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Ex Vivo Perfusion With Methylprednisolone Attenuates Brain Death-induced Lung Injury in Rats

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Background. The onset of brain death (BD) leads to the deterioration of potential donor lungs. Methylprednisolone is considered to increase lung oxygenation capacity and enhance the procurement yield of donor lungs, when applied in situ, during donor management. However, whether BD-induced lung damage is ameliorated upon treatment with methylprednisolone during acellular ex vivo lung perfusion (EVLP), remains unknown. We aimed to investigate whether the quality of lungs from brain-dead donors improves upon methylprednisolone treatment during EVLP. **Methods.** Rat lungs were randomly assigned to 1 of 3 experimental groups (n = 8/group): (1) healthy, directly procured lungs subjected to EVLP; (2) lungs from brain-dead rats subjected to cold storage and EVLP; and (3) lungs from brain-dead rats subjected to cold storage and EVLP with 40 mg methylprednisolone added to the perfusate. Ventilation and perfusion parameters, histology, edema formation, metabolic profile, and inflammatory status of lungs were investigated. **Results.** Methylprednisolone treated lungs from brain-dead donors improved positive inspiratory pressures needed to maintain tidal volumes of 7 mL/kg of body weight, which was 25.6 ± 5.8 cm H_2O in untreated lungs and 18.0 ± 3.0 cm H_2O in methylprednisolone treated lungs, after 6 h EVLP. Furthermore, dynamic lung compliance increased upon methylprednisolone treatment, with values of 0.11 ± 0.05 mL/cm H_2O versus 0.18 ± 0.04 mL/cm H_2O after 6 h of EVLP. Methylprednisolone treatment ameliorated the amount of lung edema, as corroborated by a reduction of 0.7 in the wet/dry ratio. Although glucose consumption levels were comparable, the BD-induced cumulative lactate production decreased from 0.44 ± 0.26 to 0.11 ± 0.16 mmol/L upon methylprednisolone treatment. Finally, BD-induced inflammatory status was reduced upon methylprednisolone treatment compared to untreated lungs from brain-dead donors, as reflected by lower proinflammatory gene expression levels of IL-1β, IL-6 and MCP-1, and IL-6 perfusate levels. **Conclusions.** We showed that methylprednisolone treatment during EVLP attenuates BD-induced lung injury.

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INTRODUCTION

Despite an increase in donor lung procurement rates over the last years, half of the cadaveric lung donors are considered unsuitable for transplantation.[1] One of the main factors that contribute to decreased lung quality is lung damage caused by pathophysiological changes upon brain death (BD). With the onset of BD, a catecholamine storm occurs and a proinflammatory environment is created, which eventually leads to pulmonary edema formation.[2]

A newly developed strategy to increase the yield of lung donation is ex vivo lung perfusion (EVLP). This technique provides an opportunity to assess and test donor lungs with questionable quality, in a safe setting for the potential recipient. In addition, EVLP might serve as a promising treatment platform to improve donor lung quality.[3]

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Methylprednisolone is considered to increase lung oxygenation capacity and enhance the procurement yield of donor lungs when applied in situ.[4] Also, the anti-inflammatory properties of methylprednisolone might dampen the BD-induced immune response.[5] Yet, the application of methylprednisolone treatment in the donor remains a subject of debate, mainly due to contradictory results on quality of abdominal organs.[6,7] In some countries methylprednisolone treatment is recommended in all donors, whereas in other countries only potential lung donors are treated.[5] Furthermore, negative side effects such as steroid-induced hyperglycemia may detrimentally affect organ function.[8,9] In clinically applied EVLP, methylprednisolone is conventionally added to the acellular perfusate. However, whether BD-induced lung damage is ameliorated upon treatment with methylprednisolone during EVLP remains unknown.

We aimed to investigate whether quality of lungs from brain-dead donors improves upon methylprednisolone treatment during EVLP. To this end, we induced BD in rats, and after 3 h donor stabilization and 1 h cold storage (CS), we subjected the procured lungs to EVLP for 6 h, in which methylprednisolone was added to the perfusate or omitted.

MATERIALS AND METHODS

Experimental Outline

Lungs from donor rats were randomly assigned to 1 of 3 experimental groups (n = 8/group, Figure 1): (1) healthy, directly procured lungs subjected to EVLP; (2) lungs from brain-dead rats subjected to CS and EVLP; and (3) lungs from brain-dead rats subjected to CS and EVLP with 40 mg methylprednisolone added to the EVLP perfusate.

Rats

Male inbred Lewis rats (Harlan Laboratories, Melderslo, The Netherlands) with a weight of 350–450 g were used. Rats were fed standard rat chow ad libitum, and received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985) and the Dutch Law on Experimental Animal Care. The Institutional Animal Care and Use Committee of the University of Groningen provided consent for the experiment.

