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Custodiol-MP for ex vivo lung perfusion – A comparison in a porcine model of donation after circulatory determination of death

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Abstract

Introduction: Ex vivo lung perfusion (EVLP) is an established technique to evaluate and eventually recondition lungs prior to transplantation. Custodiol-MP (C-MP) solution is a new solution, designed for clinical machine perfusion, that has been used for kidneys. The aim of this study was to compare the effects of EVLP with Custodiol-MP on lung functional outcomes to the gold standard of EVLP with Steen Solution™.

Material and Methods: In a porcine EVLP model of DCDD (Donation after Circulatory Determination of Death), lungs were perfused with Steen Solution™ (SS, $n=7$) or Custodiol-MP solution supplemented with 55 g/l albumin (C-MP, $n=8$). Lungs were stored cold for 4 h in low potassium dextran solution and subsequently perfused ex vivo for 4 h. During EVLP pulmonary gas exchange, activities of lactate dehydrogenase (LDH) and alkaline phosphatase (AP) as well as levels of lactate in the perfusate were recorded hourly.

Results: Oxygenation capacity differed significantly between groups (averaged over 4 h: SS 274 ± 178 mmHg; C-MP 284 ± 151 mmHg $p=0.025$). Lactate dehydrogenase activities and lactate concentrations were significantly lower in Custodiol-MP perfused lungs.

In a porcine model of DCDD with 4 h of EVLP the use of modified Custodiol-MP as perfusion solution was feasible. The use of C-MP showed at least comparable lung functional outcomes to the use of Steen Solution™. Furthermore C-MP perfusion resulted in significantly lower lactate dehydrogenase activity and lactate levels in the perfusate and higher oxygenation capacity.

Keywords

Lung transplant, organ preservation, ex vivo lung perfusion, Custodiol-MP, animal models, artificial lung, mechanical ventilation, pulmonary function, affordable artificial organs

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Introduction

For patients with end stage pulmonary disease, lung transplantation (LuTx) remains the only therapeutic option.[1,2] However, due to organ shortages the number of patients on the waiting list exceeds the number of available organs suitable for transplantation by far.[3] The EVLP (ex vivo lung perfusion) technology has been clinically confirmed as effective procedure for the evaluation and reconditioning of marginal organs. It even offers therapeutic options leading to the utilization of lungs otherwise not suitable for transplantation.[4,5]

Protocols frequently used for EVLP like Lund and Toronto use Steen Solution™ for perfusion.[3] Steen Solution™ is designed as a modified low potassium dextran solution with 70 g/l human serum albumin as major adjuvant.[5,6]

Lungs undergoing ischemia present a typical postischemic reperfusion injury (LIRI).[7] Since primary graft dysfunction (PGD) due to LIRI is common in lung transplantations, the development of a safe and reliable technique preventing LIRI would contribute to the improvement of transplanted lungs.[7–9]

EVLP procedures aim to improve and protect lung quality by using adequate perfusion solutions for nutrition, preservation and improvement of the lung tissue. Hence, the composition of the perfusion solution used for EVLP seems to be crucial for EVLP's success.[2,6,7,10,11]

The composition of the perfusion solution directly influences colloid osmotic pressure and antioxidative properties to reduce ROS (reactive oxygen species) formation and prevent the organ from excessive reperfusion injury.[2,11] Recent studies have investigated antioxidative properties of Steen Solution™ and discovered a specific downregulation of NOX_2 activity. This finally resulted in ROS reduction and decreased inflammatory cytokine expression in vitro.[2,11] ROS strongly contribute to the promotion of LIRI, a main cause of Primary Graft Dysfunction (PGD).[2,8,9]

Although the mechanisms of ROS-dependent lung injury have not yet been fully identified, it is well known that iron-dependent cell damage is the main pathway of cold-induced tissue injury during reperfusion.[2,11,12]

Custodiol-MP is a modified version of Custodiol-N Preservation Solution, specially designed for machine perfusion settings. Custodiol-N itself is based on histidine-tryptophan-ketogluterate (HTK) solution (Custodiol®) which in Germany is standard for cardiac preservation.[13,14] Standard lung preservation solutions are Perfadex® and Celsior.[15] Comparable and more widespread solutions worldwide are St. Thomas or University of Wisconsin solution (UW) for heart preservation.[16] A major difference of Custodiol-N, compared to HTK solution, was the addition of the iron chelators LK 614 and deferoxamine to prevent ROS formation. Further changes include the addition of the simple amino acids glycine and alanine to inhibit ischemic cell injury. Modified Custodiol-N Solution (supplemented with Dextran 40) was shown to be successful in kidney perfusion and even cold storage of lungs.[12,17–19]

In a recent study, we were able to show that modified Custodiol-N is also suitable for normothermic EVLP and associated with higher oxygenation capacity compared to EVLP with Steen Solution™.[7] Custodiol-MP was developed as a modification of Custodiol-N providing a ready-to-use version of the solution fitting clinical standards.

The aim of this study, performed to prepare a potential clinical use of Custodiol-MP, was to compare lung functional outcomes during 4 h of EVLP using either Steen Solution™ or Custodiol-MP supplemented with 55 g/l of human serum albumin in a porcine model of DCDD.

Materials and methods

Animals

The experiments were supervised by the central animal laboratory at the University of Duisburg-Essen. Sixteen mature domestic male hybrid pigs (BW 30–35 kg) were selected for the study. All animals were checked by general examination for signs of respiratory diseases prior to the experiment. Additionally, all experimental lungs were sampled and checked by real time PCR analysis for common porcine diseases potentially affecting lung functional outcome. Animals with multiple positive results were excluded from the analysis ($n = 1$). 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). As the animals did not receive medical treatment prior to euthanasia, the study is designed as an organ procurement only. This was reported to local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW) according to applicable law (§ 1 VTMVO).

