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Ex-vivo Lung Perfusion and Its Role in Experimental Studies

Tuğba Coşgun[1], Gül Dabak[2], Ömer Şenbaklavacı[1]

[1]Department of Thoracic Surgery, Acıbadem University Atakent Hospital, İstanbul, Turkey
[2]Department of Chest Diseases, Acıbadem University Atakent Hospital, İstanbul, Turkey

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Abstract

Lung transplantation is an accepted treatment modality for end-stage lung diseases. However, the donor pool is not getting larger in comparison to the number of patients on the waiting list. Of the patients on the waiting list for lung transplant, more than a quarter dies while waiting. Utilization of marginal donors became an issue, and ex-vivo lung perfusion (EVLP) was started to be performed to solve this problem. Besides that, success in lung transplantation has not reached a satisfactory level for various reasons including ischemia reperfusion injury. Treatment options against these reasons that cause low success of lung transplantations are being investigated by many centers. Nowadays, EVLP that was initially developed to re-evaluate marginal donors has become a method for studies in experimental models. In our article, we want to review EVLP models in experimental studies.

Keywords: *Ex-vivo* lung perfusion, ischemia reperfusion injury, lung transplantation

INTRODUCTION

To date, lung transplantation is an accepted and a preferred treatment modality for end-stage lung diseases (1-3). But ischemia reperfusion--related injury, allograft rejection, bronchiolitis obliterans, and chronic graft rejection are still important and detrimental problems in lung transplant medicine (2). The gap between the limited number of donor organs and the increase in the number of patients on the waiting list is increasing rapidly. The acceptance of lung donors is still 20%–30% so far. The scarcity of appropriate organs leads to improper utilization of marginal donors. One of the solutions to increase the number of donor organs is the meticulous evaluation of donor organs after cardiac death (2-4). This is why a method called *ex-vivo* lung perfusion (EVLP) was developed. The usefulness of EVLP on lung transplantation is firstly because this model makes it possible to evaluate the lung perfusion and functions of the body in the pre transplant period in marginal organs. By this method, we can make the right decision to implant. Second area of utilization is by preserving the graft after harvesting over long time intervals. Third area provides a platform for the development of additional therapies especially decreasing ischemia reperfusion (IRI). In this review, we aimed to provide the usage of EVLP on the development of treatment strategies in experimental studies and share our experiences with colleagues in the related field (5).

General Perspective of Organ Culture Systems

The experimental sciences have routinely used *in-vivo* and *in-vitro* models for stimulation of physiology and diseases. As a result of integration of physics and chemistry into *in-vitro* and *in-vivo* biomedicine, study on cell cultures and analyzing them at the molecular level became possible. The aim of *ex-vivo* experimental studies of organs is to create a system to stimulate human pathophysiology. Sys-

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Corresponding Author
Tuğba Coşgun
E-mail: tugba_cosgun@hotmail.com

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tems of long-term whole-organ cultures are called organomatics (6). Studies on cytokine release or production by using whole blood is an example of *ex-vivo* system (7). This area became a much used method for solid organs also (8). Details of studies on EVLP are eluciated in the 'Examples for Study Methods' section.

History

Evaluation and treatment of donor lungs before transplantation is a revolutionary step in the history of lung transplantation. Since its introduction, development of EVLP technique has gone through some steps.

In general, the *ex-vivo* perfusion of solid organs depends mainly on the cardiac studies, developed by Carrel and Lindbergh (9) in 1935. Then, Jirsch et al. (10) did some studies in 1970 aiming at the evaluation of distant harvested organs and their preservation. The first utilization of EVLP technique, which was being studied for a long period of time, was done by Steen et al. (11) in 2000 on a non-heart-beating donor (NHBD).

Clinical Experiences

Ex-vivo lung evaluation was first done in 2000 at the Cardiothoracic Department of University of Lund (Lund, Sweden) by Steen et al. (11) in a patient who had cardiac resuscitation after myocardial infarction. Family consent and harvesting took 65 minutes.

Wierup et al. (12) reported that they performed six lung transplantations after the evaluation of nine rejected donors in 2006 with a 3-months survival rate of 100% from Lund University in 2006. Although the initial method was used for only *ex-vivo* evaluation of NHBD, this also enabled the treatment of marginal and rejected donors (13).

To date, EVLP is a method used not only for evaluation of NHBD but also for the evaluation and treatment of marginal organs of brain-dead patients (14, 15). Primary indications are slippery slopes such as decreasing PaO_2/FiO_2 rates (300 mmHg), detecting pulmonary edema by physical examination and imaging, lower compliance parameter, high blood transfusion history, and longer duration of cardiac arrest (60 minutes) (16).

