

Original Article

Porcine slaughterhouse lungs for ex vivo lung perfusion - a pilot project

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Received March 24, 2020; Accepted May 28, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Ex vivo lung perfusion (EVLP) is an emerging technique for evaluation and eventual reconditioning of donor lungs. Before clinical use experiments with laboratory animals are standard. It was the aim of this study to compare lungs evaluated with EVLP from laboratory animals with slaughterhouse lungs and to investigate the potential use of a slaughterhouse lung model for ex vivo lung perfusion as an alternative for the use of laboratory animals. In a porcine model of Donation after Circulatory Determination of Death (DCDD) 16 lungs were obtained either from regular slaughterhouse animals (SL n = 8) or from laboratory animals in organ procurements (SS n = 8). Lungs were flushed and stored cold for four hours in Perfadex Plus™ and subsequently perfused ex vivo with Steen Solution™ for up to four hours. During 4 hours of EVLP lung functional parameters and activities of lactate, lactate dehydrogenase (LDH) and alkaline phosphatase (AP) in the perfusate were recorded hourly. Histological samples were taken and evaluated for Lung Injury. Lungs showed no significant difference in oxygen capacity in between groups (ΔPO_2 averaged over 4 hours: SL 293 ± 187 mmHg SS 247 ± 199 mmHg). LDH concentration was significantly higher in slaughterhouse lungs (SL $438,5 \pm 139,8$ U/l, SS $258,42 \pm 108,4$ U/l $P \leq 0,01$). We conclude that the use of slaughterhouse lungs for EVLP was feasible with no significant disadvantages compared to standard organ procurement lungs regarding lung functional outcomes. With the use of slaughterhouse lungs animal experiments in EVLP research could be successfully reduced.

Keywords: Lung transplantation, EVLP, organ harvesting, slaughterhouse, pig, Steen Solution

Introduction

Lung transplantation is an established therapy for patients with end stage pulmonary diseases [1]. However, organ shortage is a problem resulting in waiting list mortality up to 19% [2], while currently only 15% of the donor lungs available are considered appropriate for transplantation [3]. The use of the EVLP system as a platform for evaluation, reconditioning and even therapy of marginal grafts could be the key strategy to overcome organ shortages by improving the usage of grafts that are already available [4].

Before clinical use and to proof safety and efficacy of new techniques, these are first tested in animal models. Research on EVLP [5] to further optimize protocols, different types of machines,

perfusion solutions or optimized ventilation settings and therapies [6] is still necessary in laboratory animal models [7-9], before transition to the clinical practice is possible.

It is unclear and little information is available about the feasibility and efficacy of using slaughterhouse material for lung transplantation research and no information for EVLP [10].

First attempts to use organs for slaughterhouse pigs were carried out by the group of Grosse-Siestrup et al. performing the first multi organ harvesting from slaughterhouse pigs investigating the potential use of multiple organ harvesting for the use in isolated hemoperfused organ models [11]. Based on this, the group developed a model of isolated autologously hemoperfused porcine slaughterhouse lungs where

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the lungs are normothermally perfused with blood through the pulmonary artery and ventilated in a self-made perfusion circuit consisting of two circuits connected via a dialysis module for up to 135 minutes [10]. However, this experimental setup does not meet today's standards for performing ex vivo machine perfusion and is therefore not an adequate substitute for currently used EVLP research models. There is a description of a EVLP slaughterhouse model carried out by a Japanese research group where ex vivo perfusion was performed with slaughterhouse lungs in order to evolve a model with higher perfusion flow rates over 4.0 l/min to simulate adult human lung conditions [12]. The study neither applied today's clinical standard methods for EVLP (e.g. use of established protocol, system and supplies) nor provided detailed functional data or compared lung functional outcomes of slaughterhouse lungs to regularly used DCDD laboratory animal models for EVLP.

It was the aim of this study to compare lungs evaluated with EVLP from laboratory animals with slaughterhouse lungs and to investigate the potential use of a slaughterhouse lung model for ex vivo lung perfusion as an alternative for the use of laboratory animals.