Chemicals

Low-potassium dextran solution (LPD) (Perfadex Plus™, Xvivo, Denver, Co, USA) and Steen Solution™ were acquired from XVIVO Perfusion (Gothenburg, Sweden). Dr. F. Köhler Chemie (Bensheim, Germany) provided Custodiol-MP base solution. According to the manufacturer's suggestions, 55 g/l of low-salt human serum albumin (HSA; 300 ml Human-Albumin 20%, CSL Behring GmbH, Marburg, Germany) and 35 ml of 5% glucose were added to one bag, that is, 765 ml Custodiol-MP base solution. In addition, 3000 I.U. of Heparin (Heparin-Natrium-25000-ratiopharm®, ratiopharm® GmbH, Ulm, Germany) and 500 mg Methylprednisolone (Urbason® solubile forte 250 mg, Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) were added. The composition of the final perfusate is shown in Table 1.

Table 1. Composition of the perfusion solutions.

	Custodiol-MP base solution	Custodiol-MP(final)**
Sodium	18.8	Approx. 47#
Potassium	8.4	5.8
Magnesium	11.5	8.0
Calcium	0.06	0.04
Chloride	26.2	<45#
Histidine	110.0	76.5
N-Acetylhistidine	77.4	53.8
Sucrose	41.8	29.1
α-Ketoglutarate	1.6	1.1
Aspartate	4.2	2.9
Glycine	14.6	10.2
Alanine	8.4	5.8
Tryptophan	2.6	1.8
Arginine	4.5	3.1
Deferoxamine (μmol/l)	,*	25
LK 614 (μmol/l)	,*	7.5
Dextran (g/l)	–	–
Albumin (g/l)	–	54.5
Caprylate	–	4.4
N-Acetyltryptophan	–	4.4
Pyruvate	4.2	2.9
Glucose	–	8.8
Phosphate	0.6	0.4
pH	7.0	7.0
Osmolarity## (mosmol/l)	335	<312

Values are given in mmol/l unless stated otherwise.
*To be added from the lyophilisate provided with Custodiol-MP.
**After addition of the lyophilisate, 35 ml 5% glucose solution and 300 ml human albumin 20% (CLS Behring GmbH, Marburg, Germany).
#Depending on albumin lot.
##Calculated osmolarity.

Experimental groups, surgical process and porcine EVLP

Sixteen male domestic hybrid pigs (bodyweight 35 ± 5 kg) were sedated with ketamine (30 mg/kg BW i.m.; Ursotamin®, Serumwerk Bernburg AG, Bernburg, Germany) and xylazin (2 mg/kg BW i.m.; Xylavet®, cp-pharma®, Burgdorf, Germany) until they tolerated manipulation of the ear. Subsequently, they were anesthetized intravenously using midazolam (0.5 mg/kg BW i.v.) (midazolam-ratiopharm®, ratiopharm® GmbH, Ulm, Germany) and ketamine (30 mg/kg BW i.v.) after placing a catheter in the ear vein. Euthanasia in deep anesthesia was performed with a 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 procedure and did not receive heparin or other additional medication. After cardiac arrest was confirmed by auscultation, sternotomy was performed. Lungs were harvested using a standard operative technique as described elsewhere and randomized into two groups.[20–22] Lungs were flushed antegrade and retrograde with 2 l of cold (4°C) Perfadex Plus™ solution and stored on ice in a standard preservation bag for 4 h. Subsequently, EVLP was performed with the XVIVO XPS™ perfusion system using the Toronto protocol with modified ventilation settings to achieve a gentle lung ventilation. As standard volume controlled ventilation is associated with ventilator-induced lung injury.[23,24] pressure controlled ventilation was applied instead. All of the settings are listed in Table 2. Lungs in the standard group were perfused with Steen Solution™ (SS, $n=7$), while lungs in the second group (C-MP, $n=8$) were treated with modified Custodiol-MP solution for perfusion.

Monitoring and measurements

Lung function

During 4 h of EVLP pulmonary arterial/venous oxygen pressure and lactate concentration were measured hourly by blood gas analysis of the perfusate. XVIVO PGM Disposable Sensors continuously measured pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR). Dynamic

Table 2. Ventilation settings for EVLP.

Ventilator settings	Recruitment settings	Standard settings
PEEP	10	6
Pcontrol	12	8
Pramp	300	300
FiO2	21%/100%	21%
I:E	2:1	2:1

and static compliance (C_{sta} & C_{dyn}) as well as peak airway pressure (P_{peak}) data was recorded hourly.

Lactate dehydrogenase, alkaline phosphatase

Lactate dehydrogenase activity was measured in the initial effluent of cold-stored lungs and hourly in the perfusate with a clinical standard chemistry analyzer (VITALAB Selectra E, Vital Scientific NV, Dieren, NL) as a general marker for cell damage. Alkaline phosphatase activity was measured every hour to assess pneumocyte type 2 injury[25] (zAP, ADVIA Clinical Chemistry, Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Lactate, electrolytes. Lactate levels and levels of relevant electrolytes (Natrium, Kalium, Calcium and Chloride) were measured hourly in the perfusate using a clinical blood gas analysis device (ABL 700, Radiometer, Copenhagen, Denmark)

Wet/Dry ratio

Edema formation was evaluated by the water content of the lung tissue after 4 h of EVLP. Therefore, tissue samples were taken from the right lower lobe, weighed, dried for 24 h at 60°C and weighed again. The ratio is represented by the quotient $weight_{wet}/weight_{dry}$.

Histology

Lung tissue samples were taken from each lobe of the lungs and fixed in 4% paraformaldehyde for 48 h. Fixed tissue samples were serially dehydrated in an ethanol to xylol gradient and subsequently embedded in paraffin. Paraffin blocks were sectioned at 7 µm, dewaxed, rehydrated and washed before staining with either Haemalaun and Eosin (HE) staining or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL-TMR). Samples were analyzed using a Leica DMIRE 2 microscope. HE-stained samples were randomly sampled and examined by light microscopy at 400× magnification blinded to the examinator. A Lung Injury Score (LIS) according to the severity of 8 parameters: Interstitial edema, intra-alveolar edema, arteriolar thickening, vascular thrombosis, hemorrhage, cell infiltration, intraalveolar fibrin deposition and necrosis was assigned. Four severity grades were differentiated for each of the parameters conforming to the method employed by Lopes Medeiros et al.[26] This resulted in scores with a value between 0 and 24. TUNEL-TMR-stained samples were analyzed by fluorescence microscopy and photographed at 400× magnification. Ten random fields were chosen per slide to compare the number of bright red apoptotic cells with the total number of cells.

