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ORIGINAL ARTICLE

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Cardiopulmonary bypass increases endothelial dysfunction after pulmonary ischaemia-reperfusion in an animal model

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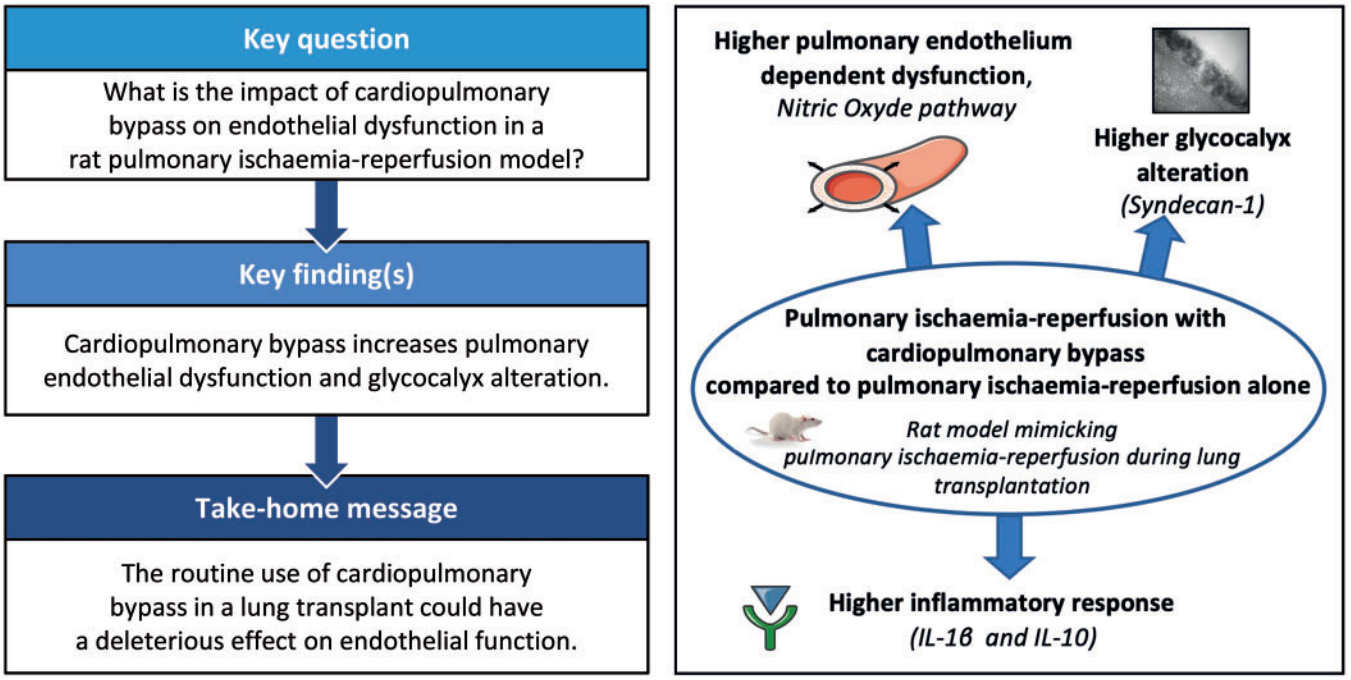

Abstract

OBJECTIVES: Endothelial dysfunction during ischaemia-reperfusion (IR) is a major cause of primary graft dysfunction during lung transplantation. The routine use of cardiopulmonary bypass (CPB) during lung transplantation remains controversial. However, the contribution of CPB to pulmonary endothelial dysfunction remains unclear. The objective was to investigate the impact of CPB on endothelial dysfunction in a lung IR rat model.

METHODS: Rats were allocated to 4 groups: (i) Sham, (ii) IR, (iii) CPB and (iv) IR-CPB. The primary outcome was the study of pulmonary vascular reactivity by wire myograph. We also assessed glycocalyx degradation by enzyme-linked immunosorbent assay and electron microscopy and both systemic and pulmonary inflammation by enzyme-linked immunosorbent assay and immunohistochemistry. Rats were exposed to 45 min of CPB and IR. We used a CPB model allowing femoro-femoral support with left pulmonary hilum ischaemia for IR.

RESULTS: Pulmonary endothelium-dependent relaxation to acetylcholine was markedly reduced in the IR-CPB group (10.7 ± 9.1%) compared to the IR group (50.5 ± 5.2%, $P < 0.001$), the CPB group (54.1 ± 4.7%, $P < 0.001$) and the sham group (80.8 ± 6.7%, $P < 0.001$), suggesting that the association of pulmonary IR and CPB increases endothelial dysfunction. In IR-CPB, IR and CPB groups, vasorelaxation was completely abolished when inhibiting nitric oxide synthase, suggesting that this relaxation process was mainly mediated by nitric oxide. We observed higher syndecan-1 plasma levels in the IR-CPB group in comparison with the other groups, reflecting an increased degradation of glycocalyx. We also observed higher systemic inflammation in the IR-CPB group as shown by the increased plasma levels of IL-1β, IL-10.

CONCLUSIONS: CPB significantly increased the IR-mediated effects on pulmonary endothelial dysfunction. Therefore, the use of CPB during lung transplantation could be deleterious, by increasing endothelial dysfunction.