Materials and methods

Animals

The study was supervised by the central animal laboratory of the University of Duisburg-Essen. All animals received human care in compliance with the "Principles of Laboratory and Animal Care" and the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). For the experiments mature domestic male hybrid pigs were divided in two groups. All animals were specially chosen by a veterinarian in cooperation with the associated farmer prior to the experiments. They were marked with spray paint and checked by general examination for signs of respiratory diseases. Additionally, tissue samples of every experimental lung were taken and checked for typical porcine diseases that can affect lung functional outcomes by real time PCR. Animals with multiple positive results were excluded from the analysis (n = 1). Because the animals did not receive medical

treatment prior to euthanasia, the present study is designed as an organ procurement only, which was reported to the local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) according to applicable law (§ 1 VTMVO).

Experimental groups, surgical process and porcine EVLP

For the experiments 16 male domestic pigs were used. For the standard group (SS) eight male domestic hybrid pigs (weight 35± 5 kg, ~9 weeks) were sedated with ketamine (30 mg/kg BW i.m.) (Ursotamin®, Serumwerk Bernburg AG, Bernburg, Germany) and xylazine (2 mg/kg BW i.m.) (Xylavet®, cp-pharma®, Burgdorf, Germany). Then they were anesthetized intravenously with midazolam (0.5 mg/kg BW i.v.) (midazolam-ratiopharm®, ratiopharm® GmbH, Ulm, Germany) and ketamine (30 mg/kg BW i.v.) using an i.v. catheter placed in the ear vein. In deep anesthesia the animals were euthanized using potassium chloride overdose (7.45%, 1.7 ml/kg BW i.v.) (Kaliumchlorid 7.45%, B. Braun Deutschland GmbH & Co.KG, Melsungen, Germany). Pigs were not ventilated during the process and did not receive any additional medication. After cardiac death was confirmed sternotomy was performed. Lungs were harvested using standard operative technique as described elsewhere and flushed with 2 liters of 4°C cold Perfadex Plus™ Solution (Perfadex Plus™, XVIVO Perfusion, Gothenburg, Sweden) [13-15]. One pig had to be excluded from the analysis due to multiple positive results for typical porcine diseases that can affect lung functional outcomes by real time PCR.

For the second group (SL n = 8) eight male domestic hybrid pigs which were matching the animal groups from the standard group were selected by a veterinarian in collaboration with the cooperating farmer and were specially marked with paint spray for follow up. These pigs were reared and fattened regularly in groups according to applicable law for pig farming (section 4, TierSchNutzTV, according to EU Council Directive 2008/120/EC). The fattened pigs (BW ≈ 100 kg, ~6 months) were transported to the slaughterhouse and slaughtered by exsanguination after CO₂ stunning. Slaughterhouse pigs went through standard slaughtering procedure including surface treatments like

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Table 1. Modified ventilator settings

Ventilator settings	Settings until full development of the lung/ <i>Recruitment settings</i>	Settings after full development of the lung
PEEP	10	6
Pcontrol	12	8
Pramp	300	300
FiO ₂	21%/100%	21%
I:E	2:1	2:1

scalding and singeing in accordance to official laws to avoid any kind of disturbance of the meat production. After the carcass has been eviscerated, the thoracic organs, including lung, heart and liver could be removed from the slaughtering line. Lungs were checked for visible signs of trauma, aspiration or pneumonia and subsequently excluded from the selection if abnormalities were present. The heart lung block was separated from attached organs (diaphragm, liver, kidneys) and prepared for perfusion according to standard procedure [16]. Subsequently the lungs were flushed antegradely and retrogradely with 2 liters of 4°C cold Perfadex Plus™ solution through the pulmonary artery and left atrial cuff. The warm ischemic time was between 30 to 40 minutes depending on the slaughtering speed. In both groups lungs were stored in Perfadex Plus™ solution in a standard preservation bag for 4 hours on ice. EVLP was performed using the XVIVO XPS™ perfusion system using the Toronto protocol with modified Ventilation settings. We used pressure controlled ventilation to achieve a gentle lung ventilation strategy, as standard volume controlled ventilation may expose the lungs to ventilator-induced injury [9, 17]. The settings are listed in **Table 1**. In both groups EVLP was performed using a standard circuit (XVIVO, Gothenburg, Sweden) which was primed according to standard protocol with Steen Solution™ (Steen Solution™, XVIVO Perfusion, Gothenburg, Sweden) as perfusion solution, 500 mg methylprednisolone (Urbason®, Sanofi-Aventis, Frankfurt, Germany) and 3000 IU Heparin (Heparin-Natrium-25000-ratiopharm®, ratiopharm® GmbH, Ulm, Germany).