Protein isolation, gel electrophoresis, and immunoblotting

For protein isolation, tissue samples were shock-frozen in liquid nitrogen and grinded with mortar and pestle. Subsequently, 150 µl of Solution A (50 mM Tris-HCl pH 8.0; 150 mM NaCl) were added and grinding was continued until the slurry was completely thawed. After transferring the slurry into a reaction tube, 150 µl solution B (50 mM Tris pH 8.0; 150 mM NaCl; 2% (*v/v*) Triton X-100; 1 % (*w/v*) sodium deoxycholate; 0.2 % (*w/v*) SDS; 2 × Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland) and 2 × PhosSTOP phosphatase inhibitor (Roche, Basel, Switzerland) were added and the suspension was sonicated for 15 min on ice in an ultrasonic bath. The suspension was centrifuged (27000 *g*, 15 min, 4°C) and the supernatant collected. Protein concentrations was determined using BCA (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Equal amounts of total protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Protran 0.2 µm, GE Healthcare, Chalfont St. Giles, Buckinghamshire UK). Caspase 3 (1:2000; #9662, Cell Signaling, Leiden, Netherlands) and 4-Hydroxynonenal adducts (1:2000; ab46545, Abcam, Cambridge, UK) were detected using specific antibodies and a horse-radish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (A8275, Sigma-Aldrich, St. Louis, Missouri, USA). Signals were visualized using ECL reaction (Pierce ECL Western Blotting substrate, Thermo Scientific, Waltham, Massachusetts, USA) and a Fusion Pulse 6 Imager (VILBER LOURMAT, Eberhardzell, Germany).

Statistical analysis

Results were tested for normal distribution using the Kolmogorov Smirnov test. Normally distributed data was analyzed with a Student's t-test. For non-normally distributed data the Mann–Whitney *U* test was applied. Moreover variance analysis of repeated measurements (with Greenhouse-Geisser correction if required) with subsequent Bonferroni adjusted post-hoc analysis were applied to evaluate differences between groups over time concerning functional outcomes. All results are

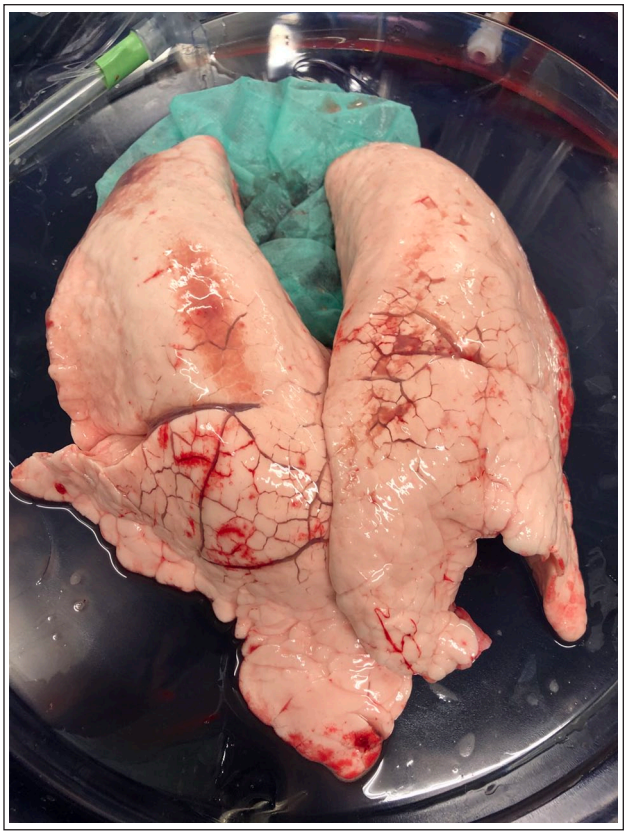

Figure 1. Typical lung appearance after 4 h of EVLP. Minor fluid accumulation in the lower lobes. Lungs appear well ventilated and show typical tissue consistence.

expressed as mean ± standard deviation. Differences of $p < 0.05$ were considered significant. Statistical analysis was performed using SPSS Statistics 26 (IBM, Armonk, New York, US).

Results

General macroscopic

One standard lung had to be excluded from the analysis due to multiple positive results for porcine diseases. All lungs were tested positive for porcine circovirus in various subclinical titer levels. Lungs of both groups showed a medium grade discoloration directly after lung retrieval. To wash out residual blood from the vascular bed flush perfusion was performed which improved coloration. In general, lungs showed the typical spongy lung consistence and were well ventilated after 4 h of EVLP (Figure 1). A minor intratracheal fluid accumulation could be detected in three out of seven SS and three out of eight C-MP lungs. A major fluid accumulation was present in two out of seven SS and one out of eight C-MP lungs at the end of the experiment. This was likely caused by interstitial edema formation in the lower lung lobes. In accordance

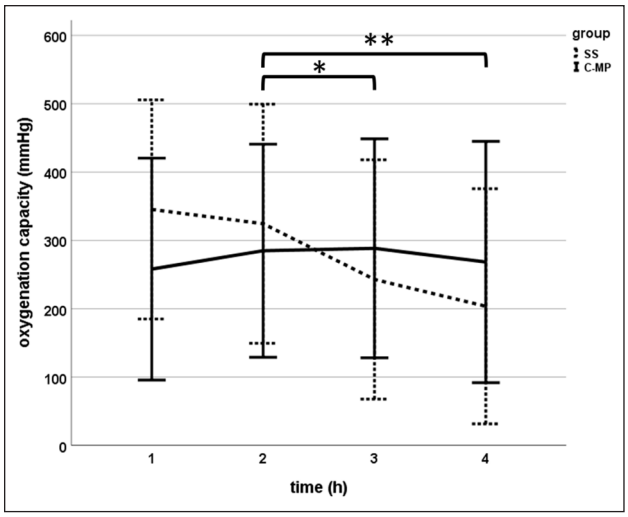

Figure 2. Mean oxygenation capacity (ΔpO_2) over 4 h of EVLP. Measured in the perfusate with blood gas analysis. Results are expressed as arithmetic mean ± standard deviation shown as error bars ($p = 0.044$).

with this SS lungs needed to be supplemented with 321 ± 267 ml of additional Steen Solution™ in the fourth hour whereas C-MP lungs only had to be supplemented with additional 122 ± 178 ml of Custodiol-MP.

