Assessment of Ischemia–Reperfusion Injury and Early Acute Rejection in Experimental Lung Transplantation After Prolonged Ischemia

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OBJETIVE: To assess ischemia–reperfusion injury and early acute rejection of the lung subjected to ischemia for 10 hours.

MATERIAL AND METHODS: Fifteen of 30 Sprague–Dawley rats underwent transplantation of a left lung that had been subjected to ischemic times of 4 (n=5), 6 (n=5), or 10 hours (n=5). The cardiopulmonary block was removed from the donor, the left lung was dissected, and the transplant was carried out using the cuff technique. The cardiopulmonary block was extracted after 48 hours. We assessed postoperative progress, ischemia–reperfusion injury and acute rejection of the transplanted and contralateral lungs. Statistical probabilities were analyzed using the χ^2 and Fisher exact tests.

RESULTS: Clinical course was not worse after an ischemic time of 10 hours (*P*=.711). No significant differences were observed in histological markers of ischemia–reperfusion injury and acute rejection or in clinical course in relation to the different ischemic times; nor was clinical course related to the presence or severity of lesions or rejection. Similarly, acute rejection was unrelated to ischemia–reperfusion injury (*P*>.05).

CONCLUSIONS: In this study, a prolonged ischemic time of 10 hours was not associated with ischemia–reperfusion injuries, with more severe acute rejection, or with a worse clinical course. Acute rejection was also unrelated to the presence or severity of ischemia–reperfusion injury.

Key words: *Lung transplantation. Ischemic time. Experimental surgery.*

Valoración de la lesión de isquemia-reperfusión y del rechazo agudo precoz en el trasplante pulmonar experimental con tiempo de isquemia prolongado

OBJETIVO: Valorar la lesión de isquemia-reperfusión y el rechazo agudo precoz del pulmón sometido a un tiempo de isquemia de 10 h.

MATERIAL Y MÉTODOS: Se han utilizado 30 ratas Sprague-Dawley, en 15 de las cuales se realizó un trasplante pulmonar izquierdo con tiempos de isquemia de 4 h (n = 5), 6 h (n = 5) y 10 h (n = 5). Del donante se extrajo el bloque cardiopulmonar, se disecó el pulmón izquierdo y se efectuó el implante con la técnica de manguitos *(cuffs)*. A las 48 h se extrajo el bloque cardiopulmonar. Se valoraron la evolución postoperatoria, la lesión de isquemia-reperfusión y el rechazo agudo del χ^2 pulmón trasplantado y del contralateral. El análisis estadístico se realizó con el test de la χ^2 y el test exacto de Fisher para el cálculo de probabilidades.

RESULTADOS: Los animales trasplantados con un tiempo de isquemia de 10 h no tuvieron peor evolución clínica (p = 0,711). No se observaron diferencias significativas entre los parámetros histológicos de lesión de isquemia-reperfusión y de rechazo agudo con los distintos tiempos de isquemia, ni en la evolución clínica por la presencia y gravedad de éstos. Tampoco se observó que el rechazo agudo se relacionara con la lesión de isquemia-reperfusión (p > 0,05).

CONCLUSIONES: En nuestro estudio, el tiempo de isquemia pulmonar prolongado de 10 h no se asocia ni a lesiones de isquemia-reperfusión y rechazo agudo más graves ni a una peor evolución clínica. El rechazo agudo no se relaciona con la presencia ni con la gravedad de la lesión de isquemia-reperfusión.

