g/l of dextran 40 (Sigma), to examine the
Table 3: Composition of preservation solutions.

<table>
<thead>
<tr>
<th></th>
<th>LPDG</th>
<th>LPGD+C(^{2+})</th>
<th>LPD</th>
<th>LPG</th>
<th>DMEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^{+})</td>
<td>139</td>
<td>145</td>
<td>134</td>
<td>132</td>
<td>110</td>
</tr>
<tr>
<td>K(^{+})</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5.3</td>
</tr>
<tr>
<td>Cl(^{-})</td>
<td>138</td>
<td>144</td>
<td>126</td>
<td>125</td>
<td>110</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>----</td>
<td>5.0</td>
<td>----</td>
<td>----</td>
<td>1.8</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>HCO(_{3})^{−}</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>33</td>
</tr>
<tr>
<td>PO(_{4})^{2−}</td>
<td>0.8</td>
<td>0.9</td>
<td>0.7</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
<td>5.0</td>
<td>----</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Dextran 40 (g/l)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>295</td>
<td>321</td>
<td>271</td>
<td>269</td>
<td>316</td>
</tr>
</tbody>
</table>

All the values were measured from prepared solutions. Selected chemical components are listed for comparison (mM). D10 is prepared by mixing 10% fetal bovine serum with DMEM.
effect of dextran 40; low potassium dextran (LPD) solution (i.e. no glucose); low potassium glucose (LPG) solution (i.e. no dextran 40) and LPDG solution with 5 mM of Ca$^{2+}$, to examine the contribution of Ca$^{2+}$. The clinical laboratories at the Toronto General Hospital confirmed the actual concentrations of the solutions; these are listed in Table 3. All preservation solutions were exposed to ischemia for varying periods of time at 4°C and 100% O$_2$ preservation gas followed by 2 h of reperfusion.

**Preservation Gases**

The concentrations of the preservation gas used were 1) 100% O$_2$ 2) 95% O$_2$ with 5% CO$_2$, 100% room air and 95% room air with 5% CO$_2$. The gas composition of the modified incubator was checked before and after simulated ischemia to assure there was no gas leaking. All preservation gases were tested using a preservation period of 24 h at 4°C in LPDG followed by 2 h of reperfusion. Standard culture media D10 and DMEM were also used to preserve cells but only at 4°C with 100% O$_2$, or with 95% O$_2$ plus 5% CO$_2$, followed by reperfusion.

**Measurement of pH**

Measurement of pH was performed on preservation solutions following ischemia using a CIBA-Corning 278 Blood Gas System (CHIRON Inc., Markham, ON.).

**Statistical Analysis**

Statistical analysis was performed using SigmaStat 3.0 (Jandel Scientific, San Rafael CA). A two-way ANOVA was used followed by Student-Newman Keuls *post hoc*
testing for all the time course studies of cell detachment. A one-way ANOVA was used for the comparisons of dextran 40, calcium and preservation gases. P values less than 0.05 were considered significant. All data are shown as the mean ± Standard Error (SE) from a minimum of 3 separate experiments.
RESULTS
RESULTS

Aerobic hypothermic ischemia and reperfusion induce cell detachment

A549 cells exposed to 100% O₂ and preserved at 4°C in LPDG preservation solution (ischemia) showed a decrease in the number of cells that remained attached to the cell culture plate after 12 h ischemia (Fig. 4a). Following this, the amount of cell attachment leveled off. Reperfusion further reduced cell attachment as there was significantly (p<0.05) less cell attachment after 24 h ischemia in LPDG and 2 h reperfusion, than after ischemia (24 h) only (Fig. 4b). Also there was significantly (p<0.05) more cell attachment after 6 h ischemia followed by reperfusion compared to 24 h ischemia and reperfusion using LPDG as the preservation solution (Fig. 4b). This suggests that cell attachment after ischemia and reperfusion decreases in a manner dependent on ischemic time. Furthermore, reperfusion exacerbated cell detachment compared to ischemia only.