BD Induction, Lung Procurement, and EVLP

The BD procedure was adapted from the experimental BD model described by Kolkert et al.[10] Briefly, rats were subcutaneously anesthetized with ketamine hydrochloride (75 mg/kg, Alfason B.V., Woerden, The Netherlands) and medetomidine (0.5 mg/kg, Orion Pharma, Mechelen, The Netherlands). Thereafter, anesthesia was continued with subcutaneous boluses of ketamine hydrochloride/medetomidine mixture administered every 15 min, at one-fourth of the initial dose. The right femoral vessels were cannulated for mean arterial pressure measurements and fluid administration. Mean arterial pressure was stabilized >80 mm Hg by administration of Hydroxyethyl starch (HAES sterile 100 g/L, Fresenius Kabi, Bad Homburg, Germany) and saline (Baxter B.V., Utrecht, The Netherlands), with a maximum of 2 mL HAES and saline per hour. A craniotomy was performed in prone position and a 4F Fogarty catheter (Edwards Lifesciences LLC, Irvine, CA) was inserted in the epidural space. Thereafter, rats were placed in supine position, tracheotomized, and intubated with a 14G polyethylene tube (Klinject, KLINIKA Medical GmbH, Usingen, Germany). Lungs were pressure-regulated volume control ventilated (Babylog 8000 ventilator, Draeger, Luebeck, Germany). Ventilation settings were as follows: tidal volume (VT) 7 mL/kg of body weight (BW), positive end-expiratory pressure (PEEP) 3 cm H_2O, inspiratory/expiratory ratio 1:1, and fraction of inspired oxygen (FiO_2) 0.5. Directly after intubation, a recruitment maneuver was performed. PEEP was increased to 15 cm H_2O at a maximum positive inspiratory pressure (PIP) of 20 mm H_2O. Respiratory rate was increased to 150/min for 10 min for preoxygenation before BD induction, and thereafter reduced to a frequency of 133/min. BD was induced by inflation of the Fogarty catheter over 60 s and confirmed by the absence of corneal reflexes 30 min after BD induction. Brain-dead rats were stabilized for 3 h. In group 1, the non-BD healthy control, femoral vessel cannulation was omitted and lungs were immediately procured after intubation.

Before lung procurement, the respiratory rate was reduced to 60 breaths/min followed by a recruitment maneuver as described before. A median laparothoracotomy was performed and 1000 IU heparin (Leo Pharma B.V., Amsterdam, The Netherlands) was injected into the right ventricle. The pulmonary artery was cannulated and lungs were flushed with Perfadex (XVIVO Perfusion, Gothenburg, Sweden) for 2 min on a pressure of 15 mm Hg. Thereafter, lungs were procured and cold-stored in Perfadex on ice for 1 h, with a PEEP of 5 cm H_2O.

Next, lungs were placed on the EVLP platform. After initial recruitment, ventilation was continued with a VT of 4 mL/kg of BW and a respiratory rate of 60/min. PEEP was set at 5 cm H_2O and FiO_2 was 0.21. Lungs were reperfused at room temperature with Steen solution (XVIVO Perfusion, Gothenburg, Sweden) supplemented with 6 g BSA (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 0.12 g cefuroxime

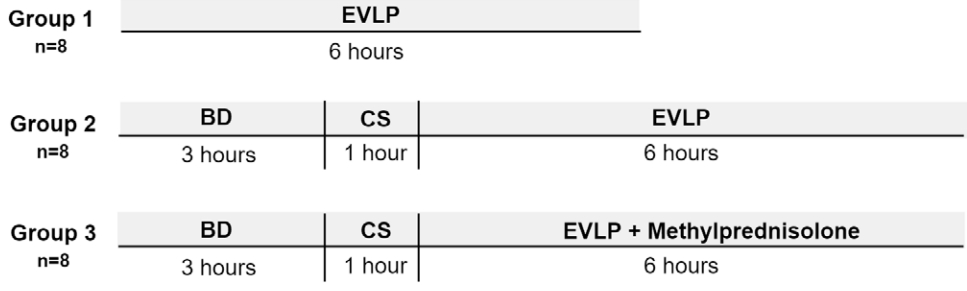

FIGURE 1. Outline of the study. Lungs from donor rats were randomly assigned to 1 of 3 experimental groups: (1) healthy, directly procured lungs subjected to 6 h EVLP; (2) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP; and (3) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP with 40 mg methylprednisolone added to the perfusate. BD, brain death; CS, cold storage; EVLP, ex vivo lung perfusion.

(Sandoz, Almere, The Netherlands), at an initial perfusion pressure of 9 mm Hg. The water bath was started to gradually increase perfusate temperature to 37 °C. In group 3, 40 mg methylprednisolone (40 mg/ml, Pfizer, Capelle aan den IJssel, The Netherlands) was added to the perfusate. The methylprednisolone dose was chosen to approach the dilution in the circulating perfusate as applied in clinical EVLP models.[11] After 10 min of reperfusion, VT was increased to 7 mL/kg of BW and perfusion pressure to 12 mm Hg. Lungs were perfused for 6 h. During EVLP, glucose levels of the perfusate in the reservoir were measured and corrected with glucose solution (50 g/L, Baxter B.V.,) in the case of levels <9 mmol/L. PIP required to ventilate with 7 mL of BW was noted over time. Dynamic lung compliance (C_{dyn}) was calculated by the equation C_{dyn} = VT/(PIP – PEEP). Perfusion flow was determined by measuring the amount of out flowing perfusate over 1 min. Perfusate was collected at baseline, 15 min, 30 min, and subsequently every hour after the start of reperfusion. At the end of EVLP lungs were clamped with a PEEP of 10 cm H_2O and placed on ice. The right and left main bronchi were ligated and the right upper and lower lobe were snap-frozen in liquid nitrogen. The right middle lobe was used to determine the wet/dry (W/D) ratio and the left lung lobe was formalin-fixed and paraffin embedded.

Oxygenation Capacity and Metabolic Profile of EVLP Lungs

Blood gas analyses were performed (ABL90 blood gas analyzer) to measure oxygenation capacity, glucose, and lactate levels of ex vivo perfused lungs. Before sample taking, FiO_2 was increased to 1 and the perfusate was deoxygenated for 5 min with a gas mixture of 6% O_2, 8% CO_2, and 86% N_2. Glucose consumption by the lung was calculated by the equation $\Delta Glucose = Glucose_{inflow} - Glucose_{outflow}$. Lactate production by the lung was calculated by $\Delta Lactate = Lactate_{outflow} - Lactate_{inflow}$. Subsequently, cumulative glucose consumption and lactate production were calculated over time.