Oxygenation capacity

The pO_2 course over the experiment remained stable and slightly increased in the Custodiol-MP group while it initially started higher but continuously decreased in the Steen Solution™ group (Figure 2). Comparison of the ΔpO_2 showed a statistically significant difference between measurements ($p = 0.044$) and also a significant difference when compared between groups ($p = 0.025$). ΔpO_2 increased by 4% in the C-MP group while lungs treated with SS showed a reduction of 41% (SS: 1 h 345 ± 160 mm Hg, 4 h 204 ± 172 mm Hg; C-MP: 1 h 258 ± 162 mm Hg, 4 h 268 ± 176 mmHg). Bonferroni adjusted post hoc analysis revealed a significant difference between the second and third ($p = 0.024$) and the second and forth hour ($p = 0.006$) of measurements.

PVR

Pulmonary vascular resistance (PVR) was generally lower in Custodiol-MP perfused lungs compared to lungs treated with SS. Compared over the entire course of the experiment (all measurements of PVR) there was a significant difference between both groups ($p = 0.026$). With regard to the time course there was no statistically significant differences between groups. PVR increased in both groups over time, by 60% in the C-MP group and 37% in the SS group (SS 1 h

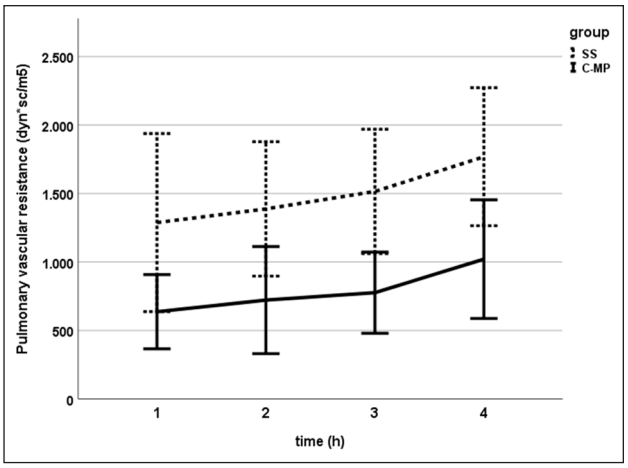

Figure 3. Pulmonary vascular resistance (PVR) over 4 h of EVLP. Results are expressed as arithmetic mean ± standard deviation shown as error bars.

1288 ± 650 dyn*sec/m^5, 4 h 1768 ± 503 dyn*sec/m^5; C-MP 1 h 637 ± 270 dyn*sec/m^5, 4 h 1021 ± 433 dyn*sec/m^5; Figure 3).

pPeak and compliance

Due to pressure-controlled ventilation mode the volume-depending parameters peak airway pressure (pPeak) and compliance could only be evaluated for each group but not compared between groups. Within SS and C-MP perfusion groups no statistically significant difference between pPeak measurements was observed. The pPeak was generally higher in the C-MP group and remained stable over the time course of the experiment. Levels decreased by only 3% in the SS group and 11% in the C-MP group (SS: 1 h 20.4 ± 3.4 cm H_2O, 4 h 19.8 ± 2.2 cm H_2O; C-MP: 1 h 23.7 ± 2.8 cm H_2O, 4 h 21.3 ± 3.5 cm H_2O).

During the 4 h of perfusion the dynamic compliance decreased by 22% in the SS and 15% in the C-MP group (SS: 1 h 18.6 ± 6.4 ml/cm H_2O, 4 h 11.7 ± 5.3 ml/cm H_2O; C-MP: 1 h 16.5 ± 9.4 ml/cm H_2O, 4 h 14.6 ± 5.3 ml/cm H_2O). There was no significant difference between treatment groups when analyzed over the time course of the experiment.

Biochemistry

Lactate levels were significantly lower in the C-MP group (SS 7.6 ± 1.8 mmol/l; C-MP 3.9 ± 2.1 mmol/l; $p < 0.01$; Figure 4(a)). Lactate levels as well as lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activity (see below), were analyzed for the third hour, since samples taken during the fourth hour were diluted differently after addition of perfusion solution (see above). There were no significant differences in electrolyte concentrations (K^+, Na^+, Cl^-, Ca^{2+}) over the course of the experiments between the groups.

Cell injury

General cell injury was assessed by LDH measurements in the initial effluent after cold storage and the perfusate for every hour. The mean perfusate LDH activity was significantly lower in lungs treated with C-MP compared to lungs perfused with SS (SS 390.0 ± 140.5 U/l; C-MP 252.3 ± 11.8 U/l; $p = 0.032$; Figure 4(b)).

To assess the damage of pneumocytes type 2 AP activity was measured in the initial effluent and hourly in the perfusate. Mean AP activity during perfusion was lower in C-MP lungs (SS 7.2 ± 5.2 U/l; C-MP 2.8 ± 0.9 U/l) but this difference wasn't statistically significant (Figure 4(c)).

Activities of LDH and AP in the first concentrated effluent after cold storage are represented by PA values. As all lungs were treated identically until the end of cold storage, PA values were similar for both treatment groups (and higher than more diluted values during perfusion; Figure 4(b) and (c)). However, both values were constantly lower in the C-MP group over the time course of perfusion.