Monitoring and measurements

Lung function: During 4 hours of EVLP hourly recruitments of the lung were performed follow-

ing the modified Toronto Protocol as mentioned above. Pulmonary arterial/venous oxygen capacity and lactate concentration were measured by blood gas analysis of the perfusate hourly. Pulmonary artery pressure, pulmonary vascular resistance (PVR), dynamic and static compliance (C_{sta} & C_{dyn}) and the peak airway pressure (P_{peak}) were measured continuously by XVIVO PGM Disposable Sensors and recorded hourly.

Lactate dehydrogenase, alkaline phosphatase, lactate: As a Marker for cell damage lactate dehydrogenase (LDH) levels were measured hourly in perfusate using a clinical chemistry analyzer (VITALAB Selectra E, Vital Scientific NV, Dieren, NL).

For pneumocyte type 2 injury levels of alkaline phosphatase (AP) were measured hourly (zAP, ADVIA Clinical Chemistry, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Wet dry ratio: Tissue Samples of the right lower lobe were taken, weighted and dried for 24 h at 60°C to analyze the water content of the lung tissue regarding edema formation. The ratio is the quotient weight wet/weight dry.

Histology: Lung tissue samples were taken from each lobe of the lung and fixed in 4% PFA for 48 h. Tissue samples were serially dehydrated using an ethanol to xylol gradient and subsequently embedded in paraffin. Paraffin blocks were sectioned at 7 µm, dewaxed, rehydrated and washed. Haemalaun staining was performed for 5 minutes. Samples were analyzed using a Leica DMIRE2.

Statistics: Comparisons were made between standard group (SS n = 7) and slaughterhouse lung group (SL n = 8) hourly for lung functional parameters and at the end for wet dry ratio. Results were checked for normal distribution using the Kolmogorov Smirnov test. For comparison of normally distributed data the students-t test was used while for non-normally distributed data the Mann Whitney U test was used. All results are expressed as mean ± standard deviation. Differences were considered significant at the level of $P < 0.05 = *$ and $P < 0.01 = **$. Statistical analysis was performed using SPSS Statistics 22 (IBM, Armonk, New York, US).

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Figure 1. Left side: slaughterhouse lung after retrieval from the slaughtering line. High-grade atelectasis. Right side: Same slaughterhouse lung after 4 hours of EVLP. No signs of atelectasis, minor signs of edema formation in the lower lobes. Lung appears well ventilated and shows typical lightweight and spongy consistence.

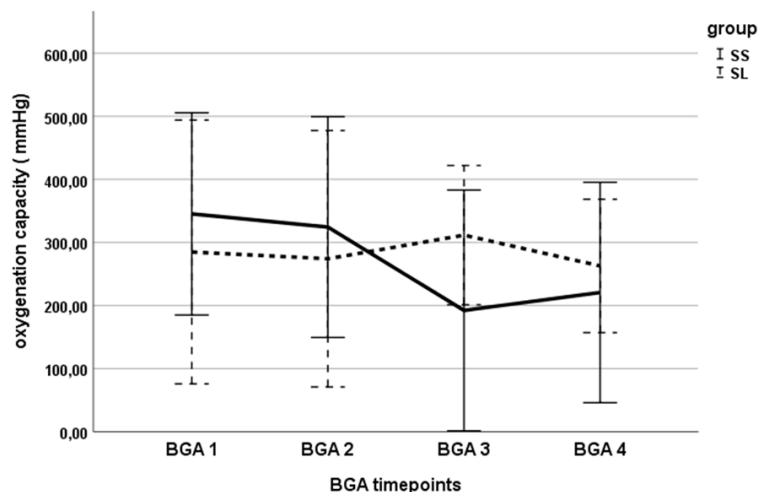


Figure 2. Mean oxygenation capacity (ΔPO_2) averaged over 4 hours of EVLP. Results are expressed as arithmetic mean \pm standard deviation shown as error bars.