Short-term and mid-term outcomes of retrospective studies on clinical experiences of EVLP began to be announced. These studies specified that selected donor lungs rejected for transplantation can be used after EVLP, and short-term and mid-term results of transplantation after EVLP and without EVLP groups were comparable (17, 18). As a consequence of these studies, the interest of the industry in related technology increased, and many current studies from different companies producing devices for lung perfusion are underway. Some of them are HELP (human EVLP from Toronto) trial, Novel trial from United States, INSPIRE and EXPAND trial from Europe and United States, the DEVELOP UK from United Kingdom, and Vienna trial (19).

Today, mobile EVLP is another accentuated issue. However, most of them are still underway, there are trials that advocate that continuous, mobile, normothermic perfusion is superior to a combination of intervals of cold ischemic preservation and normothermic evaluation (19).

Primary Graft Dysfunction

Despite the improvements on management strategies, rate of primary graft dysfunction (PGD) (ischemia reperfusion injury) after lung transplantation is 10%–30% and still affects the mortality and morbidity largely (20). A 30-day mortality rate in patients with PGD ranges between 24.5% and 36.4% according to its severity (21). Besides this, patients with 12 months' survival after severe PGD have lower pulmonary functions and higher rates of bronchiolitis obliterans (22). Therefore, studies on preventing IRI (ischemia reperfusion injury) are important for increasing the success of lung transplantations.

Increase of reactive oxygen species and polymorphonuclear leukocytes, higher pulmonary vascular resistance (PVR), bad oxygenation, and edema formation are important clues of IRI (23). These parameters and assessment method in EVLP are explained later.

Techniques

The fundamental principles of EVLP system are more or less the same in clinical or experimental studies, regarding the duration of EVLP (short or long) and also in experimental EVLP, regarding the use of small or large animals. The EVLP circuit is composed of one reservoir, one centrifugal pump, membrane oxygenator (exchanger), and leukocyte filter (Figure 1) (24). Steen solution which is the most important part of the circuit contains human albumin and dextran. It provides optimal physiologic colloidal osmotic pressure and thus prevents pulmonary edema. Initially, ABO-compatible erythrocyte suspension was added to Steen solution (Steen Solution; Vitrolife AB, Gothenburg, Sweden) until it reached 15% hematocrit level (12, 13, 25). In those days, imipenem 0.5 mg (Tienam; Merck Sharp & Dohme, Sollentuna, Sweden), insulin 20IU (Actrapid; Novo Nordisk, Bagsvaerd, Denmark), and heparin 10,000 IU (Heparin; Leo Pharma, Malmo, Sweden) were also added into the circuit (12, 13, 25).

While donor lungs are connected to the EVLP system, right ventricle and left atrium are dissected and blood is emptied. Left atrium and pulmonary artery cannulation is done, making sure that no hematoma is left (Figure 2). At the beginning, the pressure of left atrium was adjusted to "0." The system is desired, and the shunt connection is clamped and perfusion begins. While starting EVLP, the temperature is kept at 25°C and at low flow (starting from 100 mL/min). The temperature of the system is gradually increased, and at 32°C, ventilation starts. Flow is also increased step-by-step and the pulmonary arterial pressure is adjusted to be less than 20 mmHg. Tidal volume was traditionally adjusted to 100 mL/kg/min, positive-end expiratory pressure (PEEP) to 5 cmH_2O, and respiratory frequency is 16 per minute (25, 26).

Long-Term EVLP Studies

Long-term EVLP studies have been milestones for experimental research. In this way, treatments can be better understood both physiologically and from the perspective of long-term effects. Long-term EVLP was first done by Erasmus et al. (15). In that study, NHBD protocols were used and groups that were topically cooled and groups that were flush-perfused after ventilator switch off were compared. In addition to showing that flush perfusion yields better graft survival, this study proved that circuit-induced injury developed during the 6-hour *ex-vivo* perfusion. This results in increased PVR and airway pressure (15). After that, modifications which will enable long-term EVLP were developed.

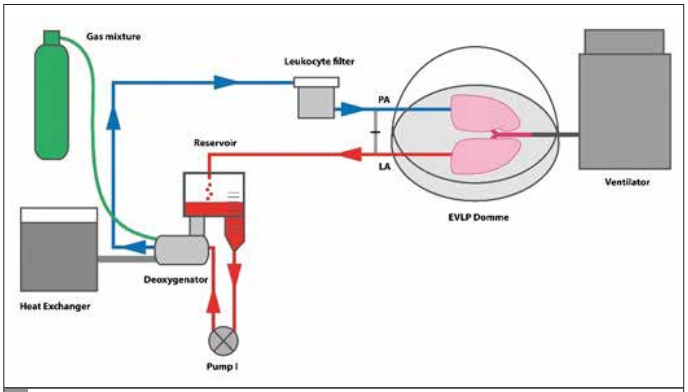

Figure 1. Schema circuit of EVLP. Figure is adapted from Inci et al. (23)
LA: left atrium; PA: pulmonary artery; EVLP: *ex-vivo* lung perfusion