Lung Edema

The severity of lung edema was investigated by the W/D ratio of the lung tissue. The right middle lung lobe was collected in an Eppendorf tube and weighed before and after drying for 24 h at 100 °C. The W/D ratio was calculated by the equation W/D ratio = (weight predrying – weight Eppendorf tube)/(weight postdrying – weight Eppendorf tube).

Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription polymerase chain reaction analyses were performed to detect proinflammatory gene expression levels in lungs. Total RNA was isolated from the snap-frozen lung tissue using Trizol (Invitrogen Life Technologies, Breda, The Netherlands), according to the manufacturer's instructions. Integrity of total RNA was analyzed by gel electrophoresis and RNA was treated with DNAse I (Invitrogen) to remove genomic DNA. RNA was transcribed into cDNA by adding M-MLV Reverse Transcriptase (Invitrogen) in the presence of dNTPs (Invitrogen), after initial incubation with Oligo-dT primers (Invitrogen). Gene expression analyses were performed at mRNA level by TaqMan low-density array. Designed primer sets (Table 1) were loaded with 5 µL cDNA (2 ng/µL) and SYBR green (Applied Biosystems, Foster City, CA). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System, which measures SYBR green emission. The PCR reaction consisted of 40 cycles at 95 °C for 15 s and 60 °C for 60 s, after initiation for 2 min at 50 °C and 10 min at 95 °C. Dissociation curve analyses were performed to ensure amplification of specific products. All samples were measured in triplicate. Gene expressions were normalized to housekeeping genes *Ppia* and *Eif2b1* and gene expression values were calculated by the ΔΔCt method.[12]

IL-6 ELISA

Protein levels of IL-6 in the EVLP perfusate were quantified by sandwich ELISA, according to manufacturer's instructions (R&D systems, Abingdon, United Kingdom). Briefly, maxisorp 96-well plates were coated overnight with the capture antibody (4.0 µg/mL). After plates were blocked with reagent diluent for 1 h, and samples were incubated for 2 h. The detection antibody was diluted in reagent diluent and 2% heat-inactivated normal goat serum, to an end concentration of 400 ng/mL. After the detection antibody was incubated for 2 h, Streptavidin-Horseradish Peroxidase was added and incubated for 20 min in the dark. Substrate solution was incubated for 35 min in the dark, and thereafter, stop solution was added. Appropriate washing steps were applied between incubations, and all incubation steps were performed at room temperature. The amount of reacted substrate was measured at an optical density of 450 nm (VICTOR-3, 1420 multilabel counter, PerkinElmer, Waltham, MA).

Lung Morphology

Formalin-fixed and paraffin-embedded lung sections (4 µm) were stained with hematoxylin and eosin to assess lung morphology. Lung morphology was quantified based on a previously described lung injury score with inclusion of alveolar septal thickening.[13] Per lung section, 10 snapshots were scored in a blinded manner on 400× magnification for 5 independent variables: (1) inflammatory cell influx in interstitium and alveolar space; (2) thickening of the alveolar septa; (3) intraalveolar and extraalveolar hemorrhage; (4) intraalveolar edema; and (5) overinflation. The variables were scored from 0 to 4: 0 = negative, 1 = slight, 2 = moderate, 3 = high, and 4 = severe. Total lung morphology scores were calculated by the sum of the scored variables.

Statistics

Statistical analyses were performed with IBM SPSS Statistics 26 (IBM Corporation, New York, NY). Data from multiple observations over time were analyzed with mixed-model analyses of variance tests to analyze the effect of group and time on ventilation and perfusion parameters. As follow-up tests, 1-way analyses of variance's with post hoc Bonferroni tests were performed to test differences between groups at specific time points. To determine differences of dependent variables between multiple groups, Kruskal–Wallis tests were performed, followed by Mann–Whitney U post hoc tests. *P* values of <0.05 were considered statistically significant and results are presented as mean ± SD.

RESULTS

Methylprednisolone Treatment Beneficially Affects Lung Ventilation Performance of Lungs From Brain-dead Donors

Ventilation and perfusion parameters during EVLP were compared between groups to investigate whether

TABLE 1.

RT-qPCR primers

Primer	Gene	Forward primer	Reverse primer	Amplicon (bp)
TNF-α	Tumor necrosis factor-alpha	AGGCTGTCGCTACATCACTGAA	TGACCCGTAGGGCGATTACA	67
IL-1β	Interleukin-beta	CAGCAATGGTCGGGACATAGTT	GCATTAGGAATAGTGCAGCCATCT	75
IL-6	Interleukin-6	CCAACTTCCAATGCTCTCCTAATG	TTCAAGTGCTTTCAAGAGTTGGAT	89
MCP-1	Monocyte chemoattractant protein-1	CTTTGAATGTGAACTTGACCCATAA	ACAGAAGTGCTTGAGGTGGTTGT	78
C3	Central complement component 3	CAGCCTGAATGAACGACTAGACA	TCAAAATCATCCGACAGCTCTATC	96

RT-qPCR, quantitative reverse transcription polymerase chain reaction.

