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Establishing an economical and widely accessible donation after circulatory death animal abattoir model for lung research using ex vivo lung perfusion

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Abstract

Background: Ex vivo lung perfusion (EVLP), is a platform that allows simultaneous testing and treatment of the lungs. However, use of EVLP is costly and requires access to lab animals and accompanying facilities. To increase the use of EVLP for research, we developed a method to perform EVLP using abattoir procured lungs. Furthermore, we were also able to significantly decrease costs.

Methods: Six pair of lungs were procured from abattoir sheep. The lungs were then flushed and stored in ice for 3 h. A low-flow (20% of cardiac output) approach, a tidal volume of 6 ml/kg bodyweight and total perfusion time of 3 h were chosen. Perfusion fluids and circuits were self-made. Lung biopsies, perfusate collection, respiratory values, circulatory pressures were recorded and hourly blood gas analyses were performed.

Results: Mean pO₂ remained stable from 60 min (49.3 ± 7.1 kPa) to 180 min (51.5 kPa ± 8.0), p = 0.66. Pulmonary artery pressure remained ≤15 mm Hg and the left atrial pressure remained between 3 and 5 mm Hg and peak respiratory pressures ≤20 cmH₂O. Lactate dehydrogenase increased from start (96.3 ± 56.4 U/L) to the end of perfusion (315.8 ± 85.0 U/L), p < 0.05. No difference was observed in ATP between procurement and post-EVLP, 129.7 ± 37.4 μmol/g protein to 132.0 ± 23.4 μmol/g, p = 0.92.

Conclusions: Sheep lungs, acquired from an abattoir, can be ex vivo perfused under similar conditions as lab animal lungs with similar results regarding e.g., oxygenation and ATP restoration. Furthermore, costs can be significantly reduced by making use of this abattoir model. By increasing accessibility and lowering costs for experiments using lung perfusion, more results may be achieved in the field of lung diseases.
1 | INTRODUCTION

Before new drugs, medical machines or interventions can be put into clinical practice, their safety and efficacy must first be tested on animals. However, reasons for not using animals solely bred for research are multifold. Furthermore, high financial costs are involved and there are ethical considerations especially if an alternative is possible. Finally, the EU as a whole is moving toward replacement, reduction and refinement in using animals bred for research, more commonly known as the 3 R’s principle.1

Using animal parts procured from an abattoir fits well into this new vision. As the animals are already bred for consumption, once they are terminated, the organs can be used for research without additional animal harming. Abattoir procurement also has the benefits of veterinarians that are on-site, and complete use of the rest of the animal. Sheep have proven themselves to be an excellent model for investigating human lung function and pathology, thanks to their similar pulmonary physiology and anatomy. To be more precise, it has been shown that distribution of respiratory epithelial cells, mast cells, and airway smooth muscle are analogous.2,3

To be comparable with the human EVLP model, it is important that animal lungs are also able to withstand static cold storage (SCS), this is usually around 3–4 h in most studies. Other clinical conditions that must be met are sufficient PaO2/FiO2 ratio’s, pulmonary artery ≤15 mm Hg and left atrial pressures 3–5 mm Hg (PAP and LAP) as well as peak airway pressures ≤20 cmH2O (PawP). We expected sheep lungs to be able to meet these requirements.4,5

We aimed to develop a low-cost and widely available donation after circulatory death (DCD) lung research model, with the use of ex vivo lung perfusion (EVLP) and sheep lungs sourced from an abattoir.

2 | MATERIALS AND METHODS

2.1 | Logistics and development of sourcing abattoir lungs

We selected a small-to-medium-sized abattoir. Reasons for not choosing a large industrial abattoir were due to distance and slower procurement of lungs. We chose sheep (weighing between 25 and 35 kg) instead of pigs for one essential reason: pigs are scalded in boiling water baths to remove hair, leading to aspiration and prolonged warm ischemia of 30 min. Sheep were stunned using a captive bolt pistol to the head, subsequently exsanguinated and a median sternotomy was performed. The lungs were removed with traction on the trachea and without touching the lungs themselves. The lungs were then put in a box with a sterile sheet over it. The lungs could be retrieved within 10 min. In total, we used 30 sets of sheep lungs to practice and develop the model before starting inclusions.

