In Situ Cooling in a Lung Hilar Clamping Model of Ischemia-Reperfusion Injury

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Experimental models for studying transplantation have up to now been unable to isolate reperfusion injury with minimal surgical manipulation and without the interference of graft rejection. Six pigs were subjected to left hilum preparation only (control group), and eight pigs were subjected to left hilum preparation plus *in situ* cooling ischemia and reperfusion of the lung (experimental group). The hilum was dissected free from other tissues in both groups. Lung preservation was achieved by antegrade flush perfusion *via* the left pulmonary artery. Pulmonary veins were clamped at the left atrium and a vent was created. The left main bronchus was clamped. Lung temperature was maintained at 4°–8°C, while core temperature was kept at 38°C. After 3 hrs of cold ischemia the clamps were removed and the lung was reperfused. Elevated pulmonary vascular resistance and local and systemic aspects of ischemia-reperfusion syndrome were consistently reproduced. This large-animal model of *in situ* unilateral lung cold ischemia with warm reperfusion proved to be very reliable in reproducing all aspects of ischemia-reperfusion injury. It excludes the interference of rejection and extensive surgical manipulation. We therefore propose its use in experimental studies investigating pharmaceutical or cooling modifications affecting lung ischemia-reperfusion outcomes. Exp Biol Med 231:1410–1420, 2006

Key words: Lung transplantation; ischemia-reperfusion injury; experimental model; animal; *in situ* cooling; hilar clamping model

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Introduction

Lung transplantation is a well accepted treatment for patients with end-stage pulmonary disease (1). Reperfusion injury (RI) is the mechanism mostly responsible for early graft dysfunction, which remains one of the major causes of early morbidity and mortality (2–4). The specific pathophysiology of RI in lung transplantation has not been fully evaluated and understood (2, 3, 5).

Much experimental data have duplicated the typical clinical and histologic pictures of ischemia-reperfusion injury (IRI) in transplant models with 2 to 6 hrs of storage (5). Others and we in our previous experimental work with pigs have been using a single lung transplantation model (6, 7). Hyperacute rejection is not the major challenge in the early phase in clinical practice due to ABO blood group matching, and preoperative screening for antibodies against common antigens has mostly eliminated this problem. However, the incidence of acute rejection is approximately 5% of early mortality, directly related to graft rejection (8), and it remains an unknown parameter in experimental allotransplantation (9). Autotransplantation models eliminate the rejection associated with allotransplantation, but have shown several technical limitations. The *in situ* hilar clamping models have up to now carried the limitation of using only warm preservation. Our aim was to create a steady and reproducible experimental model that could reveal several parameters associated with the mechanisms of RI (e.g., impaired gas exchange, elevated pulmonary vascular resistance, local and systemic aspects of reperfusion syndrome) using cold storage (10, 11), with minimal possible surgical manipulation (2, 5). We achieved the reproduction of IRI without the interference of the pathology concerning graft rejection.

Materials and Methods

Fourteen female landscape white pigs with a body weight of 20–25 kg, mean weight 22 (±3.5) kg, were used.

All animals received human care in accordance with the Principles of Laboratory Animal Care, the *Guide for the Care and Use of Laboratory Animals* prepared by the National Institute of Laboratory Animal Resources (published by the National Institutes of Health; publication 85–23, revised 1985) and the guidelines of the European Union (86/609). The University of Patras Ethical Committee approved the experimental protocol.

Anesthesia. The animals were premedicated with midazolam (5 mg) and atropine sulfate (0.5 mg), both given by intramuscular (im) injection. A venous line was established by puncturing an auricular vein. Induction of anesthesia was performed with sodium thiopental (250–400 mg) intravenously (iv). The animals were initially intubated with a 6.5-mm internal diameter orotracheal tube followed by a tracheostomy to gain better access to the tracheobronchial tree and facilitate bronchoscopy (Olympus P10; Tokyo, Japan). A volume-controlled mechanical ventilation was used (Servo Ventilator 900 C; Siemens-Elema, Solna, Sweden). Initially, tidal volume was set to 10 ml/kg body wt and respiratory rate to 14 breaths per min with an FiO_2 of 0.5. The respirator settings were subsequently adjusted to achieve a pCO_2 of 40–45 mm Hg and arterial oxygen saturation by more than 95%. Maintenance anesthesia was administered iv as a continuous infusion of sodium thiopental (5 $mg \cdot kg^{-1} \cdot hr^{-1}$), and fentanyl (1 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) (Abbott infusion system Lifecare 5000). For muscular relaxation, 4 mg of pancouronium bromide was added iv as appropriate. Perioperative antibiotic medication consisted of a cefuroxime (750 mg iv) bolus injection. Central and core temperatures were recorded (12). Ringer's lactate solution was administered to maintain the pulmonary capillary wedge pressure at 15 mm Hg. Furosemide was given iv when the pulmonary capillary wedge pressure exceeded 15 mm Hg (13).

Surgical Technique. Following dissection of the right femoral artery and vein, a central venous catheter was inserted in the right femoral vein using the Seldinger technique and a Swan-Ganz catheter was subsequently placed. An arterial catheter for invasive blood pressure monitoring was placed in the right femoral artery in the same fashion. A Foley catheter was introduced by cystostomy. The animal was placed in the right lateral position. A posterolateral thoracotomy incision was performed in the left fifth intercostal space. The incision was extended anteriorly toward the sternum. Bleeding was meticulously controlled throughout the surgical procedure. Two or three ribs were removed to achieve a wide opening exposure for careful and safe manipulation of the left lung. With the lung retracted inferiorly, the pleura overlying the left pulmonary artery was opened followed by careful dissection of the left pulmonary hilum. The hemiazygos was ligated and all lymphatic nodes of the hilum, paratracheal, and aortopulmonary window space were removed. The left main pulmonary artery and bronchus were isolated in the pulmonary hilum. The lung was elevated superiorly, exposing and dividing the inferior pulmonary ligament. The inferior pulmonary vein was isolated. The pericardium was opened and the origin of the left pulmonary vein was dissected and isolated (Fig. 1). A tape was passed around the left main bronchus, which was stripped free of all nearby bronchial arterial branches in order to ablate the bronchial circulation. During this procedure the lymphatic drainage of the lung was practically stopped. The pulmonary veins were further dissected at their entrance into the left atrium and the hemiazygos was dissected intrapericardially.