Palabras clave: *Trasplante pulmonar. Tiempo de isquemia. Cirugía experimental.*

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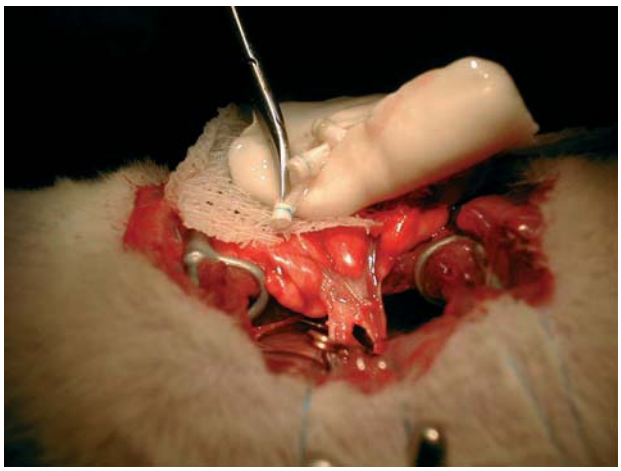

Figure 1. Anastomosis using the cuff technique and fixation with 6/0 ligatures

Introduction

Currently, the maximum permissible ischemic time for a lung graft in clinical situations is unknown and though it is accepted as being between 6 and 8 hours, it is not surprising that longer periods may be tolerated. While some authors have found that ischemia lasting over 5 hours predicts lower survival rates for transplant recipients,[2] others have observed that ischemic times of up to 6 hours do not have a significant effect.[1] One group has demonstrated that good preservation was possible for 4 hours and that a longer ischemic time (up to 6 hours) predicted an acceptable but worse response to implantation.[3] Longer ischemic times (8 hours) have been linked to decline in lung function in the immediate postoperative period.[4]

However, these findings have not been unanimously supported and some have found associations between survival after lung transplantation and other parameters such as the donor's age.[5] These authors confirmed that early death after transplantation was associated with the combination of donor age and ischemic time prolonged for over 6 hours. In contrast, when comparing 20 single-lung transplants from a single donor (donor pairs) in which ischemic time was significantly less in the first lung implanted (mean [SD], 164 [53] minutes) than in the second (377 [53] minutes), Glanville et al[6] found that ischemic time did not affect duration of intubation, early gas exchange, stay in hospital, or 1-year survival. However, they found more episodes of acute rejection in the first 3 months and a higher incidence of infection in the group with a shorter ischemic time.

Given the lack of agreement between authors who have studied donor lung ischemic time and its relation to complications such as ischemia–reperfusion injury and acute rejection, it is difficult to have a clear view of the problem. However, transplant groups continue their fight against time. This study aimed to respond to the question of how ischemic time affects ischemia–reperfusion injury and acute rejection.

Materials and Methods

Thirty male Sprague–Dawley rats, 15 donors and 15 recipients, weighing between 300 and 400 g, were used. Humidity, temperature, light, and ventilation conditions were constant and controlled at all times in the animal laboratory. The animals were randomly divided into 3 groups according to graft ischemic time: group 1, ischemic time of 4 hours; group 2, 6 hours; and group 3, 10 hours. Each group comprised 5 donor and 5 recipient animals.

Fifteen lung transplants, 5 per group, were undertaken using the cuff technique.[7] All experiments, manipulations and surgical procedures were performed in accordance with the guide for the handling and care of experimental animals (Spanish Royal Decree 223/1988) and the guide for the care and use of laboratory animals of the National Research Council of the United States of America, 1996.

Extraction of Donor Lung

The donor animal was anesthetized intraperitoneally with sodium thiopental (60 mg/kg). A tracheostomy was carried out and the animal was ventilated with a tidal volume of 1 mL/100 g. After median laparotomy, 100 U/100 g of sodium heparin were injected into the inferior vena cava, and a median sternotomy was performed. After removal of the thymus, the abdominal aorta, thoracic inferior vena cava and both atria were sectioned. At that moment, the donor animal suffered cardiorespiratory arrest and ischemic time began. The lungs were perfused with physiological saline solution at 4ºC through the pulmonary artery. The final step in this stage involved sectioning and ligating the trachea, while the lung was partially inflated before cardiopulmonary block extraction. The left pulmonary artery, pulmonary vein, and bronchus were then dissected and the hilum was distally sectioned. The cuffs and venous puncture catheters (Abbocath, Sligo, Republic of Ireland), cut in 3 mm cylinders with a 1.5 mm base and rear wing, were then positioned. The lung was kept at 4ºC in cold physiological saline solution until implantation.

