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TITLE:

Isolated Lung Perfusion System in the Rabbit Model

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KEYWORDS:

perfusion-system, rabbit model, pulmonary surgery, technique, physiology, lung preservation, edema.

SUMMARY:

The isolated rabbit lung preparation is a gold standard tool in pulmonary research. This publication aims to describe the technique as developed for the study of physiological and pathological mechanisms involved in airway reactivity, lung preservation, and preclinical research in lung transplantation and pulmonary edema.

ABSTRACT:

The isolated lung perfusion system has been widely used in pulmonary research, contributing to elucidate the lungs' inner workings, both micro and macroscopically. This technique is useful in the characterization of pulmonary physiology and pathology by measuring metabolic activities and respiratory functions, including interactions between circulatory substances and the effects of inhaled or perfused substances, as in drug testing. While *in vitro* methods involve the slicing and culturing of tissues, the isolated *ex vivo* lung perfusion system allows to work with a complete functional organ making possible the study of a continuous physiological function while recreating ventilation and perfusion. However, it should be noted that the effects of the absence of central innervation and lymphatic drainage still have to be fully assessed. This protocol aims to describe the assembly of the isolated lung apparatus, followed by the surgical extraction and cannulation of lungs and heart from experimental lab animals, as well as to display the perfusion

technique and signal processing of data. The average viability of the isolated lung ranges between 5–8 h; during this period, the pulmonary capillary permeability increases, causing edema and lung injury. The functionality of preserved pulmonary tissue is measured mainly based on a capillary filtration coefficient (Kfc) criterion, used to determine the extent of pulmonary edema through time.

INTRODUCTION:

Brodie and Dixon first described the *ex-vivo* lung perfusion system in 1903 [1]. Since then, it has become a gold standard tool for studying the physiology, pharmacology, toxicology, and biochemistry of the lungs[2,3]. The technique offers a consistent and reproducible way to evaluate the viability of lung transplants, and to determine the effect of inflammatory mediators such as histamine, arachidonic acid metabolites, and substance P, among others, as well as their interactions during pulmonary phenomena such as bronchoconstriction, atelectasis, and pulmonary edema. The isolated lung system has been a key technique in unveiling the important role of the lungs in the elimination of biogenic amines from general circulation[4,5]. Additionally, the system has been used to evaluate the biochemistry of pulmonary surfactant[6]. Over the last few decades, the *ex-vivo* lung perfusion system has become an ideal platform for lung transplantation research[7]. In 2001 a team lead by Stig Steen described the first clinical application of the *ex-vivo* lung perfusion system by using it to recondition the lungs of a 19-year-old donor, who was initially rejected by transplantation centers due to its injuries. The left lung was harvested and perfused for 65 min; afterward, it was successfully transplanted into a 70-year-old man with COPD[8]. Further research into lung reconditioning using the *ex-vivo* perfusion led to developing the Toronto technique for extended lung perfusion to assess and treat injured donor lungs[9,10]. Clinically, the *ex-vivo* lung perfusion system has shown to be a safe strategy to increase donor pools by treating and reconditioning sub-standard donor lungs, presenting no significant difference in risks or outcomes against standard criteria donors[10].

The main advantage of the isolated lung perfusion system is that the experimental parameters can be evaluated in a complete functional organ that preserves its physiological function under an artificial laboratory setup. Furthermore, it allows the measurement and manipulation of pulmonary mechanical ventilation to analyze the components of pulmonary physiology such as airway resistance, total vascular resistance, gas exchange, and edema formation, which to date cannot be measured precisely *in vivo* on lab animals[2]. Notably, the composition of the solution with which the lung is perfused can be fully controlled, enabling the addition of substances to evaluate their effects in real-time and sample collection from perfusion for further study[11]. Researchers working with the isolated lung system should bear in mind that mechanical ventilation causes decay of the pulmonary tissue shortening its useful time. This progressive fall in mechanical parameters can be significantly delayed by hyperinflating the lungs occasionally during the time of the experiment[4]. Still, the preparation cannot usually last more than eight hours. Another consideration for the *ex-vivo* lung perfusion system is the absence of central nervous regulation and lymphatic drainage. The effects of their absence are not yet fully understood and could potentially be a source of bias in certain experiments.

The isolated lung perfusion system technique can be performed in the rabbit model with a high degree of consistency and reproducibility. This work describes the technical and surgical procedures for the implementation of the *ex-vivo* isolated lung perfusion technique as developed for the rabbit model at Instituto Nacional de Enfermedades Respiratorias in Mexico City, intending to share the insights and provide a clear guide on key steps in the application of this experimental model.

PROTOCOL:

The isolated perfusion system in the rabbit model has been widely used in the Bronchial Hyperresponsiveness Laboratory at the Instituto Nacional de Enfermedades Respiratorias. The protocol includes New Zealand rabbits with an approximate weight of 2.5–3 kg. All animals were kept in standard vivarium conditions and *ad libitum* feeding in compliance with the official Mexican guidelines for laboratory animals (NOM 062-ZOO-1999) and under the Guide for the Care and Use of Laboratory Animals (1985). All the animal procedures and animal care methods presented in this protocol were previously approved by the Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias.

NOTE: The preparation of the isolated lung perfusion system involves the deliberate death of an animal under anesthesia and via euthanasia.

1. Equipment and preparation of apparatus.

1.1 Equipment arrangement:

1.1.1 Set up an operating table with size according to the weight of the rabbit.

1.1.2 Mount the cover of the artificial thorax on the steel column with the glass chamber underneath and the ventilator with a roller pump by the sides.

1.1.3 Ensure that the cover is easily inclined to have the tracheal cannula in line with the trachea to allow a faster connection.

1.2 Artificial thorax:

NOTE: It is an essential part of the system. It consists of a water-jacketed glass chamber sealed by a special cover. The cover works as the organ holder with the connections to cannulate the trachea and vessels embedded in it.

1.2.1 Set up a venturi jet operated by compressed air to generate the negative pressure inside the artificial thorax.

NOTE: The ventilation control module (VCM) allows separate adjustments of inspiratory and end-expiratory pressures as well as respiration rate and the ratio of inspiratory duration to total cycle duration.

1.3 Apparatus:

1.3.1 Ensure that a normally working apparatus consists of a main steel column mounted on a base plate holding the artificial thorax, with the pneumotachometer and weight transducer located above it and behind the preheating coil with a bubble trap.

1.3.2 Connect one differential pressure transducer to the pneumotachometer and another to the chamber pressure. Set a different couple of pressure transducers behind the thorax to measure perfusion and venous pressures.

1.3.3 Connect the changeover stock below the oxygenator with a level electrode and the ventilation system beside the apparatus.

2. Surgical extraction of the cardiopulmonary block.

2.1 Anesthesia:

2.1.1 Use a combination of a sedative (xylazine) and a barbiturate (pentobarbital).

NOTE: Different anesthetic cocktails can be used with no effect on experimental outcomes.

2.1.2 First, sedate the healthy New Zealand rabbits with a single intramuscular injection of xylazine hydrochloride (3–5 mg/kg). Ensure that the rabbits remain calm and relaxed to allow further manipulation after a few minutes of the injection.

2.1.3 Following sedation, use the marginal (lateral) ear veins as access to anesthetize the rabbits with an intravenous injection of pentobarbital sodium (28 mg/kg).

2.2 Monitoring:

2.2.1 To avoid insufficient anesthesia or excessive depression of cardiac and respiratory functions, monitor the following parameters. To assess the depth of anesthesia, perform a toe pinch test.