During these steps of the surgical procedure particular care was taken to minimize manipulation of the fragile ventilated left lung. The proper position of the Swan-Ganz catheter in the right pulmonary artery was verified by palpating the left pulmonary artery. The pulmonary catheter was found to be positioned correctly (right main pulmonary artery) in all experiments. Heparin was given iv (300 IU/kg). A purse-string 6–0 Prolene suture (Ethicon, Hamburg, Germany) was placed in the pulmonary artery. An 18-gauge arterial cannula was carefully inserted in the pulmonary artery taking care not to penetrate the lumen. The artery was proximally occluded by a tourniquet. A side-biting clamp was placed on the left atrium and an incision was made for fluid drainage (vent) at the junction of the left pulmonary veins and the left atrium. The left lung was then flushed with cold University of Wisconsin (UW) solution (60 ml/kg). Pulmonary artery pressure during flushing was kept at approximately 15 mm Hg (Fig. 2). Ventilation was continued during flush perfusion. Venting from the pulmonary vein was continued until fresh preservation fluid exited the vent site, and the left lung acquired a pale color ensuring that blood had been fully flushed out by the UW solution. After completion of flush perfusion, the main left bronchus was cross-clamped with a bronchial clamp and the lung was kept semi-inflated. The lung was left *in situ* and covered with cold swabs with flaked ice inside a custom-made isothermic and waterproof bag. The temperature was measured continuously in the left interlobar space and the lung parenchyma using a needle probe (Lutron TM-905, Dual Channel Thermometer). When temperatures exceeded 7°C, additional cold normal saline and ice were applied to the towels over the isothermic bag, which isolated the lung from the pleural space. Excess cold water and melted ice drained through the very small space between the hilar structures and the bag surrounding the lung in the pleural cavity. Warm normal saline at 38.5°C was added to the pleural space in a controlled manner (whenever the central temperature dropped to less than 36.5°C the warm infusion rate increased) and maintained the central temperature at 37.86° ± 1.42°C (Fig. 3). At the same time a suction system was applied to the pleural space in order to remove the excess normal saline. Additionally, a conductive heating system was used to maintain the animal's normal core temperature (Operatherm 202W; KanMed, Stockholm, Sweden). Total ischemic time of the left lung was set at 3 hrs.

Reperfusion was initiated by removing the tourniquet

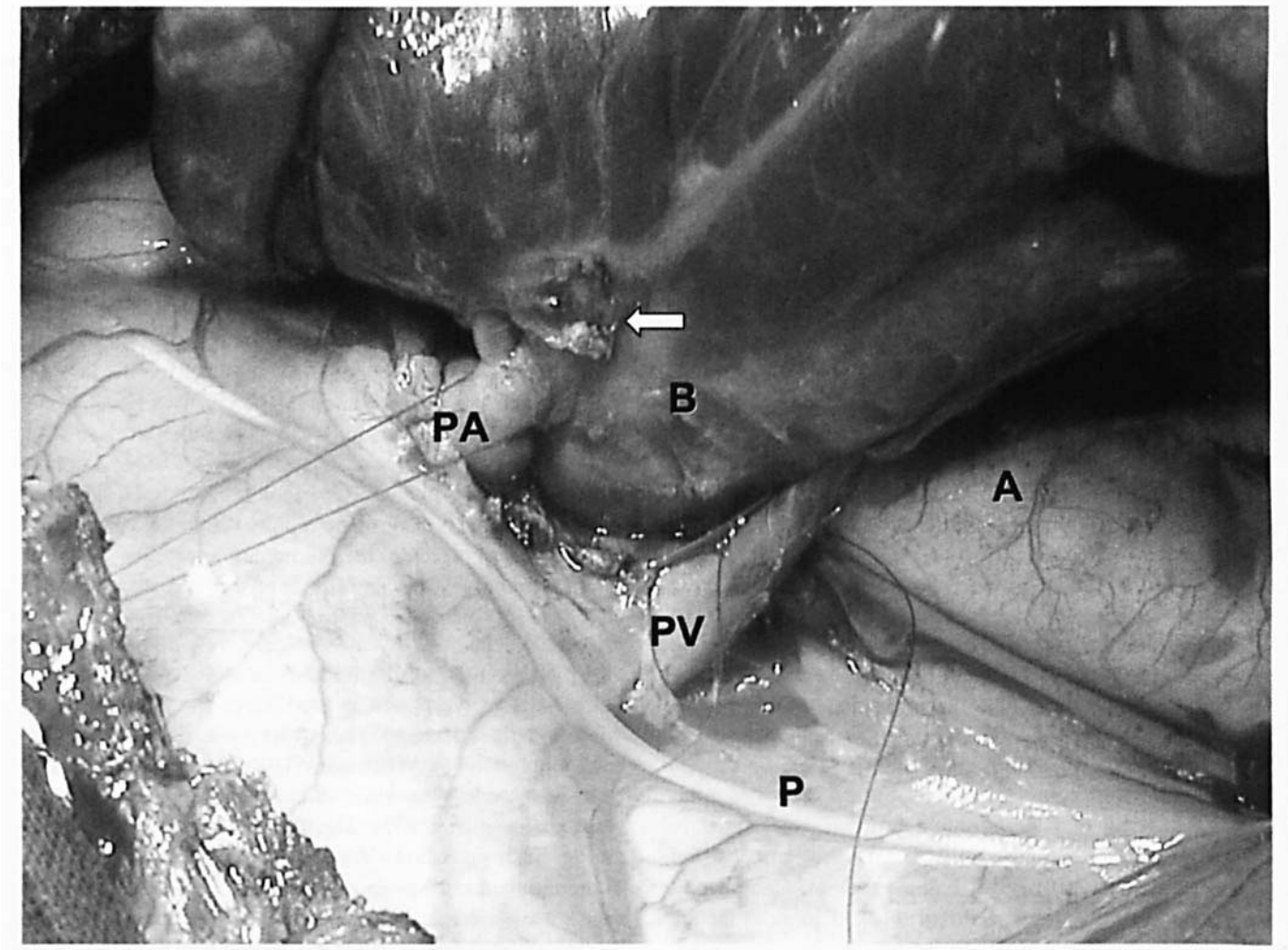

Figure 1. Intraoperative surgical view of the left lung hilum (control group). A, aorta; PV, pulmonary veins; PA, pulmonary artery; B, left main stem bronchus; P, phrenic nerve. White arrow denotes a legate bronchial artery.

from the pulmonary artery. Careful lung ventilation was commenced after removing the bronchial clamp. Venting from the left pulmonary vein was continued until fresh red blood exited the vent site; ensuring that UW preservation fluids had been flushed out by the pulmonary circulation. Subsequently, the incision of the left atrial vent was repaired with a 6–0 Prolene suture (Ethicon), the left atrial clamp was removed, and ventilation to the left lung was restored (14, 15).

Experimental Groups. Animals were randomly assigned to two groups. In Group C (control), animals underwent the same anesthesia, surgical procedure, and preparation of left hili without ischemia and subsequent manipulation. The total time of observation was 360 mins after preparation of left hili. In Group E (experimental) the left lung was cold flush–preserved and then left ischemic for 3 hrs followed by reperfusion as described above. The observation period after reperfusion was 180 mins.

Hematologic Values. The hematocrit values (packed cell volume, PCV) were measured from venous samples by microcentrifuge. The estimation points were after instrumentation and before thoracotomy (base), after 3 hrs of ischemia and after 3 hrs of reperfusion.

Cardiopulmonary Assessment. Assessment of cardiopulmonary function was facilitated by the following measurements: heart rate, cardiac output by thermodilution, pulmonary artery pressure, capillary wedge pressure, central venous pressure, arterial pressure, continuous SVO_2 measurement, arterial blood gases, and urine output (16, 17). Pulmonary vascular resistance index (PVRI) was calculated according to the following formula: (MPAP – LAP)/CI × 80, where MPAP is mean pulmonary artery pressure, LAP is left atrial pressure, and CI is cardiac index. CI was calculated as (CO/BSA), where CO is cardiac output and BSA is body surface area.