Results

General macroscopic

One standard lung had to be excluded from analysis due to multiple positive results for porcine disease and massive fibrinous pleuropneumonia when thorax was opened. All lungs were tested positive for porcine circovirus in various subclinical titer levels. Lungs from organ procurements showed low grade of hemostasis and discoloration of the right lower lobe directly after explantation. After cold stor-

age there were no macroscopical changes. All slaughterhouse lungs showed medium- to high-grade discoloration and atelectasis directly after they were removed from the slaughtering line (**Figure 1**). Flush perfusion was performed to wash out residual blood from the vascular bed, which improved coloration. Comparing the standard to the slaughterhouse group ventilation in 3 out of 8 times took longer to fully inflate slaughterhouse lungs due to high atelectasis formation. After 4 hours of EVLP both groups showed typical lung consistence and were well ventilated (**Figure 1**). Minor intratracheal fluid accumulation could be detected in three slaughterhouse and three standard lungs. High fluid accumulation was seen in two slaughterhouse lungs. These lungs could only be perfused for 2 hours.

Oxygenation capacity

Between slaughterhouse and standard lungs was no statistically significant difference for ΔPO_2 (SS 274.54 ± 178.06 mmHg SL 286.38 ± 162.72 mmHg). In both groups ΔPO_2 slightly decreased over four hours (**Figure 2**). There was no statistically significant difference for ΔPO_2 when compared between groups hour by hour

(SS 1 h 345.31 mmHg 4 h 165.61 mmHg; SL 1 h 282.61 mmHg 4 h 262.84 mmHg) (**Figure 3**). Within the SS or the SL group there were no significant differences at the individual time points.

Pulmonary vascular resistance (PVR) was significantly higher in slaughterhouse lungs (SS 1458.18 ± 533.04 dyn*sc/m⁵, SL 1995.43 ± 985.38 dyn*sc/m⁵; ($P = 0.024$)), but showed no difference within each group. PVR increased by 23.76% in the slaughterhouse group and 33.25% in the standard group.

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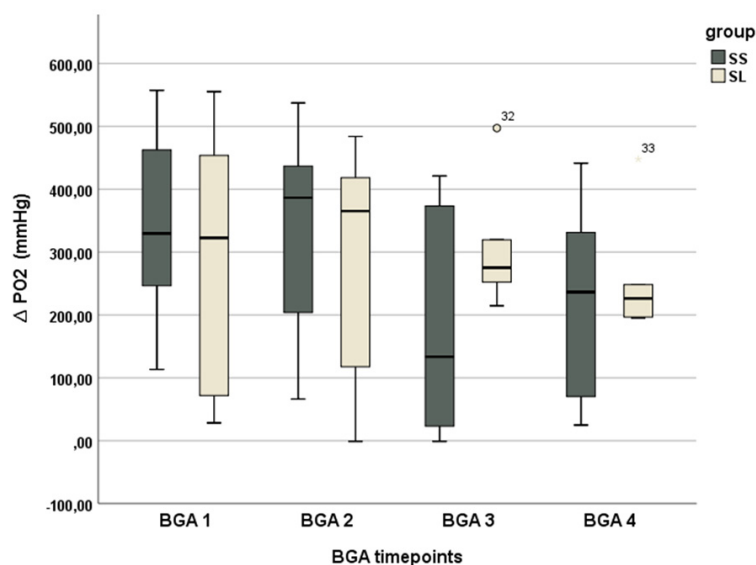


Figure 3. Mean ΔPO_2 averaged over 4 hours of EVLP for standard and slaughterhouse lungs. Results are expressed as boxplots showing the Interquartile Range (body) and the minimum and maximum (whiskers). Outliers are shown as points.