In order to determine the efficacy of LPDG, it was compared to the standard culture media DMEM and D10. Cells exposed to ischemia alone in LPDG had significantly (p<0.05) more cell attachment at 18 h and 24 h compared to D10 and DMEM at the same time points (Fig. 4a). This difference persisted after reperfusion.

Cell attachment was also assessed on cells that were kept in LPDG, DMEM or D10 but maintained at 5% CO₂/95% room air and 37°C (standard culture conditions) for periods of time equaling their ischemic time plus 2 h to account for reperfusion time (i.e. for 24 h ischemia, 24 h+2 h=26 h). The cell attachment remained similar in all groups except during the evaluation of the 26 h control where there was significantly (P<0.05) greater cell attachment in the D10 group versus the LPDG group (Fig. 4c).
Figure 4: Aerobic hypothermic ischemia and simulated reperfusion decreased cell attachment in a time dependent manner. Cells were treated as described in Figure 2. Standard culture media DMEM and D10 were used for comparison to LPDG for various periods of ischemia and reperfusion. Cell attachment is expressed as a percentage of the control (time=0) of each group. (A) Cells exposed to ischemia (6, 12, 18 and 24 h) at 4°C and 100% O₂ and preserved in LPDG, DMEM and D10. # p<0.05 LPDG vs. D10 and DMEM. (B) Cell attachment following reperfusion (2 h) after varying periods of ischemia (4 °C, 100% O₂). # p<0.05 for LPDG vs. DMEM and D10. * p<0.05 vs. 24 h reperfusion in LPDG. † p<0.05 vs. 24 h ischemia only in LPDG. (C) Standard culture controls maintained at 5%CO₂/95% room air and 37°C for periods of time equaling their ischemic time plus 2 h to account for reperfusion (i.e.for 24 h of ischemia, 24 h+2 h=26 h). ‡ p<0.05 D10 vs. LPDG. Data are mean ±SE for a minimum of 3 separate experiments.
Cell attachment provides an indirect quantification of cell viability

Cells were stained using FDA/PI staining to evaluate cell viability. Cells stained green by FDA/PI staining in situ are alive (Fig. 5a) while cells stained red by Propidium Iodide are dead (Fig. 5b). Cultures were assayed for viability on both cells that were attached to the culture plate and those that were detached. The viability of attached cells preserved for 24 h, 100% O₂ and 4°C followed by reperfusion was 99% for cells preserved in LPDG, whereas viability decreased to 32% and 18% when preservation was in DMEM or D10. For the detached cells receiving the same treatments the viability was 18%, 5% and <1% for LPDG, DMEM and D10, respectively (Fig. 6). These data confirm that cell attachment is an indirect indicator of cell viability. In other words, the cell detachment due to ischemia and reperfusion was mainly due to cell death.

