Tacrolimus Prevents Mechanical and Humoral Alterations in Brain Death–induced Lung Injury in Pigs

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Abstract

Rationale: Donor brain death–induced lung injury may compromise graft function after transplantation. Establishing strategies to attenuate lung damage remains a challenge because the underlying mechanisms remain uncertain.

Objectives: The effects of tacrolimus pretreatment were evaluated in an experimental model of brain death–induced lung injury.

Methods: Brain death was induced by slow intracranial infusion of blood in anesthetized pigs after randomization to tacrolimus (orally administered at 0.25 mg · kg−1 twice daily the day before the experiment and intravenously at 0.05 mg · kg−1 1 h before the experiment; n = 8) or placebo (n = 9) pretreatment. Hemodynamic measurements were performed 1, 3, 5, and 7 hours after brain death. After euthanasia of the animals, lung tissue was sampled for pathobiological and histological analysis, including lung injury score (LIS).

Measurements and Main Results: Tacrolimus pretreatment prevented increases in pulmonary arterial pressure, pulmonary vascular resistance, and pulmonary capillary pressure and decreases in systemic arterial pressure and thermodilution cardiac output associated with brain death. After brain death, the ratio of Pao2 to Fio2 decreased, which was prevented by tacrolimus. Tacrolimus pretreatment prevented increases in the ratio of IL-6 to IL-10, VCAM1 (vascular cell adhesion molecule 1), circulating concentrations of IL-1β, and glycosyl-derivates molecules. Tacrolimus partially decreased apoptosis (Bax [Bcl2-associated X apoptosis regulator]–to–Bcl2 [B-cell lymphoma-2] ratio [P = 0.07] and number of apoptotic cells in the lungs [P < 0.05]) but failed to improve LIS.

Conclusions: Immunomodulation through tacrolimus pretreatment prevented pulmonary capillary hypertension as well as the activation of inflammatory and apoptotic processes in the lungs after brain death; however, LIS did not improve.

Keywords: lung donor; neurogenic pulmonary edema; immunomodulation; cytokines

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*These authors contributed equally to this work.

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Author Contributions: All measurements and analyses were performed in a blinded manner. A.B. and B.R. conceptualized and designed the study. A.B., B.R., E.H., M.R., L.G., S.R., and L.D. performed the research. B.R. and A.B. performed the animal investigation. Pathobiological experiments, including real-time quantitative PCR, the multiplex cytokine magnetic bead panel, ELISAs, and nitric oxide assays, were performed by a team of three collaborators in duplicate (L.D., E.H., and L.G.). Two independent investigators performed acute lung injury scoring (M.R. and S.R.), terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end label analysis, and proapoptotic rate determination (L.D. and E.H.). A.B., B.R., L.D., and M.R. analyzed the data. A.B., B.R., and L.D. contributed to the new methods or models. A.B., B.R., and L.D. wrote the original draft. C.M. performed the statistical analysis. All authors have read and agreed to the published version of the manuscript.

Data sharing statement: The data sets generated during and/or analyzed in the present study are available from the corresponding author on reasonable request.

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Lung transplantation is a lifesaving therapeutic option for patients with end-stage lung disease. Although organ transplantation from donation after circulatory death has shown increasing success (1), most organs are still obtained from brain-dead donors (2).

Brain death is defined as irreversible damage to the brain stem combined with lesions of the cerebral hemispheres. The central insult, secondary to an acute, rapid, abrupt, and extreme increase in intracranial pressure, produces severe physiological and structural derangements in the peripheral organs, including lung injury, also called neurogenic pulmonary edema (3, 4). Brain death influences allograft survival, affecting the time course and severity of early graft rejection. In lung transplant recipients, brain death has been linked to the risk of developing chronic rejection (bronchiolitis obliterans syndrome) in lung transplant recipients (5). The general consensus in the field is that better characterization and quantification of a single *primum movens* of brain death–induced lung injury would show therapeutic relevance in improving early and long-term graft function after transplantation (6). Several pathophysiologic mechanisms directly implicating the autonomic response to elevated intracranial pressure have been proposed to explain the clinical syndrome of neurogenic pulmonary edema.