Keywords: Lung transplantation • Cardiopulmonary bypass • Ischaemia-reperfusion • Endothelial dysfunction • Glycocalyx • Systemic inflammation

ABBREVIATIONS

CPB	Cardiopulmonary bypass
ECMO	Extracorporeal membrane oxygenation
IL	Interleukine
IR	Ischaemia-reperfusion
LTx	Lung transplantation
NO	Nitric oxide
SNP	Sodium nitroprusside

INTRODUCTION

Lung transplantation (LTx) is the preferred treatment for terminal respiratory failure in selected patients. However, despite technical progress, morbidity and mortality remain high compared to other organ transplants [1, 2]. The primary graft dysfunction that usually occurs within 3 postoperative days is one of the main causes. Aetiologies of primary graft dysfunction are multiple, but the main contributor is the ischaemia-reperfusion (IR) that the graft undergoes [1–3]. There are formal indications for the use of cardiopulmonary bypass (CPB) in LTx, such as haemodynamic instability or severe pulmonary hypertension with right ventricular dysfunction. During LTx, the combination of anaesthesia, one lung ventilation, pulmonary artery clamping and reperfusion induces haemodynamic failure, leading to major haematosis disorders (hypoxia, hypercapnia and respiratory acidosis). The use of cardiocirculatory support could be helpful in these situations by restoring sufficient arterial pressure, reducing right ventricular pressure, controlling revascularization flow and providing oxygenation and CO_2 clearance of blood [4, 5].

However, the use of routine CPB in LTx remains controversial. Analysis of the literature shows conflicting data [6, 7]. On the one hand, arguments in favour of the routine use of CPB suggest that it optimizes surgical exposure, allows haemodynamic stability, improves blood haematosis and reduces surgical time. Also, CPB allows the control of reperfusion pressure of the transplanted lung and contributes to a decrease in shear stress, pulmonary oedema and reperfusion damage in humans [8–10]. On the other hand, arguments against CPB point to the fact that it expands the blood–air interface and contact between blood components and artificial surface. This leads to a strong inflammatory process with subsequent deleterious effects on vascular and organ functions [11]. The use of CPB is also associated with platelet dysfunction, fibrinolysis and coagulation dysfunction [12–14].

In this context, although no consensus has been reached, a number of issues need to be considered. In fact, CPB alone and IR alone are known to contribute to the development of major endothelial dysfunction, but no study has explored the impact of these 2 conditions together on the endothelial function of the pulmonary artery. Therefore, the study of such function seems promising to answer the question of CPB use during thoracic surgery with prolonged clamping of pulmonary artery. Thus, the primary objective of this study was to investigate the impact of CPB on pulmonary endothelial dysfunction in a model of pulmonary IR in rats. Secondary objectives were to explore vascular glycocalyx, systemic inflammation and pulmonary tissue inflammation.

MATERIALS AND METHODS

Animal care and study groups

Male Wistar rats (Janvier Labs, Saint Berthevin, France) weighing 400–450 g were used. Rats were housed at a constant temperature of 21°C with a 14/10 h dark/light cycle. All animals had free access to standard chow and drinking tap water. They received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the study was approved by the French 'Ministère de l'Enseignement Supérieur et de la Recherche-Direction Générale pour la Recherche et l'Innovation' (No APAFIS 2016102718162386-V2). All procedures were performed in accordance with the French Ethics Committee as well as with the guidelines of European Parliament directive No. 2010/63/EU and the council for the Protection of Animals used for Scientific Purposes under the supervision of an authorized investigator. Rats were allocated to 4 groups as follows: sham group ($n = 10$); IR group ($n = 9$) with left pulmonary IR; CPB group ($n = 9$); IR-CPB group in association ($n = 10$). A schematic representation of the study design is presented in Fig. 1.

Surgical procedure

Rats were anaesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The left carotid was cannulated with a 22-gauge catheter (Introcan® Safety B. Braun, Boulogne Billancourt, France) for continuous monitoring of blood pressure, heart rate (Labchart, ADInstruments®,Colorado, USA) and drug administration. During the entire experiment, rectal temperature was monitored (Eutech Instrument®, Eroscan Temp 4, Nijkerk Netherlands). Ketamine was injected every 30 min and heparin (500 IU/kg) after conditioning. A tracheotomy was performed with a 16-gauge catheter (Introcan Safety B. Braun, Boulogne Billancourt, France) for mechanical