Effects of chemical components on cell viability

We sought to explore the reason behind the dramatic increase in cell death observed in the cells preserved in D10 and DMEM, compared to LPDG, after prolonged preservation. This may provide useful information about factors that affect cell viability during preservation and reperfusion. The major chemical differences between DMEM and LPDG are that DMEM contains Ca²⁺ and LPDG contains dextran 40 (Table 3). Preparations of D10 containing 20 g/L of dextran 40 were therefore studied to determine the effect of dextran on cell viability. This addition, however, did not provide significantly better cellular protection, in terms of cell attachment, than D10 alone after 24 h ischemia or after ischemia and reperfusion (Fig. 7). Also, LPDG was prepared with
Figure 5: Cell viability as determined by fluorescein-diacetate/propidium iodide (FDA/PI) staining. A549 cells were stained in situ with FDA/PI and photographed under a fluorescent microscope using different filters (100x). Alive cells stained green (a) while dead cells (b) under the same field stained red. This is a representative field exhibiting the methodology.
Figure 6: Cell attachment provided an indirect representation of cell viability. Cells were exposed to 24 h of simulated ischemia at 4 °C in 100% O₂ in D10, DMEM or LPDG followed by 2 h reperfusion. Attached and detached cells were collected separately and then double stained with FDA/PI for viability. The viability of attached cells resembles the cell attachment data for the same conditions. Data are mean ± S.E. from 3 separate experiments.
Figure 7: Dextran did not confer better cellular preservation when added to D10 while adding Ca^{++} in LPGD significantly increased cell viability after reperfusion. Cells were preserved in D10 supplemented with dextran (20g/l) or LPGD supplemented with Ca^{++} (5 mM) for 24 h at 4 °C in 100% O_2 and then reperfused with D10. The presence of Ca^{++} in LPGD significantly increased cell viability after reperfusion, as determined by attachment. # p<0.05 for LPGD + Ca^{++} vs. LPGD. Data are mean ± S.E. from 3 separate experiments.
Figure 8: The removal of glucose or dextran from LPDG solution did not significantly affect cell viability. LPDG solutions without glucose (LPD) or dextran (LPG) were prepared. A549 cells were exposed to 24 h ischemia at 4 °C in 100% O₂ in one of the three preservation solutions followed by 2 h reperfusion. Cultures were quantified for cell attachment as an estimate of viability. Data are mean ± S.E. from 4 separate experiments.
5 mM of Ca$^{2+}$, and compared to LPDG alone (Fig. 7). There was no difference in cell attachment after 24 hours of simulated ischemia. However, after reperfusion of cells preserved in LPDG with 5 mM Ca$^{2+}$, cell attachment was significantly better (P<0.05) versus LPDG alone (Fig. 7).

Preparations of low potassium dextran (LPD) and low potassium glucose (LPG) solutions were tested against LPDG to determine the contributions of glucose and dextran respectively, in cell preservation (Table 3). After 24 h at 100% O$_2$ and 4°C (simulated ischemia) there was no significant change in the percentage of cells attached to the culture plate (Fig. 8). There was also no significant difference in cell attachment after ischemia and 2 h simulated reperfusion. These data were positively correlated with cell viability staining (data not shown), where the majority of cells attached to the culture plate were alive, and the majority of detached cells were dead.