According to the neurocardiac theory, the catecholamine storm accompanying the Cushing reflex (CR) leads to direct toxic myocardial injury and the development of pulmonary edema (7). The rapid severe increase in hydrostatic pressures after the catecholamine surge results in a net shift of blood volume from the systemic to pulmonary circulation, leading to the development of transudative pulmonary edema, according to the neurohemodynamic and blast proposition (8). The blast theory further posits that the extreme increase in capillary pressure induces barotrauma, damaging the capillary–alveolar membrane (4). According to the pulmonary venule adrenergic hypersensitivity hypothesis, the massive sympathetic discharge could directly affect the pulmonary capillary bed, with venoconstriction or endothelial disruption responsible for the formation of pulmonary edema (3). In addition, the early autonomous storm associated with brain death triggers the development of a systemic and pulmonary inflammatory response, which may lead, by itself, to increased pulmonary endothelial permeability and sympathetic vasoconstriction of systemic and pulmonary vessels, indirectly maintaining or worsening the condition (9).

Donor hemodynamic instability with the upregulation of *in situ* inflammatory processes and cell damage–associated signaling pathways, as well as the increased release of inflammatory cytokines and stress hormones, have all been described as factors contributing to poor transplantation outcomes (10), but the specific role played by immunity in early brain death has received less attention thus far (11). Tacrolimus, a macrolide immunosuppressant belonging to the calcineurin inhibitor family, modulates the cellular immune and nonimmune mechanisms responsible for acute lung graft rejection, such as reduced pulmonary capillary leakage and decreased leukocyte infiltration, as well as the altered release of proinflammatory cytokines (12–14).

Interestingly, calcineurin inhibitors have already been used successfully as a pretreatment in several models of acute lung injury (ALI) (15–18), but never in brain death–induced lung injury.

In the present study, we evaluated the effects of tacrolimus administered as a pretreatment in an experimental model of neurogenic pulmonary edema secondary to subdural autologous blood infusion–induced brain death in pigs (19). We focused on the relationship between the expression of inflammatory mediators, endothelial function actors, glycocalyx proteoglycans, and hemodynamic derangements and the development of ALI.

Some of the results of this study were reported in the form of an abstract at the 42nd International Society of Heart and Lung Transplantation Annual Meeting and Scientific Sessions, April 27–30, 2022, in Boston and at the American Thoracic Society International Conference 2022 Annual Meeting and Scientific Sessions, May 13–18, 2022, in San Francisco.

**Methods**

**Protocol: Animal Preparation**

Eighteen female 4-month-old pigs were premedicated, anesthetized, and paralyzed following a protocol described previously (19–22). As previously reported (19–23), the animals were equipped to measure systemic arterial pressure (Psa), pulmonary arterial pressure (Ppa), occluded Ppa (Ppao), right atrial pressure (Pra), effective pulmonary capillary pressure (Pcap), thermodilution cardiac output, and instantaneous pulmonary blood flow (Q) and Ppa. The animals were ventilated using an FIO2 of 0.4–1.0 to maintain SaO2 > 90%, a respiratory rate of 12–20 breaths · min⁻¹, and a VT of 15–25 ml · kg⁻¹ to achieve an arterial PaCO2 between 35 and 45 mm Hg and a positive end-expiratory pressure of 5 and 8 cm H2O (19–22). Balanced crystalloid- and gelatin-modified solutions were perfused (at 10 ml · kg⁻¹ · h⁻¹) to maintain Pra between 6 and 8 mm Hg. When Psa was < 65 mm Hg, noradrenaline infusion was started to maintain organ perfusion (19).

**Protocol: Brain Death Procedure**

The day before the procedure, the pigs were assigned to placebo (lactose monohydrate; BD group, n = 9) or 0.25 mg · kg⁻¹ of tacrolimus (PROGRAF [Astellas] in lactose monohydrate; BD + Tac group, n = 8) administered twice a day with blocked randomization in blocks of four animals. The assignment was centralized in sealed, opaque envelopes, and the drugs were prepared in
the pharmacy department. One hour before the experiment, the pigs were pretreated with an intravenous administration of placebo (5% glucose solution; BD group, n = 9; weight 50 ± 1 kg) or 0.05 mg·kg⁻¹·d⁻¹ tacrolimus (in 5% glucose solution; BD + Tac group, n = 8; weight 52 ± 1 kg). As previously reported, one hole was drilled in the temporoparietal cranium. Slow parenchymal infusion (0.5 ml·min⁻¹ for 240 min) of autologous blood was performed to induce brain death (19).

The administration of drugs used for anesthesia was stopped when the CR was observed, and protective ventilation was applied with a Vt of 6 ml·kg⁻¹ and a target plateau pressure of <30 cm H₂O. Periodic deep inspirations were administered to prevent atelectasis.

