Technical Modifications of the Orthotopic Lung Transplantation Model in Rats With Brain-Dead Donors

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ABSTRACT

Background: Microsurgical lung transplantation in rats has allowed us to obtain new knowledge about lung transplantation. However, some aspects in human transplantation technique still have not been included in this model, which could interfere with the clinical interpretation and extrapolation of results.

Methods: Twenty left lung transplantations were performed with a cuff technique and technical modifications, such as brain death induction, the control of ischemia time and retrograde perfusion in the donor and the controlled sequential reperfusion of the implanted lung in the recipient.

Results: Survival rate was 80%. The transplanted lungs showed proper perfusion and ventilation with good permeability of the anastomoses. Signs of ischemia-reperfusion injury were observed in all animals while mild acute rejection was seen in half of them.

Conclusions: The shown model proves valid and is very similar to the procedure carried out in humans, which would reduce the number of possible variables derived from the surgical technique when extrapolating the study results to clinical use.

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Modificaciones técnicas del modelo de trasplante pulmonar ortotópico en ratas con donantes en muerte cerebral

RESUMEN

Introducción: El trasplante microquirúrgico de pulmón en ratas ha permitido adquirir nuevos conocimientos sobre el trasplante de pulmón. Sin embargo, algunos aspectos de la técnica de trasplante en humanos aún no han sido incluidos en este modelo, lo que podría interferir en la interpretación clínica y en la extrapolación de los resultados.

Método: Se han realizado 20 trasplantes pulmonares izquierdos con la técnica de manguitos (cuff) incorporando algunas modificaciones técnicas como la inducción de la muerte cerebral, el control del tiempo de isquemia, la perfusión retrograda en el donante y la reperfusión secuencial controlada del pulmón implantado en el receptor.

Resultados: La supervivencia ha sido del 80%. Los pulmones transplantados mostraron una adecuada perfusión y ventilación con buena permeabilidad de las anastomosis. Se han observado signos de isquemia-reperfusión en todos los animales, y de rechazo agudo leve en la mitad de ellos.

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Introducción

La transplante de pulmón (LT) es una alternativa terapéutica para algunas enfermedades respiratorias terminales. Es actualmente indicado en enfermedades pulmonares avanzadas, con disfunción intersticial, y enfermedades pulmonares y enfermedades neumáticas. El uso más ampliamente aceptado es el trasplante bilateral de pulmón, realizado por caminos pulmonares. La importancia de mejorar ciertos aspectos, como la preservación, la isquemia, la reperfusión (IR) y las reacciones aguda y crónica (AR y CR), permite desarrollar modelos LT adecuados.

En el comienzo, el modelo animal experimental más utilizado fue el de perros, aunque los éticos y los problemas económicos de los animales experimentales hicieron que se desarrollaran modelos, como por ejemplo, conejos y ratones. En 1971, Asimacopoulos2,3 empezó a realizar LT en ratones, y en 1982 Wildevuur4 completó el modelo, a pesar de las dificultades que se hacían durante la operación. Algunos autores2,3 han señalado que los miembros de la Asociación Estadounidense de Investigación sobre Enfermedades Pulmonares (American Thoracic Society, ATS) y la Asociación Estadounidense de Investigación sobre Enfermedades Pulmonares (American Lung Association, ALA) han recomendado la realización de LT en animales de laboratorio.

La complejidad del procedimiento microquirúrgico en ratones mejoró en 1989 con la técnica original de Mizuta.7 Se utilizó por primera vez el sistema de sonda Teflon cuffs para la realización de anastomosis. Para esta etapa, se utilizaron catéteres intravenosos polietileno, que luego se introdujeron en dos caminos, inicialmente, Mizuta realizó la anastomosis de los vasos en el sistema de la sonda cuffs, mientras que la anastomosis de la bronquial se realizó con suturas. Luego, Mizuta8 y Reis9 realizaron la anastomosis con cuffs. La modificación en el sistema de suturas siguió el sistema de anastomosis de cuffs.