**Preservation gas mixture has a marked effect on cell viability**

To further examine the inadequate cellular preservation provided by D10 and DMEM, as well as the effect of various concentrations of O$_2$ and CO$_2$ in gas mixtures during preservation, special gas preparations were made. Cells exposed to ischemic conditions at 4°C for 24 h in D10 or DMEM with 95% O$_2$/5% CO$_2$ preservation gas had significantly (p<0.05) more cell attachment than did cells preserved in the same solutions using 100% O$_2$ preservation gas (Fig. 9). A similar, significant (p<0.05) increase in cell attachment was seen after simulated reperfusion in the D10 and DMEM groups. Furthermore, the presence of 5% CO$_2$ in the preservation gas, when using DMEM or D10 provided equivalent cellular preservation to LPDG using 100% O$_2$ preservation gas, as
Figure 9: The presence of 5% CO₂ during ischemia significantly increased cellular attachment after ischemia and after reperfusion for cells preserved in D10 or DMEM. A549 cells were preserved for 24 h at 4°C and 95% O₂/5% CO₂ in D10, DMEM and LDPG followed by reperfusion. In the D10 and DMEM groups there was significantly superior cellular preservation, as defined by cell attachment vs. ischemia using 100% O₂. Furthermore, preservation in D10 and DMEM with 95% O₂/5% CO₂ preservation gas was equivalent with LDPG using 100% O₂ preservation gas. * p<0.05 compared with 100% O₂ preservation gas. # p<0.05 vs. reperfusion following ischemia in LDPG using 100% O₂ preservation gas. Data are mean ± S.E. from 3 separate experiments.
Figure 10: Varying concentrations of O₂ and CO₂ in the preservation gas provided equivalent cellular preservation after 24 h at 4 °C in LPDG preservation solution. Cells underwent simulated ischemia at 4 °C for 24 h in LPDG with varying concentrations of preservation gases: 100% O₂, 95% O₂/5% CO₂, 100% room air and 95% room air/5% CO₂ followed by simulated reperfusion. No significant differences in cell viability, as defined by cell attachment, were seen between preservation groups. # p<0.05 vs. simulated reperfusion in 100% O₂. Data are mean ± S.E. from 3 separate experiments.
determined by cell attachment (Fig. 9). These results were confirmed by FDA/PI staining for cell viability (data not shown). Cells preserved in LPDG were similarly studied after 24 h of ischemia with a variety of preservation gas concentrations. No significant differences between groups were observed, with respect to cell attachment, after either ischemia or reperfusion (Fig. 10). However, there was a significant decrease (p<0.05) in cell number, post reperfusion, for cells preserved in 100% O₂. This decrease was not seen in cells preserved for 24 h in LPDG and exposed to 95% O₂/5% CO₂, 100% room air, or 100% room air and 5% CO₂.
DISCUSSION
We have developed a novel cell culture model that simulates the clinical process of lung transplantation. Most cellular models for I/R injury are not suitable for lung transplantation-related studies, because of the differences in ischemic conditions between transplantation and other clinical situations as well as between lungs and other organs preserved for transplantation. These differences are outlined in the introduction section. For example, in I/R injury triggered by stroke or myocardial infarction, ischemia is hypoxic and occurs at body temperature while the reperfusion phase is characterized by the reintroduction of oxygenated blood. In transplantation, all organs are maintained without blood flow under hypothermic conditions. However, the lungs are inflated with O₂ and are able to maintain aerobic metabolism during ischemia while other donor organs must undergo anaerobic metabolism (19). To our knowledge, the model described in the present study is the first cell culture model to combine aerobic, hypothermic ischemia with oxygenated warm reperfusion, a protocol closely mimicking the current clinical practice of lung preservation and transplantation.

Critique of the model

The use of cell culture as a tool to study pathological changes associated with I/R injury is extremely useful as the extracellular conditions can be easily controlled compared to in vivo studies (107). However, the simplicity of this model obviates factors that are present in vivo. This model excludes the contribution of blood borne factors that are present in I/R injury clinically and in animal models. The role of leukocytes has been
defined in injurious pathways. They are responsible for the production of cytokines such as TNFα, INFγ and IL-2, all of which contribute to the inflammation process associated with I/R injury (68, 108). Furthermore, the activation of complement has been implicated in the pathogenesis of I/R injury (73). Moreover, neutrophil generated reactive oxygen species are known to contribute to the morbidity associated with I/R injury (63). These factors, which are present in blood, are not present in this cell culture model. However, this model will allow the addition of exogenous cytokines or complement to elucidate the contributions of these factors to the pathogenesis of I/R injury. Furthermore, a co-culture system can be further developed based on this model to determine the contributions of different cell types to I/R injury.

Human organ allo-transplantation also involves activation of the immune system. Lung allo-transplantation is associated with the upregulation of MHC molecules and this response may serve to contribute to the injurious effects of inflammatory cytokines (108). The upregulation of MHC has been associated with particularly severe I/R injury and may correlate with increased incidences of allograft rejection (61). While a potential weakness, this can also be viewed as strength of this model because the confounding factors that the immune system present in I/R injury are eliminated.