### Hemodynamic Data Acquisition and Analysis

Each data set included blood gas analysis; the acquisition of Psa, Ppa, Ppao, Pra, and Q values using a Biobox acquisition system (Biomedisoft); and lung biopsies as previously reported (19). The data sets were analyzed with the Biomedisoft program (Biomedisoft); and lung biopsies as previously reported (19). The data sets were analyzed with the Biomedisoft program (Biomedisoft); and lung biopsies as previously reported (19). The data sets were analyzed with the Biomedisoft program (Biomedisoft); and lung biopsies as previously reported (19).

### Biological and Histological Assessments

#### Real-time quantitative PCR

Real-time quantitative PCR experiments were performed according to a previously reported protocol (19–22) for PPET-1 (preproendothelin-1), ETA (type A endothelin receptor) and ETB (type B endothelin receptor), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), PTGIR (prostaglandin I₂ receptor), Tie2 (TEK receptor tyrosine kinase), HO-1 (hemoglobin oxygenase-1), IL-6 and IL-10, VCAM1 (vascular cell adhesion molecule-1), proapoptotic Bax (Bcl2-associated X apoptosis regulator) and antiapoptotic Bcl2 (B-cell lymphoma-2) and BclXL (Bcl2-like 1) molecules, and RPL4 (ribosomal protein L4) mRNA sequences (Table 1). Relative mRNA expression quantification was normalized to the housekeeping gene (RPL4) (24).

#### Table 1. Primers Used for Real-Time Quantitative PCR in Porcine Lung Tissue

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPET-1</td>
<td>5-TCCTGCTTCTCCCTGTAGTGA-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-TGTCAGAGTGGTGTAGCCA-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-TTTATCTGGCCATCCCTCA-3</td>
</tr>
<tr>
<td>ETA</td>
<td>5-GCCTTTGCGCTATTTGCA-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-CCCTTACCCAGAGTATT-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-GCACCAGCAATAAGCATGAT-3</td>
</tr>
<tr>
<td>ETB</td>
<td>5-CTTCTGCTTTGCCTTACCA-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-CCGGTATCTAGACCCAAAGG-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-CTGCAATGGATAATCAGCTGACC-3</td>
</tr>
<tr>
<td>NOS3 or eNOS</td>
<td>5-AGCTTCCTGATCGATGACCAAGCAA-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-ATGATCCGCGCCTTCACC-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-AAGACCGAGGTCAGGAT-3</td>
</tr>
<tr>
<td>NOS2 or iNOS</td>
<td>5-CTTCCGCCCCTCCACCAC-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-GGCTCTCTGTTATCCCGAT-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-CAGCATGCCAGGATTGT-3</td>
</tr>
<tr>
<td>PTGIR</td>
<td>5-GACCTGCCCCCTTCAAAGT-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-CCACCCAGGAAGGAAGAGG-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-AGTACCCATCCAGGAAGG-3</td>
</tr>
<tr>
<td>Tie2</td>
<td>5-TCACTAACATTCCCTGCTG-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-TGAGACACCCCTTCTTGGA-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-GGAATTCTACGGTGAGAGG-3</td>
</tr>
<tr>
<td>Bax</td>
<td>5-TCCTGGAGACCACTGAG-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-CCAGTGAGATGAGACG-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-GACTTTGCGAGATGTC-3</td>
</tr>
<tr>
<td>Bcl2</td>
<td>5-TCTCCGGAGGACACTCTG-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-GCCAAGGAAATGAGACT-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-ACATCTTCCCCAGT-3</td>
</tr>
<tr>
<td>BclXL</td>
<td>5-TGGTGGCGCTTTTCTCTTC-3</td>
</tr>
<tr>
<td>Sense</td>
<td>5-AGATGCGACTCCAATACCT-3</td>
</tr>
<tr>
<td>Antisense</td>
<td>5-AAAACCAAGGAGAGCTTCGG-3</td>
</tr>
<tr>
<td>RPL4</td>
<td>5-CATTCCGCTGAGGAGGCTCA-3</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** Bax = Bcl2-associated X apoptosis regulator; Bcl2 = B-cell lymphoma-2; BclXL = Bcl2-like 1; eNOS = endothelial nitric oxide synthase; ETA = endothelin type A receptor; ETB = endothelin type B receptor; HO-1 = hemoglobin oxygenase-1; iNOS = inducible nitric oxide synthase; NOS2 = nitric oxide synthase 2; NOS3 = nitric oxide synthase 3; PPET-1 = preproendothelin-1; PTGIR = prostanstaglin I₂ receptor; RPL4 = ribosomal protein L4; Tie2 = TEK receptor tyrosine kinase; VCAM1 = vascular cell adhesion molecule 1.