Body surface area (BSA) was calculated according to the formula (16): BSA (m^2) = weight $(gr)^{2/3} \cdot K \cdot 10^{-4}$, where $K = 9$ for pigs.

Static compliance (Cstat), a measure of the "stiffness" of the lung, was also assessed. For Cstat estimation the following formula was used: Cstat = Vt/(Ppp – PEEP),

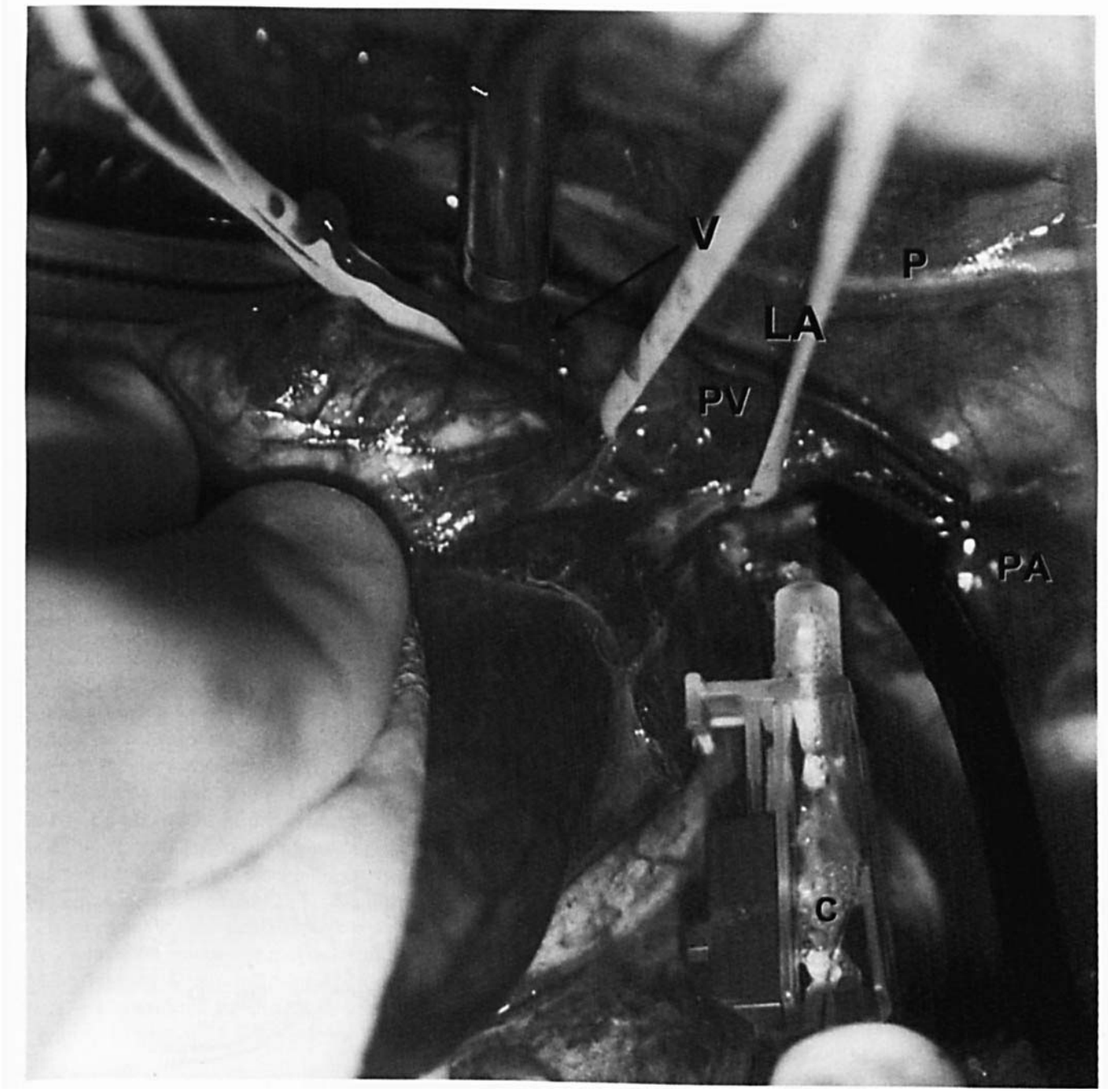

Figure 2. Hilar structures. Antegrade flushing through the pulmonary artery and venting from the left atrium near to the pulmonary veins. Note the clamp on the left atrium and the tape proximally occluding the pulmonary artery (experimental group). PV, pulmonary veins; PA, pulmonary artery; LA, left atrium; V, vent; C, pulmonary artery cannula; P, phrenic nerve.

where Vt is tidal volume in milliliters, Ppp is the plateau pressure in centimeters of water, and PEEP is the positive end expiratory pressure in centimeters of water.

Throughout reperfusion, all animals received mechanical ventilation with an FiO_2 of 1.0, a PEEP of 5 cm H_2O, a rate of 16 breaths/min, and a tidal volume of 15 ml/kg.

The evaluation time points were set at the start of the experiment; after completion of instrumentation; after hilar preparation; 60, 120, and 180 mins after ischemia; and 60, 120, and 180 mins after reperfusion.

Histologic Studies. A pulmonary biopsy was performed from the left upper lobe after thoracotomy, from the left lower lobe after 3 hrs of reperfusion (Group E) and from the left lower lobe of Group C. Every sample was examined histopathologically using both hematoxylin-eosin staining and Karnovsky's solution as previously described (18). Assessment was performed in a blinded fashion. Lesions were scored according to a semiquantitative scale, where 0 = no changes, 1+ = focal mild subtle changes, 2+ = multifocal mild changes, 3+ = multifocal prominent changes, and 4+ =