Figure 2. Circuit of EVLP after cannulation Green cannula is sutured with prolene. Yellow cannula is tied with silk
EVLP: *ex-vivo* lung perfusion

It was the Toronto Lung Transplant Group in 2008 that defined 12-hour EVLP utilization (16). In their study, long-term normothermic EVLP was applied and variations concerning better *ex-vivo* perfusion were defined (16, 19). The principles of perfusion, which can be named as Toronto technique, are principally as follows:

1. Step-by-step approach to normothermic period
2. Flow should be gradually increased till 40% of the estimated cardiac output
3. Usage of acellular perfusion fluid
4. Adjustment of left atrial pressure to "4" mmHg instead of "0" mmHg

EVLP in Experimental Research

The value and importance of EVLP is increasing in lung transplant studies. Nowadays, EVLP is more often preferred for experimental studies concerning lung IRI. One of the reasons is confounding factors, such as surgical or anesthetical details which cannot be controlled by the protocol, have fewer effects on lung functions and the rate of IRI during EVLP studies. In EVLP studies, harvesting procedure and connection to the circuit can be performed in a standardized fashion, whereas lung transplant models can be affected by factors such as operation time and volume of bleeding (19).

Experiments can be ended during posttransplant observation period due to reasons such as hemodynamic instability, and this situation provides advantages for *ex-vivo* experimental models. Another advantage of EVLP models is that two animals are needed for transplant models, but for EVLP models, only one animal for harvesting is enough to evaluate IRI. Hence, EVLP models are more beneficial in terms of cost and utilization of fewer animals.

Russell and Burch defined 3R rules in ethical principles in 1959. These principles are Replacement (using small animals whether it is possible), Reduction (using minimal number of animals to get significant values), and Refinement (minimizing factors such as pain and stress during and before experiment) (27). EVLP models have advantages in terms of the second principle.

In studies performed with EVLP, if small animals such as rabbit, rat, and Guinea pig are used, then using 5–8 animals in per group and up to 50 animals in total can be preferred (28). Usually, 4–6 animals are preferred in each group for large animals like pig, sheep, and dog.

Small-Animal models

This group, which includes rats, mice, Guinea pigs, and rabbits, has advantages such as lower costs compared with groups having large animals. In addition to the low costs of animals, utilization of less perfusion solutions also provides cost-effectiveness. Because the anatomy is larger in rats, Guinea pigs, and rabbits, surgical procedures can be applied more easily than on mice. Although surgical microscope is necessary for mice, it is not mandatory for the others. Despite the fact that shorter perfusion times are favored in this model, there are studies increasing in this period (29).

Large-Animal Models

The most important advantage of these animals is their similarity with human beings with regard to physiology and anatomy. The most beneficial group is the one with pigs. For the investigators, there are different advantages of working on pigs. Since these are large animals that can be compared with humans, parameters of ventilation (tidal volume, PEEP) can be adjusted as they are applied on patients. Besides there are factors concerning the similarities in immune system and in biology which in turn lead to better clinical reflections in human population. If a medication is planned to be used in the study, its dosage adjustment can be done in a more specific style. Besides, it enables a greater confidentiality on the results of biologic parameters which will let us determine the success of the study. Another advantage is the applicability of similar technical apparatus (28).

Their disadvantage is negative cost-effectiveness of perfusion solution, drugs, etc. Also, limitation of the number of cases lowers the reliability of the study.

Ex-vivo lung perfusion experiments on rats and pigs are summarized in Table 1.

Table 1. Parameters to be adjusted during EVLP on rats and pigs

	Rat	Pig
Tidal, mL/kg	4-10 mL/kg	6-8 mL/kg
PEEP, cmH_2O	2-6 cmH_2O	5 cmH_2O
Flow rate	20% of CO	40% CO
Estimated cardiac output	25-50 mL/min for 100 mg	100 mL/min per kg
Pulmonary artery pressure, mmHg	5-10	10-15
Left atrium pressure, mmHg	3	4

CO: Cardiac output; EVLP: ex-vivo lung perfusion; PEEP: positive-end expiratory pressure

Injury

Initial injury is generally required to evaluate the treatment results especially in EVLP studies. Cold ischemia after harvesting is a widely accepted method. Furthermore, certain medications can be applied to induce injury. Inci et al. used acid aspiration to promote lung injury and studied the effect of surfactants. For this procedure, 5 mL/kg betaine HCL/pepsin was bronchoscopically instilled (24). Another application of EVLP is to assess organs of NHBD (30, 31). This is why, death before harvesting should be initiated in the experimental setup. For this purpose, ventilation switch off and potassium chloride can be given intravenously.