2.2 | Lung preservation on site

After procurement, lungs are immediately intubated and inflated with a bag valve set to 10 PEEP and a release valve set to 40 cmH2O. Simultaneously, lungs were inspected for cuts, leaks, pneumonia (dark purple area’s), consolidations, severe bruising, and any other suspicious irregularities. If lungs inflated properly and could be easily ventilated without setting off the release valve, the PEEP was turned to 5 and the trachea was clamped. After passing the first inspection, lungs were brought to an improvised back table. There, lungs were quickly freed from excess tissue such as the thymus, esophagus, diaphragm and liver. At the same time, the heart was removed giving access to the pulmonary artery and the pulmonary veins. This allowed ante- and retrograde flushing by means of a 14Fr Foley catheter connected to the flush bag. Lungs were then put in a plastic bag, that covered a bowl with ice water, before starting 1 L of antegrade Prismasol2 flush. After antegrade flushing, the plastic bag was replaced and 1 L of retrograde Perfadex Plus flush was started. Five thousand IU/L of heparin (Leo Pharma, Amsterdam, The Netherlands) was added to each bag pre-flush. Lastly, lungs were submerged in their own retrograde effluent. A second bag containing ice water was put around as a second layer. Both bags were then covered with ice in a styrofoam box. Then the lungs were transported to the perfusion setup in our hospital.

2.3 | Preparing perfusion fluid

Buffered dextran-albumin (BDA) perfusion fluid was made a day before each experiment. Two liter of demi-water is collected in a beaker and set on a magnetic stirrer. The following is then mixed in bovine serum albumin (140 g), dextran 40 (10 g), NaCl (10.06 g), glucose anhydrous (3.60 g), KCl (0.68 g), NaH2PO4 monohydrate (0.29 g), CaCl2 dihydrate (0.44 g), MgCl2 (0.22 g), NaHCO3− (2.52 g)
and titrated to pH 7.4 with NaOH 1 M solution. The final solution was then stored in the fridge until the next day.

2.4 | **Perfusion setup**

A Lung Assist 2.0 (Organ Assist, Groningen, The Netherlands) was used as perfusion machine and a Servo-I (Maquet, Göteborg, Sweden) for the ventilation. A flow sensor, in- and outflow temperature, and a level-sensor are included in the Lung Assist. PA and LA pressures were measured using a homemade double line pressure sensor system connected to homemade software suitable for any laptop. A FLIR T1040 thermography camera was used to create a 1 h, 1 min interval (temperature) time lapse of the lungs. Each experiment, a new homemade perfusion set including reservoir (Organ Assist, Groningen, The Netherlands), oxygenator (LivaNova, Houston, Texas), pump rotor (Medos, Linkoping, Sweden), and PA-LA shunt was primed with our BDA solution. (See Figure 1) 10 000 IU heparin, 94 mg dexamethasone and 1500 mg ce- furoxime were added in the priming phase.

2.5 | **Ex vivo lung perfusion**

One hour pre-EVLP, PA and LA cannulas were attached to the lungs while maintaining static cold storage (SCS). After 3 h of SCS, samples were taken and perfusion was started by first retrograde flushing 100 ml of effluent and discarding it. The set was then connected and antegrade perfusion was started in supine position. We choose a low-flow approach of 20% of the cardiac output. After stabilizing the pressures, lungs were put into prone position for the remainder of the experiment. Machine temperature was increased from 20 to 37°C within 30 min. Ventilation was started after outflow temperature reached 32°C. Lungs were recruited within the first 5 min of starting ventilation, afterward recruitment was regularly performed half an hour before each evaluation phase. Recruitment consisted of a 1-min period of 10 PEEP instead of 5 PEEP. Perfusion phases and settings are presented in Table 1. Total perfusion time was 3 h.