methylprednisolone affects performance of lungs procured from brain-dead donors. PIP required to maintain ventilation at a tidal volume of 7 mL/kg of BW, showed an interaction between time and treatment group ($P = 0.008$). PIP increased over the perfusion period, and from 3.5 h after reperfusion onward, this increase in PIP was higher in untreated lungs from brain-dead donors, than in healthy donor lungs (Figure 2A), with PIP levels of 25.6 ± 5.8 cm H_2O versus 17.1 ± 3.4 cm H_2O after 6 h of EVLP ($P = 0.005$). Methylprednisolone treatment of lungs from brain-dead donors attenuated the BD-induced PIP increase to 18.0 ± 3.0 cm H_2O ($P = 0.012$) after 6 h of EVLP, comparable to values of healthy donor lungs ($P = 1.000$). Dynamic lung compliance values showed a main effect for time ($P = 0.000$) and treatment group ($P = 0.021$), yet no interaction between time and group was observed ($P = 0.589$, Figure 2B). C_{dyn} worsened over time, and from 4 h after reperfusion onward, C_{dyn} values were lower in untreated lungs from brain-dead donors than in healthy donor lungs, with C_{dyn} values of 0.11 ± 0.05 mL/cm H_2O versus 0.20 ± 0.05 mL/cm H_2O after 6 h of EVLP ($P = 0.013$). Methylprednisolone attenuated C_{dyn} decrease in lungs from brain-dead donors to 0.18 ± 0.04 mL/cm H_2O after 6 h of EVLP ($P = 0.036$), comparable to values of healthy donor lungs ($P = 1.000$). Nonetheless, no effect of time ($P = 0.075$) nor treatment group ($P = 0.365$) was observed in oxygenation status of donor lungs on EVLP, as reflected by the PaO_2/FiO_2 ratio (Figure 2C). Perfusion flow of lungs on EVLP decreased over time ($P = 0.000$), but was not affected by methylprednisolone treatment ($P = 0.267$, Figure 2D). Collectively, these results indicate that methylprednisolone treatment during EVLP beneficially affects lung ventilation performance of lungs from brain-dead donors.

Methylprednisolone Does Not Ameliorate Histological Lung Injury, Yet Reduces the Quantity of Edema Formation in Lungs From Brain-dead Donors

Subsequently, we assessed whether lung morphology is affected by methylprednisolone treatment during EVLP. Overall histological evidence of lung injury was higher in lungs from brain-dead donors subjected to EVLP than healthy donor lungs subjected to EVLP ($P = 0.012$, Figure 3A-D). This was mainly the result of higher inflammatory cell influx in lungs from brain-dead donors than in lungs from healthy donors ($P = 0.002$). Qualitative evidence of edema formation in hematoxylin and eosin stained lung tissue was similar between groups ($P = 0.798$). However, quantitative measurements by means of W/D ratio showed that methylprednisolone reduced the amount of lung edema in lungs from brain-dead donors with 0.7 (5.5 ± 0.4 versus 6.2 ± 0.4, $P = 0.013$), even lower than healthy donor lungs (5.5 ± 0.4 versus 6.1 ± 0.3, $P = 0.018$). These results suggest that histological lung injury does not improve upon methylprednisolone treatment during EVLP, yet the amount of pulmonary edema is reduced in methylprednisolone treated lungs from brain-dead donors.

Methylprednisolone Attenuates Lactate Production by Lungs From Brain-dead Donors

Considering that pulmonary lactate production is increased in acute lung injury, we investigated the metabolic profile of ex vivo perfused lungs.[14,15] The amount of cumulative glucose consumption was comparable in all 3 groups ($P = 0.348$, Figure 4A). In contrast, cumulative lactate production was higher by untreated lungs from brain-dead donors, than by healthy donor lungs (0.44 ± 0.26 mmol/L versus 0.14 ± 0.10 mmol/L, $P = 0.014$, Figure 4B). Methylprednisolone treatment decreased cumulative lactate production by lungs from brain-dead donors to 0.11 ± 0.16 mmol/L ($P = 0.017$), comparable to healthy donor lungs ($P = 0.547$). Taken together, these results suggest that a shift to anaerobic metabolism occurs in lungs from brain-dead donors, which is attenuated by methylprednisolone treatment during EVLP.

Methylprednisolone Downregulates the BD-induced Proinflammatory Response

Since the process of BD leads to a proinflammatory state of the donor and subsequently donor organs, we investigated the effect of methylprednisolone on proinflammatory gene expression levels of ex vivo perfused lungs (Figure 5A–E).[16-18] Overall, proinflammatory gene expressions were higher in lungs from brain-dead donors compared to healthy donor lungs. Nevertheless, significance was reached only in IL-1β gene expression ($P = 0.002$), in contrast to TNF-α ($P = 0.085$), IL-6 ($P = 0.224$), and MCP-1 ($P = 0.277$). Complement C3 gene expressions were similar between lungs from healthy donors and lungs from brain-dead donors ($P = 0.749$). Methylprednisolone downregulated gene expression levels of IL-1β ($P = 0.009$), IL-6 ($P = 0.006$), and MCP-1 ($P = 0.002$) in lungs from brain-dead donors, when compared with untreated lungs from brain-dead donors. To confirm the anti-inflammatory effect of methylprednisolone on a protein level, IL-6 perfusate levels were measured over time and an interaction between time and treatment group was observed ($P = 0.026$). IL-6 perfusate levels increased over the perfusion period and from 3 h onward, this increase was higher in the perfusate of untreated lungs from brain-dead donors than in the perfusate of methylprednisolone treated lungs (51 631.06 ± 34 635.20 pg/mL versus 17 067.59 ± 12 418.92 pg/mL after 6 h of EVLP, $P = 0.047$, Figure 5F). All together, these results show that the