Edema formation

The absolute lung weight after 4 h EVLP did not differ significantly between groups. The difference in absolute lung weight before and after the experiments reflects the absolute weight gain, which was not statistically significant either (SS 319.3 ± 274.9 g; C-MP 415.9 ± 188.7 g). The Wet/Dry ratio was assessed as a quotient of weight before/after 24 h of drying at 60°C and tended to be lower in Steen Solution™ perfused lungs (SS 5.92 ± 1.48; C-MP 6.67 ± 0.62). No statistically significant difference could be observed between groups.

Histology

Lung injury scores showed no statistically significant differences between the two perfusion groups (SS 5.52 ± 1.29; C-MP 5.46 ± 1.01; Figure 5(a)). Similarly, no statistically significant differences could be detected when directly comparing the ratings of the individual parameters. Examples of histological findings used for lung injury scoring are displayed in Figure 6.

TUNEL-TMR

The number of apoptotic cells was calculated as percentage of TUNEL-TMR positive cells out of all cells in the evaluated field. Both groups showed similar numbers of total cells (SS 22.10^3 ± 8.*10^3/mm^2; C-MP 21*10^3 ± 4*10^3/mm^2), with a significantly bigger proportion of apoptotic cells in the C-MP treated group (SS 3 ± 2; C-MP 6 ± 2; Figure 5(b)).

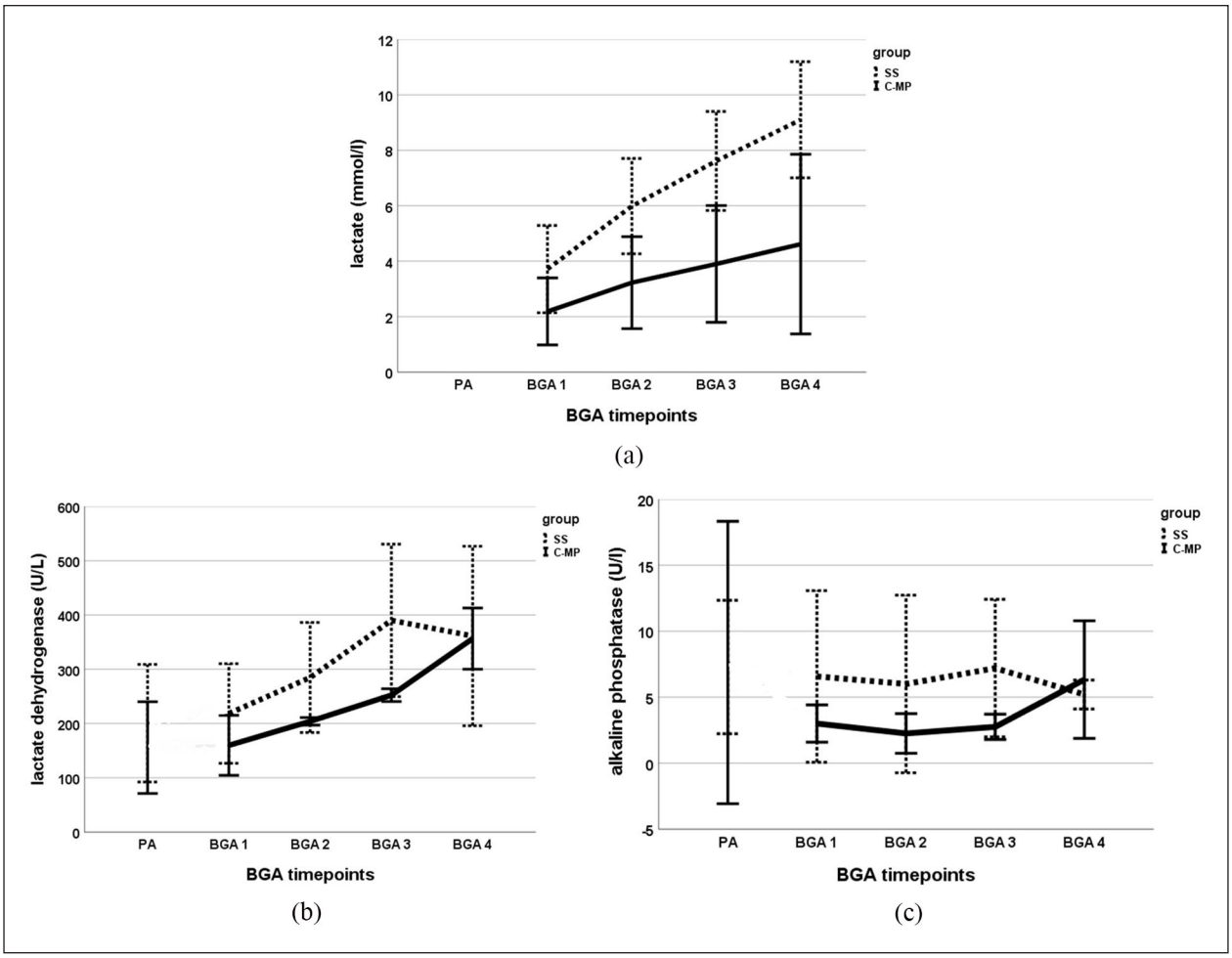

Figure 4. Lactate concentration (a) and activities of lactate dehydrogenase (b) and alkaline phosphatase (c) in perfusate during 4 h of EVLP. Results from different sampling points (BGA = blood gas analysis) expressed as arithmetic mean ± standard deviation shown as error bar. LDH activities and lactate levels were significantly higher in the SS group (LDH $p = 0.032$; Lac $p < 0.01$). LDH activities were generally lower in the C-MP group. Initial effluent levels obtained at the beginning of EVLP (PA) indicate similar cold storage injury in both groups. 4-h values are influenced by different dilutional effects (Steen Solution™: 321 ± 267 ml; Custodiol-MP 122 ± 178 ml).