Due to pressure controlled ventilation mode the volume depending parameters peak airway pressure (pPeak) and compliance could only be evaluated within the groups and not in between groups. Within SL pPeak was significantly higher in the first hour compared to the second hour (SL 1 h 23.07 ± 1.2 cmH₂O; SL 2 h 21.6 ± 0.8 cmH₂O). Despite that pPeak remained stable during the experiment in both groups (SS 19.86 ± 2.75 cmH₂O; SL 22.66 ± 1.8 cmH₂O). Dynamic compliance decreased over 4 hours EVLP within the standard group but the difference did not reach statistical significance. Within the slaughterhouse group was a significant decrease in compliance between the second and fourth hour (SL 2 h 35.79 ± 7.12 ml/cmH₂O; SL 4 h 19.06 ± 9.00 ml/cmH₂O ($P = 0.022$)). In General Cdyn within SL decreased over time (SL 1 h 38.97 ± 22.15 ml/cmH₂O; SL 4 h 19.06 ± 9.00 ml/cmH₂O).

Lactate levels were lower in slaughterhouse lungs but did not reach statistically significant difference to standard lungs (averaged over 4 hours: SS 6.29 ± 2.55 mmol/l; SL 5.45 ± 1.58 mmol/l) (**Figure 4**).

Cell injury

For assessment of general cell injury LDH concentration levels were measured hourly.

Specifically for lung cell pneumocytes type 2 damage AP activities were measured hourly. Mean LDH levels were significantly higher in the slaughterhouse group (averaged over 4 hours SS 272.58 ± 135.42 U/l; SL 407.61 ± 131.15 U/l ($P \leq 0.01$)). The levels increased by 61.38% in the slaughterhouse group over time and in the standard group by 90.73% (SS 0 h 189.38 ± 105.14 U/l, 4 h 361.2 ± 165.32 U/l; SL 0 h 317.63 ± 138.69 U/l, 4 h 512.6 ± 67.34 U/l) (**Figure 4**). AP levels were lower in SL lungs but the difference was not significant (averaged over 4 h: SS 7.84 ± 6.80 U/l; SL 6.00 ± 2.90 U/l). In the standard group, AP levels decreased 66.16% and increased 16.67% in the slaughterhouse

group (SS 0 h 7.86 ± 4.97 U/l 4 h 5.2 ± 1.1 U/l; SL 0 h 6.00 ± 3.74 U/l 4 h 7.00 ± 2.12 U/l) (**Figure 4**).

Edema formation

The Wet Dry ratio did not differ significantly between the groups. But the W/D ratio tended to be lower in SS lungs (SS 5.92 ± 1.48 ; SL 7.27 ± 1.51). Absolut lung weight compared within the groups was significantly higher after the EVLP workout in both groups (SS $P = 0.021$; SL $P = 0.003$). Both groups gained weight nearly equally. In the standard group the averaged lung weight gained 69.88% and 74.63% in the slaughterhouse group (SS 456.86 g to 776.14 g; SL 755.88 g to 1320.00 g).

Histology

For studying epithelial cell integrity, both groups were analyzed in regard to edema, presence of erythrocytes, fluid accumulation and septal integrity. Lung samples of both groups showed similar degrees of mild epithelial and perivascular edema (**Figure 5**). However, lungs of the slaughterhouse group seem to show a slightly higher number of atelectatic areas than standard lungs (**Figure 6A**). While presence of erythrocytes in the alveolar spaces could be found in individual lungs from both groups, the lung that