2.2.2 Ensure that the mucous membrane is pink. Blue or gray shades indicate hypoxia.

2.2.3 Ensure that the heart rate is between 120–135 beats/min, and that the body temperature does not drop below 36.5 °C.

2.3 Animal placement:

2.3.1 Shave the rabbit's torso and place the animal on the operating table in supine position. Place the ventilation system near the table, behind the rabbit's head, to permit connecting the cannulae quickly after tracheotomy to avoid tissular damage.

2.4 Incision and tracheotomy:

2.4.1 Dissect the skin with a ventral median line incision of 3–5 cm from the diaphragm up to the neck.

2.4.2 With the operating scissors, cut the anterior 2/3 of the trachea between the cartilage rings to insert the tracheal cannula through the tracheal fibrous membrane.

2.4.3 Insert a 5 mm (outer diameter; OD) tracheal cannula through the tracheal fibrous membrane and use a 4-0 silk suture to fix it carefully.

2.4.4 Place either forceps or tweezers underneath the trachea to ensure the cannula did not bend against the trachea.

2.5 Positive-pressure ventilation:

2.5.1 As long as the lungs remain outside the artificial thorax, use a small species respiration pump to ventilate a positive pressure in order to avoid lung collapse during the surgery.

2.5.2 Initiate ventilation through the tracheal cannula connected to the respiration pump quickly after tracheotomy and before the thorax is opened.

2.5.3 Set the tidal volume at 10 mL/kg.

NOTE: Depending on the experiment setup and artificial thorax model, provide positive-pressure ventilation by either the same ventilation pump used to provide negative-pressure or a different one, granting a quick re-cannulation.

2.6 Thoracotomy and exsanguination:

2.6.1 To access the thoracic cavity, use a scalpel or scissors to open the thorax wall and perform a medial sternotomy up to the upper third of the thorax.

2.6.2 Hold the thorax halves open by two retractors. Several lung flaps usually surround the heart.

2.6.3 Localize the superior and inferior vena cava and refer them with threads.

2.6.4 Prior to the exsanguination of the animal, identify the right ventricle and inject 1000 UI/kg of heparin.

2.6.5 Immediately after the injection, ligate the superior and inferior vena cava with the pre-looped thread and perform exsanguination.

2.7 Heart-lung harvest:

2.7.1 Harvest the cardiopulmonary block gently and quickly. Use direct digital dissection or spring scissors to separate the connective tissue so as to remove the lungs from the thorax.

2.7.2 Dissect the vasculature in the area, as well as the esophagus.

2.7.3 Cut through the manubrium sterni to extend the medial sternotomy towards the tracheal cannula, releasing the trachea on both sides from connecting tissue.

2.7.4 Now, resect the trachea above the tracheal cannula. Gently pull up the cannula in a craniocaudal axis as the dorsal fixation of the trachea and lungs is resected.

2.8 Cannulation:

2.8.1 Lift the isolated lungs out of the thorax and carefully place them over a sterile gauze on a petri dish.

2.8.2 To prevent atelectasis, ventilate the lungs using positive-pressure ventilation with positive end-expiratory pressure (PEEP) set at 2 cmH_2O.

2.8.3 Remove the ventricles by cutting them off the heart at the level of the atrioventricular groove.

2.8.4 After opening the two ventricles, introduce the OD 4.6 mm pulmonary artery cannula for the rabbit with a basket through the right pulmonary artery and introduce the OD 5.9 mm left atrium cannula for the rabbit with the basket through the mitral valve into the left atrium.

2.8.5 Use a 4-0 silk suture in the pulmonary artery and left atrium to fix the cannulaes. Include the surrounding tissues in the ligatures of the pulmonary artery and left atrium to avoid the distension of these structures.

2.8.6 Inject 250 mL of saline isotonic solution through the arterial cannulae to flush the remaining blood from the vascular bed.

3. Perfusion technique.

3.1 Setup:

3.1.1 Place the isolated lungs carefully into the lung chamber.

3.1.2 Attach the trachea to the transductor on the cover of the chamber.

3.1.3 Connect the cannulated vessels to the perfusion system.

3.1.4 Close the chamber and secure it with the rotary lock.

NOTE: The recirculating perfusion circuit consists of an open venous reservoir, a peristaltic pump, a heat exchanger, and a bubble trap.

3.1.5 At this point, attach the chamber lid and switch over a stopcock to switch from positive to negative pressure ventilation. To check the negative pressure ventilation of the lungs and airtight closure of the chamber, inspect the respiratory excursion of the lung and chamber pressure on the pressure gauge.

3.1.6 Perfuse the lungs with 200 mL of artificial blood-free perfusate (a Krebs-Ringer bicarbonate buffer containing 2.5% of bovine albumin).

3.1.7 Start the perfusate flow at 3 mL/min/kg, then slowly step up the flow over a 5-min period to 5 mL/min/kg. Reach a flow of 8 mL/min/kg over the next 5 min and then after another 5-min period reach a maximum flux of 10 mL/min/kg. Take care of avoiding air from getting into the circuit.

NOTE: Maintain the pH and the temperature of the perfusate within physiological ranges (pH 7.4–7.5; temperature, 37 °C–38 °C). To adjust the pH, add $NaHCO_3$ (1N) or increase the flow of carbon dioxide. Alternatively, use HCl (0.1N) to acidify.

3.2 Parameters:

3.2.1 Check whether the predetermined perfusion and ventilation parameters are set as required.

3.2.2 Ventilate the lungs with humidified air at a frequency of 30 bpm, a tidal volume of 10 mL/kg, and an end-expiratory pressure (Pe) of 2 cmH_2O.

NOTE: The pulmonary arterial pressure (0–20 mmHg) corresponds to the height of the liquid level in the oxygenator or reservoir in centimeters above the pulmonary trunk, while the pulmonary venous pressure corresponds to the height of the pressure equilibration chamber above the left atrium. Both values can be modified. Note that left atrium pressure is also 0–20 mmHg.

3.3 Achieving zone 3 conditions:

3.3.1 Use the two catheters connected to side ports of the cannulae secured in the pulmonary artery, left atrium, and pressure transducers to measure the arterial (Pa) and venous (Pv) pressures.

3.3.2 Zero-reference the pressures at the level of the lung hilum.

3.3.3 Conduct the experiments under zone 3 ventilation conditions. To achieve this, wait for 10–15 min to obtain an equilibrium characterized by an isogravimetric state.

3.3.4 Ensure that the venous pressure is higher than the alveolar pressure (Palv) and the arterial pressure remains higher than both (Pa > Pv > Palv) for Zone 3 conditions to occur.

3.3.5 Ensure that the lungs' weight remains constant and arterial and left atrial pressures are stable to achieve zone 3 conditions to open up a maximum number of pulmonary vessels and maintain the microvascular bed content during the experiment.

NOTE: The measurement of Kfc as an indicator of pulmonary edema has no variation between a manual and an automatic perfusion system.

3.4 Electronic control and signal processing: Ensure that the respiratory flow, weight changes, microvascular pressure, tidal volume, vascular resistance, among others, are registered on a multiple central electronics unit that integrates signals coming from the transducers and displays them on the evaluation system.

REPRESENTATIVE RESULTS:

The isolated lung perfusion system allows organ manipulation for biopsy, sample collection from perfusion, and real-time data collection of physiological parameters. The isolated system can be used to test many hypotheses involving different functions and lung phenomena, from metabolic and enzymatic activity to edema formation and preservation periods for lung transplants.