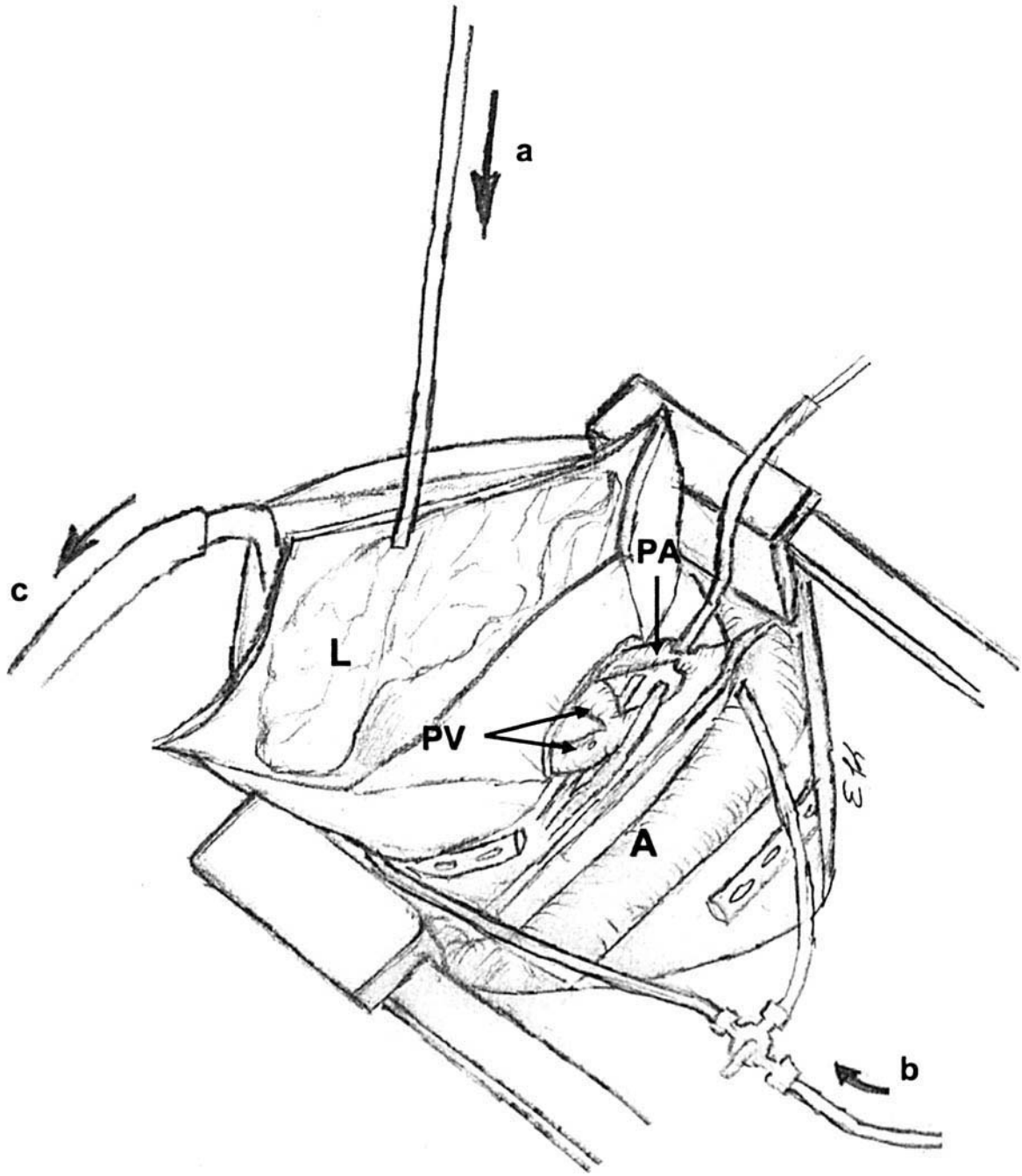

Figure 3. Diagram of cold conservation of the left lung. Note the cold normal saline delivered inside the isothermic waterproof bag through cannula "a." Note the warm normal saline added into the pleural space by cannula "b" to maintain normal core temperature. The excess of the pleura water is aspirate. c, aspiration catheter; PV, pulmonary veins; PA, pulmonary artery; L, left lung; A, aorta.

extensive prominent changes (19). The evaluation procedure assessed the degree of interstitial edema, intra-alveolar edema, extravascular granulocyte infiltration, pulmonary hemorrhage, emphysema-like lesions, desquamation of alveoli cells, and atelectasis.

Extravascular Water. Extravascular lung water (in grams per gram of blood-free dry lung weight) was measured according to the method described by Drake and colleagues (20). Two biopsy specimens were taken at the end of the experiment; one from the left lung of the experimental group and one from the left lung of the control group (control biopsy). Both specimens were obtained from the lower lobe (20).

Statistical Analysis. All values were expressed as mean measurements ± SD. Comparison of mean values of continuous data representing the same group at different times before and after ischemia or reperfusion were made by the paired-samples *t* test procedure. A low significance

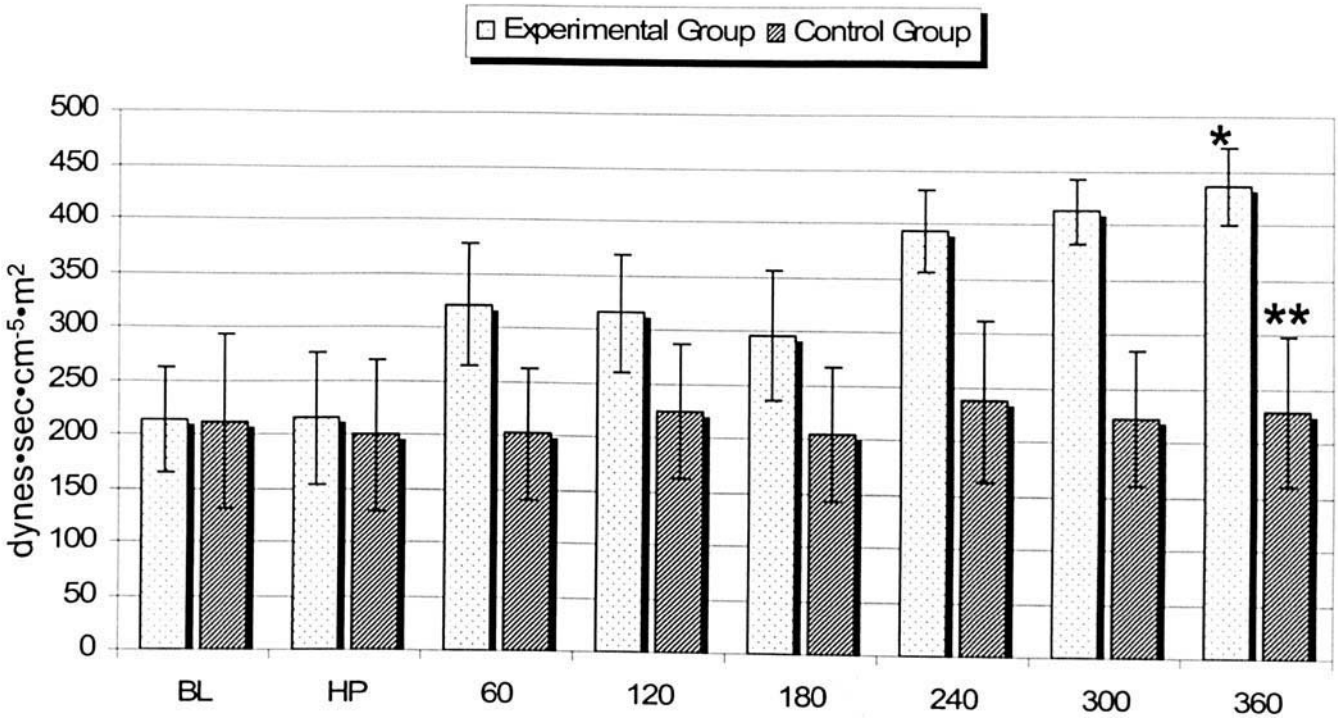

Figure 4. Values of pulmonary vascular resistance index as a function of time (dynes*sec*cm^{-5}*m^2). Error bars represent standard deviation (multiplier 1). Estimation points: after completion of instrumentation (baseline) (BL); after hilar preparation (HP); after 1, 2, and 3 hrs of ischemia (60, 120, and 180 mins); and after 1, 2, and 3 hrs after reperfusion (240, 300, and 360 mins). Asterisks denote statistical differences among groups from basal levels of each group. (*$P = 0.000$, **$P = 0.117$)

value of the t test ($P < 0.05$) indicated a significant difference between the two variables. If the confidence interval for the mean difference did not contain zero, this also indicated that the difference was significant. Comparisons of mean values of different groups at the same time were made with the independent-samples t test. Comparisons of continuous data among the groups were performed by repeated-measures ANOVA. Significance tests were performed and P values < 0.05 were considered significant. To evaluate the groups in a nonparametric way, we used the Mann-Whitney U test and when the possibility was $P < 0.05$, we considered the groups dissimilar. Analyses were performed using SPSS for Windows, release 11.0.1 (SPSS Inc., Chicago, IL).