Desde entonces, algunos autores10,11 han presentado modificaciones en la técnica quirúrgica para el desarrollo de diferentes aspectos de LT y mejorar los modelos experimentales, aunque no se incluyen los aspectos clínicos de LT, como la muerte cerebral (BD), la perfusión retrograda en el corazón y la muerte en el momento de la isquemia.

En nuestro estudio, presentamos un nuevo protocolo de anestesia y algunas modificaciones en la preservación y la implantación del pulmón en ratones con el objetivo de reproducir la técnica actualmente utilizada en humanos. Nuestro objetivo es reducir los posibles cambios en la técnica quirúrgica y la extrapolar los resultados a la clínica.

Métodos

Animales

Cincuenta y dos ratones de Sprague-Dawley machos consangüíneos (20 donantes y 20 receptores), con un peso de 300 g y 400 g fueron alojados bajo condiciones normales. Todos los animales fueron sometidos a las recomendaciones del “Manual de la unión europea para el uso de la medicina animal en el campo de la investigación y la sección de laboratorios” de la “Asociación Española de Investigación en Enfermedades Pulmonares (AEIP)”. La investigación fue aprobada por el Comité Ético para el Experimentación Animal y el bienestar del animal en nuestro centro.

Inducción de la muerte en el cerebro (BD)

Los animales fueron sometidos a anestesia con 0.25 mg/kg de ketamina (Ketalar®; Pfizer, Madrid, Spain) y 50 mg/kg de ketamina (Ketalar®; Pfizer, Madrid, Spain). El antecúrtico del donante se realizó con una anestesia local, y la intubación orotraqueal se realizó con un Abbocath® 14G catéter. Luego se realizó la ventilación con un ventilador Evita 4 (Dräger®; Hamburg, Alemania) con presión media (Pm) de 10 cmH2O y respiración continua (CR) hasta el minuto 12–14 cmH2O y la inspiración/expiración (I/E) ratio de 1:2, una respiración de 60 rpm, con una solución de oxígeno (FiO2) de 0.21 y una presión postoperatoria de 2 cmH2O.

Para la inyección de BD, se utilizó un catéter de Fogarty® (LeMaitre®, Burlington, MA, USA). La inyección de BD se realizó con una velocidad de 20 μl/min de solución salina. La inyección de BD fue realizada con un catéter de Fogarty® (LeMaitre®, Burlington, MA, USA) en el momento de la muerte cerebral (Fig. 1). La temperatura corporal se mantuvo cerca de 36,5 °C con un electrocardiómetro eléctrico. BD fue confirmado por el reconocimiento de la aparición de las arritmias, el disminución de la actividad electroencefalográfica y la dilatación de los pupilas.12,13

Extracción del pulmón

Conclusión: El modelo que presentamos es válido y similar al procedimiento que se realiza en humanos, lo que reduciría el número de posibles variables derivadas de la técnica quirúrgica a la hora de extrapolar los resultados a la clínica.
perfusion was carried out through the left auricle (10–12 ml of LPDG from 20 cm high).

When the perfusion of the lungs was homogenous, we proceeded with the ligation and transection of the trachea with the lung semi-inflated at 50% of total lung capacity (10–15 cmH₂O), the extraction of the cardiopulmonary block with traction separating it from the esophagus and the transection of the supra-aortic trunks, thoracic aorta, vena cava and the pulmonary ligaments. The cardiopulmonary block was placed on a Petri dish with cold LPDG, and we dissected the artery, left bronchus and vein using a 10× optical microscope (Carl Zeiss®, Germany) with transection distal to the hilum of said structures for separating the left lung. A microclamp was placed in the bronchus to maintain the lung in a state of semi-inflation (Figs. 2 and 3).