Figure 1 displays a diagram of the fully assembled isolated lung perfusion system along with the ventilation system and the computed data acquisition. The perfusion component of the system ensures that the perfusate is constantly flowing through the isolated lungs. The pulmonary artery is cannulated to provide inflow perfusion, while perfusate outflow is provided by cannulating the left atrium of the heart. The perfusate is passed using the roller pump so that perfusate passes through the heat exchanger, then through the bubble trap into the pulmonary artery, and finally into the lung vascular bed. The ventilation component of the system allows the ventilation medium to flow constantly past the distal end of the pneumotachometer directly via the tracheal cannula into the lungs.

Figure 2 shows the concentration of MAO (**Figure 2A**) and 5-HT (**Figure 2B**) in an isolated lung preserved at 4 °C through 24 h. Serotonin and monoamine oxidase levels were determined from intravascular fluid samples obtained at different times and analyzed by ELISA. 5-HT concentration peaked after 15 min of preservation and then decreased during the next 6 h. Afterward, perfusion levels showed a non-statistically significant increase up to the 24th hour. MAO levels showed a similar behavior, peaking after 15 minutes of preservation, then decreasing during the next six hours up to the 24th hour[12]. **Figure 3** shows 5-HT and MAO release rates, expressed as a percentage of the initial value, measured through 24 h in an isolated lung preparation at 4 °C. During the first hour of preservation, 5-HT levels rose higher than MAO and decreased within 6 h after being recaptured by endothelial cells and platelets as well as MAO mediated catabolism[12].

Figure 4 shows NEP, and ACE enzymatic activity through time in an isolated lung preparation. NEP (optic densities/mg protein/min) activity (**Figure 4A**) was determined by spectrophotometric analysis using N-Dansyl-D-Ala-Gly-p-nitro-Phe-Gly as NEP substrate followed by enalapril addition to inhibit ACE. ACE (optic densities/mg protein/min) activity (**Figure 4B**) was determined by spectrophotometric analysis using enalapril as ACE substrate, followed by phosphoramidon addition to inhibit NEP. Since both solutions contained enalapril, ACE activity was calculated as the difference in fluorescence between samples with and without enalapril[13].

Figure 5 shows the effect of pulmonary preservation in capillary permeability (mKfc) through a period of 24 h in the isolated lung perfusion system in the rabbit model. A control group (n = 6), assessed immediately after harvesting, had an mKfc of 2.8 ± 0.8 (mL/min/cmH_2O/g) standard error, in contrast, the perfused lung suffered a progressive increase on mKfc scoring 7.5 ± 1.4 (n = 6) at 6 h, 10.8 ± 2.3 (n = 6) at 12 h and reached 16.3 ± 2.5 (n = 6) after 24 h of preservation[13].

Figure 6 shows the effect of different additives in the capillary permeability of the isolated lung perfusion system under diverse conditions. A sudden pressure increment of 10 cmH_2O is made through a partial obstruction of the venous outflow to measure the permeability of the capillary bed through the capillary filtration coefficient (Kfc). To measure the Kfc, partially clamp the outflow tubing that goes out of the left ventricle to the Krebs reservoir. Then, maintain the partial clamp for 3 min making sure that the pressure increment reaches 10 cmH_2O. Stop the clamping and continue the normal flow. This maneuver will be registered as an increment of the arterial pressure and a lung weight augmentation. This last parameter is considered the Kfc.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram for the isolated lung perfusion system. This figure has been modified from Hugo Sachs Elektronik (HSE), Harvard Apparatus[14].

Figure 2: Concentration of serotonin (5-HT) and monoamine oxidase (MAO) involved in lung metabolism and vascular permeability. The concentration of (**A**) MAO and (**B**) 5-HT in an isolated lung preserved at 4°C through 24 h.

Figure 3: Release rates of serotonin (5-HT) and monoamine oxidase (MAO). The release rates of 5-HT and MAO, expressed as a percentage of the initial value, measured through 24 h in an isolated lung preparation at 4 °C.

Figure 4: Enzymatic activity of Neutral endopeptidase (NEP) and Angiotensin-converting enzyme (ACE). Enzymatic activity of (**A**) NEP and (**B**) ACE through time in an isolated lung preserved at 4 °C through 24 h.

Figure 5: Effect of pulmonary preservation in capillary permeability (mKfc). The data shows the effect of pulmonary preservation in capillary permeability (mKfc) through a period of 24 h in the isolated lung perfusion system in the rabbit model.

Figure 6: Effect of different additives in the capillary permeability. The effect of different additives in the capillary permeability of the isolated lung perfusion system under diverse conditions.

DISCUSSION:

This work displays a general view of the isolated lung perfusion system, an essential technique in pulmonary physiology research. The isolated lung perfusion system offers a great degree of versatility in its uses and allows the evaluation of several parameters relevant in the testing of a wide range of hypotheses[15]. An isolated lung system is a tool with worldwide presence that, in the last decade, has further established its relevance for organ-specific evaluations and also expanded its usefulness as an extension of state-of-the-art technologies and novel therapies involving mesenchymal stem cells[16] and CRISPR/Cas9 genome engineering[17], among others. Current *ex vivo* lung perfusion research areas broadly cover anti-inflammatory strategies, ventilation injury management and prevention, anti-rejection treatment, and anti-pulmonary edema performance[15].

A proper assembly of the apparatus is required to guarantee correct data recollection. As shown in **Figure 1** the whole system consists of a negative pressure wet chamber attached to a ventilation system and a perfusion system that mimics the respiratory and circulatory functions of the lungs, respectively. Both systems are connected to a data acquisition system that allows the addition of measurement devices that can be tailored for the needs of any protocol. The surgical process of harvesting the cardio-pulmonary block should be performed quickly, preferably by experienced personnel, to avoid additional tissue injury to maintain the lung as intact as possible so the physiological function can continue without further interference during the experiment. The system also allows for real-time perfusion sample collection that can be used to determine the effect of certain molecules in different pulmonary functions (for instance, heparin effect on pulmonary preservation).

In order to achieve a proper distribution of the perfusion flow among pulmonary vessels, namely capillaries, zone 3 conditions should be procured. Zone 1 conditions are defined as the region where the arterial pressure drops below the alveolar pressure, typically approaching atmospheric pressure. When this happens, the capillaries flatten, making blood or perfusion flow impossible. Under normal circumstances zone 1 cannot exist since arterial pressure is enough to guarantee flow distribution. However, zone 1 conditions can appear if arterial pressure drops, or alveolar pressure increases (as it does during positive pressure ventilation). Zone 1 conditions lead to an unperfused ventilated lung that is incapable of performing a gas exchange. In zone 2 conditions, arterial pressure is higher than alveolar pressure. However, the venous pressure remains below the alveolar pressure resulting in a perfusion flow determined by the difference between arterial and alveolar pressures. This behavior can be modeled using a Starling resistor. Zone 3 conditions are determined by the difference between arterial and venous pressures. The increase in perfusion flow in zone 3 occurs because the capillaries distend, conditioning the opening of a maximum number of pulmonary vessels.

The system´s unit consists of seven modules: two analog transducer amplifier modules (TAM-A) equipped with an analog LED bar graph signal to monitor dynamic signals (blood pressure, respiratory airflow, contraction force, etc.), one digital transducer amplifier module (TAM-D) with a digital numeric display designed to monitor slow-changing pulsatile signals; a servo controller for perfusion module (SCP) that works together with TAM-A and TAM-D amplifiers for perfusion control of isolated organ perfusions using the peristaltic pump, the pump speed can be set in constant pressure mode or manually controlled through the SCP; an edema balance module (EBM) that measures lung weight; a ventilation control module (VCM) to control positive and negative pressure ventilation, and a timer counter module (TCM) that can be set to trigger the VCM to perform deep inspiration cycles.