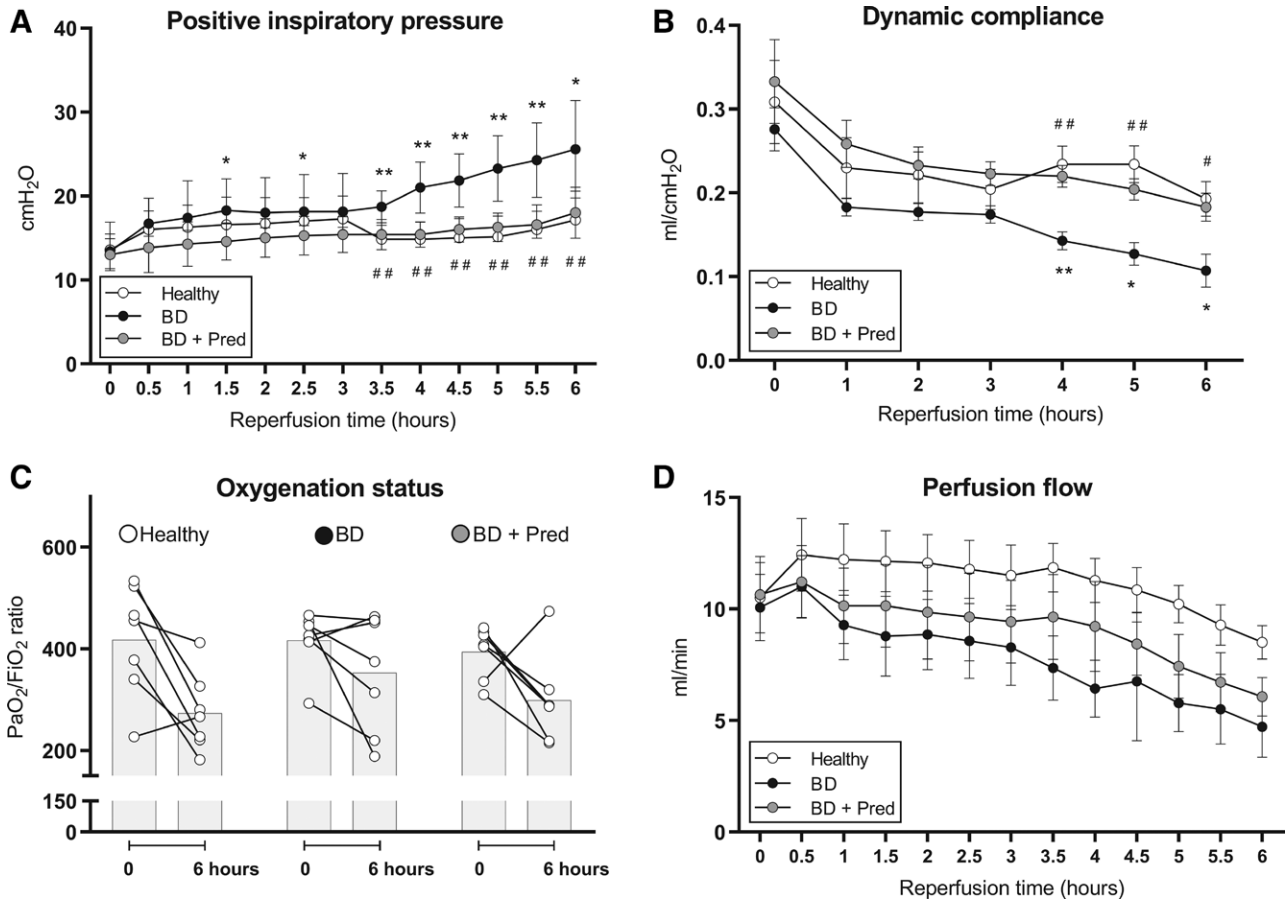

FIGURE 2. Ventilation and perfusion parameters during EVLP. Lungs from donor rats were randomly assigned to 1 of 3 experimental groups: (1) healthy, directly procured lungs subjected to 6 h EVLP; (2) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP; and (3) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP with 40 mg methylprednisolone (Pred) added to the perfusate. A, Positive inspiratory pressures required to maintain tidal volumes of 7 mL/kg of body weight. B, Dynamic lung compliance of lungs during EVLP. C, Oxygenation status of lungs during EVLP, reflected by Pao_2/FiO_2 ratio. D, Perfusion flow of lungs during EVLP. $^{*}P < 0.05$ in BD lungs vs BD + Pred lungs, $^{**}P < 0.01$ in BD lungs vs BD + Pred lungs, $^{\#}P < 0.05$ in BD lungs vs healthy lungs, $^{\#\#}P < 0.01$ in BD lungs vs healthy lungs. BD, brain death; CS, cold storage; EVLP, ex vivo lung perfusion.

proinflammatory response in lungs from brain-dead donors is attenuated upon methylprednisolone treatment during EVLP.

DISCUSSION

In vivo methylprednisolone administration is a generally accepted treatment to improve lung quality during donor management. However, the wide effect range of methylprednisolone might be accompanied by adverse systemic side effects and the effect on quality of abdominal organs is debated.[6,7] Therefore, methylprednisolone treatment in an isolated setting might be preferable. In this study, we aimed to investigate whether quality of lungs from brain-dead donors is improved upon ex vivo methylprednisolone treatment. We showed that BD-induced lung injury is attenuated upon methylprednisolone treatment during EVLP.