### Multiplex cytokine magnetic bead panel assay

Circulating concentrations of IL-6, IL-10, IL-1α, and IL-1β were determined using a cytokine magnetic bead panel assay (MILLIPLEX MAP; Merck Millipore) as previously published (19). ELISAs and nitric oxide (NO) assays. Quantitive endothelin-1 and hyaluronan (R&D Systems) and heparan sulfate proteoglycan (MyBioSource) ELISA kits were used to determine circulating concentrations. Total nitrite concentrations were determined using the Measure-IT High-Sensitivity Nitrite Assay Kit ( Molecular Probes) according to the manufacturer’s instructions.

**Immunohistochemistry:** terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end label. Lung epithelial cells undergoing apoptosis were detected by...
terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end label staining using the ApopTag Detection Kit (EMD Millipore), as previously described (19, 22, 25).

**Lung injury scoring.** ALI was scored as previously reported (26). Briefly, hematoxylin and eosin–stained sections were analyzed for neutrophil infiltration, airway epithelial cell damage, interstitial edema, hyaline membrane formation, hemorrhage, and the total lung injury score as the sum of those criteria. Each criterion was scored on a scale of 0–4, where 0 = normal, 1 = minimal change, 2 = mild change, 3 = moderate change, and 4 = severe change. Two independent investigators performed all counts in duplicate in a blinded manner.

**Statistical Analysis**

The sample size for the study groups was calculated using PASS sample size software for medical research (NCSS Statistical Software). The number of animals needed to achieve power > 80% for repeated-measures ANOVA (two groups, five times, and interaction) was calculated. The power analysis showed that with eight animals, we reached power of 0.9857 for the hemodynamic variables, indicating an increase of more than 1 SD between treated and control groups. A minimum of five animals per group was obtained. Analyses were performed using Prism version 5 (GraphPad Software Inc.). All data are expressed as mean ± SEM. The data were analyzed using a two-factor (group × time) repeated-measures ANOVA with time and the interaction group × time. When the F ratio reached significance, the Fisher protected t test was performed for Bonferroni correction for multiple comparisons (27). Using univariable linear regression analyses, we tested the relationships between the hemodynamic and respiratory, endothelial, inflammatory, and apoptotic variables and the occurrence of histological lung injury assessed by the ALI score using the Poon procedure (28). A P value <0.05 was considered to indicate statistical significance. For the easiest readability of tables and figures, only significance symbols for comparisons between the interventional and noninterventional groups and intragroup repeated measures for baseline versus CR + 7 hours are shown.

**Results**

The baseline hemodynamic parameters were similar between the two study groups (Table 2 and Figure 1). After 60–90 minutes of autologous blood infusion, intracranial pressure exceeded 80 mm Hg and remained higher than Psa throughout the remainder of the protocol. In the BD and BD + Tac groups, all animals presented the CR before hemodynamic evaluation. One animal from the BD + Tac group died during blood parenchymal infusion used to induce brain death because of ventricular fibrillation.

**Hemodynamic Characterization**

In the BD group, heart rate, Q (not illustrated), mean Ppa, and Pcap increased from 1 hour after the CR (CR + 1 h), Psa decreased, and noradrenaline increased (Table 2 and Figure 1). Q increased 1 hour after the CR; after progressively decreasing, it reached basal values 5 hours after the CR (CR + 5 h) (not illustrated) and lower than basal values after 7 hours (CR + 7 h) (Table 2). Increased pulmonary vascular resistance (PVR), Ppao, and Pra associated with decreased arteriolar compartmental resistance were observed from the third hour after the CR (CR + 3 h) with progressive aggravation until the end of the protocol (CR + 7 h) (Table 2 and Figure 1). Interestingly, Ppao and Pra remained within physiological ranges. Both 0-Hz and characteristic impedance increased from 5 hours after the CR (Figure 1).

Seven hours after the CR (CR + 7 h), tacrolimus pretreatment prevented changes in Q, mean Ppa, PVR, arteriolar compartmental resistance, and 0-Hz and characteristic impedance but failed to prevent an increase in Ppao compared with that in the BD group (Table 2 and Figure 1). In the BD + Tac group, mean Ppa and Pcap remained increased during the protocol compared with basal values but were lower than those observed in the BD group (Figure 1). Tacrolimus also prevented the decrease in Psa and reduced the need for noradrenaline throughout the protocol (Table 2).