Evaluation of Results

Physiological Parameters

Evaluation is mostly done on an hourly basis. Depending on the practice of different centers, some maneuvers are undertaken 5–10 minutes prior to evaluation. Tidal volume is adjusted to 10 mL/kg, respiratory frequency to 10, and FiO_2 to 1.0 (16, 26, 32). Pulmonary artery pressure, left atrium pressure, peak airway pressure, and dynamic and static compliance parameters are recorded (33). Partial oxygen pressures are recorded, and delta values of pulmonary artery and left atrium oxygenation are evaluated.

Measuring permeability of lungs in those studies is very important. Thus, wet/dry ratio calculation may provide important clues. After measuring wet lung mass, lungs are dried in the oven preferably at a temperature of 60°C, and daily measurements are done until weight is stabilized. But in small tissues, this method may be erroneous (34). An indirect method of measuring edema in the lung thus lung permeability is injecting Evans blue intravenously and measuring extravascular lung volume ındex (EVLVI) is not preferred in EVLP studies. Measuring protein levels in bronchoalveolar lavage is another feasible method.

Evaluation of Inflammation

Presence of neutrophils in bronchoalveolar lavage is a marker of inflammation. Studying with multiple samples and different methods helps understand the underlying mechanism and the efficacy of the study. Myeloperoxidase is a substance which is found mainly in the neutrophils in all myeloid cells. It enables the study of tissue cultures. Measurement of neutrophil elastase level is another method of evaluation. Proinflammatory mediators again give clues about inflammation and acute lung injury. Tumor necrosis factor (TNF), interleukin (IL)-1, IL-8, are macrophage inhibitor factor (MIF) are frequently tested important mediators. These parameters can be measured by ELISA (Enzyme-Linked ImmunoSorbent Assay), and levels of bronchoalveolar lavage fluid in both tissue and fluid can be determined (34).

Histological Evaluation of Tissues

Histologic evaluation of tissues is an important tool in the evaluation of lung injury. Hematoxylin and eosin is the preferred dye for this purpose. Centers may have different evaluation schemes. It is important that all samples should be evaluated by the by only one blind pathologist for each study pathologists. While evaluating, criteria for comparison should not be changed. It was mentioned earlier that there might be differences between protocols of different centers. According to the protocol of 2011 Workshop of American Thoracic Society, 20 randomized high-power field examinations were suggested; 400× magnification should be performed. In every area, the number of neutrophils in alveolar space, the number of neutrophils in the interstitial area, formation of hyaline membranes, proteinaceous debris formation, and alveolar septal thickness are assessed from 0–2. Those values are replaced on a formula and a constant score is determined (34). Different dyes can be chosen according to the purpose of the study and the mechanism of action.

Although general evaluation criteria have been listed here, different modifications can be done according to the treatment schemes and their effectiveness.

Examples for Study Methods

Maneuvers and treatments on the better functioning of microcirculation can be developed in EVLP studies. Inhaled nitric oxide and beta-adrenergic drugs were used in different studies. Another utilization of inhalation treatments is the one concerning surfactant (4, 30). Anti-inflammatory drugs, antiapoptotic drugs, antibiotics, and immunosuppressants are added to the perfusate for treatment purposes. The advantage of EVLP studies is enabling their high-dose treatments without toxic side effects. In a study from Toronto, two groups were put on EVLP for 12 hours and high-dose antibiotics were applied on one group (ciprofloxacin 400 mg or azithromycin 500 mg, vancomycin 15 mg/kg and meropenem 2 g) (35). It was proved that some anti-inflammatory agents can improve lung functions during EVLP (36). Treatments which can affect formation of microthrombi through their effect on circulatory system were developed. Thrombolytic and fibrinolytic agents were tested (37-39). Improvements in oxygenation and PVR were demonstrated. Afterward, these treatments were applied on humans and they yielded successful results.

Studies concerning gene and cellular therapies during EVLP are being performed. Toronto Lung Transplant Group has shown in a study that functions of rejected donor lungs could be improved by gene therapy and biomarkers could be decreased by this method (40). In another study, mesenchymal cell cultures were applied on EVLP lungs which were previously injured by *Escherichia coli* endotoxin. These cell cultures helped restore the permeability of endothelial barrier and alveolar fluid balance (41).

CONCLUSION

Lung transplantation is an established treatment option for patients with end-stage lung diseases. One of the limitations of this treatment

is the shortage of donors and its associated consequence, the significant number of patients who die while on the waiting list (42). Because the utilization of marginal donors became an issue to solve this problem, EVLP was developed. But today, EVLP is not only an established method to reassess marginal donor lungs but also a platform to deliver therapeutics on donor lungs outside the body. Since EVLP is easily applicable and standardizable, it comprises a greater place in experimental studies.

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