2.6 | **Sampling and evaluation phase**

Arterial and venous blood gas analyses were performed hourly during perfusion, and analyzed using an ABL90 Flex, (Radiometer, Zoetermeer, The Netherlands) Ten minutes prior to sampling, evaluation settings were initiated: FiO₂ was set to 100%, respiratory frequency was increased from 7 to 10/min, de-oxygenation gas (mixture of 86% N₂, 8% CO₂ and 6% O₂) 4 L/min was turned on, and biopsies were taken from pre-determined regions at set times. See Figure 2.

2.7 | **Alkaline phosphatase and lactate dehydrogenase**

Alkaline phosphatase (AP) activity was measured in the perfusate as a type 2 pneumocyte cell damage marker.⁶

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FIGURE 1  Schematic ex vivo lung perfusion circulation setup
Lactate dehydrogenase is a cytoplasmic enzyme and was also measured in the perfusate as a general lung tissue damage marker.\textsuperscript{7}

### 2.8 Adenosine triphosphate, wet-to-dry weight, and histology

Biopsies were divided into three parts. The first part was stored in sonification solution (SONOP containing 0.372 g EDTA in 130 ml H\textsubscript{2}O and NaOH at pH 10.9 + 370 ml 96\% ethanol) for later ATP measuring. The second part was fixed in neutral buffered formalin and embedded in paraffin. Paraffin sections were cut at 3 micron and stained with hematoxylin and eosin (H&E) and Martius Scarlet Blue (MSB) for lung injury scoring. The slides were then scored (0–1) for presence of edema, interstitial abnormalities, and peribronchial abnormalities. The final biopsy part was put into a 1.5 ml Eppendorf, which was then put into an Eppendorf oven (Avantor, VWR, Analog Heatblock, Amsterdam, The Netherlands) for 24 h at 100\degree C to determine wet-dry weight.

### 2.9 Statistical analysis

Normally distributed continuous variables were analyzed using the Student’s or paired \( t \)-tests. The Mann–Whitney \( U \)-tests were performed to compare non-normally distributed data. All data are expressed as mean \( \pm \) standard deviation or as median and range, unless stated otherwise. For nominal variables, either the \( \chi^2 \) test or the Fisher’s exact test was used; variables are expressed in percentages and numbers. A \( p \)-value <0.05 was considered significant. All calculations were performed using the IBM SPSS Statistics 25.0 software (IBM Corp., Armonk, NY, USA).
3 | RESULTS

3.1 | Gaseous exchange during ex vivo lung perfusion

Mean arterial blood gas pO₂ remained stable from T60 (49.3 ± 7.1 kPa) to T180 (51.5 kPa ± 8.0), \( p = 0.66 \). Mean pCO₂ also did not significantly change between T60 (5.36 ± 0.33) and T180 (5.04 ± 1.02), \( p = 0.52 \) (See Figure 3A,B).

3.2 | pH and metabolite composition in perfusate during ex vivo lung perfusion

Mean pH is 7.22 ± 0.06 at baseline and decreases the most in the first hour to 7.02 ± 0.05, \( p < 0.001 \). No significant decrease is observed between T60 (7.02 ± 0.05) and T180 (6.98 ± 0.07), \( p = 0.42 \) (See Figure 3C).

Mean lactate at baseline is 2.48 ± 0.21 mmol/L and increases the most in the first hour of perfusion to 3.27 ± 0.26 mmol/L, \( p < 0.001 \) (See Figure 3D).

Mean glucose starts at 9.98 ± 0.33 mmol/L at baseline and is consumed the fastest in the first hour of perfusion (8.83 ± 0.41 mmol/L at T60), \( p < 0.001 \) (See Figure 3E).