**Table 2. Hemodynamic Evaluation in Pigs with Brain Death on the Basis of Tacrolimus Pretreatment**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BD (n = 9)</th>
<th>BD + Tac (n = 8)</th>
<th>P Value</th>
<th>BD (n = 9)</th>
<th>BD + Tac (n = 8)</th>
<th>P Value</th>
<th>CR + 7 h</th>
<th>CR + 7 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats·min⁻¹</td>
<td>78 ± 7</td>
<td>73 ± 3</td>
<td></td>
<td>118 ± 7</td>
<td>96 ± 11</td>
<td>&lt;0.001*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q, L·min⁻¹·m⁻²</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td></td>
<td>2.1 ± 0.2</td>
<td>3.2 ± 0.4</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psa, mm Hg</td>
<td>113 ± 5</td>
<td>118 ± 7</td>
<td></td>
<td>67 ± 2</td>
<td>110 ± 13</td>
<td>*</td>
<td>&lt;0.01#</td>
<td></td>
</tr>
<tr>
<td>Ppao, mm Hg</td>
<td>7.4 ± 0.5</td>
<td>8.7 ± 0.6</td>
<td></td>
<td>10.6 ± 0.6</td>
<td>11.5 ± 1.0</td>
<td>*</td>
<td>&lt;0.01#</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>Pra, mm Hg</td>
<td>5 ± 0</td>
<td>5 ± 1</td>
<td></td>
<td>8 ± 1</td>
<td>6 ± 1</td>
<td>*</td>
<td>&lt;0.01#</td>
<td></td>
</tr>
<tr>
<td>NA need, μg·kg⁻¹·min⁻¹</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>*</td>
<td>0.13 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>*</td>
<td>&lt;0.01†</td>
<td></td>
</tr>
</tbody>
</table>

*Definition of abbreviations: BD = brain death group; BD + Tac = brain death + tacrolimus group; CR = Cushing reflex; HR = heart rate; NA = noradrenaline; Ppao = pulmonary arterial pressure; Pra = right atrial pressure; Psa = systemic arterial pressure; Q = instantaneous pulmonary blood flow.

**Belhaj, Dewachter, Hopkens, et al.: Tacrolimus for Brain Death–Associated Lung Injury**
During resuscitation, balanced crystalloid- and gelatin-modified solutions were perfused to maintain \( P_{\text{ra}} \) between 6 and 8 mm Hg (15 ± 1 vs. 13 ± 1 \text{ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) in animals from the BD vs. BD + Tac groups; \( P > 0.05 \). When \( P_{\text{sa}} \) was <65 mm Hg, noradrenaline infusion was started to maintain organ perfusion (0.13 ± 0.04 vs. 0.03 ± 0.01 \text{µg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) in animals from the BD versus BD + Tac groups; \( P = 0.02 \) (Table 2).

### Lung Function Characterization

Brain death was associated with a decreased \( P_{\text{ao}_2}/F_{\text{li}_2} \) ratio from 1 hour after the CR (CR + 1 h) and with reduced total thoracic compliance from CR + 3 hours (Table 2). Pretreatment with tacrolimus completely prevented the changes in \( P_{\text{ao}_2}/F_{\text{li}_2} \) and total thoracic compliance (Figure 2). The \( P_{\text{ao}_2}/F_{\text{li}_2} \) ratio \( R^2 = 0.5702; P < 0.001 \) and total thoracic compliance \( R^2 = 0.1115; P < 0.01 \) were inversely correlated with \( \text{Pcap} \) (Table 2).

### Endothelium-Derived Vasoactive Molecules and Glycocalyx

Brain death was associated with decreased pulmonary gene expression of the ET-1 (endothelin 1) precursor \( PPET-1 \) from the fifth hour after the CR (CR + 5 h), with a nonsignificant trend toward increased endothelin receptor gene expression \( P = 0.07 \), evaluated as the provasoconstrictor ETA-to-ETB ratio, while pulmonary gene expression of the prostacyclin receptor \( \text{PTGIR} \) decreased, and \( \text{eNOS} \) expression tended (but not significantly) to increase at the seventh hour after the Cushing-s reflex (CR + 7 h; \( P = 0.06 \)) (Figure 3A). Tacrolimus pretreatment completely prevented these pulmonary gene expression alterations (Figure 3A). At the systemic level, the serum concentration of ET-1 was increased in the BD group (CR + 7 h), while NO concentrations (evaluated as serum nitrite concentrations) decreased after brain death (Figure 3B). Pretreatment with tacrolimus prevented the decrease in circulating NO concentrations but not the increase in ET-1 concentrations (Figure 3B). Pulmonary gene expression of \( PPET-1 \) (Figure 3A) and circulating serum concentrations of ET-1 (Figure 3B) were inversely and positively correlated with \( \text{Pcap} \), respectively. Conversely, the pulmonary gene expression of \( \text{eNOS} \) (Figure 3A) and circulating serum NO concentrations (Figure 3B) were positively and negatively correlated with \( \text{Pcap} \), respectively. These findings strongly suggest the differential regulation of these vasoactive signaling pathways at the systemic level and locally in the lungs.