The high global prevalence of pulmonary and respiratory affections and the limitations of current therapeutic options are forcing a greater demand for lung transplants, as it remains the gold standard treatment for patients with terminal lung disease[18]. The *ex-vivo* lung perfusion system represents an excellent platform to test targeted therapies in both basic and clinical research. On a clinical level, the *ex-vivo* perfusion system can be used to evaluate graft tissue outside the body, allowing to test the isolated organ before transplant, helping to gather clinical data for a more precise prognostic on the effectiveness of the transplant. Rational use of the isolated lung perfusion system could to help optimize lung transplant surgery, making them a safer and more elective procedure. The isolated lung model is also useful in the basic research of advanced diagnosis and therapy techniques such as instillation of mesenchymal stem cells and other immune-mediated therapies; many reports have shown the potential of the *ex-vivo* perfusion technique as a platform to make further research on pulmonary preservation in the development of techniques to avoid ischemia-reperfusion injury and pulmonary edema, prolonging organ viability[15]. Some troubleshooting steps and limitations associated to the isolated lung model are mainly the short-available time of this technique for possible edema generation induced by lymphatic drain limitation as well as the systemic effect of the technique. The capillary filtration

coefficient (Kfc) determination is a reliable criterion to measure the functionality of preserved pulmonary tissue and establish the extent of edema through time. No difference has been found between the manual and automatic determinations of Kfc[19].

As the use of the isolated lung perfusion system popularizes and new therapies change the clinical landscape, the *ex-vivo* perfusion technique is becoming an elective choice to improve patient outcomes in different pulmonary pathologies, as well as to increase the pool of potential lung donors without compromising recipient safety, promising a new era in pulmonary preservation and lung transplant. The emergence of the Covid-19 pandemic and the increase of COPD's prevalence[18,20] in the global population highlights the need for further basic research into pulmonary physiology, pulmonary preservation, and lung transplant, as well as preclinical research of novel therapies with views towards translational medicine. Furthermore, the *ex-vivo* rabbit model is an accessible and practical model to train residents and students in the area of pulmonology, particularly those involved with thoracic surgery and ECMO. Any laboratory involved in respiratory or thoracopulmonary research protocols is encouraged to consider the isolated lung perfusion system as part of their daily tools for their experiments.

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DISCLOSURES:
The authors declare no conflicts of interest.

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Figure 1

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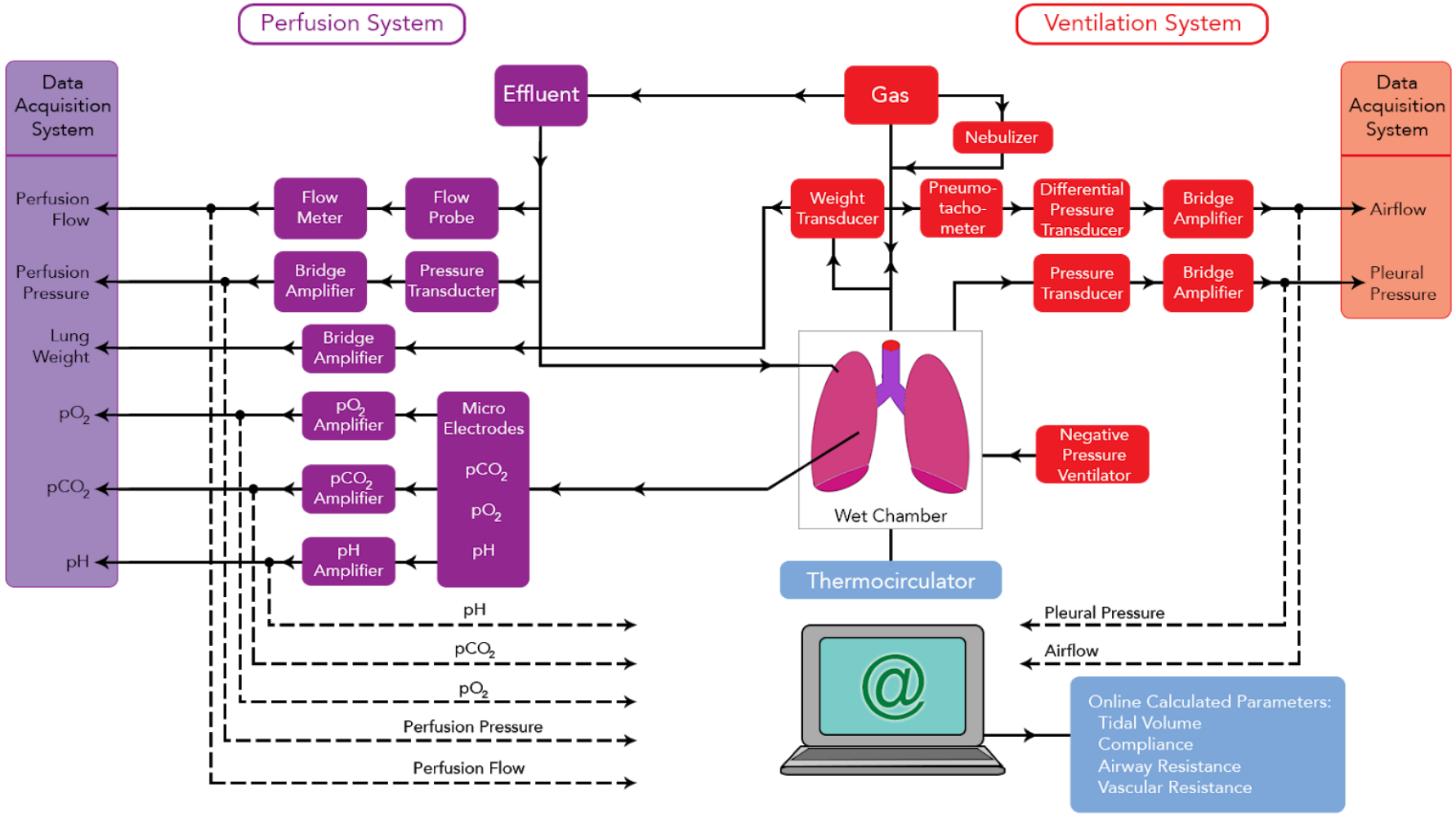

Figure 1.