The improved C_{dyn} of lungs upon methylprednisolone treatment during EVLP probably reflects the impact of alveolar fluid clearance in the donor lung, as described before in literature.[19] Although less edema was present upon methylprednisolone treatment in our study, this observation did not result in improved Pao_2/FiO_2 levels. Oxygenation status is traditionally considered most important when evaluating lung function. However, when measured in an acellular perfusate and open system during EVLP, the reliability of this test is questioned. Since only a few molecules of oxygen can significantly change Pao_2 values in plasma-like solutions, lung compliance is suggested as a more accurate parameter for assessing lung quality.[20]

Methylprednisolone treatment during EVLP was suggested to limit a shift to anaerobic metabolism in our study, which possibly occurred in untreated lungs from brain-dead donors. Lactate levels are often used as a marker of poor prognosis and in clinically performed EVLP, in the presence of methylprednisolone, lactate levels are described to increase over time. However, it is suggested that this lactate increase represents physiologic lactate accumulation in a setting with reduced lactate clearance because lactate levels do not correlate with transplantation outcomes.[21] Since a clinical comparative study with and without methylprednisolone treatment has not been performed, it is unknown whether lactate levels would be detrimentally increased in human EVLP lungs, in the absence of methylprednisolone. We speculate that the routine administration of methylprednisolone in clinically performed EVLP limits the BD-induced anaerobic shift. This theory might explain unsuitability for lactate as a marker of poor prognosis, in an EVLP setting in the presence of methylprednisolone.

The increase in inflammatory cells as suggested by histological injury scores of lungs from brain-dead donors

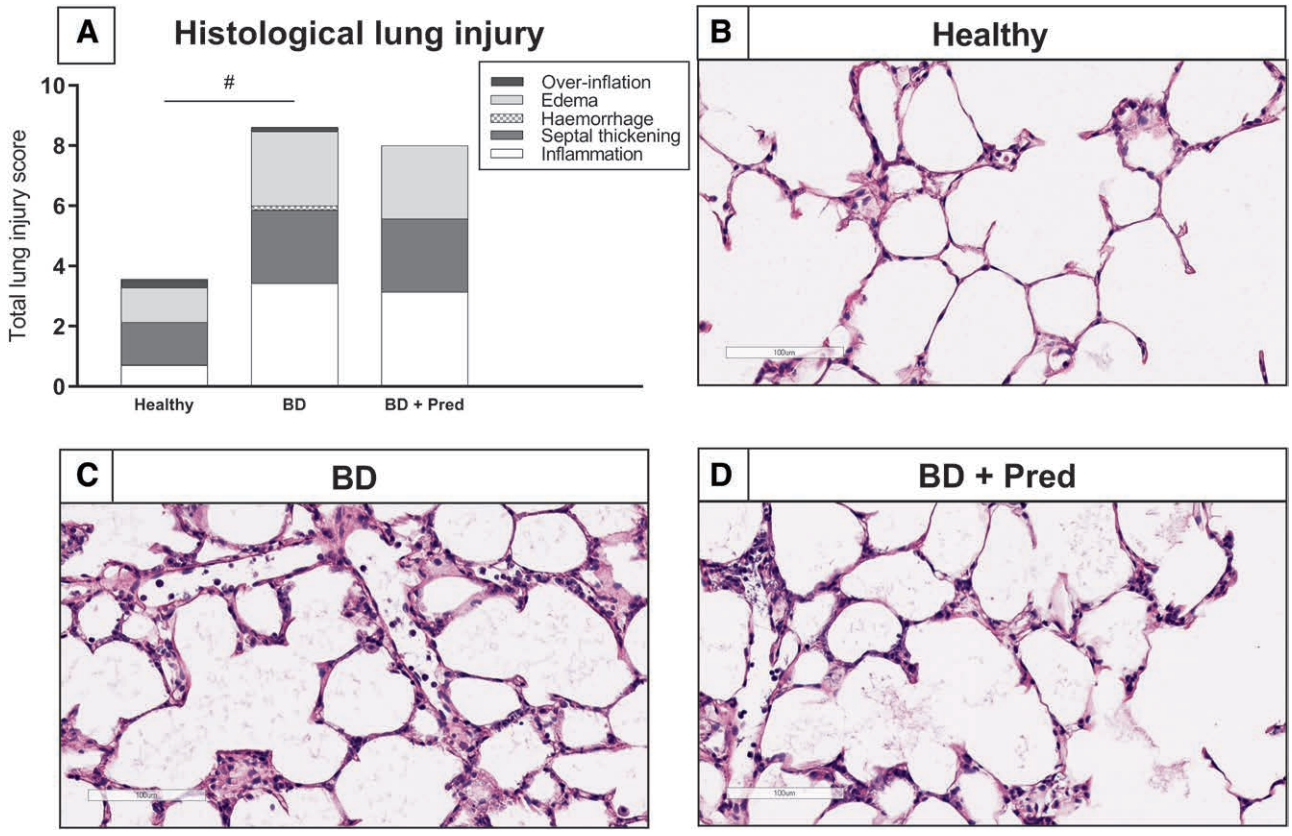

FIGURE 3. Lung morphology of lungs after EVLP. Lungs from donor rats were randomly assigned to 1 of 3 experimental groups: (1) healthy, directly procured lungs subjected to 6 h EVLP; (2) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP; and (3) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP with 40 mg methylprednisolone (Pred) added to the perfusate. Lung morphology scores were assessed after 6 h of EVLP by means of hematoxylin and eosin staining. A, Quantification of lung morphology scores in hematoxylin and eosin stained lung slides. B–D, Representative hematoxylin and eosin stained slices of healthy donor lungs, untreated lungs from brain-dead donors, and methylprednisolone treated lungs from brain-dead donors, after 6 h of EVLP. $^{\#}P < 0.05$ in BD lungs vs healthy lungs. BD, brain death; CS, cold storage; EVLP, ex vivo lung perfusion.