To assess endothelial cell injury, we further evaluated pulmonary \( \text{Tie2} \) and \( \text{HO-1} \) expression after brain death. Lung gene expression of the endothelial \( \text{Tie2} \) receptor decreased from the third hour after the CR, while \( \text{HO-1} \) expression increased (Figure 3A). Pretreatment with tacrolimus was associated with decreased lung expression of \( \text{Tie2} \) already present at the base and globally failed to restore lung expression of \( \text{Tie2} \) and \( \text{HO-1} \) (Figure 3A). Pulmonary gene expression of the endothelial \( \text{Tie2} \) receptor and cell stress–induced \( \text{HO-1} \) were negatively and positively correlated with \( \text{Pcap} \), respectively (Figure 3A).
Brain death was associated with increased circulating serum concentrations of hyaluronan and heparan sulfate ($P = 0.06$), two important glycocalyx constituents released in the systemic circulation after deterioration of the protective endothelial glycocalyx, which was completely prevented by tacrolimus pretreatment (Figure 3B). Circulating serum concentrations of hyaluronan and heparan sulfate were correlated with Pcap (Figure 3B).

Inflammation
Brain death was associated with a progressive increase in pulmonary IL-6 gene expression throughout the protocol from the first to the seventh hours after the CR, while IL-10 gene expression remained stable (data not shown). Thus, the proinflammatory IL-6–to–IL-10 ratio increased in the lungs in the BD group 7 hours after the CR (Figure 4). This effect was associated with decreased pulmonary gene expression of iNOS from the third hour and with increased VCAM1 expression from the fifth hour after the CR (Figure 4). At the systemic circulating level, serum concentrations of proinflammatory IL-6–to–IL-10 ratio, IL-1α ($P = 0.06$), and IL-1β were significantly increased 7 hours after brain death (Figure 4). Tacrolimus pretreatment completely prevented the local increase in the proinflammatory IL-6–to–IL-10 ratio in the lungs (because of reduced pulmonary IL-6 gene expression in the BD + Tac group compared with that in the BD group) but failed to prevent an increase in the proinflammatory IL-6–to–IL-10 ratio, as well as increases in IL-1α and IL-1β at systemic serum concentrations (Figure 4). Altered pulmonary gene expression of iNOS and VCAM1 was also prevented by tacrolimus pretreatment (Figure 4).

Pulmonary expression of the proinflammatory IL-6–to–IL-10 ratio and VCAM1 was positively correlated with Pcap, whereas iNOS gene expression was inversely correlated with Pcap (Figure 4). Circulating serum concentrations of the proinflammatory IL-6–to–IL-10 ratio, IL-1α, and IL-1β were also positively correlated with Pcap (Figure 4).

Lung Activation of Apoptotic Processes
The lung proapoptotic Bax–to–Bcl2 and Bax–to–BclXL ratios increased 7 hours after the CR (Figure 5). Pulmonary gene expression of proapoptotic Bax mitochondrial members remained stable ($P > 0.05$), while pulmonary expression of antiapoptotic Bcl2 (0.05 < $P < 0.01$) and BclXL ($P < 0.001$) increased in the BD group 7 hours after the CR.
Figure 3. Pathobiological characterization of pulmonary endothelium-derived vasoactive molecules and glycocalyx in pigs with brain death on the basis of pretreatment with tacrolimus. (A) Relative lung mRNA content for ET-1 (endothelin-1) precursor, ppET-1 (preproendothelin-1), provasoconstrictor ET-A-to-ET-B ratio, eNOS (or NOS3), endothelial TiE2 (TEK receptor tyrosine kinase), and the vascular protector HO-1 (heme oxygenase-1) in total lung homogenates at baseline (Base) and 3, 5, and 7 hours after the Cushing reflex (CR) after preventive tacrolimus administration (brain death + tacrolimus group [BD + Tac], n = 8) or not (brain death group [BD], n = 9). Correlations between ppET-1, eNOS, TiE2 receptor, and HO-1 and pulmonary capillary pressure (Pcap) are presented. (B) Serum concentrations of vasoconstrictor ET-1, nitrite (as a surrogate of vasodilatory nitric oxide concentration), glycocalyx proteoglycans, hyaluronan, and heparan sulfate in pigs at Base and 7 hours after the CR (CR + 7 h) in the placebo-pretreated (BD, n = 9; red bars) and tacrolimus-pretreated (BD + Tac, n = 8; blue bars) brain death groups. Correlations between these endothelial parameters and Pcap are presented. Values are expressed as mean ± SEM. *P < 0.05 (BD vs. BD + Tac) and †P < 0.05 (Base vs. CR + 7 h). Red denotes P < 0.05 in the BD group, and blue denotes P < 0.05 in the BD + Tac group. CR = Cushing reflex; eNOS = endothelial nitric oxide synthase; ET-A = type A endothelin; ET-B = type B endothelin; PTGIR = prostaglandin I2 receptor.
To assess whether this upregulation in upstream mitochondrial apoptotic pathways was accompanied by the completion of apoptosis, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end label staining was performed to evaluate the degree of apoptosis in the lungs. Brain death was associated with a 5-fold increase in the lung apoptotic rate 7 hours after the CR, showing diffuse apoptosis within the lung parenchyma (Figure 5). Tacrolimus pretreatment prevented the brain death–induced increase in the apoptosis rate in the lung parenchyma (Figure 5).
Pulmonary proapoptotic gene ratios (Bax/Bcl2 and Bax/BclXL) and the lung proapoptotic rate were positively correlated with Pcap (Figure 5).