Implantation in the Recipient

The recipient was anesthetized with subcutaneous medetomidine (0.25 mg/kg), intraperitoneal ketamine (50 mg/kg) and intramuscular atropine (0.7 mg/kg). The animal was ventilated through an orotracheal tube with the same tidal volume as during extraction. The pulmonary hilum was dissected through a left posterolateral thoracotomy, and the pulmonary artery and vein and the bronchus were identified. Ligatures and microvascular clips were positioned, vessels were sectioned, and an intravascular flush with heparinized saline solution was performed. Subsequently, the bronchus was cut and anastomosis was undertaken with the cuffs (Figure 1). Finally, after ventilating and slowly reperfusing the implanted lung, a pleural drain was inserted and the thoracotomy was closed. Intramuscular atipamezole (1.25 mg/kg), medetomidine antagonist and intramuscular buprenorphine (0.05 mg/kg) were administered. Suction through the drain was used to return negative pressure to the pleural cavity and the drain was removed when the animal was breathing spontaneously.

Assessment Procedures

All the animals were sacrificed after 48 hours. The cardiopulmonary block was removed and perfused through the trachea with 1% formaldehyde. The lung was embedded in paraffin, longitudinal cuts were made and the sections stained

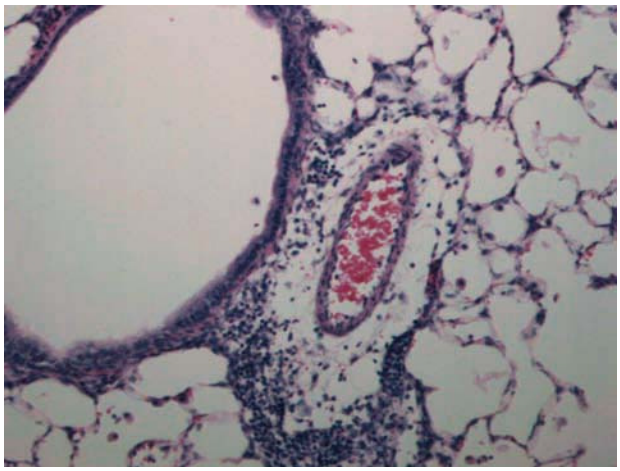

Figure 2. Perivascular lymphocytic inflammatory infiltrates (type A2 acute rejection) in a lung with mild edema that was transplanted after 4 hours of ischemia

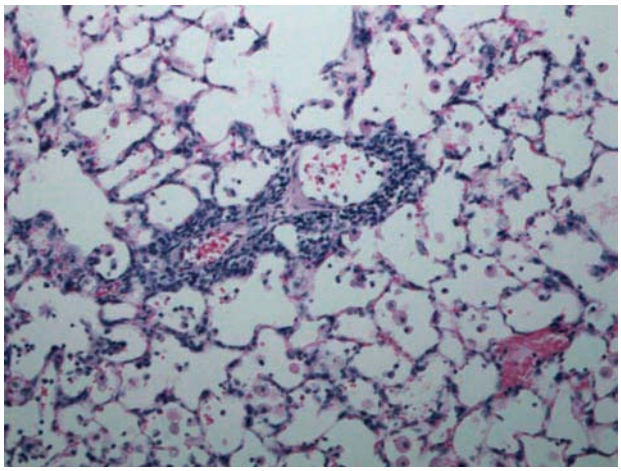

Figure 3. Perivascular and peribronchiolar lymphocytic inflammatory infiltrates (type A2 acute rejection) in a lung with moderate edema that was transplanted after 10 hours of ischemia

with hematoxylin–eosin. Samples were examined under an optical microscope, which revealed signs of ischemia–reperfusion injury (peribronchial and perivascular edema, vascular congestion, acute inflammatory infiltration [neutrophils] and bleeding), as well as acute rejection in accordance with the international guidelines for posttransplant pulmonary rejection.