The cuffs were constructed with an Abbocath® 16G catheter (1.7 mm in diameter). Each cuff had a body and a 1.5 mm-long tab. The tab was used to hold the cuff with a clamp during the implantation. First, the pulmonary artery was passed through the cuff. The proximal end was everted over the cuff and held in place firmly with a non-absorbable 7/0 ligature (Fig. 4). The same process was carried out with the bronchus and the pulmonary vein, respectively, and then the lung was stored in cold LPDG until the moment of its implantation, in our case 2 h after clamping the thoracic aorta.

Implantation in the Receptor

The receptor animals (n=20) were anesthetized with 0.25 mg/kg medetomidine sc, 50 mg/kg ketamine im and 0.7 mg/kg atropine im (Atropine®, Braun, Barcelona, Spain). Once anesthetized, the rats were intubated with an Abbocath® 14G. The receptor animals were ventilated in the same manner as the donors.

After shaving the left hemithorax, left thoracotomy was carried out in the fourth intercostal space and the lung was removed from the thoracic cavity by means of a metallic paper clip. The pulmonary hilum was dissected in order to identify the artery, the bronchus and the pulmonary vein. The bronchial arteriolar plexus was cauterized together with the arteriole of the pulmonary ligament, under the vein. Long 6/0 polypropylene ligatures were placed, as well as microclamps (Yasargil® 4 mm) on each one of the structures, as close as possible to the heart. The artery and vein were cut proximally and tangentially to the heart and an intravascular lavage was done with heparinized plasma. Later, the bronchus was also transected.

The donor lung was placed on the native lung of the recipient and was frequently irrigated with a cold preservation solution until the anastomoses were completed with the cuffs. In doing so, each cuff was introduced into the corresponding structure through the orifice made. Once inside, it was set in place transitorily with a microclamp placed over the tab in order to facilitate the definitive anastomosis with ligatures, and then the microclamp was removed.

First of all, the arterial anastomosis was done, followed by the bronchial (Fig. 5), and the sequential reperfusion of the donor lung was completed. The microclamp proximal to the heart was withdrawn transitorily from the bronchus to allow for the ventilation of the lung with a PEEP of 5 cmH₂O eliminating atelectasis. Then, the pulmonary artery was slowly reperfused by opening the microclamp proximal to the artery until it was observed that a few drops of blood flowed out the pulmonary vein (Fig. 6). The artery and the bronchus were clamped once again and we proceeded with
the anastomosis of the vein. Slow, progressive reperfusion was carried out by opening the vein microclamp, then the bronchus and finally the artery.

Once the donor lung was implanted, the native lung was removed from the receptor and an 8-Fr pleural drainage catheter was inserted and connected to syringe for aspirating, and the thoracotomy was closed. Afterwards, we administered 0.25 mg/kg im atipamezole (Antisedan®, Pfizer, Madrid, Spain), an antagonist of medetomidine, and 0.05 mg/kg sc buprenorphine (Buprex®).

Schering-Plough, Madrid, Spain). When the rat began spontaneous breathing, the thoracic and orotracheal tubes were withdrawn.

Post-op Follow-up and Evaluation of the Transplantation

Rats that survived surgery were sacrificed under general anesthesia with 0.25 mg/kg sc medetomidine and 50 mg/kg im ketamine at 7 (n = 8) and 90 days (n = 8). After clamping the artery and
the right pulmonary bronchus, the cardiopulmonary block was removed with the lung inflated. The block was set in formaldehyde through the trachea and was included in paraffin for the histological study. In our case, the animals received no immunosuppressant treatment during the study period.

The samples obtained from the transplanted lung were stained with hematoxylin–eosin. Signs of IR injury were evaluated, such as alveolar interstitial edema, vascular congestion, alveolar hemorrhage and neutrophilic infiltration, and the IR injury was classified as mild, moderate or severe. AR signs were also evaluated, such as the distribution of the perivascular and peribronchial mononuclear infiltrate in accordance with the International Rejection Classification, as were signs of CR, such as bronchial and bronchiolar dilation, areas of vasculitis and fibrosis. The anastomoses were also inspected visually in order to determine their macroscopic permeability.