Figure 2

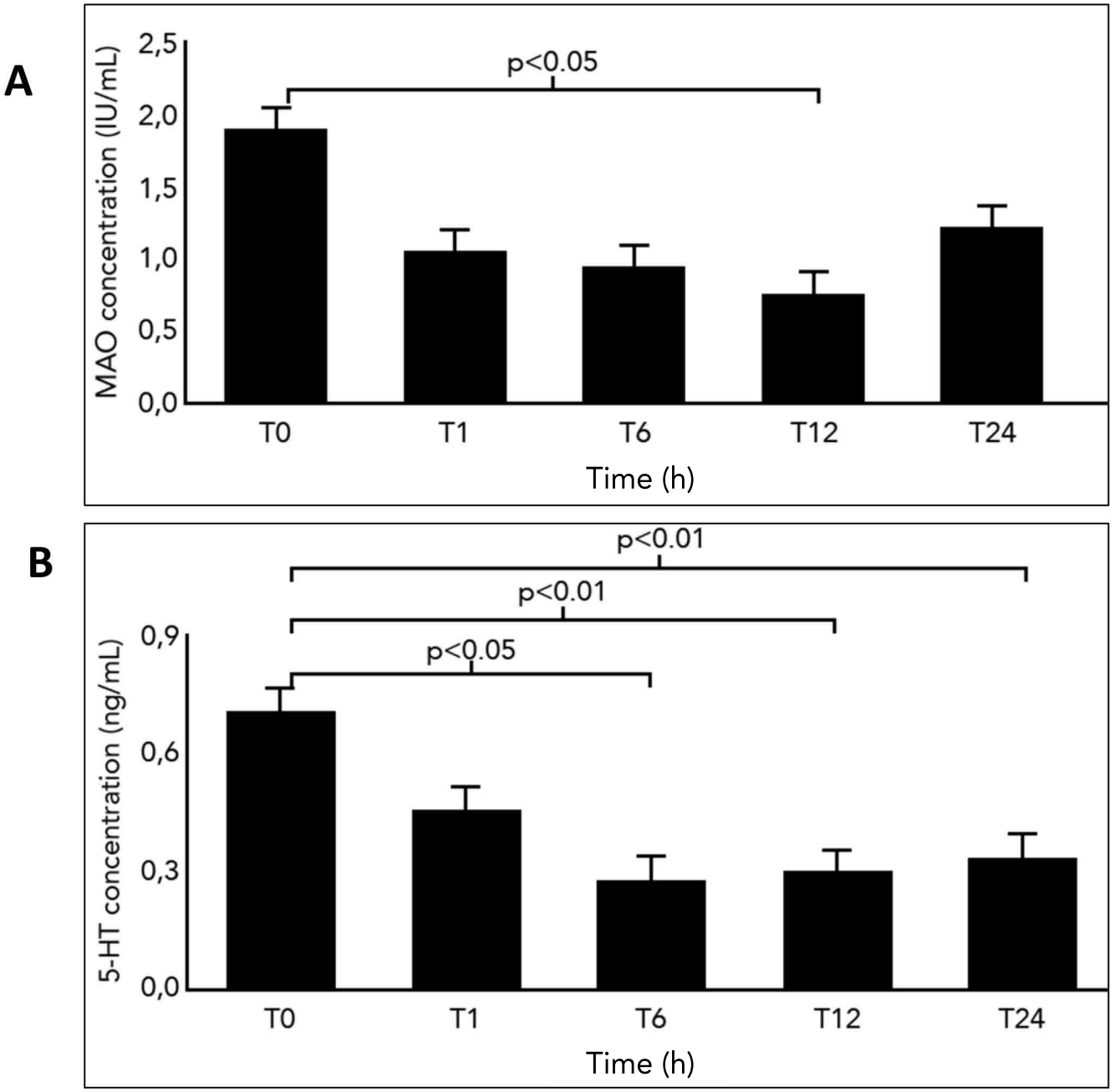

Figure 2.

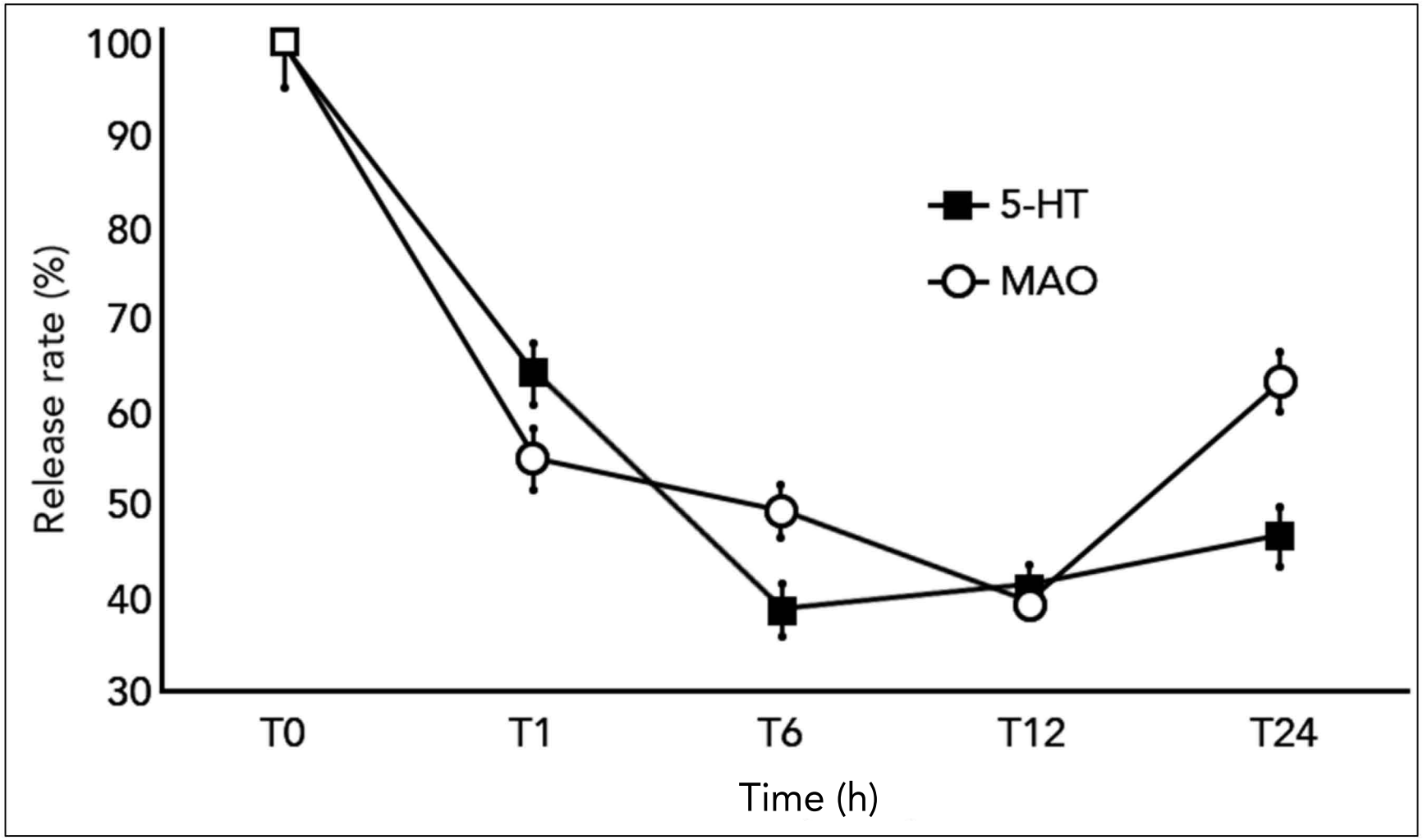

Figure 3.