As mentioned the importance of using type II cells lies in the importance of that cell type in the overall setting of the lung. While type II cells only constitute 10-20% of the alveolar epithelial cells in the lung they differentiate into type I cells as well as produce surfactant (83). The maintenance of surfactant in the lung during the transplantation procedure has been shown to be extremely important with respect to lung function post transplantation (84, 86, 87). In our studies we employed A549, a human
epithelial cell line of type II origin. The cells were originally procured from the tissue of a human alveolar carcinoma. They maintain a human karyotype and contain multilamellar bodies, typical of type II cells (109). The cells also synthesise pulmonary surfactant phospholipids (110). Furthermore, the cell line has been used in other investigations as a representative cell to study drug interactions (111). A transformed cell line, such as A549, is not perfect for mimicking biological systems because the genetic mutations in the cell that allow it constantly propagate do not represent typical non-cancerous cells. However, A549 cells seem to remain stable for up to 1000 generations and are easily maintained in the laboratory (109). The use of primary cultured type II cells from rats would be another option, however, maintaining homogeneity, adequate cell yields and the complexity of the cell extraction make the use of a primary culture for type II cells difficult (112).

While the model we used does not include blood borne or immune factors it does represent a model for the analysis of I/R injury in lung transplantation. I/R injury is extremely complex. With further improvement, this model allows for the control of various components of I/R injury such as shear stress, mechanical ventilation, immune factors, cytokines and leukocytes to determine which aspect is contributing in what capacity to produce I/R injury. Controlling these factors is extremely difficult in animal models and impossible in clinical lung transplantation. However, these factors can be used as an adjunct to the existing model to determine their contributions.

Furthermore, the model we employed is very simple, inexpensive and rapid to perform. This makes it an ideal model to test hypotheses before proceeding to more expensive, laborious animal models.
Validation of the model

In this model, with a short period of ischemia (6 h), 4°C and 100% O₂, followed by reperfusion, it is important to note that there was equivalent and minimal cell detachment in D10, DMEM or LPDG preservation groups (Fig 4a, 4b). However, as preservation times were prolonged, cell viability was decreased in all groups, especially in D10 and DMEM groups after ischemia or reperfusion. This phenomenon has been shown in animal models. Prolonged ischemic preservation results in increased cell death which is correlated with a decrease in lung function (79). Furthermore, viability was further reduced after reperfusion (p<0.05), in the LPDG group preserved for 24 h at 4°C in 100% O₂. This is similar to what has been noted in animal studies, where reperfusion itself exacerbates the injury after ischemia [Serrick, 1996 #12]. Also, these results simulate the current lung transplantation practice where short preservation times, in contrast to prolonged preservation times, yield adequate lung function after transplantation (26).

EFFECTS OF CHEMICAL COMPONENTS

As the preservation time was extended to 18 h and 24 h, LPDG afforded significantly better cell viability than did either DMEM or D10 after ischemia or reperfusion (Fig. 4a, 4b). In an effort to delineate the mechanisms by which LPDG provides superior cell preservation compared to standard culture media (D10 or DMEM), we attempted to determine which chemical component is critical for cell viability.
Effect of glucose

Date and coworkers have shown that glucose plays a protective role in lung preservation, as determined by arterial PO$_2$ post reperfusion (19). Glucose has also shown to be beneficial when used in conjunction with oxygenated cardioplegia in an isolated perfused rat heart model (113). In this model, there was no significant difference in cell viability after preservation or reperfusion in LPDG compared to LPD (Fig. 7). This suggests, that the beneficial effect conferred by glucose is not through direct cytoprotection.

Effect of dextran 40

Dextran 40 has also been shown to confer superior oxygenation post-reperfusion in a dog model of lung transplantation (33). However, in the present study, subtraction of dextran 40 from LPDG solution did not make a significant difference on cell attachment after ischemia or reperfusion (Fig. 8). Also, dextran 40 as an additive to the standard culture medium, D10, did not confer any additional cellular protection after ischemia or reperfusion (Fig. 7). In a hypothermic cell preservation model, the use of dextran 40 in LPD also did not improve cell viability of fibroblasts compared to low potassium solution alone (97). In animal models physiological assessments are performed as primary endpoints. The proposed protection mechanism of dextran 40 is to improve microvascular flow in the capillaries of the lung and to decrease of micro-thrombi at the time of reperfusion (33). These physiological endpoints were not examined in this study. However, our results suggest that the beneficial effects dextran 40, seen in vivo, are not through direct cytoprotection.
Effect of calcium