From a clinical standpoint, we evaluated the presence of tachypnea, respiratory failure and post-surgical infections during the post-op period. In all cases, chest radiographs were taken at 55 kV and 3.2 mA (Siemens Mobilett II®; Siemens España, Madrid, Spain) immediately after surgery as well as 7 and 90 days, before the animal was sacrificed.

Results

Mean surgical time was 48.5 ± 2.0 min for the extraction and 59.2 ± 4.2 for the implantation. Mean time for the complete procedure was 107 ± 6.2 min.

No post-op infections were observed. Four of the recipient animals (two from the 7-day group and two from the 90-day group) presented tachypnea and hemoptysis due to severe edema and died within the first 48 h. One animal presented tachypnea for the first 24 h. The other animals presented no clinical problems, and the survival rate was 80% (Table 1).

In the histologic study, signs of IR injury were observed after 7 days in all the transplanted lungs (n=8): mild (n=5), moderate (n=2) and severe (n=1); signs of AR were seen in 6 animals (A1=3 and A2=3). After 90 days, in all the animals (n=8) a fibrotic lung stump was observed, and microscopically we viewed bronchial dilation with areas of vasculitis and fibrosis accompanied by severe AR (A4), indicating the presence of active CR. No histological changes were observed in the non-transplanted lung. The inspection of the cuffs showed in all animals adequate bronchial and vascular lumen without signs of stenosis or thrombosis.

Chest radiography showed adequate lung expansion with an increase in density of the transplanted lung related to the presence of IR injury after 7 days and CR after 90. One animal presented mild left pneumothorax and another presented contralateral pneumothorax immediately after surgery, with no clinical repercussions.

Discussion

LT is still a challenge for patients and medical transplantation groups, and there are still important areas requiring basic and clinical research. To face these challenges, the American National Heart, Lung, and Blood Institute (NHLBI) recommends considering the following points: (a) to identify essential biomarkers, based on proteomic and genomic studies that can predict the presence of CR; (b) to develop relevant animal models that better reproduce human diseases in order to research the pathogenesis of lung injury after transplantation; and (c) to support and promote research covering the range of graft dysfunctions, including CR.

In this direction, following the recommendations of the NHLBI, the experimental model that we have developed in this study is based on previous reports of cuff techniques7–9 with modifications in the preservation and implantation techniques such as the induction of BD, control during the ischemia time and retrograde perfusion in the donor and controlled sequential reperfusion in the lung of the recipient, in addition to an improvement in the percentage of survival of 80% compared with other transplantation models in rats.11 This is an attempt at developing animal models that reflect what happens in human diseases according to the aforementioned international recommendations.

The organs used for transplantation come mainly from BD donors.15 The majority of the donors suffer considerable damage in the central nervous system due to subarachnoid hemorrhage or traumatic brain injury before being declared BD.16,17 BD induces autonomic and hemodynamic alterations as well as alterations in the neurohormonal state of the donor16,17 that can induce lung edema and intra-alveolar hemorrhage and should be taken into account in experimental LT models in order to simulate a model that is closer to reality.

Different animal models have been described that evaluate the effects caused in donated organs due to either rapid-onset or gradual-onset BD13,16,18,19 used in transplant studies for kidneys,20 liver21 and very rarely in LT22 (Table 2). In our case, we have used the slow BD model, with a gradual increase of the intracranial pressure in order to reduce the hemodynamic instability of the donor13 and the subsequent degree of lung edema, which in the case of rats is aggravated by the liability of the pulmonary vascular permeability. In fact, in donor BD rats, a severe pulmonary edema is frequently produced more than 2 h after the induction of BD; this is why we do the LT with 2 h of ischemia time.22

In our model, orotracheal intubation with an Abbocath® 14G minimizes peritracheal air leak and ensures better ventilation of the rat during surgery, as there are no endotracheal tubes for rats. The removal of the donor lung by mid-sternotomy,23 unlike Mizuta,2 facilitates the extraction of the thoracic organs and minimizes any possible injury. Similar to transplantation in humans, the clamping of the aorta gives us precise control of the duration of the ischemia, which is not controlled in other proposed models.7,9 This also acts as a mechanical factor favoring cardiac arrest, similar to what happens in humans. In our model, as in that by Reis,4 we prefer 16G cuffs in order to minimize the risk of tearing the hilar structures, especially the pulmonary vein. The reperfusion and permeability have been shown to be good; therefore, we consider them to be adequate for animals between 300 and 400 g.