Results

All the animals survived the given period of 3 hrs.

Hematologic Analysis. The baseline PCV value in the control group was 29.7% ± 3.14%, (range, 26.5%–35.7%), after 3 hrs of ischemia the PCV was 29.14% ± 2.83% (25.3%–34.2%), and after 3 hrs of reperfusion the PCV was 28.96% ± 2.58% (24.8%–33.5%). Baseline PCV in the experimental group was 29.6% ± 1.78% and at the end of the observation period it was 28.32% ± 1.4%. The PCV value was therefore stable during the entire experimental procedure in both groups (the difference among the estimation points was not significant, $P > 0.561$).

Hemodynamics. Heart rate, mean arterial pressure, central venous pressure, and cardiac index were all monitored continuously during the period of the protocol and the values remained constant with no use of inotropes.

In the experimental group the mean baseline PVRI was measured at approximately 214.22 ± 48.84 dynes · sec · cm^{-5} · m^2. During the entire ischemia period the PVRI increased from a mean of 295.20 to 320.19. During the entire postreperfusion period a highly significant increase in PVRI was observed: at 3 hrs after reperfusion a 102% increase was observed compared with baseline values (*$P = 0.000$).

The differences among the estimation points of the control group were not statistically significant (**$P = 0.117$) for the entire observation period (Fig. 4).

Respiratory Parameters—Lung Mechanics. We evaluated the Cstat as described above. In the experimental group, the mean baseline Cstat value after instrumentation (BL) was 20.92 ± 1.7. After the hilar preparation (HP), the mean Cstat was 20.61 ± 0.36 ml/cm H_2O. During the entire postreperfusion period a highly significant ($P = 0.000$) drop in Cstat was observed: 17.95 ± 0.86, 15.47 ± 1.65, and 13.9 ± 1.17 for the first, second, and third hrs respectively, after reperfusion. In the control group, the Cstat value remained constant (Fig. 5).

Figure 6 shows the mean values for the PO_2 to FiO_2 ratio after completion of instrumentation, after hilar preparation, and during the ischemia and reperfusion period (blood samples were taken from the femoral artery).

In the experimental group during the reperfusion period

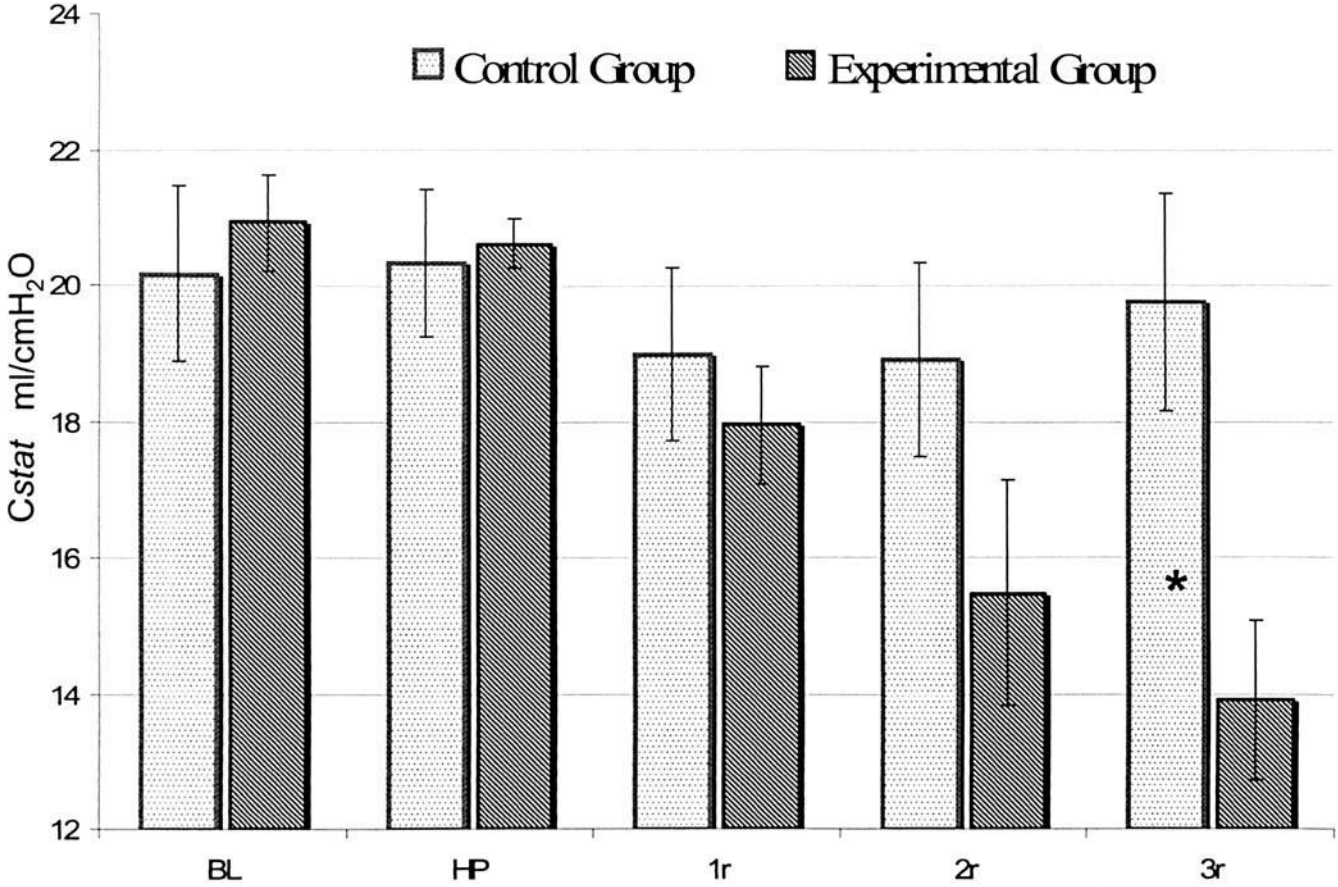

Figure 5. Values of static compliance (Cstat in ml/cm H_2O) at the start and during the ischemia period. Error bars represent standard deviation (multiplier 1). Estimation points: after completion of instrumentation (BL); after hilar preparation (HP); and after 1, 2, and 3 hrs after reperfusion for the experimental group or 240, 300, and 360 mins after HP for the control group (1r, 2r, and 3r, respectively). Asterisk denotes a statistically significant difference from basal levels of Cstat in the experimental group (*$P = 0.000$).

there was a significant decrease ($P = 0.001$) in the PO_2 to FiO_2 values; 256.28 ± 26.48, 259.57 ± 35.26, and 263.34 ± 41.53 respectively, compared with the values after completion of instrumentation 492.4 ± 58.9 (baseline), after hilar preparation, and during the ischemia.