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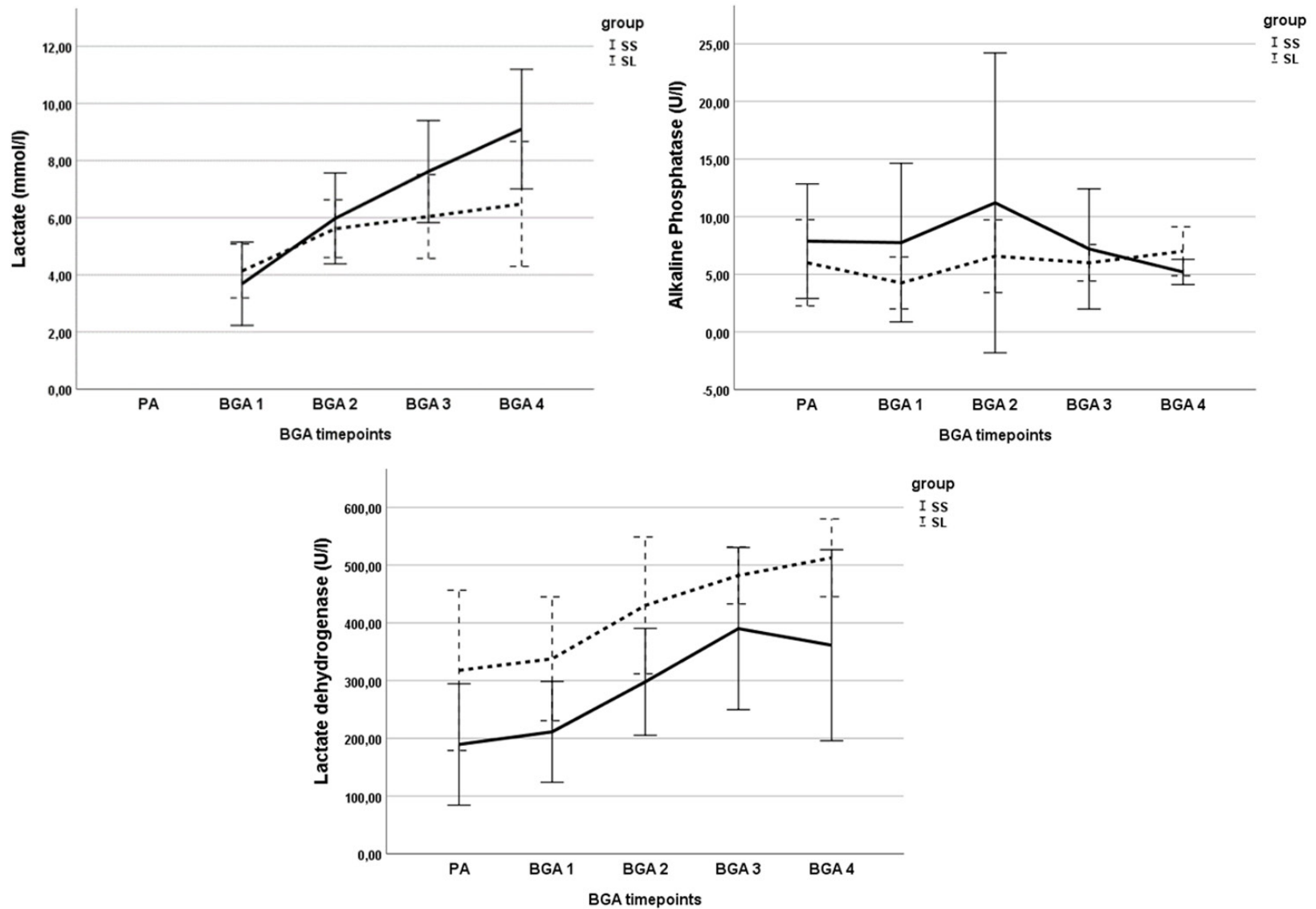


Figure 4. Lactate concentration and activities of lactate dehydrogenase and alkaline phosphatase in perfusate during 4 hours of EVLP. Results expressed as arithmetic mean \pm standard deviation shown as error bar. LDH levels were significantly higher in the SL group ($P < 0.01$). AP and Lactate levels were lower in the SL group but the difference did not reach statistical significance.