An important part of the extraction process in humans is the anterograde and retrograde organ preservation. During extraction, the flow volume and rate should guarantee homogenous perfusion of the lung. This should be carried out with the lungs ventilated in order to avoid areas of atelectasis and poor perfusion.24 In our model, we have reached optimal perfusion with an Abbocath® 16G using cold low-potassium dextran solution, which is currently the most widely used in clinical practice. The introduction of retrograde perfusion, which has not been reported in other rat models, is important because anterograde perfusion has been shown to be incomplete as it obviates the bronchial circulation.25 Retrograde preservation can be safely done with lower pressure and volume than those used in anterograde perfusion due to the pulmonary vascular lability of this type of animals. This makes our model more similar to what happens in clinical practice (Table 3).
Evolution of the Lung Transplantations in Rats.

<table>
<thead>
<tr>
<th>Study</th>
<th>Anastomosis</th>
<th>Technique</th>
<th>Cuff Length</th>
<th>Cuff Diameter</th>
<th>Surgery, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asimacopolous, 1971</td>
<td>B–A–V</td>
<td>Suture</td>
<td>–</td>
<td>–</td>
<td>Not described</td>
</tr>
<tr>
<td>March, 1982</td>
<td>V–A–B</td>
<td>Suture and suture</td>
<td>2 mm (body = 1, tab = 1 mm)</td>
<td>–</td>
<td>240</td>
</tr>
<tr>
<td>Mizuta, 1988</td>
<td>V–A–B</td>
<td>Cuffs (Teflon)</td>
<td>3 mm (body = 2, tab = 1 mm)</td>
<td>1.65 mm</td>
<td>100.7 ± 4.8</td>
</tr>
<tr>
<td>Reis, 1995</td>
<td>A–V–B</td>
<td>Suture</td>
<td>–</td>
<td>–</td>
<td>108.65 ± 4.18</td>
</tr>
<tr>
<td>Zhang, 2008</td>
<td>B–A–V</td>
<td>Suture</td>
<td>–</td>
<td>–</td>
<td>78.4 ± 5.2</td>
</tr>
<tr>
<td>Sugimoto, 2009</td>
<td>A–B–V</td>
<td>Cuff (Teflon)</td>
<td>Not described</td>
<td>No described</td>
<td>50</td>
</tr>
<tr>
<td>Current study</td>
<td>A–B–V</td>
<td>Cuff (Teflon)</td>
<td>3 mm (body = 1.5 and tab = 1 mm)</td>
<td>1.65 mm</td>
<td>107.7 ± 6.2</td>
</tr>
</tbody>
</table>

The anastomosis column shows the order of the anastomoses in the surgery (B: bronchus; A: pulmonary artery; V: pulmonary vein). The surgery time is expressed in minutes (average ± standard deviation).

Table 3

Technical Comparisons Between the Lung Preservation in our Experimental Model and Lung Transplantation in Clinical Practice.