Postoperative clinical complications were assessed for tachypnea (>100 breaths/min), vocalizations or stridor, anomalous or orthopneic postures, hemoptysis, and infection of the surgical wound. All animals underwent a chest x-ray (Siemens Mobilett II, Madrid, Spain) at 55 kV and 3.2 mA immediately after surgery and after 48 hours, before being sacrificed.

Statistical Analysis

SPSS software (Chicago, Illinois, USA) was used for statistical analysis. Statistical probabilities were analyzed using the χ^2 and Fisher exact tests.

Results

Effect of Ischemic Time on Clinical Course

All the animals in group 1 (ischemic time of 4 hours) had a satisfactory clinical course prior to sacrifice, except 1, which developed tachypnea and stridor. One animal in group 2 (ischemic time of 6 hours) had tachypnea and isolated hemoptysis, which we attributed to deficient preservation of the graft during perfusion; nevertheless, the animal survived until sacrifice. Clinical course for the remaining animals in this group was good. One animal in group 3 (ischemic time of 10 hours) developed tachypnea and stridor after implantation, which we also related to deficient preservation of the donor lung, and another animal had well-tolerated tachypnea. Both animals survived until sacrifice. The remaining animals in group 3 progressed satisfactorily (Table 1). We observed no surgical wound infections.

Radiographic studies revealed a case of left basal pneumothorax in the animal with mild tachypnea in group 3. In all cases we found increased diffuse density of the transplanted lung, absent in the right lung; we related it to ischemia–reperfusion injury.

Effect of Ischemic Time on Lung Injury

No significant differences were observed between histological markers indicating lung injury and the different ischemic times of 4 (Figure 2), 6 and 10 hours (Figure 3) to which implanted lungs were subjected (Table 1).

Implanted lungs with an ischemic time of 10 hours tended to be less associated with pulmonary bleeding and acute inflammatory infiltration, but the trend was not statistically significant. They did not have more severe ischemic–reperfusion injury or acute rejection. The contralateral lung effects we observed were acute inflammatory infiltration in the animal in the 4-hour

TABLE 1
Effect of Ischemic Time on Clinical Course, Ischemia–Reperfusion Injury, and Acute Rejection

Variable	Ischemic Time			*P*
	4 h	6 h	10 h	
Clinical Course				.711
Good	4	4	3	
Bad or death	1	1	2	
Edema				.711
None or mild	4	3	4	
Moderate-severe	1	2	1	
Vascular congestion				.343
None or mild	2	2	4	
Moderate-severe	3	3	1	
Bleeding				.036
No	3	1	5	
Yes	2	4	0	
Acute rejection				.343
No	2	4	2	
Yes	3	1	3	
Neutrophil infiltration				.099
No	3	5	5	
Yes	2	0	0	

TABLE 2
Effect of Ischemia–Reperfusion Injury and Acute Rejection on Clinical Course

Variable	Evolution		*P*
	Good	Poor–Death	
Edema			>.05
None or mild	8	3	
Moderate–severe	3	1	
Congestion			>.05
None or mild	6	2	
Moderate–severe	5	2	
Bleeding			.604
No	6	3	
Yes	5	1	
Acute rejection			.282
No	7	1	
Yes	4	3	
Neutrophil infiltration			.476
No	10	3	
Yes	1	1	

TABLE 3
Relationship of Edema to Severity of Lung Injury

Variable	Edema		*P*
	None or Mild	Moderate–Severe	
Congestion			>.05
None or mild	6	2	
Moderate–severe	5	2	
Bleeding			.235
No	8	3	
Yes	1	3	
Acute rejection			>.05
No	6	2	
Yes	5	2	
Neutrophil infiltration			.476
No	10	3	
Yes	1	1	

TABLE 4
Relationship Between Acute Rejection and Ischemia–Reperfusion Injury

Variable	Acute Rejection		*P*
	No	Yes	
Edema			>.05
None or mild	6	5	
Moderate–severe	2	2	
Congestion			.619
None or mild	5	3	
Moderate–severe	3	4	
Bleeding			.608
No	4	5	
Yes	4	2	
Neutrophil infiltration			>.05
No	7	6	
Yes	1	1	

ischemic time group whose clinical course was poor, with severe pulmonary edema in the transplanted lung.