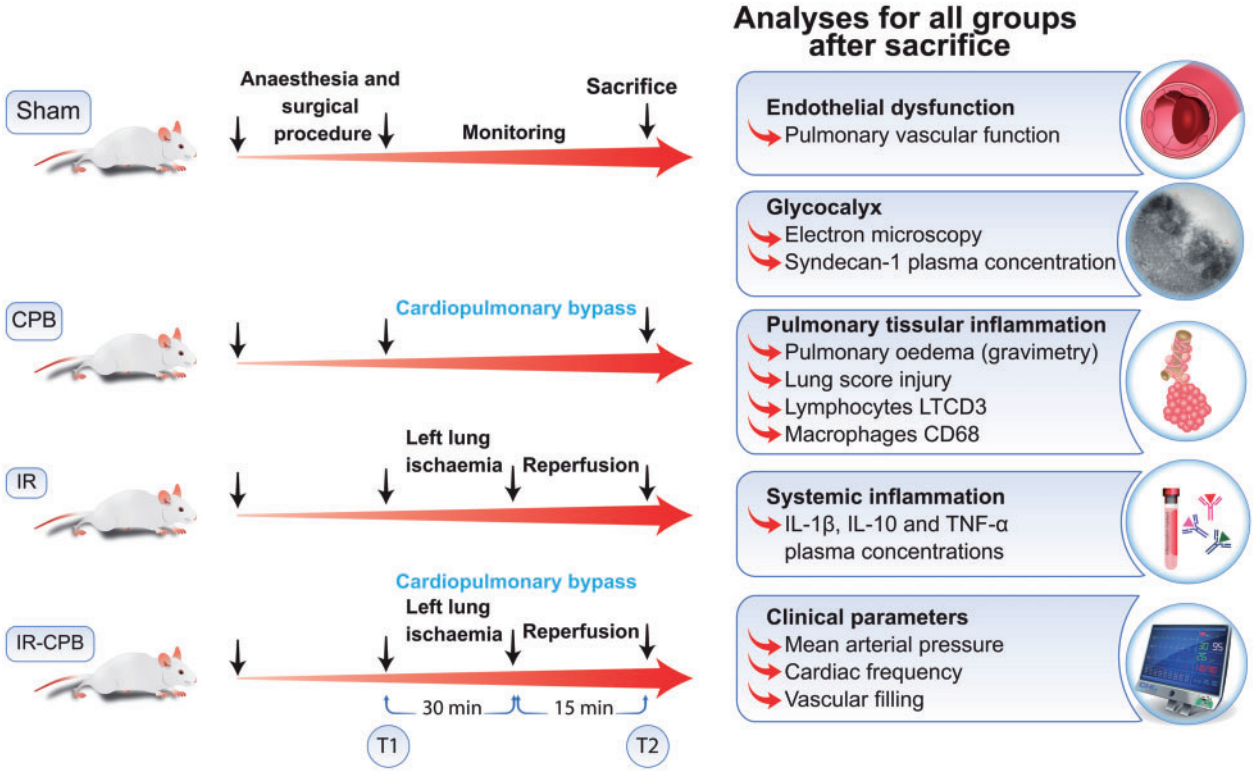

Figure 1: Schematic diagram of the experimental procedure and sampling points. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion; T1: time 1 corresponding to left lung IR with CPB and samples obtained at the end of anaesthesia-surgical procedure; T2: time 2 corresponding to samples obtained at the end of the experiment.

ventilation (Ugo Basile®, Rodent ventilator, model 7025, Varese, Italy) with a tidal volume of 6 ml/kg at a respiratory frequency of 75 cycles/min, and inhalation of air during CPB and 100% O_2 during other situations. The right femoral vein and the right femoral artery were cannulated, respectively, by a 16-gauge catheter and a 22-gauge catheter (Introcan Safety B. Braun, Boulogne Billancourt, France). For the CPB group, we used a CPB sterile circuit including an oxygenator (Kewei Medical Instrument Inc. Shenzhen, China), a roller pump (Fresenius Apparatebau, Bad Homburg, Germany), a cardiotomy reservoir consisting of a 5 ml syringe (Terumo®, Tokyo, Japan) and tubing lines. The oxygenator was connected to an oxygen tank. The circuit was aseptically set up and 'free from air bubbles' with a 4% Gelofusine® solution (B. Braun, Boulogne Billancourt, France). Circuit priming volume was 10 ml (4% Gelofusine). In the CPB group, the arterial cannula was connected to the right femoral artery and the venous cannula to the right femoral vein. The rat was placed upon a heated surface in a 30° proclive position to facilitate venous drainage. The CPB outflow was progressively increased to 100 ml/kg/min. Vascular filling was realized with 4% Gelofusine allowing satisfactory venous return for CPB and additional infusion was performed in case of mean arterial pressure inferior to 55 mmHg [15].

For each group, a thoracotomy was performed by a left intercostal incision under the axillary hollow. In the group with pulmonary IR, the left pulmonary hilum was delicately tightened with a lasso (Gore-Tex® Suture, Gore Medical, Arizona, USA) for ischaemia and released for reperfusion. The rats were euthanized at the end of the study. Rats were exposed to 30 min of left pulmonary ischaemia (left hilum clamping) and 15 min of reperfusion with or without CPB. For the sham group, rats were euthanized 45 min after conditioning. Blood samples were carried out in all groups at 2 time points: (time 1) immediately after the surgical procedure of the animal and (time 2) before the sacrifice.

Pulmonary vascular studies

Pulmonary vascular function was assessed as previously described [16, 17,18]. The left lung was removed and placed in cold oxygenated Krebs buffer. This solution was maintained at 37°C and gassed with a mix of 95% O_2 and 5% CO_2. The pH was controlled and maintained at 7.40. A 2–3 mm segment of second-order intralobar pulmonary artery (internal diameter <700 μm) was carefully dissected and mounted on a myograph (DMT®, Aarhus, Denmark). After normalization, responses to acetylcholine (10^{-9} to 3.10^{-5} mol/l) and to sodium nitroprusside (SNP) (10^{-9} to 3×10^{-5} mol/l) were obtained in segments precontracted with phenylephrine (10^{-5} mol/l). To explore the nitric oxide (NO)-related pathways in the response to acetylcholine, vasoreactivity was also assessed after 45-min incubation with the NO synthase inhibitor L-NG-nitro-arginine (10^{-4} mol/l).

Glycocalyx analysis

Glycocalyx degradation was measured using enzyme-linked immunosorbent assay kits for syndecan-1 in plasma.

Transmission electron microscopy

Samples were processed as previously described [19]. Detailed procedures are described in Supplementary Material.

Systemic inflammation

Blood samples were collected and centrifuged at 2 time points: after rat conditioning and before sacrifice, and immediately stored

at -80°C until further analysis. Proinflammatory plasma cytokines were measured using enzyme-linked immunosorbent assay for Interleukine-1β (IL-1β) (Rat IL-1β/IL-1F2 RLB00 Quantikine, R&DSystem), tumour necrosis factor α (TNF-α) (Rat TNF-α RTA00 Quantikine, R&DSystem) and anti-inflammatory cytokines using enzyme-linked immunosorbent assay for Interleukine-10 (IL-10) (Rat IL10 RL1000 Quantikine, R&DSystem). Kits were used according to the manufacturer's instructions and the analytics were read using a plate reader at a 450 nm optical density.

Histology and immunostaining studies

For histologic studies, the left lung apex was removed and placed in Krebs solution and then in water-soluble glycols and resins (Tissue-Tek®, Microm Microtech, Brignais, France) just before snap freezing and were then stored at -80°C. Detailed procedures for methodologies are described in Supplementary Material.

Lung injury score

We used a pulmonary tissue sample from the area surrounding the pulmonary artery used for transmission electron microscopy to determine a lung injury score for each group. Detailed procedures and score are described in Supplementary Material.