In the control group the mean values of PO_2 to FiO_2 were constant (statistically significant; $P > 0.05$) during the entire observation period; ranging from 511.99 ± 86.66 to 452.25 ± 81.44.

Temperature of the Lung. The basal core temperature of the pigs at the induction of anesthesia and for 1 hr after the induction of anesthesia was 37.56° ± 0.98°C. The core temperatures of the experimental animals remained at a normal level (37.86° ± 1.43°C) for the entire observation period. There was no statistical difference between the intralobal and lung parenchyma temperatures; the mean value of these was used as the lung temperature. The lung temperature during the flush perfusion period dropped quickly and remained at the optimum temperature, which our protocol had stipulated, for the entire ischemia period that followed (Fig. 7).

Histologic Evaluation. The baseline biopsies of all animals before preservation showed normal lung tissue. At the end of the 3 hrs of reperfusion significant histologic alterations were present in the cold-ischemic lungs. These alterations consisted of edema, inflammatory cell infiltration, hemorrhagic infiltrations, and desquamation of alveoli cells. In Group E the alteration was more profound (Table 1). No intravascular thrombosis or necrosis of vessel walls was observed, which are common histologic findings of acute or hyperacute rejection. There were minimal pathologic findings in the control group (Table 1 and Fig. 8). We observed similar mild and focal subpleural emphysema-like lesions and atelectasis in both groups, probably due to manipulations during the operation.

Extravascular Water. Extravascular water (wet to dry ratio) in the Group E lungs after reperfusion was 6.29 ± 0.78 compared with 4.01 ± 0.51 in Group C ($P = 0.000$) at the same observation time.

Discussion

Lung transplantation has become a life-saving operation for patients with end-stage pulmonary disease, with a 1-yr survival of 77%–80% and a 5-yr survival of 60%–63% (21, 22). The primary risks encountered in the early days of lung transplantation were problems related to bronchial

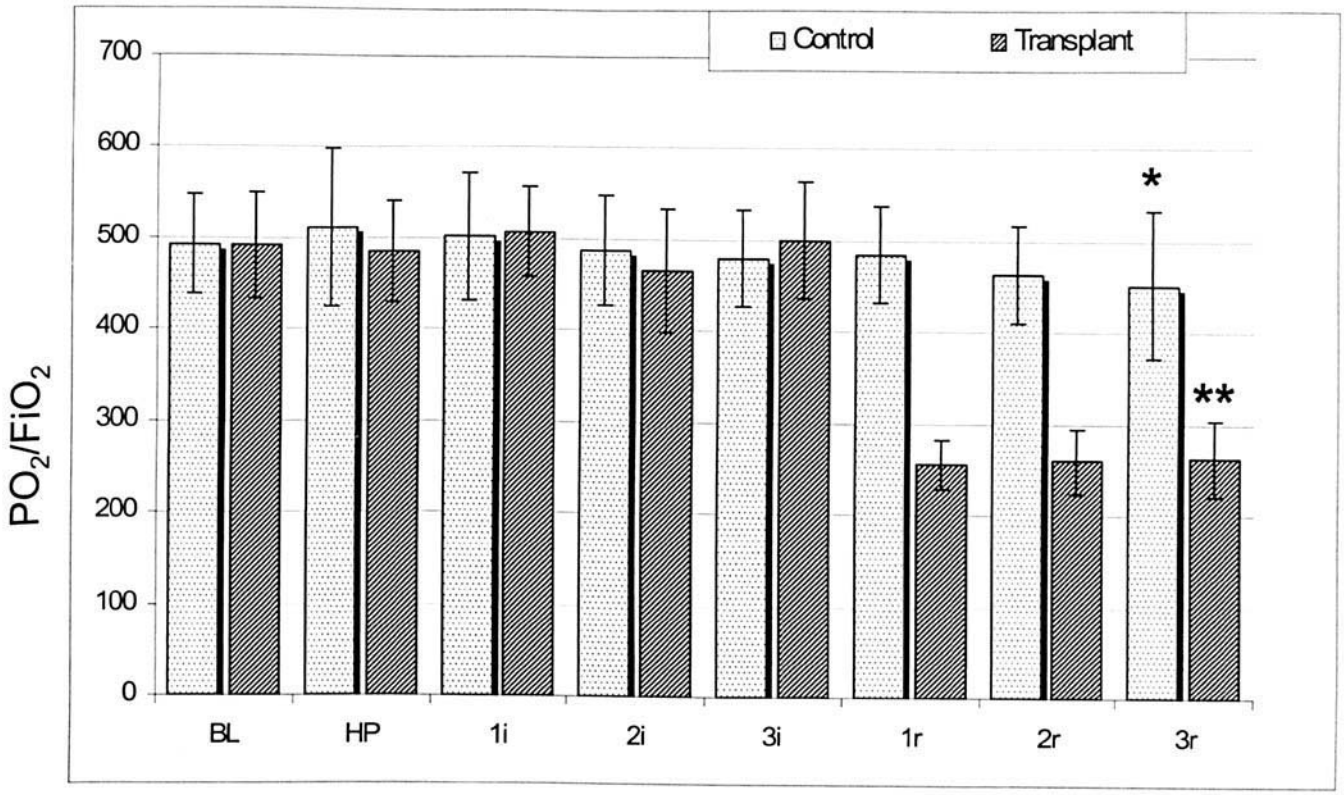

Figure 6. PO_2 to FiO_2 ratio values (in mm Hg) during the entire protocol. Each error bar represents standard deviation (multiplier 1) within the time. Estimation points: after completion of instrumentation (baseline) (BL); after hilar preparation (HP); after 1, 2, and 3 hrs of ischemia (1i, 2i, and 3i, respectively); and after 1, 2, and 3 hrs of reperfusion (1r, 2r, and 3r, respectively). Asterisks denote statistical differences among groups from basal levels of each group (*$P = 0.001$, **$P > 0.05$).

healing, infections, acute rejection, and graft failure (23, 24). The majority of these problems have now been diminished. Airway anastomoses can be safely performed. Infections can be better controlled using known pharmaceutical substances. Acute rejection can now be properly managed in various ways (3).

The incidence of acute rejection is higher after lung transplantation compared with rejection in other solid organs, and approximately 5% of early mortality is directly related to graft rejection (25). Rejection could be identified as *hyperacute* rejection, which occurs in minutes or hours; *accelerated* rejection, which evolves in hours or days; and *acute* rejection, which occurs over days or weeks (26).