The addition of 5 mM Ca\textsuperscript{++} to LPDG provided better cellular protection (p<0.05) after 24 h of ischemia and 2 h of reperfusion (Fig. 7). However, calcium has been identified as an injurious agent in ischemia of the heart and liver (\textsuperscript{114}). Furthermore, the use of a calcium channel blocker has been shown to be protective in I/R injury of the lung (\textsuperscript{43}), and, disruptions in calcium homeostasis are known to be a causative factor in cell membrane damage during ischemic kidney injury (\textsuperscript{115}). Typically, this is manifest as a massive calcium overload inside the cell (\textsuperscript{116}). In our study, there was no significant difference in cell viability for cells undergoing preservation in LPDG vs. LPDG+Ca\textsuperscript{++}. However, the cell viability was significantly higher after reperfusion for cells preserved in LPDG+Ca\textsuperscript{++}. In light of the evidence that implicates calcium as an injurious agent in ischemic injury this result seems paradoxical. However, Ca\textsuperscript{++} deprivation followed by the reintroduction of calcium containing media, such as D10 used in this study, may result in an increased permeability to calcium and a massive Ca\textsuperscript{++} influx, resulting in cell death. This is known as the “calcium paradox” (\textsuperscript{117}). It is possible that adding a small amount of Ca\textsuperscript{++} to the preservation solution avoided the cell damage associated with the reintroduction of calcium.
1. NORMAL

\[ [\text{Ca}^{++}] = 10^{-3} \text{ M} \]

\[ [\text{Ca}^{++}] = 10^{-7} \text{ M} \]

2. \text{Ca}^{++} \text{ DEPLETION}

\[ [\text{Ca}^{++}] = 10^{-5} \text{ M} \]

\[ [\text{Ca}^{++}] = 10^{-7} \text{ M} \]

3. \text{Ca}^{++} \text{ REINTRODUCTION}

\[ [\text{Ca}^{++}] \]

\[ [\text{Ca}^{++}] \]

**Figure 11: The calcium paradox.** (Panel 1) The normal physiological calcium concentration of \(10^{-7} \text{ M}\) inside the cell and \(10^{-3} \text{ M}\) outside the cell. (Panel 2) Depicts the removal of calcium from the medium bathing the cells. (Panel 3) Following calcium depletion, the reintroduction of calcium to the medium bathing the cells results in a massive influx of calcium into the cell. Subsequently, this can induce cell injury or death.
EFFEKT OF pH

Interestingly, using 95% O₂/5% CO₂ significantly protected cells in both DMEM and D10 groups after ischemia or reperfusion (Fig. 9). In fact, the cell attachment and viability became equivalent to that of the LPDG group, preserved at 100% O₂. While the mechanism for the poor preservation seen in the culture media using 100% O₂ is unknown, CO₂ may be necessary to provide adequate buffering for the culture media. Standard culture medium, DMEM, uses bicarbonate as the buffering system while LPDG uses phosphate. When maintained at 4°C with 100% O₂ for 24 h, the pH of D10 and DMEM rises into the basic range (7.8), while the pH of the LPDG solution falls into the acidic range (6.9). The presence of 5% CO₂ in the preservation gas maintained the pH of D10 and DMEM in the slightly acidic range (7.2). Although the pH of LPDG solution dropped below the physiological range, previous studies have shown that extracellular acidosis provides cytoprotection for cells in a variety of injury models including I/R injury (95, 118-121). Furthermore, it has been shown that oxygenation of bicarbonate buffered St. Thomas cardioplegia solution, using 100% O₂ resulted in the solution becoming alkalotic because H⁺ ions produced were consumed to balance the CO₂ concentration according to the following equation (122).

\[ \text{H}_2\text{O} + \text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \]

Moreover, when the cardioplegia solution was bubbled with 95% O₂/5%CO₂ it became acidotic. This acidotic solution conferred superior postischemic functional recovery
Therefore, it seems that CO₂ is necessary for prolonged organ preservation if the preservation solution used depends on bicarbonate for buffering.