Clinical parameters

Invasive blood pressure and heart rate were measured continuously during the procedure with the PowerLab® device (AD Instruments, Colorado, USA). Lung oedema was measured using dry weights after 5 days at 65°C. Mean arterial pressure was maintained between 50 and 60 mmHg post CPB using 4% Gelofusine, if necessary.

Statistical analysis

All statistical analyses were performed with GraphPad Prism® 6.0 (GraphPad Software Inc. California, USA). Because no data were available for the possible effect of CPB on pulmonary vascular reactivity (primary outcome), we performed a simulation, using our historical data, of the effect of CPB on mesenteric vascular reactivity to demonstrate statistical significance ($P < 0.05$) with a minimal power of 80%. The minimal expected effect on relaxation between IR and IR-CPB groups was fixed at 40%. At least, 5 rats per group were thus required. For all significant differences concerning primary end points, *a posteriori* powers higher than 80% were also checked. Before applying parametric tests as a one-sample *t*-test or analysis of variance followed by Tukey's multiple comparison test, the Gaussian distribution of data was assessed using the Kolmogorov–Smirnov test. For non-parametric distribution, the Mann–Whitney test or Kruskal–Wallis test, followed by Dunn's multiple comparison test, was used.

All results are expressed as mean ± standard deviation, except for vascular studies, results are expressed as mean ± SEM. A result with *P*-value <0.05 was considered to be statistically significant. For statistical analyses of the glycocalyx degradation, the systemic inflammation, the immunohistology, the lung score injury and the clinical parameters, analysis of variance was used, followed by Tukey's multiple comparison (*post hoc*) testing.

RESULTS

Association of pulmonary ischaemia-reperfusion and cardiopulmonary bypass increases pulmonary endothelial dysfunction

Contractile responses of pulmonary arteries to phenylephrine were not different between the 4 groups after 45 min (Fig. 2A). Pulmonary endothelium-dependent relaxation to acetylcholine was markedly reduced in IR-CPB rats (10.7 ± 9.1%) compared to the IR group (50.5 ± 5.2%, $P < 0.001$), the CPB group (54.1 ± 4.7%, $P < 0.001$) and the sham group (80.8 ± 6.7%, $P < 0.001$). Both CPB and IR groups showed a decrease in pulmonary endothelium-dependent relaxation compared to sham ($P < 0.01$). No difference was observed between CPB and IR groups (Fig. 2B). The study of pulmonary endothelium-independent relaxation to SNP showed no difference between groups when compared to sham, suggesting no alteration of smooth muscle cells (Fig. 2C).

Role of nitric oxide

To investigate pathways involved in endothelium-dependent relaxation in our study, we incubated pulmonary arteries with L-NG-nitro-arginine to limit NO production by inhibiting NO synthase. First, we observed that endothelium-dependent relaxation of pulmonary arteries in sham decreased from 80.8 ± 6.7% to 17.2 ± 5.2% ($P < 0.01$). Second, in IR-CPB, IR and CPB groups, vasorelaxation was completely abolished when inhibiting NO synthase (Fig. 2D).

Implications of glycocalyx

We did not observe any significant differences between groups at time 1 (Fig. 3A). We found significantly increased plasma levels of the glycocalyx marker syndecan-1 at time 2 in the IR-CPB group compared to IR (IR-CPB: 22.1 ± 2.5 μg/ml; IR: 18.2 ± 2.2 μg/ml, $P < 0.001$). No difference was observed in CPB or IR groups when compared to sham (Fig. 3B).

To further examine the implications of glycocalyx in endothelial dysfunction, pulmonary artery sections were examined by transmission electron microscopy. Accordingly, our qualitative analysis of the glycocalyx of the pulmonary artery showed visual injuries of this structure in IR, CPB and IR-CPB groups when compared to sham. Glycocalyx damage seemed higher when CPB was associated with IR than with IR, CPB or Sham (Fig. 4).

Inflammatory response

We did not observe any significant difference between the groups at time 1 for IL-1β, IL-10 and TNF-α (Fig. 5A, C and E). IL-1β levels were significantly higher at time 2 in the CPB group compared to the sham group (sham: 64.7 ± 54.6 pg/ml; CPB: 523.2 ± 382.4 pg/ml, $P < 0.01$). We did not identify any significant differences between the CPB group and the IR-CPB group. We also observed a significant increase in IL-1β levels in the CPB-IR group compared to the IR group (IR-CPB: 591.4 ± 319.9 pg/ml; IR: 116.6 ± 70.1 pg/ml, $P < 0.01$) (Fig. 5B). For IL-10, we did not show any significant difference between the sham group and the CPB and IR groups or between the CPB and IR-CPB groups. In contrast, IR-CPB significantly increased plasma concentrations of IL-10 compared to the IR group (IR-CPB 234.8 ± 50.8 pg/ml;