During lung transplantation the graft is submitted to ischemia-reperfusion, surgical manipulation, and immune attack. Activation of the inflammatory cascade and its interaction with the pulmonary vascular endothelium is the major mechanism of IRI that causes the damages observed during transplantation. The lung tissue is modified by these assaults in such a way that the graft becomes more susceptible to rejection. Cellular and humoral immune damage is less likely to occur in normal cells, which are generally more resistant than transformed, infected, or activated target cells. The abovementioned alterations to the graft induce the process of destruction (26). At present, graft failure related to IRI has been identified as the major cause of early mortality (23). Experimental investigation of IRI could prove to be useful in identifying specific factors involved in this process.

Our results indicate that it is obvious that the IRI and the rejection procedures could be overlapping both pathophysiologically and chronically. The lack of suitable experimental models analogous to the usual clinical situations, without the interference of hyperacute or acute rejection, has limited the progress in eliminating IRI during lung transplantation (27). Various experimental models have been proposed for the evaluation of IRI (28). Transplantation procedures from one animal to another animal (allotransplantation) have the disadvantage that the qualitative impairment of ventilation and pulmonary perfusion is the result of the double trauma of surgery (removal and reinsertion) and the ischemia-reperfusion. Moreover, there is the interference of hyperacute or accelerated rejection, which is initiated at the moment of reperfusion due to the inflammatory cascade. To eliminate the factors associated with the rejection process, autotransplantation or reimplantation models have been proposed (5). The reimplantation procedures (mostly historical), in which the lung is severed and reattached, have the advantage that they can achieve ischemia and storage at various temperatures. However, the primary disadvantage is the difficulty of the surgical technique in reanastomosing the stumps of

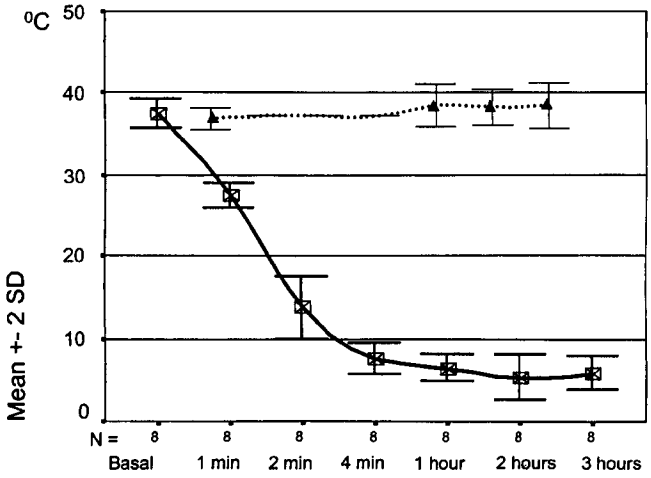

Figure 7. Temperature values. Data are summaries of separate variables. Error bars represent standard deviation (multiplier 2). Estimation points: after induction of anesthesia; 1, 2, and 4 mins after starting the flush perfusion, respectively; and 1, 2, and 3 hrs of ischemia period, respectively. The round dotted line denotes the core temperature of the pig and the solid line denotes the lung temperature.

the pulmonary veins. Early historical experiments suggested that there was an increase of PVR due to anastomotic technique of the vascular attachments. Later, it was shown that meticulous anastomotic techniques minimize these problems but do not eliminate them (29). The hilar clamping models in which complete hilar stripping and clamping of the pulmonary artery vein and bronchus is achieved minimize the manipulation of the graft.

Our aim was the development of an experimental animal model that was able to isolate the IRI from the acute rejection and permit the study of the former process in more detail. This model could provide an environment in which reproduction of the early malfunction of a preserved and ischemic lung would allow an extensive investigation and appropriate modifications for diminishing IRI. A large-animal model (that anatomically resembles humans closely) for studying IRI could help in a better understanding of the pathophysiology of this injury (5).

Up to now the experimental hilar clamping models that have been proposed permit only warm ischemia and storage. An extensive search of the literature revealed that Demertzis *et al.* (30) have reported a model with mild cold ischemia. The limitations of that model were the high temperature of lung preservation at 15°C and the duration of 2 hrs of ischemic graft preservation. However, we have not found literature reports of *in situ* cooling of the lung at 4°–8°C while keeping the heart at normal temperature.

The model presented here combines several beneficial features. The two immediate rejection processes, hyperacute and accelerated, were eliminated, allowing the ischemia-reperfusion element to be isolated and specifically studied. Major hazards such as the issue of extensive surgical manipulation and the potential interference of rejection have been eliminated. In our model, to further diminish the manipulation of the lung parenchyma during dissection and ischemia, an extensive thoracoplasty was utilized. The process of the ischemia-reperfusion pathology was the only factor affecting the graft. However, we emphasize that this is a surgically challenging model and during the period of the learning curve we had two deaths due to surgical or anesthetic problems.

This model demonstrated the ability to maintain important variables such as viscosity (from hematocrit at constant temperature and blood flow), core temperature, and hemodynamics at a steady-state level in order to achieve more specific comparisons (Fig. 7). It will also allow the possibility of modifying any of the variables in a relatively easy manner (including lung temperature, time intervals, and the inclusion of a variable warm-ischemic period). This could facilitate the study of these modifications on the respective outcomes. Moreover, this model has revealed the systemic aspects of IRI such as the elevated PVRI (Fig. 4), the decrease of oxygenation (decreased PO_2 to FiO_2 ratio) (Fig. 6), and the decrease of lung compliance (Fig. 5). The accuracy of the PVRI measurements may be compromised by not having occluded the right pulmonary artery; thus forcing the animals to depend on the function of the left lung. Occlusion of the right pulmonary artery, while it was feasible in our model, was deemed to be an unnecessary surgical manipulation that could significantly affect the hemodynamic stability of the Group E animals. The plot of PVRI shows that during ischemia (when the pulmonary circulation depended on the right lung) the PVRI value increased by 47%, and subsequently, during reperfusion, increased by a further 37%. We can hypothesize that the elevation of PVRI concerns the entire lung circulation (left

Table 1. Histologic Findings in Experimental and Control Groups[a]

Histologic alterations	Experimental group (n = 8)	Control group (n = 6)
Interstitial edema	(5/++) (2/+++) (1/++++)	(1/+) (1/++)
Intra-alveolar edema	(3/+) (3/++) (2/+++)	(1/+) (2/++)
Extravascular granulocyte infiltration	(1/++) (5/+++) (2/+++)	(2/+) (1/++)
Pulmonary hemorrhage	(2/+) (1/++) (1/+++)	(1/+) (1/++)
Desquamation of alveoli cells	(3/+) (2/++) (1/+++)	(1/+)

[a] The number in front of the crosses denotes the number of occurrences. Histologic alterations are scored as follows: + = focal mild subtle changes, ++ = multifocal mild changes, +++ = multifocal prominent changes, ++++ = extensive prominent changes.