3.3 | Lung injury markers measured during ex vivo lung perfusion

After flushing the lungs at procurement (post-flush), mean AP is 6.8 ± 6.2 U/L. AP activity is decreased after cold storage (non-significant) and after 1 h of perfusion. A significant increase can be seen between T60 and T180, \( p < 0.05 \) (See Figure 4A).

Lactate dehydrogenase significantly increased from post-flush (110.2 ± 92.7) and T60 (96.3 ± 56.4) to the end of perfusion (315.8 ± 85.0), respectively \( p < 0.01 \) and \( p < 0.05 \) (See Figure 4B).

3.4 | Peak airway pressure, dynamic lung compliance (Cdyn), and pulmonary vascular resistance

PAP remained <15 mmHg and LAP between 3 and 5 mmHg. Peak pulmonary airway pressures remained <20 cmH₂O throughout perfusions, except during recruitment periods, which are not included in the graph. (See Figure 5A) After starting ventilation Ppeak already increased significantly at T60, respectively, 13.5 ± 1.1 to

![Figure 3](https://via.placeholder.com/150)  
**Figure 3** PaO₂ (A) and pCO₂ (B) in kPa. pH (C), lactate (D) and glucose (E) in mmol/L at baseline and at 1, 2, and 3 h on ex vivo lung perfusion
18.3 ± 1.8, p < 0.01. The Ppeak only decreases after recruitment, shown at T90 (14.7 ± 1.4, p < 0.01). This pattern can be seen to repeat itself until the end. Dynamic lung compliance can be seen to present itself inversely due to its relation to Ppeak, dynamic lung compliance = tidal volume/(Ppeak – PEEP) (See Figure 5B).

Pulmonary vascular resistance (PVR) can be seen to decrease to 1026.7 ± 509.3 dynes/s/cm^5 during the initial ventilation phase. After the 1 h mark, the PVR slowly increases again to 1656.7 ± 787.6 dynes/s/cm^5 and eventually lowers to 1280.3 ± 354.4 dynes/s/cm^5 (See Figure 5C).

3.5 | Adenosine triphosphate measurements

Adenosine triphosphate levels are shown in Figure 6A. No significant difference was observed between pre-flush and...
post-EVL"P, 129.7±37.4 nmol/g to 132.0±23.4 nmol/g, \( p = 0.92 \).

Compared with moment of procurement (pre-flush), the only almost significant change was a decrease in ATP at post-flush, respectively 129.7±37.4 nmol/g to 76.2±32.4 nmol/g, \( p = 0.05 \). ATP levels were higher \(( p < 0.05 \) at post–cold ischemic time (CIT) (113.3±37.2 nmol/g) compared with post-flush.

3.6 | Wet-to-dry weight

Mean wet-to-dry-weight analyses showed a significant increase in fluid concentrations. From 81.5% at procurement to 85.7% after 3 h of perfusion, \( p < 0.05 \). (See Figure 6B) A total increase of 4.2%, which for 52.4% (2.2%) can be attributed to the flushing and cold storage period (81.5% to 83.7%). Post-CIT to the end of perfusion resulted in a total 2.0% increase, 83.7% (post-CIT) to 85.7% (T180), \( p < 0.05 \).

3.7 | Hematoxylin and eosin and Martius Scarlet Blue staining scores of lung samples

Histology review as well as slide staining scores did not show significant differences between the time points for edema \(( p = 0.30 \), interstitial abnormalities \(( p = 0.15 \), or peribronchial abnormalities \(( p = 0.50 \). (See Figure 7).

3.8 | Temperature

Real-time localized temperature change, as visualized on the FLIR T1040 thermography camera, was used to assess the perfusion pattern. (See Figure 8) This aided in optimally positioning the lungs for equal perfusion of the lobes, as well as visualizing any notable circulatory shortcoming. A representative photo series, from the last inclusion, between T0 to T50 is shown. As seen in all our acceptable lungs, both sides warm up evenly. Furthermore our low-flow strategy can be seen to adequately perfuse the whole lung.