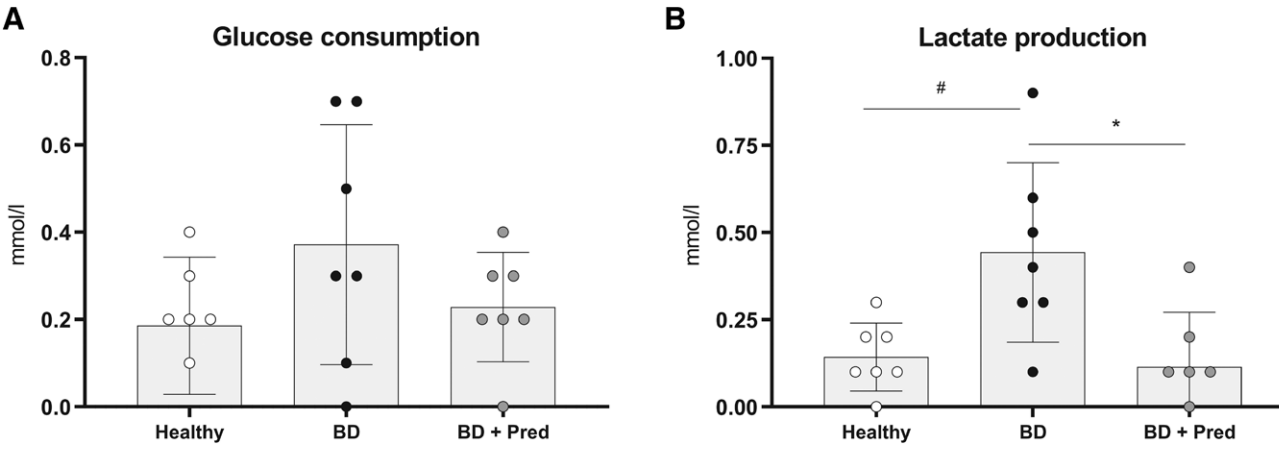

FIGURE 4. Metabolic profile of lungs during EVLP. Lungs from donor rats were randomly assigned to 1 of 3 experimental groups: (1) healthy, directly procured lungs subjected to 6 h EVLP; (2) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP; and (3) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP with 40 mg methylprednisolone (Pred) added to the perfusate. A, Cumulative glucose consumption by lungs during EVLP. B, Cumulative lactate production by lungs during EVLP. $^{*}P < 0.05$ in BD lungs vs BD + Pred lungs, $^{\#}P < 0.05$ in BD lungs vs healthy lungs. BD, brain death; CS, cold storage; EVLP, ex vivo lung perfusion.

compared to healthy donor lungs, probably reflect the earlier recruitment of inflammatory cells to the donor lung during the BD period. The ability of EVLP to wash out these donor-derived leukocytes into the perfusate has been described in the literature before.[22] Yet in our model, the appearance of inflammatory cell influx after 6 h of EVLP was not decreased upon methylprednisolone treatment. Nevertheless, the down-regulated levels of proinflammatory gene expressions upon