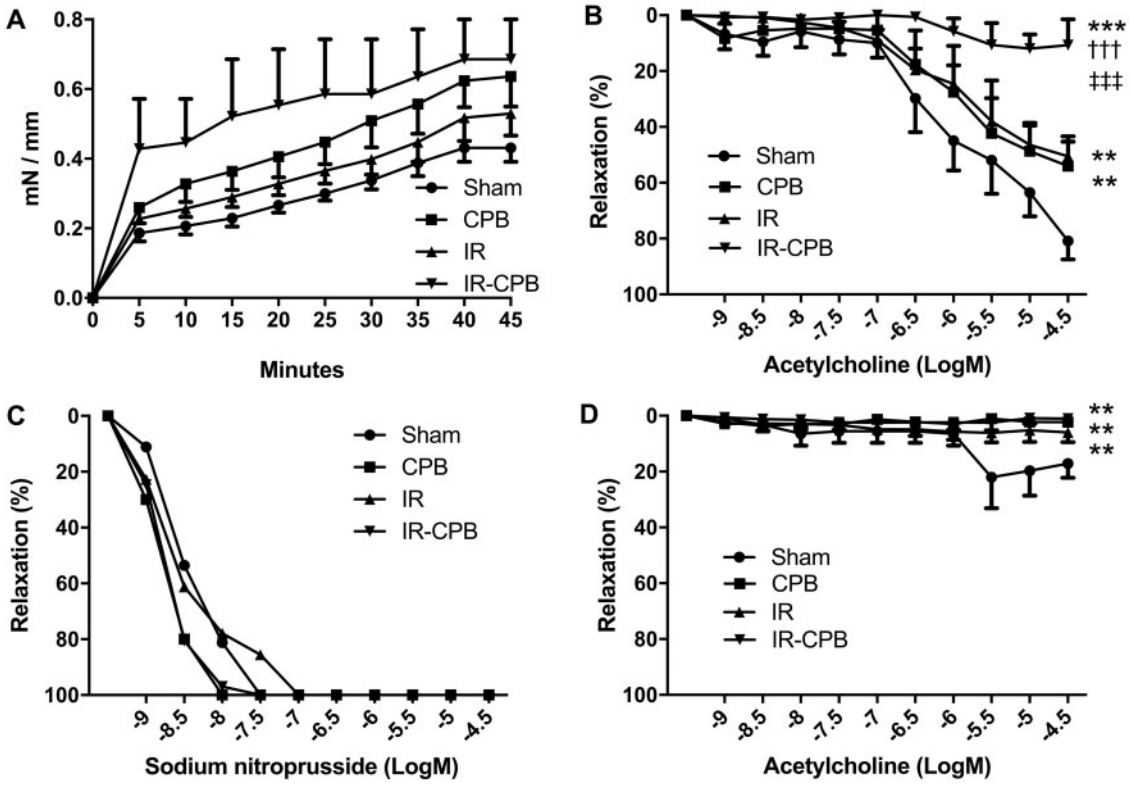

Figure 2: *In vitro* vascular responses. (**A**) Contractile responses to phenylephrine. (**B**) Relaxation to acetylcholine after precontraction with phenylephrine (endothelium-dependent relaxation). $^{**}P < 0.01$ vs sham. $^{***}P < 0.001$ vs sham. $^{\dagger\dagger\dagger}P < 0.001$ vs CPB. $^{\ddagger\ddagger\ddagger}P < 0.001$ vs IR. (**C**) Relaxation to sodium nitroprusside after precontraction with phenylephrine (endothelium-independent relaxation). (**D**) Relaxation to acetylcholine after precontraction with phenylephrine with L-NNA. $^{**}P < 0.01$ vs sham. For statistical analyses, analysis of variance was used for repeated measurements, followed by Tukey's multiple comparison (*post hoc*) testing. The concentrations of acetylcholine and sodium nitroprusside were expressed by the logarithmic decimal value of their molarity (**B**–**D**). A result with $P < 0.05$ was considered to be statistically significant. For (**B**–**D**), responses are expressed as percentage relaxation of phenylephrine-induced precontraction (mean ± SEM). ($n = 5$ per group). For more clarity, errors bars for each group in Figure (**C**) are not represented. CPB: cardiopulmonary bypass; L-NNA: L-NG-nitro-arginine; IR: ischaemia-reperfusion; Log [M]: logarithm decimal of molarities.

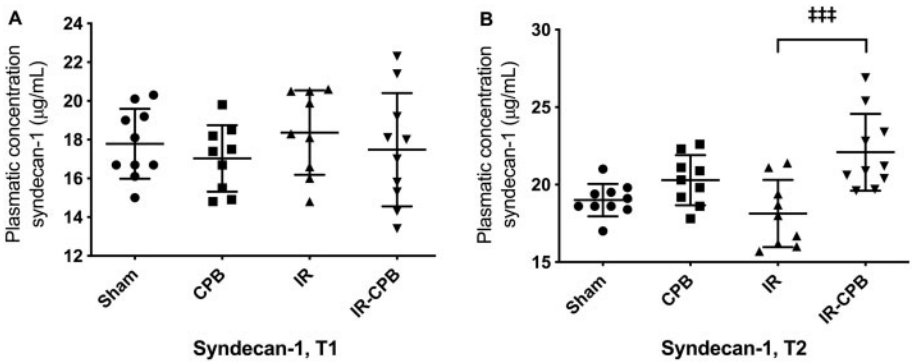

Figure 3: Plasmatic glycocalyx degradation. (**A**) Plasma concentration of syndecan-1 at T1. (**B**) Plasma concentration of syndecan-1 at T2. $^{\ddagger\ddagger\ddagger}P < 0.001$ vs IR. For statistical analyses, ANOVA was used, followed by Tukey's Multiple comparison (post hoc) testing. A result with p <0.05 was considered to be statistically significant. Data are presented as mean ± SD. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion; T1: time 1; T2: time 2.

IR; 137.7 ± 36.8 pg/ml, $P < 0.01$) (Fig. 5D). Plasma TNF-α concentration was significantly higher in the CPB, IR, IR-CPB groups compared to the sham group (respectively $P < 0.001$; $P < 0.001$, $P < 0.001$ vs sham) (Fig. 5F).

Histology

To determine whether leucocytes are involved in inflammatory changes, immunohistology was performed on the left lung in each group (Fig. 6A–D). The analysis of the lung sections revealed greater macrophage infiltration in the IR-CPB group than in the CPB group (IR-CPB 782 ± 185 macrophages/µm^2; CPB 439 ± 154 macrophages/µm^2, $P < 0.01$), but no significant variation concerning the infiltration of T-lymphocytes between groups (Fig. 6E and F).