There were 5 cases of type A2 acute rejection (2 animals in group 1, 1 in group 2, and 2 in group 3) and 2 cases of type A1 acute rejection (1 animal in group 1 and 1 in group 3).

Effect of Ischemia–Reperfusion Injury and Acute Rejection on Clinical Course

The clinical progression of the transplanted animals revealed no significant changes either due to the severity of the edema or to the presence and severity of other signs of ischemia–reperfusion injury and acute rejection (Table 2).

Relationship Between Ischemia–Reperfusion Injury and Acute Rejection

The severity of the edema was not significantly related to a more severe degree of vascular congestion, inflammatory infiltration, or acute rejection, although a nonsignificant tendency to bleed was observed (Table 3). Acute rejection was unrelated to the presence or severity of the remaining markers indicative of ischemia–reperfusion injury (Table 4).

Discussion

Lung injury caused by ischemia and reperfusion and acute rejection continues to be a significant cause of early morbidity and mortality after lung transplantation.[2,8] One of the most controversial aspects of lung transplantation in humans is the maximum ischemic time that the organ can tolerate prior to transplantation.[2,6] At present, it is generally accepted that it must be between 6 and 8 hours.[1,3-6] However, there are reasonable doubts about the feasibility of exceeding these limits.[2,5,6] We consider that the data presented here are preliminary, but they do indicate that the lung could tolerate longer ischemic times than those currently accepted as the limit, and in fact we have successfully performed lung transplants in experimental conditions without seeing signs of more severe ischemia–reperfusion injury and acute rejection, as have other authors.[9,10]

Pulmonary edema is considered the best indication of tissue damage arising from ischemia–reperfusion injury. It is responsible for transplanted lung dysfunction, and clinical progression after a lung transplant is related to ischemic time. Experimental studies in rats 48 hours after lung transplantation reveal a variable degree of edema and alveolar congestion, mainly from mononuclear white blood cell infiltrates, bleeding and necrosis, in addition to signs of acute rejection.[11]

In this study, we did not observe a correlation between the severity of ischemia–reperfusion injury and a worse clinical course in the transplanted animals. This could be attributable to the fact that we performed left-lung transplants and that the right lung has an important functional respiratory reserve in these animals. This could be a limitation when assessing the clinical implications of our study.

The rat is a good model for studying acute rejection and its relation to ischemia–reperfusion injury, since acute rejection makes an early appearance in other mammals, such as dogs or humans.[11] A possible link between the presence and degree of acute rejection and the severity of ischemia–reperfusion injury has been put forth repeatedly. Shiraishi et al[12] reported that, though ischemia–reperfusion

injury predisposes the development of acute rejection, the severity of the latter was not related to the former. Our data supports these findings, since we have identified no association between ischemia–reperfusion injury and the presence or severity of acute rejection. A tendency toward accompanying bleeding indicates that tissue damage in cases of severe ischemia–reperfusion injury is considerable and associated with vascular changes in the capillary filtration coefficient.[13,14]

Contralateral lung effects are a common finding in single lung transplants or in reperfusion studies of a single lung.[15] We have not found this association in our series. We have only been able to observe the presence of severe edema in the transplanted lung when contralateral lung complications appear.

Not only did lungs implanted after a prolonged ischemic time (10 hours) not develop a greater degree of pulmonary injury, but they also tended to bleed less and have less acute inflammatory inflammation, although those trends were not statistically significant.

We conclude that a prolonged ischemic time of 10 hours is not associated with more severe ischemia–reperfusion injury or acute rejection or with a worse clinical course. Acute rejection is not linked to the presence and severity of ischemia–reperfusion injury. Further studies will be required to determine the maximum ischemic time that lungs can tolerate. This could help increase the supply of donors from outlying regions to transplant centers.

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