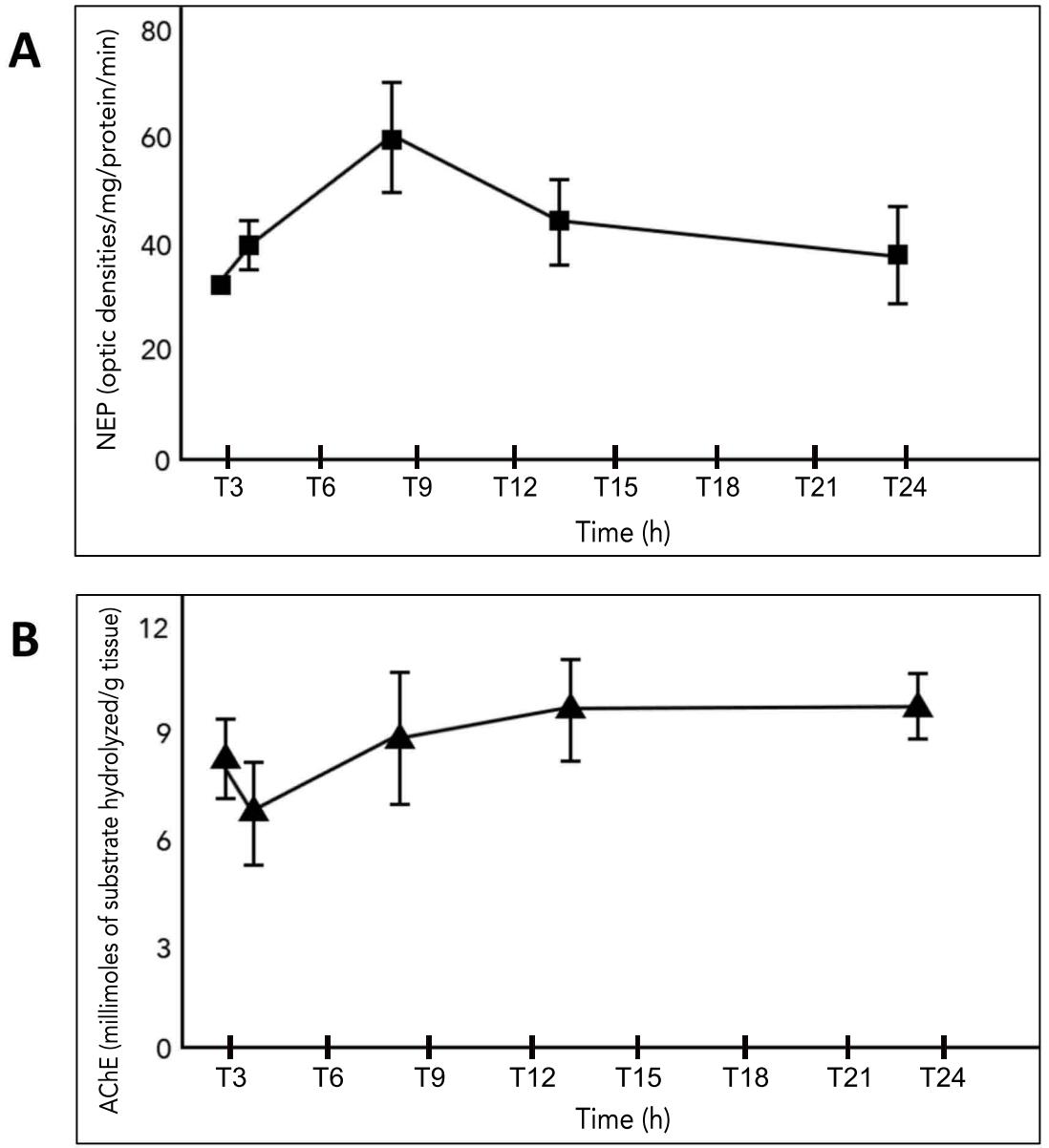

Figure 4.

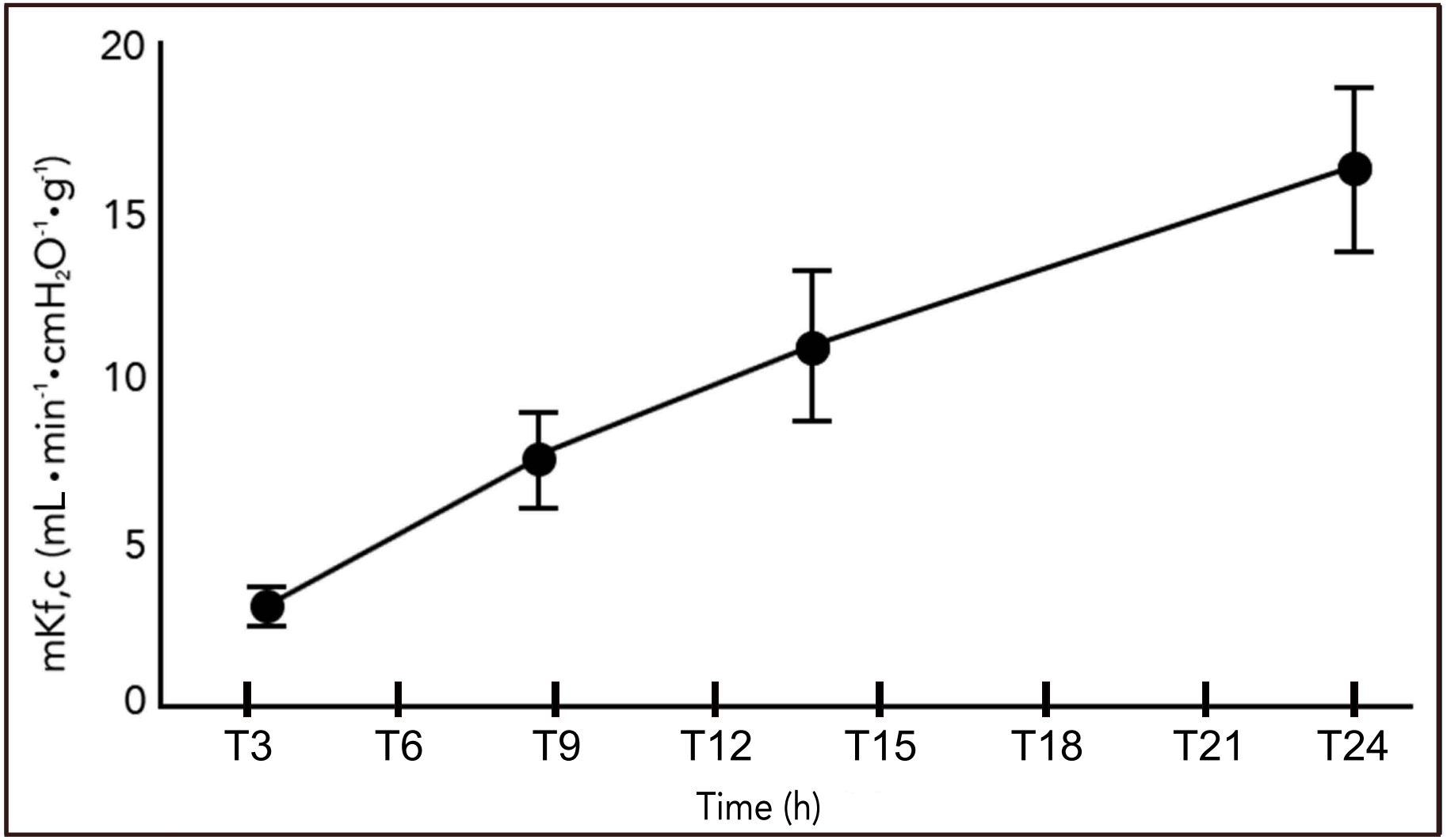

Figure 5.