The mechanisms by which alkalosis is cytotoxic are unknown. However, a high extracellular pH will tend to draw protons from the inside of the cell to the outside. This will tend to increase the respiration rate of the mitochondria in an effort to re-establish the proton gradient. This increased rate of respiration will use more oxygen and in turn produce injurious oxygen free radicals. This increase in free radicals will over saturate the superoxide dismutase (SOD) system and result in mitochondrial damage. In that study, cells that over expressed SOD counteracted these effects. At alkaline pH mitochondrial structural and DNA damage also occurred (124).

Furthermore, the Na⁺/H⁺ exchanger protein can perform the extrusion of H⁺ ions from the cell during alkalosis. This will result in an increase in cytosolic Na⁺ which can cause Ca²⁺ to enter the cell via the Na⁺/Ca²⁺ exchanger resulting in calcium mediated cell death. Inhibitors of Na⁺/H⁺ exchange have been used successfully to ameliorate both lung I/R injury as well as transient intracellular Ca²⁺ elevation associated with Na⁺ extrusion (121, 125). Our results suggest that alkalosis is indeed harmful to the cells and perhaps the mechanism of cytotoxicity is through one of these mechanisms or a combination of both.

For the LPDG group stored at 100% O₂, acidosis appeared to be protective. Acidosis inhibits the activity of the Na⁺/H⁺ exchanger protein (121). Therefore it may afford cytoprotection in this system by not allowing Na⁺ entry, which in turn will not allow Ca²⁺ entry, which can result in cell death, or water entry that will result in cell swelling. Furthermore, acidosis during ischemia can inhibit various phospholipases that
are activated at physiologic and alkalotic pH. These phospholipases have been implicated in cell membrane damage that is seen during ischemia (126). Cellular acidosis may also effectively reduce the activity of intracellular enzymes, by altering the dissociation of enzyme amino acids. This may aid the cell in slowing down metabolic reactions, something that organ preservation is dependent on.
Figure 12: The cytotoxic effects of alkalosis. (A) Under normal physiological conditions the mitochondrion produce an $H^+$ gradient in order to produce cellular ATP. (B) Under alkalotic conditions, as pictured, the $H^+$ ions are transported from the mitochondrial membrane to the exterior of the cell via the $Na^+/H^+$ antiporter. (C) $Na^+$ enters via the same antiport. The extrusion of $H^+$ ions effectively depolarizes the mitochondrial membrane. In an effort to reestablish the $H^+$ gradient the mitochondrial respiration rate increases. This produces oxygen free radicals (not shown) that can overwhelm the Super Oxide Dismutase system.
EFFECT OF PRESERVATION GAS

Reactive oxygen species during lung transplantation provide a source of cellular injury (63). This could be a result of the hyperoxic conditions during lung preservation, as well as the use of 100% O₂ during reperfusion. Therefore, we tested the effect of different gases on cellular preservation for 24 h using LPDG solution. We showed equivalent cellular preservation, defined by cell attachment to the culture plate, after 24 h of ischemia and after reperfusion, using preservation gases of 100% O₂, 95% O₂/5% CO₂, 100% room air, or 95% room air/5% CO₂ (Fig. 10). The significant reperfusion-induced decrease in cell attachment and viability, seen in 100% O₂, was not significant in other groups, employing lower O₂ concentrations in the preservation gas. Thus, decreasing O₂ concentration during lung preservation should be considered in the amelioration of I/R injury of lung transplants.