<table>
<thead>
<tr>
<th></th>
<th>Our Experimental Model</th>
<th>Clinical LT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain death</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Type of ventilation</td>
<td>Orotracheal</td>
<td>Orotracheal</td>
</tr>
<tr>
<td>Ventilatory parameters</td>
<td>TV = 10 ml/kg</td>
<td>TV = 8–10 ml/kg</td>
</tr>
<tr>
<td></td>
<td>P_{E}OT _{AT} = 12–14 cmH_2O</td>
<td>P_{E}OT _{AT} = 20–30 cmH_2O</td>
</tr>
<tr>
<td></td>
<td>RR = 60 rpm</td>
<td>RR = 8–10 rpm</td>
</tr>
<tr>
<td></td>
<td>PEEP = 2 cmH_2O</td>
<td>PEEP = 0–5 cmH_2O</td>
</tr>
<tr>
<td></td>
<td>I/E: 1:2</td>
<td>I/E: 1:2 or 1:4</td>
</tr>
<tr>
<td>Heparinization</td>
<td>Yes; intravenous</td>
<td>Yes; intravenous</td>
</tr>
<tr>
<td>Prostaglandin E1</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Incision</td>
<td>Sternotomy</td>
<td>Sternotomy</td>
</tr>
<tr>
<td>Ischemia time</td>
<td>Clamping of the aorta</td>
<td>Clamping of the aorta</td>
</tr>
<tr>
<td>Perfusion</td>
<td>Anterograde and retrograde</td>
<td>Anterograde and retrograde</td>
</tr>
<tr>
<td>Extraction</td>
<td>Cardiopulmonary block</td>
<td>Cardiopulmonary block</td>
</tr>
<tr>
<td>Lung extraction</td>
<td>Semi-inflated</td>
<td>Semi-inflated</td>
</tr>
</tbody>
</table>

In our case, the extraction of the cardiopulmonary block is similar to what is done in humans, unlike what is described in other experimental models where only the left lung is extracted. This enables us to maintain the ventilation of the lung with FiO_2 0.21 and PEEP 2 cmH_2O until the ligature of the trachea and extraction of both lungs semi-inflated to 50% of their total capacity, which is achieved with an air pressure of 12–15 cmH_2O. The dissection of the left hilum with a microscope minimizes the risk of vascular injury and enables the vessels and the bronchus to be cut as long as possible for the placement of cuffs and the later transplantation. The clamping of the left main bronchus before its transaction at the carina allows us to maintain the lung ventilated during its preservation prior to implantation.

The anesthesia protocol, especially in the receptor, allows for the animal to be awakened at any moment without it being necessary to metabolize the anesthesia, add extra doses or use inhaled anesthesia. The effects of medetomidine can be easily reverted with its antagonist, atipamezole. In our case, we associate it with ketamine in order to achieve proper sedation, relaxation and hypnosis. In addition, the administration of atropine diminishes the secretions of the bronchial tree and has palliative effects on the secondary effects caused by medetomidine. Due to the effect of the anesthesia, hypothermia may appear, which is controlled with an electric blanket under the animal; if it is not corrected, it may cause death. The administration of these three drugs has constituted in our experience a rapid, efficient and safe anesthesia protocol for LT in these animals.

The approach of the thoracic cavity through the fourth intercostal space in the recipient (unlike other authors who use the fifth intercostal space) allows us to better manipulate the pulmonary hilum for its dissection. In addition, the transaction of the pulmonary vessels and the main bronchus as far as possible from the heart and on its anterior side, without completely cutting the structure, make the anastomoses much easier. Similarly, using long ligatures behind the microclamps makes it easier to introduce the cuff and close the anastomosis, unlike what is described by Reis.

Controlled sequential reperfusion is a critical step in human transplantation, but it has not been described in other proposed experimental models. In our model, we carried out anterograde reperfusion with the ventilated lung, enabling a homogenous distribution of the blood flow of the lung with flushing of the preservation solution out the vein, similar to what happens.
during transplantations in clinical practice. In addition, it avoids the introduction of massive quantities of preservation solution within the vascular venous flow along with an increase in the lung capillary pressure and a subsequent pulmonary edema unrelated with IR injury, which may cause artifacts in the results (Table 4).

Thus, we conclude that, with the modifications that we have described, the LT technique with cuffs in rats more closely resembles what happens in human clinical practice. This could facilitate the interpretation and clinical extrapolation of the results obtained by working with these experimental models, which are essential in LT research.

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