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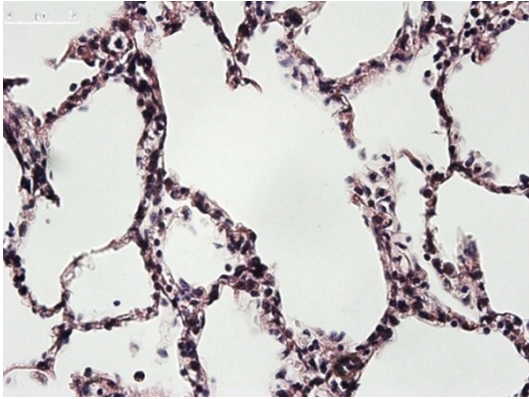


Figure 5. Mild epithelial edema. Both groups showed similar degrees of mild epithelial and perivascular edema formation.

shows the highest amount of erythrocytes in alveolar space belongs to the slaughterhouse group (**Figure 6B**). In contrast to that, the lung with the most severe destruction of alveolar cell structure was found in the standard group (**Figure 6C**). Minimal fluid accumulation in alveoli appears in both groups (**Figure 6D**). In general, all lung samples exhibit predominantly thin-walled clear alveoli.

Discussion

We have 1) compared lungs from laboratory animals with slaughterhouse lungs and 2) demonstrated, that the use of a slaughterhouse lung model for EVLP is an alternative to lungs from laboratory animals. Most important, the averaged oxygenation capacity over 4 hours did not differ significantly between the experimental groups and tended to be lower in the standard group. Regarding the time course, the difference between the groups was not significant neither. From this the conclusion can be drawn, that although the initial damage to the lung tissue is much higher in the slaughterhouse group, due to the technical procedures and potential risks of aspiration and longer warm ischemic times during the slaughtering procedure, the functional outcomes are still comparable. The decrease of the compliance occurring in both groups is likely caused by developing edema or atelectasis caused by post ischemic reperfusion injury. Which is in accordance with the increasing lung weight observed in our study.

Cell injury is associated with increased serum LDH levels [18]. LDH levels in our experiments

are increasing in both groups but significantly higher in slaughterhouse lungs. A reason for the increased LDH levels might be potentially higher stress levels before the stunning procedure and technical standard procedures like scalding and singeing causing possible trauma. These confounders may differ in between slaughterhouses with other technical equipment.

Compared to other large animal models our model is designed without any lung protective treatment before euthanasia because treatments like this would not be possible during the slaughtering process and the groups should have a comparable starting situations regarding the damage to the lung caused by hypoxia and clot formation. To keep potential lung injury caused by the slaughtering procedure as low as possible all lungs were checked for visible signs of damage caused by trauma or aspiration. Nevertheless, two lungs from the slaughterhouse group couldn't be perfused for the whole 4 hours. This may also be due to our study design as we had specially randomized tagged animals. For better comparability, this was essential, as they were fitting the animals of the standard group, but this limited the choice of lungs at the slaughterhouse. For further experiments using only slaughterhouse material, the choice is much greater and the selection of high-quality lungs can be easily increased.

In contrast to the previously introduced models for slaughterhouse lungs we used a clinical standard perfusion system and circuit designed and already in use for clinical performed ex vivo lung perfusions. Moreover, we performed EVLP maintaining standard cold ischemic time of 4 hours and normal EVLP duration of 4 hours. To the best of our knowledge, our model is the first described successfully performed ex vivo lung perfusion comparing functional outcomes of slaughterhouse lungs to regular laboratory animals in a standardized and randomized EVLP setting over 4 hours. For standardization, the trial protocol was adapted to the conditions at a slaughterhouse to intervene as little as possible in the processes of food production while maintaining a scientifically useable organ. The model could be used for technical experiments investigating pathomechanisms of reperfusion injury or general considerations about optimal lung functional outcomes under different preservation, perfusion or ventilation conditions during EVLP. The model could also be used to