Lung injury score

The histological examination (Fig. 7A–D) found a significantly higher lung injury score in CPB (3.6 ± 0.7), IR (4.1 ± 1.1) and IR-CPB (4.9 ± 1.4) groups compared to Sham (1.8 ± 0.9) (respectively

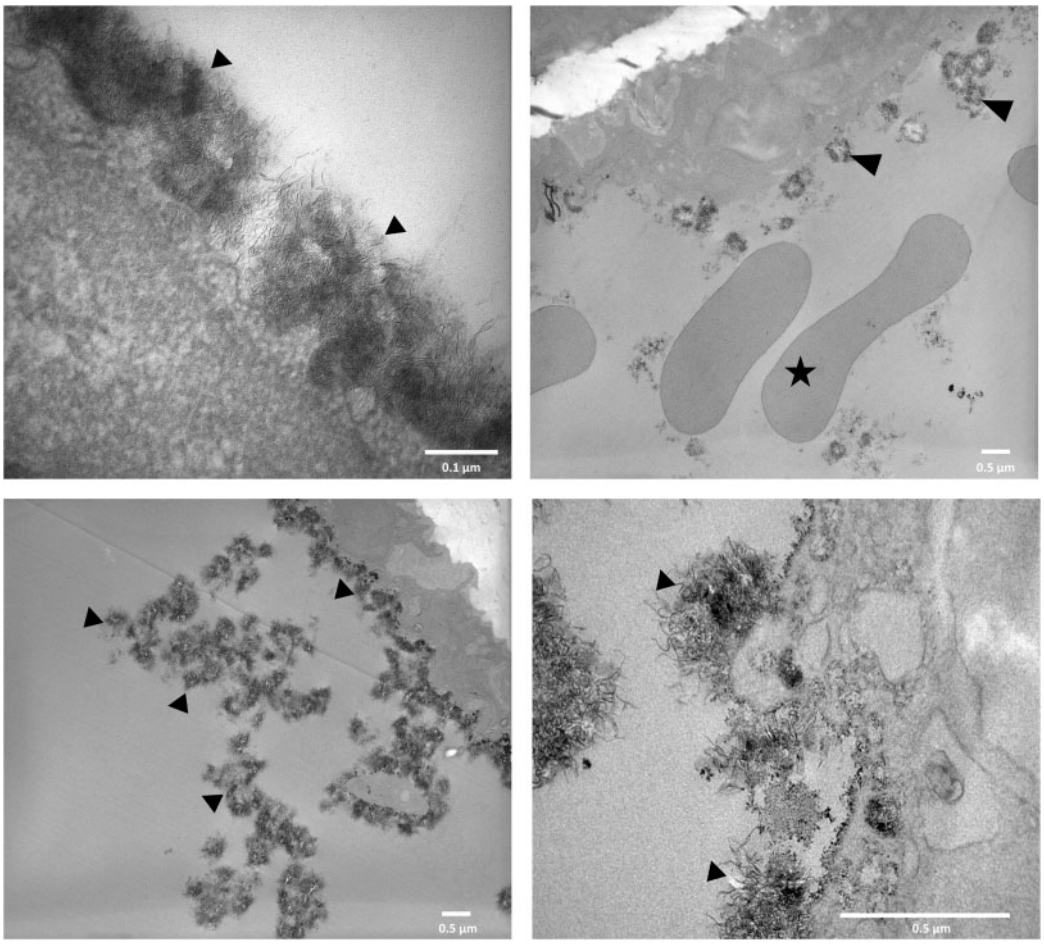

Figure 4: Visualization of pulmonary endothelial glycocalyx with transmission electronic microscopy. Glycocalyx (black head arrow) is revealed by lanthanum salts and is observed at the outer surface of the endothelium (**A**–**D**), in the lumen of artery (**C**) and at the surface of red blood cells (**B**) (black star). (**A**) Sham group, scale bar = 0.1 µm. (**B**) CPB, scale bar = 0.5 µm. (**C**) IR group, scale bar= 0.5 µm. (**D**) IR-CPB group, scale bar=0.5 µm. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion.

$P < 0.01$; $P < 0.001$, $P < 0.001$ vs sham). There was no significant difference between IR and IR-CPB groups (Fig. 7E).

Clinical parameters

We did not observe a significant difference between the IR-CPB and the CPB groups for mean arterial pressure and heart rate (Fig. 8A–D). We showed significantly higher vascular filling in the IR-CPB group than in the CPB group (IR-CPB 8.0 ± 2.2 ml; IR: 5.7 ± 1.3 ml, $P < 0.05$) (Fig. 8E). No significant difference between groups was observed for the pulmonary wet/dry ratio (Fig. 8F).

DISCUSSION

The major result of the present study is that CPB associated with IR increased endothelial dysfunction in pulmonary arteries in our rat model mimicking pulmonary IR during LTx. Lung IR and endothelial dysfunction are associated with the occurrence of primary graft dysfunction in humans. Also, CPB is associated with a systemic inflammatory response that has been clearly correlated with multi-organ failure. The routine use of CPB during LTx remains controversial [6, 7].

Mechanical circulatory support

There is still controversy about the use of CPB or extracorporeal membrane oxygenation (ECMO) as mechanical circulatory support during LTx. This debate may seem dated, but all studies are retrospective with many biases. There are no randomized prospective studies to answer this question. Both ECMO and CPB have advantages and disadvantages [20]. However, this study is the first to our knowledge to assess the impact of mechanical circulatory support on pulmonary endothelial dysfunction in an IR rat model.