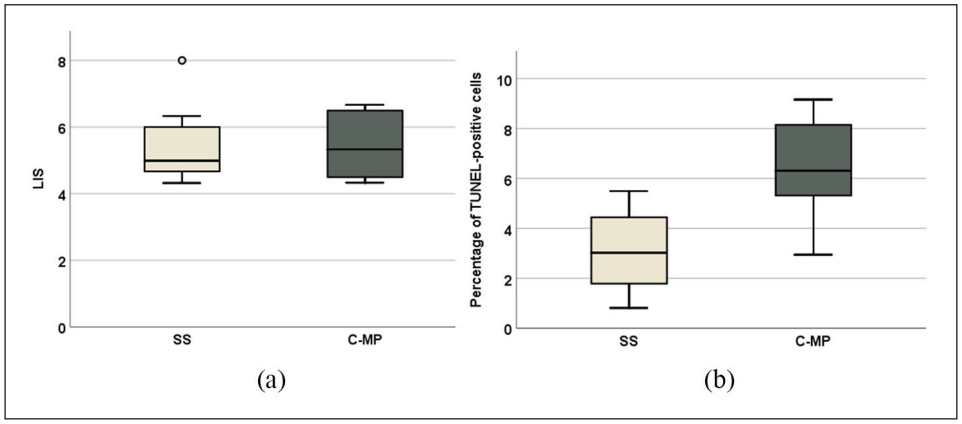

Figure 5. (a) Mean Lung Injury Score (LIS). Results are expressed as boxplots showing the Interquartile Range (body) and the minimum and maximum (whiskers). Outliers are shown as points, and (b) mean percentage of TUNEL-TMR-positive cells. Results are expressed as boxplots showing the Interquartile Range (body) and the minimum and maximum (whiskers).

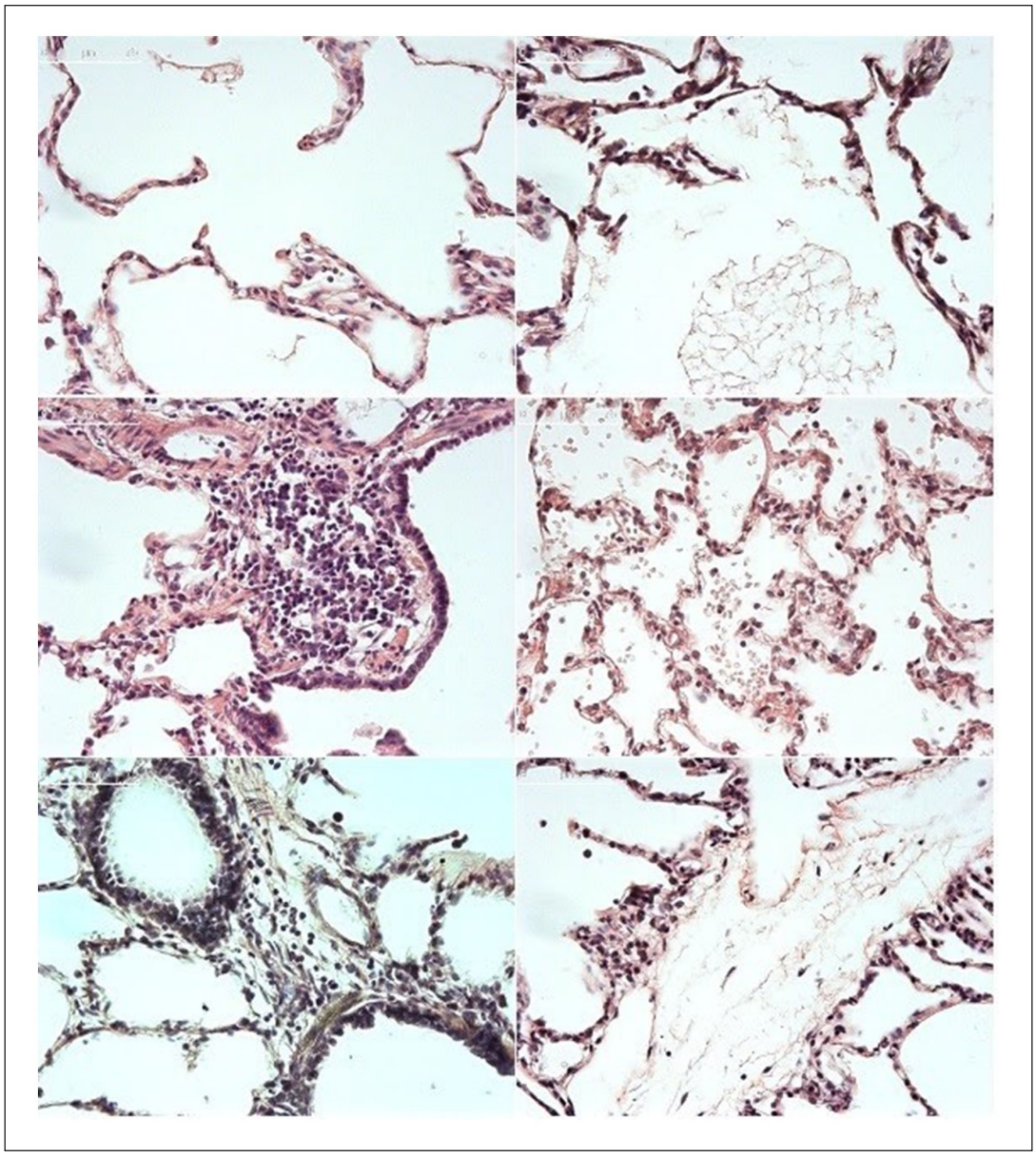

Figure 6. Examples of histological findings evaluated for the Lung Injury Score (LIS).
Top left: Mild intra-alveolar edema; Top right: Moderate intra-alveolar edema; Middle left: Severe cell infiltration; Middle right: Severe hemorrhage; Bottom left: moderate interstitial edema; Bottom right: Severe interstitial edema.

Caspase 3 activation

Procaspase 3 could be detected readily in all samples whereas cleaved caspase 3 could be detected neither after cold storage nor after EVLP (Figure 7(a)). Even after prolonged exposure no cleavage product was identified after EVLP with SS or C-MP.