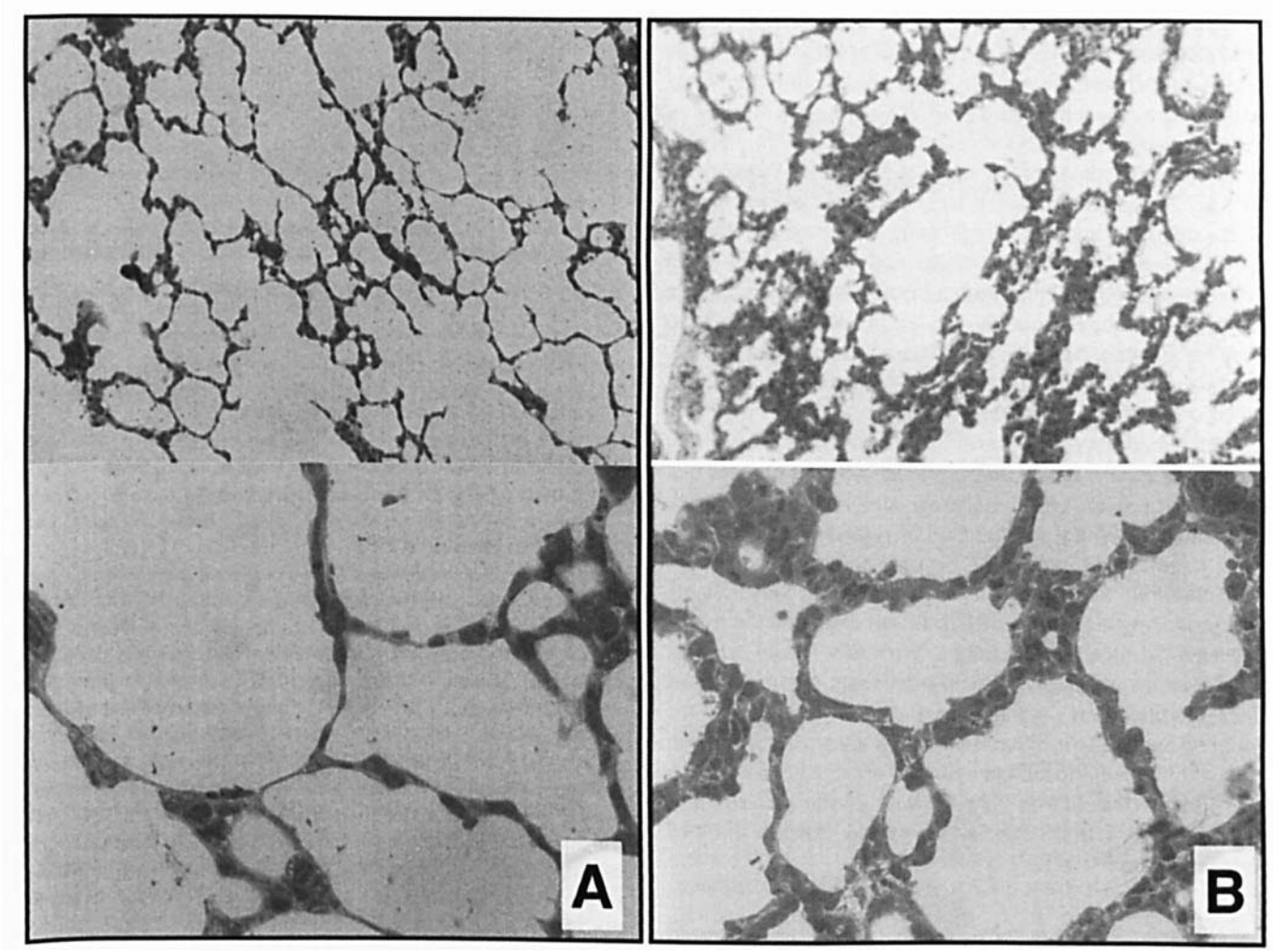

Figure 8. In (A), histology of the control lung shows only minimal alveolar-interstitial leukocyte infiltration. (B) Histology of Group E lungs after 3 hrs of reperfusion. An obvious alveolar-interstitial leukocyte infiltrate was present (1% toluidine blue in 1% borax stain, ×82 magnification (upper photos) and ×327 magnification (lower photos).

and right). This hypothesis is in accordance with the previously published finding of decreased serum nitric oxide, a well-established vasodilator, during lung transplantation (18). It is known that the PVRI can increase just from edema. The goal of fluid management during the experimental protocol was to minimize edema formation in the reperfused lung while maintaining adequate cardiac function. The effects of IRI and the practical absence of lymphatics may contribute to the development of pulmonary edema. In the model described in this paper there was no bronchial transaction and, as a consequence, there was not definitive impairment of the lymphatic drainage. We can assume that this will lessen the lung edema in the early period of reperfusion after cold ischemia.

Pulmonary capillary wedge pressure was kept as low as possible (<15 mm Hg) without compromising ventricular preload and cardiac output. The observation that the wet to dry ratio did not increase significantly in the control group could allow us to assume that the elevation of PVRI was not due to edema in the right lung.

Our histologic findings (i.e., increase in extravascular lung water, edema, inflammatory cell infiltration, hemorrhagic infiltrations, and desquamation of alveoli cells) represent the local histologic aspects of IRI (3) (Table 1 and Fig. 8). These alterations are different from hyperacute rejection alteration, which occurs immediately (within minutes to hours of vascularization of the transplanted graft) and is caused by a humoral immune response. The histologic findings of hyperacute rejection demonstrate intravascular thrombosis, necrosis of vessel walls, and infiltration with mononuclear and polymorphonuclear cells, followed by massive intravascular coagulation, lack of tissue perfusion, and graft necrosis (26). Such findings were not observed in our model.

In the clinical setting, the time between removal of the lung from the cold solution and restoration of blood flow to the lung is defined as the transplantation time. The harvest time plus the transplantation time is defined as the warm ischemic time (WIT), an important factor related to IRI. Our protocol allows the inclusion of an adjustable WIT, which could vary both in temperature and in time.

The most widely used method for organ preservation is cold storage at 4°C. We have managed to keep the ischemic period temperature close to the usual clinical situation. Cold preservation was maintained at 4°–8°C, which to the best of our knowledge, is the first to be reported in an *in situ* model. It should be noted that the core temperature remained at 37.5°C. The results obtained from this standardized model were consistent and had a high grade of reproducibility.

An *in situ* hilar clamping model, such as the one described above, needs only half the number of animals compared with a single lung allotransplantation model. In addition, there is no need for double catheters (arterial lines, pulmonary artery line, or tracheal tube). This situation is relatively cost-effective and incorporates the highest degree of ethics.

In conclusion, this novel basic science experimental model can offer a steady and reproducible environment that could be used to assess ischemia and reperfusion injury without the potential interference of rejection mechanisms. This large-animal model of *in situ* unilateral lung cold ischemia with warm reperfusion proved to be very reliable in reproducing all aspects of IRI. It also excluded the use of extensive surgical manipulation. With this model, various investigations of pharmaceutical treatments, preservation of fluid modifications, storage, and reperfusion conditions, could be applied to evaluate IRI in lung transplantation with a view to reducing local and systemic complications (1, 28). We propose this *in vivo* large-animal model of ischemia-reperfusion as a well substantiated method for the study of reperfusion injury in lung grafts.

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