Characterization of ALI

Seven hours after the CR, the ALI score was increased compared with that at baseline. Pretreatment with tacrolimus did not prevent this increase in the ALI score (Figure 6). A correlation between the ALI score and Pcap was observed (Figure 6).

Discussion

The present study showed that pretreatment with the immunosuppressor tacrolimus prevented pulmonary hypertension and pulmonary capillary hypertension, lung activation of inflammatory and apoptotic processes, and alterations in the endothelial glycocalyx barrier, with no improvement in the lung injury score in an experimental porcine model of brain death.

Brain death influences allograft survival. Better characterization and quantification of a single primum movens of brain death–induced lung injury would be of therapeutic relevance to improve graft function after transplantation (6). Accordingly, we chose to focus on the early pathomechanisms associated with brain death in the lungs. The pretreatment protocol design is widely used in the field of solid organ transplantation and pathologies involving uncontrolled production of...
circulating inflammatory cells. Immunosuppressants, such as calcineurin inhibitors, have been used as a pretreatment in several models of ALI (15, 16) and in the field of transplantation (29, 30). Our experimental protocol evaluating therapeutic intervention with tacrolimus as a pretreatment before brain death is the first step to highlight the potential therapeutic use of tacrolimus in lung allograft preservation in the condition but requires further study in a more clinically translational way after declaration of brain death.

In the present study, we modified our previously published experimental model of brain death (12) to be closer to the true clinical situation. The total amount of autologous blood injected into the brain parenchyma was decreased; therefore, the observation follow-up was extended to 7 hours after the CR. The CR was delayed, with a longer exposure time to intracranial hypertension. Central nervous system injury is associated with the systemic inflammatory response syndrome, which occurs within the first few hours after injury and is responsible for inflammatory cell mobilization and recruitment to the lungs, activation and release of inflammatory mediators, and increased pulmonary vascular permeability, all of which contribute to the development of lung injury and dysfunction (2, 31). In the present study, inflammatory markers in both the circulation and lung tissue were considerably upregulated, a finding that is consistent with the inflammatory response syndrome observed after brain death. The present experimental model of brain death in pigs provides useful information to establish the relative contribution of preventive strategies when donor hemodynamic instability coupled with the activation of lung inflammatory and cell-damage processes associated with increased release of inflammatory cytokines and activation of innate immune effector cells constitutes a major threat.

Seven hours after brain death, PVR (assessed by PVR, 0-Hz impedance, and arterial elastance) was increased, as was mean Ppa, while the characteristic impedance remained unchanged. Brain death–induced pulmonary hypertension was associated with increased systemic concentrations of ET-1 and increased lung expression of ETA-to-ETB ratio, which contribute to pulmonary hypertension (21, 32) and primary graft dysfunction (33). Indeed, the interstitial edema associated with brain death has been linked to activation of the endothelin axis (33, 34). In addition, ET-1 may act as an immune modulator, contributing to lung injury by upregulating the expression of cytokines and cell adhesion molecules (32, 35). Consistently, endothelin receptor antagonists improve donor lung function in an experimental ovine model of brain death (36). In the present study, systemic NO concentrations and lung eNOS expression decreased. This finding might indicate a physiological adaptation to attempt to counteract the increased concentrations of vasoconstrictors. In addition, NO has protective effects against oxidative stress (37), and altered NO production can play critical roles in pathophysiologic mechanisms directly or indirectly implicated in the pathogenesis of neurogenic pulmonary edema syndrome associated with brain death. Tacrolimus can prevent most of these pathobiological anomalies.