Oxidative injury

Lung tissue samples were analyzed for α-4HNE (4-hydroxynonenal) adducts as markers for oxidative injury. Two weak but potentially unspecific bands were identified in controls taken prior to cold storage (Figure 7(b)). The same pattern was observed for tissue sampled after cold storage but prior to EVLP. After EVLP with either SS or C-MP the band at approximately 70 kDa was increased (Perf.LS/LD; Figure 7(b)). However, controls with the perfusion solutions showed that this signal likely occurred due to cross-reaction of the antibody with human serum albumin present in the respective perfusion solutions. No further signals could be detected in either tissue sample, thus giving no evidence for oxidative tissue injury during EVLP with either solution.

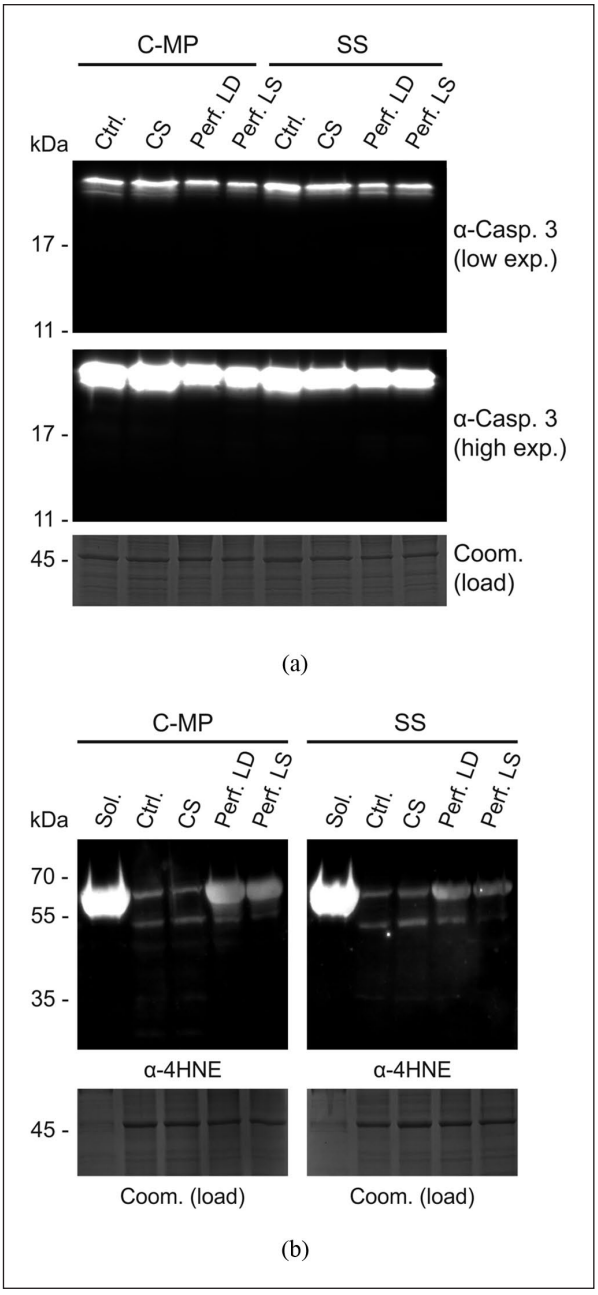

Figure 7. Total protein extracts were prepared from lung tissue samples of Lobus acc. (Ctrl., prior to cold storage; CS, end of cold storage), Lobus caudalis dexter (LD) and sinister (LS; each after perfusion with Custodiol-MP or Steen Solution™). Equal amounts of total protein (30 µg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. (a) Detection of procaspase 3 and cleaved caspase 3 is shown in a low exposition to compare the protein level of procaspase 3 and a high exposition to monitor low abundant cleavage products. Coomassie staining of a separate gel primed with each 15 µg total protein was performed to check for comparable loading, (b) Detection of 4-hydroxynonenal adducts as a marker of oxidative stress. Additionally, samples of the corresponding solution were loaded to check cross reaction of the antibody with human albumin. A second Coomassie-stained gel primed with each 15 µg total protein served as loading control.

Discussion

We successfully demonstrated the use of modified C-MP solution as perfusion solution for ex vivo lung perfusion in a porcine DCDD model. Comparison of lung functional outcomes between 4 h of EVLP with Steen Solution™ and C-MP highlights that perfusion with C-MP is feasible. Addition of iron chelators and cytoprotective amino acids to C-MP further seems to improve lung functional outcomes resulting in lower AP and LDH activities as well as lower lactate levels. Significantly lower PVR values together with reduced lactate levels in lungs perfused with C-MP suggest a positive effect on pulmonary microcirculation.

Custodiol-N solution has already been shown to improve cold static preservation of lung tissue.[17] Apart from that, we successfully used Custodiol-N in a porcine EVLP model with dextran 40 and low amounts of albumin as additional supplements.[7] In the present study C-MP perfusion solution, a variant adapted to the needs of clinical organ perfusion and with the potential to be clinically applicable, was used. The supplemented C-MP, as used here, included 55 g/l human serum albumin. Unlike dextran, human serum albumin is widely available and was therefore preferentially supplemented to maintain colloid osmotic pressure. Moreover, human serum albumin has not only scavenging and antioxidant effects[27] but can also bind free fatty acids and other lipophilic mediators released upon reperfusion.

This study shows that lung functional parameters like oxygenation capacity differ significantly between perfusion groups over an experimental period of 4 h. Overall, ΔpO_2 values were more stable and consistent in the C-MP group compared to the SS group. These findings are matching the results of a study previously performed by our group using Custodiol-N for perfusion. In contrast to other models, our study design did not include lung protective treatments such as ventilation with positive end expiratory pressure prior to euthanasia to create a substantial tissue damage resulting in a lower oxygenation capacity than in other porcine EVLP models.[28] This is the first successful porcine EVLP using C-MP as perfusion solution.