3.9 | Financial costs per experiment

Each experiment had a total cost of around €209,25 and mainly consisted of the following. (Table 2) We did not take into account the additional overhead costs needed to maintain lab animal facilities.

4 | DISCUSSION

We have proven that lungs sourced from abattoir sheep can be successfully used in an EVLP research model. It was possible to measure lung function, damage markers and metabolites. Furthermore, we were able to drastically decrease almost all costs involved. The protocol we presented here is similar to the original porcine EVLP protocol as established by Steen et al. Using 40% of the calculated living cardiac output as max flow rate, is seen in many EVLP setups. We however, used 20% of calculated CO, as we believe that this amount of perfusion provides the lung with ample perfusion and decreases shear stress on the endothelium. Additionally, we calculated the tidal volume using 6 ml per kilogram butchered bodyweight, which does not include the weight of the skin and head of the sheep. Important to note is that we did not add perfusate nor remove metabolites, to see the unadulterated effect of EVLP on sheep lungs and the perfusate. To our knowledge, we present here the most detailed and stable abattoir sourced EVLP model.
Our results show that even without refreshing our perfusate, the lungs are able to maintain stable and adequate PaO$_2$ and CO$_2$ levels. Peak airway pressure, dynamic lung compliance, and PVR levels are all similarly maintained throughout EVLP. Metabolites are overall not stable due to this lack of fluid renewal and removal. The decrease in glucose is caused by glycolysis; whereas the lactate increase is due to it being a metabolite of glycolysis. The decreased pH levels are thought to be because of a combination of physiological H$^+$ ions produced during anaerobic glycolysis and a reduced lactate clearance in ex-vivo conditions. Another changing value is the accumulative
damage done to the type-2 pneumocytes and lung cells in general, respectively presented by AP and lactate dehydrogenase. Nevertheless, the histology did not show major pathological changes or signs of degeneration in this setup. Concordantly, we found that it was possible to restore ATP levels to baseline pre-flush values. Interestingly, there was a significant increase in ATP between post-flush and post-CIT. This indicates that the lung was able to create significant amounts of ATP, without perfusion, and using only the atmospheric air (21% O₂) that was given for inflation.

FIGURE 8  Thermal imaging of the lungs from the start of perfusion with 10-min intervals
Okamoto et al. first reported on using porcine abattoir lungs for EVLP.\textsuperscript{12} They were successful in establishing perfusion for 2–4 h. We think a crucial difference that enabled them to use pig lungs, is that their supplier did not scald the pigs, which is usually impossible to prevent in the western hemisphere and most of Europe. Unfortunately, they did not present perfusate analyses, go in-depth regarding ventilatory lung outcomes, nor provided detailed descriptions, making it difficult to compare.

More recently, Kalka et al. reported on an abattoir model using eight porcine lungs (100 kg and 6 months old) and comparing those to eight laboratory procured porcine lungs (35 kg and 9 weeks old).\textsuperscript{13} Their report also lacked information for a fair understanding and comparison of the model. No comparisons between the groups pPeak and compliance could be made due to their pressure controlled approach, but no reached tidal volume data was presented as a substitute. There were also no mentions of the perfusion flow (approach) or the amount of perfusion fluid that was exchanged during EVLP. They did report dynamic compliance decreases over 50%, high standard deviation in oxygenation capacity levels, indicating large variations within each group. Intratracheal fluid accumulation was detected in 3/8 for both groups, and an additional high fluid accumulation in 2/8 in their slaughterhouse group. We suspect that their abattoir model suffers from the scalding bath, thus leading to decreased outcomes. Additionally, their pigs were CO\textsubscript{2} stunned. CO\textsubscript{2} combines with H\textsubscript{2}O to form H\textsubscript{2}CO\textsubscript{3} (carbonic acid) and leads to a metabolic and respiratory acidosis at the cellular level, as well as hyperglycaemia, hyperlactatemia, hypercapnia, and hyperkalaemia.\textsuperscript{14}

Our study, as it is, has some limitations. Firstly, we did not include a sheep EVLP control group from a laboratory-raised background. We knew from other studies that use a similar EVLP protocol that it is possible to successfully perform EVLP using animal lungs procured under laboratory environments. In the next paragraphs, we discuss possible differences.