Figure 6

Click here to access/download;Figure;Fig. 6.pdf

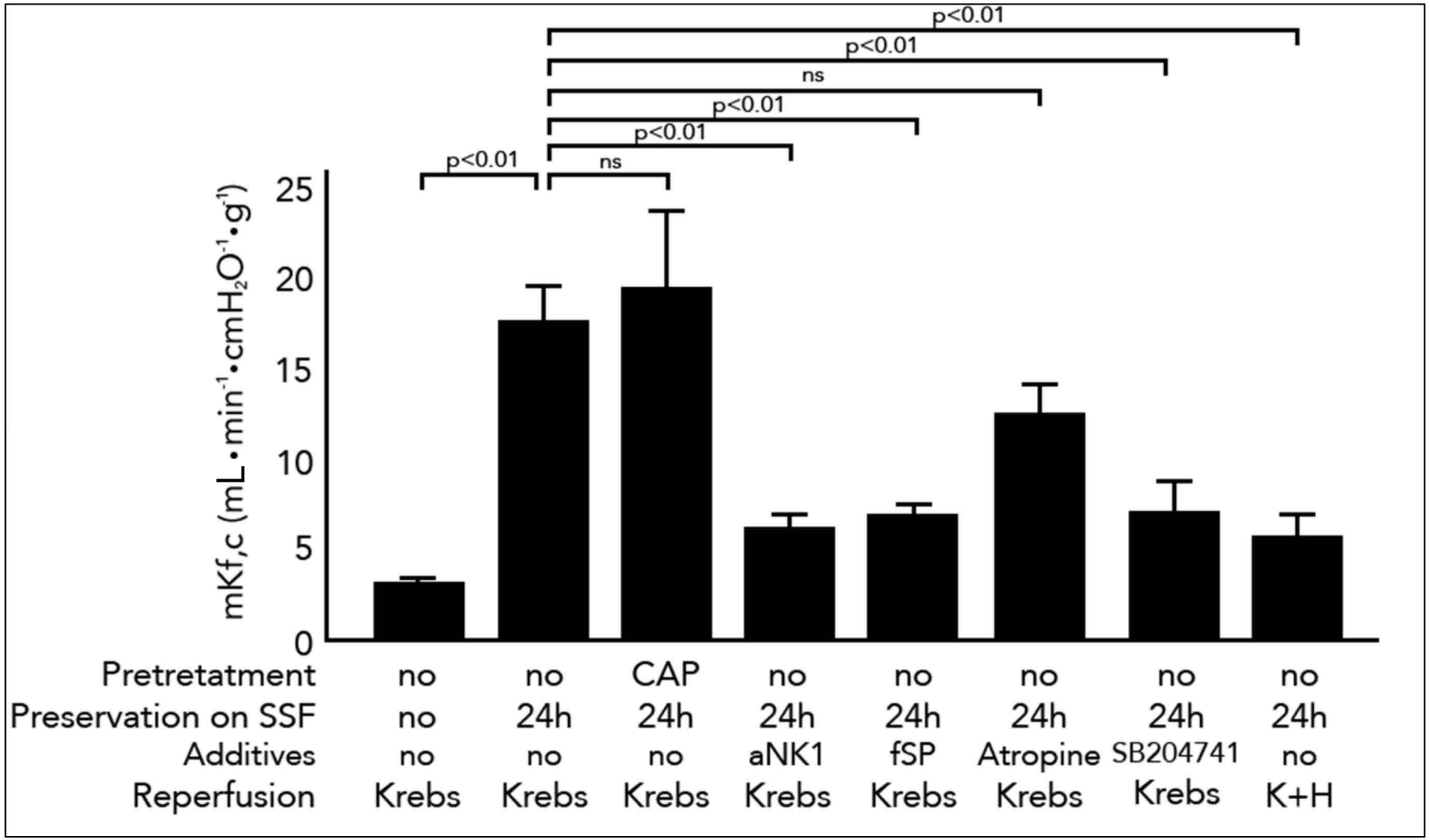

Figure 6.

Mexico City, May 16th 2021

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear professor Bajaj:

Thank you very much for your feedback on our manuscript, JoVE62734, entitled "Isolated Lung Perfusion System in the Rabbit Model". We have carefully analyzed the Reviewers' observations and our point-by-point replies to these are as follow:

Reviewer #1:

Manuscript Summary:

This is a method for ex-vivo lung perfusion in a rabbit system. This protocol can prove valuable to many investigators doing basic and translational research on pulmonary systems which will include parameters of ventilation (respiratory mechanics and gas exchange), pulmonary vascular resistance, edema formation, and tissue physiology.

Major Concerns:

None

Minor Concerns:

Only 2:

1) The graphic nature of this experimental protocol may be alarming for some viewers and the video should have a disclaimer that the preparation of the experiment will involve the deliberate death of an animal.

Reply: we appreciate your kind comments on our protocol. Regarding your first minor concern, all procedures and animal care methods were reviewed and approved by the Ethics Committee of the Instituto Nacional de Enfermedades Respiratorias, and were performed following current national and international guidelines for the Care and Use of Laboratory Animals. Still, we will be adding a disclaimer at the beginning of the video to avoid any concerns.

2) The materials list is comprehensive, though the description of the protocol in the methods and apparatus doesn't contain all parts necessary. I am not sure if this is necessary, but a bit more description in the cannulation section could be helpful, as I believe this to be a specialized technique that requires specialized equipment and is a common point of failure in our hands.

Reply: thank you for the observation, in light of your comment we´ve written a more detailed description of the cannulation technique, incision, and tracheotomy to fully address the equipment needed to perform these steps.

2.4 Incision and tracheotomy:

2.4.3 With the operating scissors cut the anterior 2/3 of the trachea in between the cartilage rings so the tracheal cannula can be inserted through the tracheal fibrous membrane.

2.4.4 Insert a 5 mm OD tracheal cannula for rabbits through the tracheal fibrous membrane and carefully fix it using 4-0 silk suture.

2.8 Cannulation:

2.8.3 Once the two ventricles are opened introduce the OD 4.6 mm pulmonary artery cannula for rabbit with basket through the right pulmonary artery and introduce the OD 5.9 mm left atrium cannula for rabbit with basket through the mitral valve into the left atrium.

2.8.4 Fix the cannulae using 4-0 silk suture in the pulmonary artery and left atrium. The surrounding tissues should be included in the ligatures of the pulmonary artery and left atrium to decrease the compliance of these structures.

Reviewer #2:

Manuscript Summary:

the manuscript describes the protocol for preparing isolated rabbit lungs. The procedure is clearly summarized with relevant figures. The procedure itself is of great importance as it allows for an ex vivo study of the lung. I think that it will be useful for other researchers to have a video manuscript of the procedure.

Major Concerns:

I have no issues with the manuscript.

Minor Concerns:

I have no issues with the manuscript.

Reply: we are glad to hear you have no concerns with our manuscript and we appreciate these and any other feedback on the protocol.

Reviewer #3:

Manuscript Summary:

Baltazar et al described the methodology of isolated rabbit lung perfusion by using the system from Hugo Sachs Elektronik. There are several major concerns as shown below.

Major Concerns:

1) There are multiple parts marked by yellow. I do not understand what the yellow mark means and it is unusual to see marked parts in a manuscript.

Reply: thank you on your feedback, taking note of your comments we´ve made several corrections and additions to the manuscript. In regard to your first concern, the yellow marks are due to the instruction: “C) Protocol length and highlighting” in the Protocol section of the “Instructions for Authors” document available on the JoVE website. The highlighted marks are the critical steps to be featured in the video.

2) 2.6

The procedure of lung procurement is described but it includes several concerns or questions from a surgical viewpoint. Was inferior vena cava cut as clinical human lung procurement?

Reply: inferior vena cava was cut after being ligated along with the aorta artery to provide exsanguination and euthanasia for the rabbit.

Was left atrium or appendage cut to release pressure in the left atrium?

The cannulation was performed through the auriculoventricular valves into the atriums after cutting both left and right ventricles. Both atriums are left untouched.

Any pulmonary artery flushing with solution in the chest?

We only flush solution through the circulation after cannulation, once the lungs are harvested.

3) 2.8.1

The following details should be included. Pressure of PEEP and duration of PEEP.

Reply: PEEP ventilation is used for as long the lungs are outside of the chest cavity during harvesting and bronchoalveolar flushing, once the lungs are inside the artificial thorax either PEEP or negative-pressure ventilation can be regulated by the ventilator according to each researcher´s needs.