Vascular dysfunction analysis

Regarding pulmonary vascular function, we first observed that IR alone and CPB alone were associated with mild endothelial dysfunction revealed by reduced vasorelaxation to acetylcholine. This dysfunction was significantly increased when IR was associated with CPB, suggesting that this association enhances the alteration of endothelial cells. Pathways involved in vasorelaxation of pulmonary arteries were explored in our study. We demonstrated the important role of NO in the sham group as NO synthase inhibition reduced maximal relaxation by nearly 75%. This

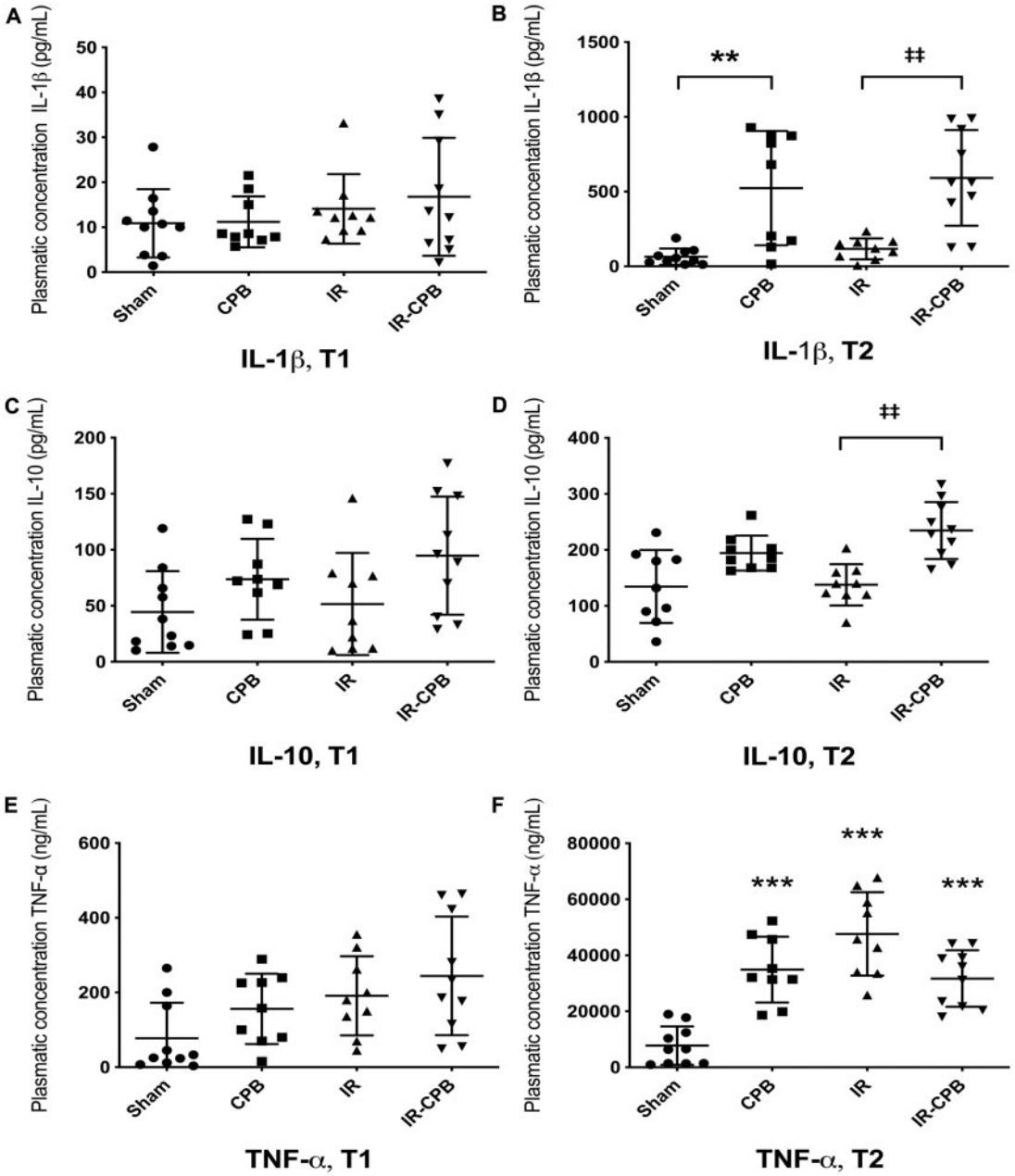

Figure 5: Systemic inflammation. (**A**) Mean value of IL-1β plasma level at T1. (**B**) Mean value of IL-1β plasma level at T2. $^{**}P < 0.01$ vs sham; $^{‡‡}P < 0.01$ vs IR. (**C**) Mean value of IL-10 plasma level at T1. (**D**) Mean value of IL-10 plasma level at T2. $^{‡‡}P < 0.01$ vs IR. (**E**) Mean value of TNF-α plasma level at T1. (**F**) Mean value of TNF-α plasma level at T2. $^{***}P < 0.001$ vs sham. For statistical analyses, ANOVA was used, followed by Tukey's Multiple comparison (post hoc) testing. A result with p <0.05 was considered to be statistically significant. Data are presented as mean ± SD. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion; T1: time 1; T2: time 2.

suggests that most of the relaxation was mediated by NO, although a small component of NO-independent response was also involved. This result suggests that there are alternative pathways involved in 25% of this relaxation, such as epoxyeicosatrienoic acid pathways. In IR-CPB, IR and CPB groups, vasorelaxation was completely abolished when inhibiting NO synthase, suggesting that these alternative pathways were abolished.

In a previous study in our laboratory concerning CPB models, we already showed an activation of oxidative stress leading to a decrease in NO bioavailability, and therefore altered endothelial-dependent relaxation in the mesenteric arteries of rats [17]. Thus, in this study, we suggest that this dysfunction may be partially explained by oxidative stress and potentiated by ischaemia. In order to test this hypothesis, it would have been pertinent to incubate vessels with antioxidants such as superoxide dismutase for at least 30 min in order to scavenge reactive oxygen species and then enhance bioavailability of NO [21]. Due to the experimental limitation regarding the duration of the surgical procedure and of functional vascular exploration, we were not able to test this hypothesis.