Furthermore, we aimed to establish a model with minimal damage, that others could use as a base for their own studies and interventions.

A second limitation was that the sheep we used, lived in big groups where airborne diseases could easily spread. As a consequence, there have been instances where no suitable lungs could be obtained on an experiment day. A related limitation, which is recognizing damaged or sick lungs. In the beginning, our lungs were sometimes damaged (cut or bruised) due to the generally practiced organ removal technique in a slaughterhouse. This was resolved by asking the butchers to be more careful when removing the lungs. Sick lungs with consolidations, purple/dark spots (pneumonia), and persistent atelectasis were also discarded.

Other challenges compared with a laboratory environment were to keep the lungs as sterile as possible during procurement and flushing. The animal was first herded into a large cage inside the abattoir where temperature ranged between 5 and 10\textdegree{}C. There seemed to be no distress as the animals were either eating or standing still before being euthanized. The animal was euthanized by means of a captive bolt pistol to the head, subsequently the carotid arteries were cut and the animal was suspended by its hind legs to exsanguinate. Quickly after the exsanguination, a median sternotomy was performed and the lungs were removed by keeping traction on the trachea while simultaneously cutting away structures attaching the lung to the thorax. The lungs were removed without actually touching the lungs themselves. The lungs were then placed on a disposable plastic sheet draped over a container and brought to the back table in the meat-packing area of the abattoir. These areas were under strict hygiene measures as it involves meat for human consumption.

This method of procurement is of course different compared with a lab animal situation where the animals are first anesthetized before shutting down ventilation after which circulatory arrest occurs due to asphyxiation. Van De Wauwer et al. showed that hypoxic circulatory arrest presented with worse pulmonary graft results compared with exsanguination.\textsuperscript{15} A longer, warm ischemic time for our model may be appropriate to induce more injuries and thus have a better comparison with asphyxiation induced death lung grafts. A related difference is that compared with our clinical perfusions, we were unable to achieve an outflow temperature of 32\textdegree{}C at T20. This delay however, did not seem to harm the lungs and has been reported to be due to initially
increased PVR. \(^1^5\) Lastly, our increased wet-to-dry-weight results are similar to the findings from other EVLP studies using lab-animals, as EVLP using cellular as well as acellular perfusates are still known to cause an increase in lung fluid content. \(^1^6\)

In conclusion, we present a method that is a more accessible and viable alternative to the dominant practice of sourcing lungs from lab animals for EVLP. Additionally, our approach lowers logistical and financial costs, while achieving similar results. Although it may take some time to set up and accustom oneself to this approach, we aim to have shown that this is worth the effort. We believe that a widespread adoption of this model can increase research output and thus increase results that may be achieved in the field of lung research.

**AUTHOR CONTRIBUTIONS**

Running experiment, data collection, critical revision of article, approval of article: Niels Moeslund and Michiel Andy Hu.

Critical revision of article, approval of article: Roland Frederik Hoffmann, Caroline Van De Wauwer, Wim Timens, Toshihiro Okamoto, and Hans Eiskjaer. Concept, critical revision of article, approval of article: Leonie Harmina Venema, Erik Alfons Maria Verschuuren, Henri Leuvenink, Michiel Erasmus.

**CONFLICTS OF INTEREST**

The authors of this manuscript have no conflicts of interest to disclose as described by the Journal of Artificial Organs.

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