Pulmonary vascular resistance was continuously lower during EVLP with C-MP compared to EVLP with SS (significant overall); the time course of PVR changes did not differ between groups. The generally lower PVR in C-MP perfused lungs suggests an improved pulmonary microcirculation either by direct vasodilation (e.g. by the relatively high Mg^{2+} concentration in C-MP, that might act as a vasodilator,[29]) and/or by improved endothelial preservation. Just as Custodiol-N or the vascular preservation solution TiProtec[30] C-MP contains the iron chelators LK614 and deferoxamine to reduce redox-active iron ions and inhibit the formation of ROS. Solutions containing these chelators have been shown to inhibit endothelial injury in cold-stored blood vessels[30] as well as cold-stored rat hearts[31] and small intestine.[32]

It is well described that cell injury is associated with increased serum LDH activity. Increased AP activity suggests injury to type 2 pneumocytes. Increasing lactate levels on the other hand are a marker for poor tissue perfusion.[33] Previous studies using Custodiol-N for lung perfusion reported lower LDH and significantly lower AP activities compared to perfusion with Steen Solution™.[7] In accordance with these studies our C-MP based model presented similar advantageous results compared to Steen Solution™ although AP activities were not significantly different. However, AP and LDH activities as well as lactate levels in C-MP perfused lungs were constantly lower than in the SS group.

Lactate levels of lungs perfused with C-MP were not only significantly lower than in lungs treated with SS but also comparable to clinically measured levels in human EVLP or even lower. However, lactate levels result from both normal production in the lung tissue as well as anaerobic glycolysis in ischemic/poorly perfused areas and therefore increase over time in EVLP circuits. Consequently, they probably shouldn't be considered as a marker of poor transplant outcomes.[34] Nevertheless, lower lactate levels suggest less ischemic areas/less disturbance of microcirculation and support a beneficial effect of C-MP on the pulmonary microcirculation as discussed above.

Unsuccessful detection of Caspase 3 activation in both perfusion groups seems to contradict the higher numbers of apoptotic cells identified by TUNEL assay in C-MP perfused lungs. However, TUNEL staining only highlights general DNA fragmentation and is therefore not specific for apoptosis. Sampling errors, especially with regard to the general heterogeneity of the injury (cf. Figure 6), but also activated alveolar macrophages or investigation of only one time point could have also distorted these results. As this study was primarily designed to assess global lung tissue injury rather than the mode of cell death, LDH and AP activities measured in the perfusate are a better representation of lung injury.

Better protection of pneumocytes, especially pneumocytes type 2 by EVLP with C-MP compared to Steen Solution™, might in part be due to direct effects of the solution on these cells. This includes the cytoprotective amino acids glycine and alanine[35] and the iron chelators mitigating ROS-dependent cell injury (although looking for 4-HNE adducts – no oxidative injury could be demonstrated with Steen Solution™ either). On the other hand, secondary effects resulting from improved microcirculation could significantly benefit cell survival (see above).

Steen Solution™ contains 70 g/l human serum albumin whereas other EVLP solutions such as OCS Solution achieve comparable results without any additional albumin.[3,36] Previous studies have shown that even low concentrations of supplemented albumin have a positive antioxidative effect on lung epithelial cells.[2] The prevention of edema formation during EVLP requires a certain oncotic power of the perfusion solution and addition of albumin is a convenient method to regulate its oncotic properties.[37]

The albumin concentration in the C-MP solution (55 g/l compared to 70 g/l in Steen Solution™) was chosen to be at the upper end of normal serum albumin levels and to be practically feasible. Our findings suggest that the different albumin concentrations in both solutions did not significantly affect the ability of the perfusion solution to prevent edema formation over 4 h of EVLP. This was demonstrated by non-significant differences in absolute lung weight gain and wet/dry ratio. Macroscopic findings like lower fluid accumulation as well as less necessary solution replenishment additionally support this hypothesis. In addition, the albumin concentration in C-MP could be increased to 68 g/l (300 ml 25% human albumin instead of 300 ml 20% human albumin for the supplementation of one bag of C-MP base solution) if required for certain pre-damaged lungs.

Strengths of this study include its strict experimental design and protocol adapted to clinical standards. We especially aimed to demonstrate the feasibility of Custodiol-MP in a clinically common setting of DCDD and standard organ procurement. Therefore, we used Perfadex Plus™ as medium of choice for cold pulmonary flush and storage to in particular enable a good comparability of effects of the perfusion solution, by creating similar starting conditions for the two groups in question. For this reason, we also chose a relatively mild model of DCDD with 4 h of standard organ preservation reflecting a category V DCDD donation following the Maastricht classifications.[38] The study is however limited by its small cohort size and its focus on the perfusion, since lungs were not transplanted.

Since for evaluation of donor lungs 4 h of EVLP are the accepted standard we applied this to our experimental designs. Nevertheless, regarding the need for longer perfusion times, for example, for possible therapeutic approaches, long-time effects of C-MP perfusion must be examined in future studies to assess its usefulness also for these applications.

Effects of C-MP used in EVLP on lung functional outcome are comparable to EVLP with Steen Solution™ in a DCDD porcine model. The use of C-MP (including iron chelators and cytoprotective amino acids) as perfusion solution with additional human serum albumin had a statistically significant impact on the accumulation of lactate and resulted in lower LDH and AP activities as markers for poor tissue perfusion and cell damage. Finally, it should be noted that, since EVLP procedures are very expensive, the use of C-MP for perfusion is very likely to be cheaper than using standard Steen Solution™. Hence, it would make EVLP economically accessible to more centers and patients.

C-MP might be considered as a suitable alternative perfusion solution for clinical ex vivo lung perfusion. However, further studies, in particular a preclinical transplant model, need to be carried out to fully assess post-transplant outcomes for perfusion only or even combined

cold storage/perfusion using Custodiol-MP to explore its potential for lung transplantations.

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The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: UR has been a consultant of Dr. Franz Köhler Chemie and is listed as one of the inventors in the patents the company holds on the preservation solutions Custodiol-N, TiProtec®, and Custodiol-MP. UR received some funding for scientific projects by the company, but this is unrelated to the current study. The other Authors declare that there is no conflict of interest and that this study is not commercially motivated.

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