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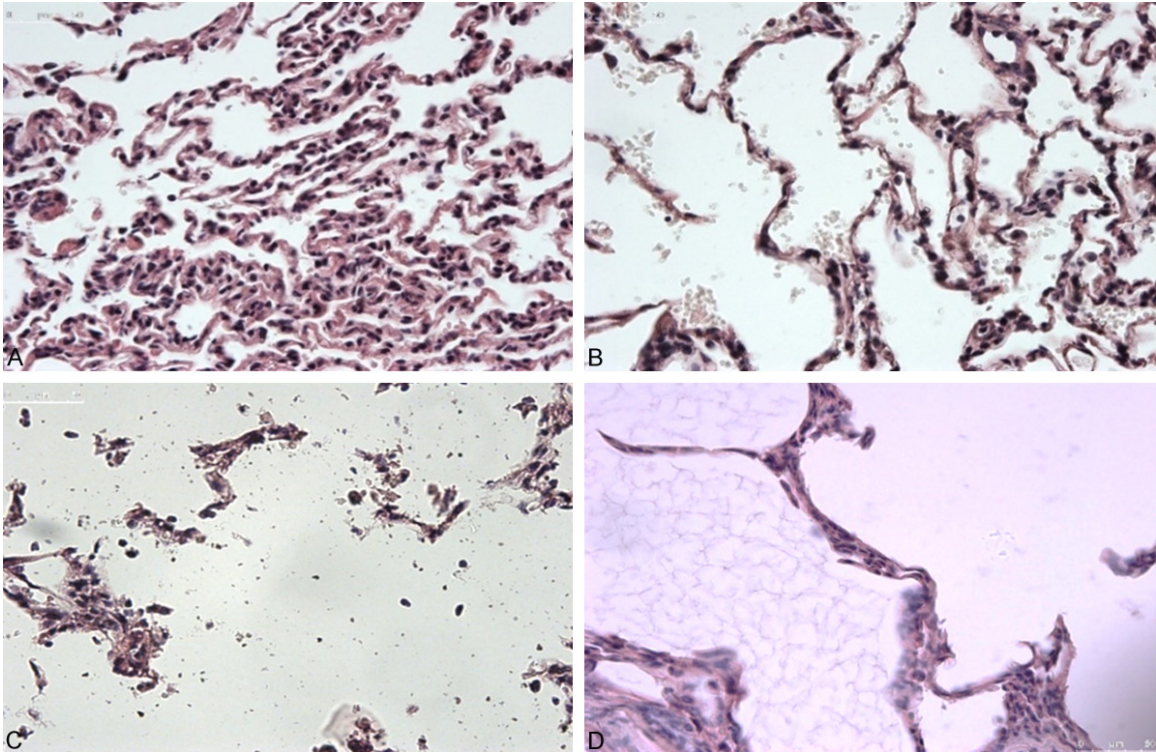


Figure 6. Histological Findings: (A) Atelectasis, (B) Erythrocytes in alveolar space, (C) Severely torn alveolar structure, (D) Fluid in alveolar sacculle.

answer questions about toxicities of novel therapeutic approaches to further reduce animal numbers. In addition, the use of slaughterhouse material for lung perfusion experiments is not defined as an animal experiment and therefore is easily and fast implementable as an alternative research model.

Large animal models have proven to be superior to small animal models because like described by Nelson et al. and Yeung et al. most physiological parameters like size, tidal volume, PEEP (pulmonary end expiratory pressure), perfusion times and maximum flow rates achieved during perfusion reflect human lung parameters very well. Clear disadvantages of large animal models remain their high costs for purchase and care as well as higher material costs, which makes each experiment more expensive and keeps the number of animals used per group low, compared to small animal models [3].

Clear advantages of slaughterhouse lungs are the widespread availability and the enormous difference in experimental costs as slaughterhouse lungs are a side product from the slaughtering industry and therefore very cheap.

Ex vivo lung perfusion is an expanding research field with a high impact on the solution of the organ shortage problem of lung transplantation. Because of the promising results of lungs reconditioned through EVLP, the influence of the research carried out to improve the EVLP system is very high. However, the potential of this technique is not fully exploited yet.

The study is strong for the standardized, experimental design. Limitations of the study are the small cohort of animals used for the experiments.

The use of slaughterhouse lungs has no profound disadvantages compared to standard lungs obtained from organ procurements in laboratory animals. Because slaughterhouse material is easily available at low costs and low administrative effort it has a great potential to increase case numbers in EVLP research and improve in particular experiments regarding optimal technical implementations. Moreover, in line with the 3R concept, the use of slaughterhouse material greatly contributes to the reduction of animals necessary for EVLP experimentations and therefore should be considered as a successful alternative to animal experiments.

Acknowledgements

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: this project was funded by the German federal Institute for Risk Assessment (BfR) under the funding number: 1328-563.

Disclosure of conflict of interest

None.

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