Pulmonary hypertension is associated with pulmonary capillary hypertension together with increased pulmonary venous resistance and Ppa. These factors have been tightly linked to the development of hydrostatic pulmonary edema (38). Interestingly, both pulmonary hypertension and pulmonary capillary hypertension are prevented by tacrolimus pretreatment. In addition, various inflammatory markers, including the lung and serum proinflammatory IL-6–to–IL-10 ratio, serum concentrations of IL-1α and IL-1β, and lung expression of VCAM1, are correlated with an increase in Pcap, suggesting that brain death–associated lung injury is linked to both increased Pcap and capillary permeability.

Hemodynamic and inflammatory alterations after brain death contribute to vascular endothelial dysfunction (39). Here, we showed decreased endothelial Tie2 receptor in the lung and increased concentrations of glycocalyx components in serum, strongly suggesting endothelial injury after brain death. This finding is consistent with previous studies implicating pulmonary endothelial dysfunction in brain death–induced lung injury (40, 41). Because the endothelial glycocalyx plays a crucial role...
in maintaining the hemostatic balance between blood coagulation and anticoagulation, endothelial cell function and glycocalyx shedding have been prioritized for further exploration (42). Interestingly, tacrolimus pretreatment largely prevents glycocalyx layer deterioration, which also likely contributes to the beneficial pulmonary vasomotor effects observed regarding endothelial function preservation (43). The use of this immunosuppressors has already been shown to preserve microcirculation through NF-κB (nuclear factor-κB) signaling blockade (44).

In the present study, tacrolimus had a major impact on the different parameters directly implicated in brain death–associated lung graft dysfunction, such as hemodynamics, in situ expression of proinflammatory mediators, and histological lung integrity. Tacrolimus pretreatment did not completely prevent the dysregulated circulatory systemic inflammatory response induced by brain death that might secondarily affect the lungs. This response could affect various signaling pathways responsible for excessive accumulation and activation of leukocytes and platelets, as well as the increased permeability of alveolar endothelial and epithelial barriers (45, 46), which could maintain or worsen lung injury (47).

In addition, tacrolimus has already been shown to attenuate ischemia–reperfusion injury through decreased free radical production and the inhibition of NF-κB (36). However, studies on the effects of tacrolimus in the context of lung ischemia–reperfusion injury have been more focused on in vivo and rodent models, not large animal models (10, 37). Tacrolimus is a cornerstone of current immunosuppressive regimens for maintenance immunosuppression in heart and lung transplant recipients. On the basis of the results of the present investigation, tacrolimus should be considered a pretreatment for lung grafts.

Here, we showed an increase in Pcap together with an altered glycocalyx layer, likely promoting the development of ALI, which is histologically characterized by congestion, hemorrhage, and inflammatory cell infiltration. This effect is associated with increased concentrations of proinflammatory cytokines (e.g., IL-6–to–IL-10 ratio), as previously reported for BAL after brain death (48, 49). ALI is characterized by the acute persistent pulmonary inflammatory response syndrome, destruction of capillary endothelial cells from capillary leakage, alterations in the epithelial glycocalyx layer, and promotion of cell injury and death (50). Taken together, the findings show that these factors contribute to the perpetuation of lung injury (48, 51) and an increased risk of chronic rejection after lung transplantation (49, 52). Tacrolimus failed to improve the ALI score, although it lowered cytokine concentrations in the lungs and serum concentrations of glycocalyx components and inhibited the activation of apoptotic processes. However, tacrolimus pretreatment could not completely reduce systemic circulating inflammation, which was likely responsible for the presence of ALI.

Conclusions

Tacrolimus pretreatment prevented pulmonary capillary hypertension and the activation of inflammatory and apoptotic processes in lung tissue but failed to improve the lung injury score observed after brain death. Notably, the present study, designed as an experimental animal model and pretreatment intervention, had limitations. Therefore, further investigations should be performed in an animal model to assess the efficacy and safety of tacrolimus (alone or in combination with other drugs) administered in a post–brain death design protocol to evaluate hemodynamics and pathobiological changes and minimize the clinical impact of brain death after lung transplantation.

Author disclosures are available with the text of this article at www.atsjournals.org.

References