2.8.1 Lift the isolated lungs out of the thorax and carefully place them over a sterile gauze on a petri dish.

2.8.2 To prevent atelectasis ventilate the lungs using positive-pressure ventilation with positive end-expiratory pressure (PEEP) set at 2 cm H_2O.

4) 2.8.4

The cannulae is pulmonary artery cannula, correct? Please specify it.

Reply: we used the term cannulae as the plural of cannula to refer to both the left atrium cannula and the pulmonary artery cannula. However, we now realize how this can be unclear so we have re-written the section to provide a more detailed description of the cannulation process:

2.8.4 Once the two ventricles are opened introduce the OD 4.6 mm pulmonary artery cannula for rabbit with basket through the right pulmonary artery and introduce the OD 5.9 mm left atrium cannula for rabbit with basket through the mitral valve into the left atrium.

2.8.5 Fix the cannulae using 4-0 silk suture in the pulmonary artery and left atrium. The surrounding tissues should be included in the ligatures of the pulmonary artery and left atrium to decrease the compliance of these structures.

5) 3.1.8

Details of step up of flow and each time should be described. This will be really helpful for readers.

Reply: we recommend stepping up the flow over a 15-minute period to gradually increase from 3 to 10 ml/min/kg. According to your suggestion, we have corrected the paragraph to mention each time we increase the flow:

3.1.8 Start the perfusate flow at 3 ml/min/kg, then slowly step up the flow to 5 ml/min/kg over a 5-minute period, reach a flow of 8 ml/min/kg over the next 5 minutes, after another 5 minutes reach a maximum flux of 10 ml/min/kg. Care must be taken to avoid air from getting into the circuit.

6) 3.2.3

Lower and upper limit of the pressure of pulmonary artery and left atrium should be included.

Reply: in the amended manuscript values were included, and the text read as follows:

3.2.3 The pulmonary arterial pressure (0-20 mmHg) corresponds to the height of the liquid level in the oxygenator or reservoir in centimeters above the pulmonary trunk, while the pulmonary venous pressure corresponds to the height of the pressure equilibration chamber above the left atrium. Both values can be modified. Note that left atrium pressure is also 0-20 mmHg.

Perfusate sampling timing needs to be added.

One of the advantages of the isolated perfused lung is that perfusate sampling can be done at any time during the perfusion and sampling timing is largely determined by the goals of the experiment. However, we usually prefer to sample every 15 minutes during the first hour of perfusion and then hourly.

Perfusion duration should be described.

An immediately harvested lung can be perfused for as long as 8 hours, however, the duration can vary according to each experiment.

How was deoxygenation? Was it done glass tube? What was the flow rate and component of gases?

No gasometry was taken since the rabbit was previously exsanguinated. Perfusion solution was used to measure metabolites present in the lungs that were perfused only with a Krebs-Ringer bicarbonate buffer solution as described in 3.1.7. Notably, Krebs solution is tested periodically to keep a pH value of around 7.3 – 7.4. When the pH value needs to be adjusted, a glass tube is used to bubble it with a mixture of 5% CO_2 and 95% O_2 (commercially available). Also, $NaHCO_3$ 1N can be used, as well as HCl 0.1N. Meanwhile, when using autologous blood from the rabbit´s exsanguination, diluted in Krebs solution, as perfusion,

blood oxygenation, and gas composition can be measured by gasometry. Accordingly, circumstances will be defined by the experimental protocol.

7) Figure 1

The circuit (especially pulmonary artery and left atrium cannula, reservoir, deoxygenation column) should be described.

Reply: the following text has been added to the amended manuscript under the “Figure 1” section:

The perfusion component of the system ensures that the perfusate is constantly flowing through the isolated lungs. The pulmonary artery is cannulated to provide inflow perfusion, while perfusate outflow is provided by cannulating the left atrium of the heart. The perfusate is passed using the roller pump, so that perfusate passes through the heat exchanger, then through the bubble trap into the pulmonary artery, and finally into the lung vascular bed. The ventilation component of the system allows for the ventilation medium to flow constantly past the distal end of the pneumotachometer directly via the tracheal cannula into the lungs.

8) Kfc

Please describe how the Kfc was measured?

Reply: to measure the permeability of the capillary bed through the capillary filtration coefficient (Kfc), a sudden pressure increment of 10 cmH 2 O is made through a partial obstruction of the venous outflow. Partially clamp the outflow tubing that goes out of the left ventricle to the Krebs reservoir. Maintain the partial clamp for 3 minutes making sure that the pressure increment reaches 10 cmH 2 O. Stop the clamping and continue the normal flow. This maneuver will be registered as an increment of the arterial pressure and a lung weight augmentation. This last parameter is considered the Kfc.

The former paragraph was added to the amended manuscript under the “representative results” section, Figure 5

9) Fig 4 and Fig 5

Timing of each data should be specified. Three time points are unclear in fig 4 and two time points are not specified in Fig 5.

This has been corrected in light of your observation.

Minor Concerns:

1) Abbreviation of MAO and 5-HT should be consistently used throughout the manuscript.

Abbreviations were uniformed along the text.

2) Were MAO and 5-HT measured in perfusate sample, correct?

Yes, that’s correct.

3) 2.2.1

No space between 36.5 °C.

Corrected thank you.

4) 1.3.1

"In sum" should be in summary in scientific writing.

Correction was made.

5) Fig 5

What does "*" or "**" mean?

This was an artifact, thank you for pointing it, it's been removed.

Dear professor Bajaj, we hope that our answers to the Reviewer's queries along with the changes done to the manuscript have help to improve our protocol and prove it useful to other researchers so that it can be considered suitable for production and publication in the innovative Journal of Visualized Experiments, JoVE.

Sincerely yours:
Jose L Arreola

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De: Laura Dalrymple (ldalrymple@harvardapparatus.com)

Para: arreolaj2002@yahoo.com.mx

Fecha: miércoles, 2 de junio de 2021 12:45 GMT-5

Hello,

Two images attached, approved for reuse, thank you!

Laura Dalrymple

Senior Designer

617-759-1631

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From: Kata Gurski <kgurski@harvardapparatus.com> on behalf of BioScience <bioscience@harvardapparatus.com>
Date: Wednesday, June 2, 2021 at 1:42 PM
To: Laura Dalrymple <ldalrymple@harvardapparatus.com>
Subject: FW: Copyright permission to reuse Figure 1 modified from HSE-HA-ISO-Lung catalog

Hi Laura – one for you, or if not, you could direct it to the correct person. Thank you!

Thanks,

Kata Gurski

Harvard Bioscience, Inc.

Salesforce Administrator / MarCom Assistant

609-513-1586 (mobile - preferred)

609-904-6051 (landline)

From: Dra. Patricia Segura Medina <psegura@unam.mx>
Sent: Tuesday, June 1, 2021 3:39 PM
To: BioScience <bioscience@harvardapparatus.com>; Webmaster <webmaster@harvardbioscience.com>
Cc: José Luis Arreola <arreolaj2002@yahoo.com.mx>
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To whom it may concern

Recently, our research group summitted a manuscript to review in order to be published in JoVE journal.

Editorial Comments was the next:

Your manuscript JoVE62734R2 "Isolated Lung Perfusion System in the Rabbit Model" has been editorially reviewed and the following comments need to be addressed before your manuscript can be formally accepted.

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Could you help me or maybe address this petition to the specific bureau for obtain this approval?

Greetings

Dra. Patricia Segura Medina

Jefe del Depto. de Investigación en

Hiperreactividad Bronquial SNI 2

Instituto Nacional de Enfermedades Respiratorias

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