Free radical damage during organ preservation and reperfusion has been well established (63, 115, 127). A free radical is a molecule that contains an odd number of electrons in its valence shell. These species are highly reactive. If two radicals react a non-radical will be formed but if a radical reacts with a non-radical another free radical will be produced. In this way, free radicals are able to participate in long chain reactions (128). Reactive oxygen species (ROS) are a type of free radical that mediate injury during lung ischemia and reperfusion and are capable of damaging lipids and damaging DNA (63). A potential source of ROS is ambient oxygen, particularly high concentrations of ambient oxygen (129, 130). The production of large amounts of ROS can overwhelm the cells endogenous mechanism to control them. These mechanisms include a group of enzymes known as the superoxide dismutases (SOD) (127). These enzymes are
responsible for converting superoxide, an ROS, to peroxide according to the following equation (128).

\[ \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2^- \]

In our study, it is possible that decreasing oxygen concentrations in the preservation gas decreases free radical production. Furthermore, the decrease in cell viability is seen using preservation with 100% O\(_2\) after reperfusion. This is similar to what Christie and co-workers have found where reperfusion, following preservation with 100% O\(_2\), initiated most of the free radical damage to lipids and DNA (63). The fact that there was not a significant decrease in cell viability after reperfusion, when less than 100% O\(_2\) concentrations were used for preservation might suggest a potential decrease in harmful ROS. However, this endpoint was not measured.

**SUMMARY**

Lung dysfunction in the early period post-transplantation continues to be a significant problem. The cellular and molecular mechanisms responsible for the pathogenesis of I/R injury are poorly understood. In this study we have designed and validated a cell culture model that can permit the study of I/R injury at the cellular and molecular level. As mentioned, I/R injury is a multifactorial problem. This model will allow the addition or subtraction of most of these factors so that contributions of particular stimuli can be elucidated.
With the validation of the model, and establishing the significance of aerobic, hypothermic preservation in lung transplantation, contributions of these factors to lung transplant-related I/R injury could be examined. This model may be a useful tool to study the cellular and molecular mechanisms associated with lung transplantation-related I/R injury. Furthermore, potential therapeutic interventions, such as antisense or gene therapy, can be tested with this model to evaluate their potential application in ameliorating I/R injury. Several examples are listed below.

**Future directions**

Future work in this area could involve investigating the cytoskeletal rearrangements associated with ischemia and reperfusion in this model. These insults clearly cause cell detachment and death to occur. Because microfilaments are so intimately involved in cell adhesion through interactions with cell adhesion molecules one would expect alterations in their structure. Furthermore, are these alterations related to ambient gas during preservation? Changes in ambient gas concentrations are known to effect the cytoskeleton and could represent a path for further investigation in I/R injury.

The role of pH in lung preservation has been poorly elucidated. The use of amiloride as an inhibitor of Na⁺/H⁺ exchanger has been investigated in an isolated perfused rat lung system (121). This model would provide an excellent platform to study pH alterations associated with ischemia, the role of the Na⁺/H⁺ exchanger and the gradual return to physiologic pH without using complicated animal models. Furthermore, as mentioned, calcium plays a pathogenic role in conjunction with pH. Intracellular calcium
concentrations could be tested in conjunction with gradual returns to physiologic pH and the use of amiloride to block Na⁺/H⁺ exchange.

Decreasing the concentration of oxygen in the preservation gas seems to impart a degree of cellular protection in this model. Reactive oxygen species are thought to mediate the damage incurred as the result of using high oxygen concentrations. However, free radical measurement was not performed. Further work could involve exploring both free radical production and the effects of free radical scavengers such as glutathione.

Endothelial cell alterations, as mentioned earlier, are also important in the pathogenesis of lung I/R injury. Upon reperfusion these cells are exposed to shear stress introduced by blood flow. Employing cold preservation in conjunction with reperfusion under a set amount of shear stress using the Parallel Plate Flow Chamber™ can simulate this effect.

Information has emerged regarding apoptosis and necrosis in the setting of lung transplantation (79, 131). The precise signal activation pathways have not been elucidated. Apoptosis and its respective pathways in I/R injury could be explored in further work.
REFERENCES
REFERENCES