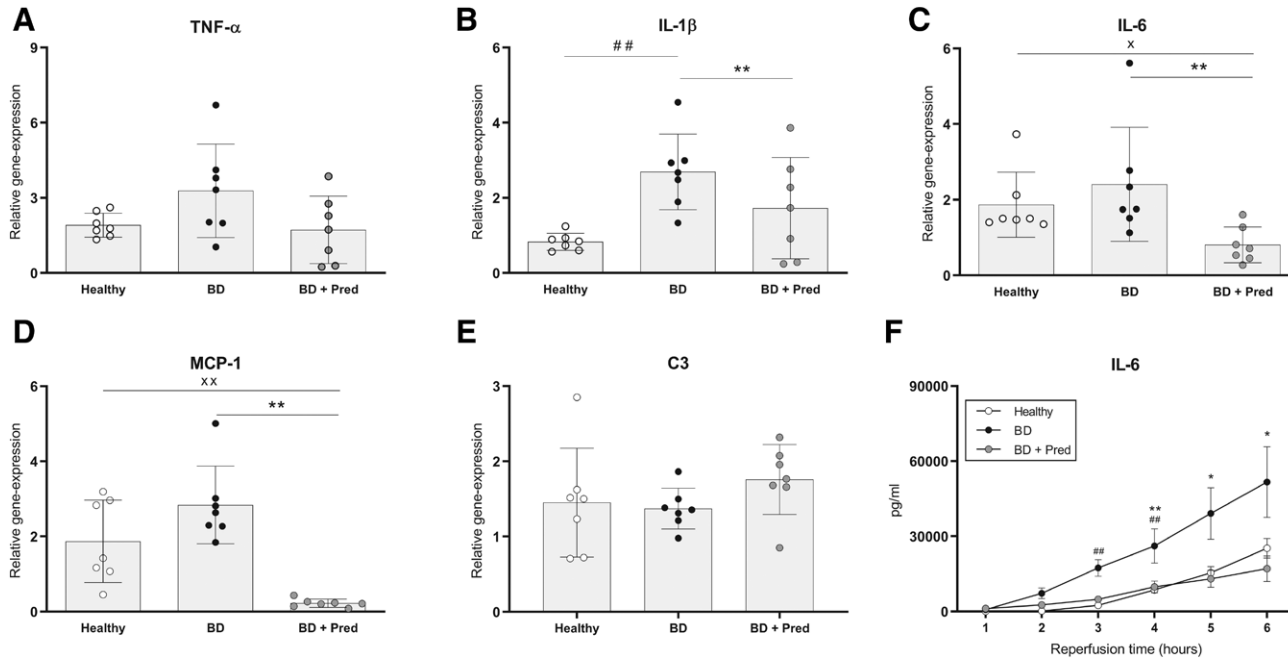

FIGURE 5. Inflammatory status of lungs during EVLP. Lungs from donor rats were randomly assigned to 1 of 3 experimental groups: (1) healthy, directly procured lungs subjected to 6 h EVLP; (2) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP; and (3) lungs from brain-dead rats (BD sustained for 3 h) subjected to 1 h CS and 6 h EVLP with 40 mg methylprednisolone (Pred) added to the perfusate. A–E, mRNA gene expression levels of proinflammatory mediators (A) TNF-α, (B) IL-1β, (C) IL-6, (D) MCP-1, and (E) C3 in lung tissue measured after 6 h of EVLP. F, Concentration of IL-6 protein in perfusate over time. $^{*}P < 0.05$ in BD lungs vs BD + Pred lungs, $^{**}P < 0.01$ in BD lungs vs BD + Pred lungs, $^{\#\#}P < 0.01$ in BD lungs vs healthy lungs, $^{x}P < 0.05$ in healthy vs BD + Pred lungs, $^{xx}P < 0.01$ in healthy vs BD + Pred lungs. BD, brain death; CS, cold storage; EVLP, ex vivo lung perfusion.

methylprednisolone treatment during EVLP probably reflect an attenuated inflammatory state of the donor lung. Although Stone et al[23] suggested that the technique of EVLP itself beneficially alters the inflammatory signaling profile of the donor lung, methylprednisolone might further contribute to the reduction of donor lung immunogenicity. Methylprednisolone is known to bind glucocorticoid receptors, which are present on most cells, including airway epithelial cells. Upon binding, activation of nuclear factor kappa B is inhibited and proinflammatory gene expression is blocked. Our findings are in line with methylprednisolone treated lungs as described by Martens et al,[24] who studied the effect of methylprednisolone in a porcine model for donation after circulatory death (DCD) donors and showed a reduction in IL-1β and TNF-α cytokine expression. Yet, it should be noted that the importance of the cytokine profile during acellular EVLP as a marker for lung injury has not yet been fully elucidated, since the absence of blood circulation or bone marrow in an EVLP circuit excludes the effect of recruited leukocytes.[25] In contrast, when the lung is transplanted in a recipient with functioning bone marrow, elevated cytokine production is associated with poor graft function.[26] In our study, BD-induced MCP-1 expression was downregulated by methylprednisolone treatment, which suggests diminished chemoattraction of recruited macrophages. Macrophage count after methylprednisolone treatment during EVLP remained unaffected in our study (results not shown).

To our knowledge, comparative studies of EVLP with and without methylprednisolone treatment with a focus on quality of lungs from brain-dead donors, have not been performed before. Noda et al[27] described their designed rat EVLP model for healthy donor lungs, and noted the necessity of methylprednisolone in the perfusate to establish a stable perfusion model. When methylprednisolone was omitted in their study, evident lung edema was noticed and perfusion for >1 h was not achieved. Martens et al[24] showed in their porcine DCD model, that lung quality was improved upon methylprednisolone treatment during EVLP. In line with our findings, C_{dyn} was ameliorated and oxygen status was unaffected in methylprednisolone treated lungs from DCD donors. However, it should be noted that the pathophysiological mechanisms of DCD donors eminently differ from brain-dead donors.[28] Besides, in the mentioned study, methylprednisolone was administered in both the donor and during EVLP. Therefore, the exact effect of methylprednisolone during EVLP only remained unknown.

Although the strong point of our study is the specific focus on the effect of methylprednisolone on BD-related lung injury, a limitation is the absence of lung transplantation in our model. Whether methylprednisolone treatment during EVLP has an extended effect on recruited leukocytes associated with reperfusion injury, remains therefore unknown. In addition, it should be noticed that in our model, methylprednisolone treatment did not improve quality of lungs compared to baseline values, in accordance with previously described rat models for EVLP.[27,29] We attribute the deterioration in quality over time in our model to the small organ size of rats. Given the fundamental differences in anatomy and physiology between small and large animal models or even human models, we believe that methylprednisolone treatment in large EVLP models might improve quality of lungs from brain-dead donors over time.[30]

Methylprednisolone treatment has been applied from the very beginning of immunosuppression in lung transplantation, and remained a corner stone in both donor management and recipient immunosuppressive strategies.[31] Traditionally,

methylprednisolone is added to the EVLP perfusate, despite that its effect on BD-induced lung injury has not specifically been studied before. Before investigating new anti-inflammatory treatment modalities, we aimed to identify potentially confounding anti-inflammatory properties of methylprednisolone on BD-induced lung injury. The current, clinically applied EVLP strategy is described to significantly increase the amount of potential human donor lungs.[32] However, other potential agents and particularly combined treatment strategies might even further enhance the quality of potential donor lungs. Most lung transplant recipients conventionally receive a triad of maintenance immunosuppression consisting of a calcineurin inhibitor, antiproliferative agent, and corticosteroid, with the goal to minimize immune-mediated injury to the donor lung.[31] It might be suggested that donor lung preconditioning with this treatment triad additionally benefits donor lung quality. Haam et al[33] already investigated the potential of the calcineurin inhibitor cyclosporine in preconditioning donor lungs during EVLP, and showed improved lung graft preservation due to anti-inflammatory and mitochondrial protective properties. Furthermore, new treatment strategies such as IL-10 gene therapy have been tested on the EVLP platform with positive results, which suggests that future donor lung optimization strategies may shift from general to a more specific approach.[34]

In conclusion, this study contributes to the current knowledge on the potential of EVLP as a treatment platform, by showing that methylprednisolone treatment during EVLP attenuates BD-induced lung injury.

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