Relaxation in response to the NO donor SNP was similar in all groups. NO released by SNP acts on smooth muscle cells to induce vasorelaxation. Our results show that IR, CPB and IR-CPB did not alter this responsiveness of smooth muscle cells to NO.

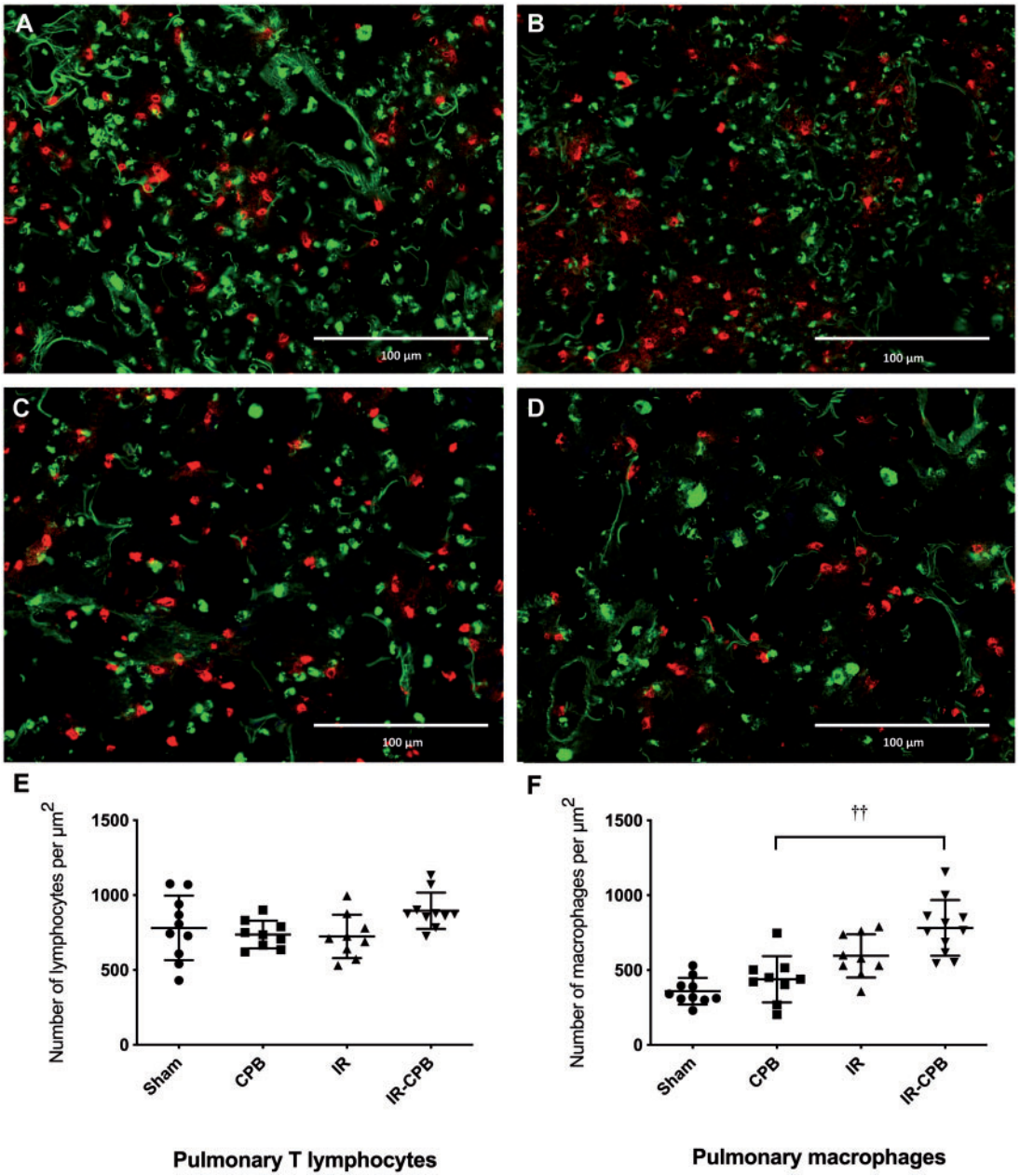

Figure 6: Immunohistology (magnification ×40, scale bar = 100 µm). Fluorescent immunohistological marking of the pulmonary parenchyma of the Sham group (**A**) the CPB group (**B**), the IR group (**C**) and the IR-CPB group (**D**). For all the groups, macrophages (CD68) are marked in green and lymphocytes (CD3) are marked in red. (**E**) Pulmonary T lymphocytes (CD3) of the left lung tissue. (**F**) Pulmonary macrophages (CD68) of the left lung tissue, $^{\dagger\dagger}P < 0.01$ vs CPB. For statistical analyses, ANOVA was used, followed by Tukey's Multiple comparison (post hoc) testing. A result with p <0.05 was considered to be statistically significant. Data are presented as mean ± SD. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion.

This confirms that the vascular dysfunction observed in our study specifically affected the endothelium with unaltered smooth muscle function.

Glycocalyx approach

In order to explain this endothelial dysfunction, we focused on possible alterations of pulmonary glycocalyx. The glycocalyx is a dynamic structure of endothelial cells that contributes to a large number of physiological functions including regulation of coagulation, oxidative stress and perception of shear stress. The glycocalyx lines the surface of the endothelial cells and also plays a major role in mediating endothelial function [22]. Its main components are proteoglycans, glycoaminoglycans and soluble proteins. Several studies reported the association between glycocalyx damage and lung injury, mainly via endothelial dysfunction [23, 24]. In our study, increased syndecan-1 concentration in the IR-CPB group indicates that glycocalyx is degraded more severely in this condition. To confirm this hypothesis, we studied glycocalyx structure by electron microscopy . In the IR, CPB and IR-CPB groups, we observed increased irregularities or total disappearance of endothelial glycocalyx in the vascular lumen compared

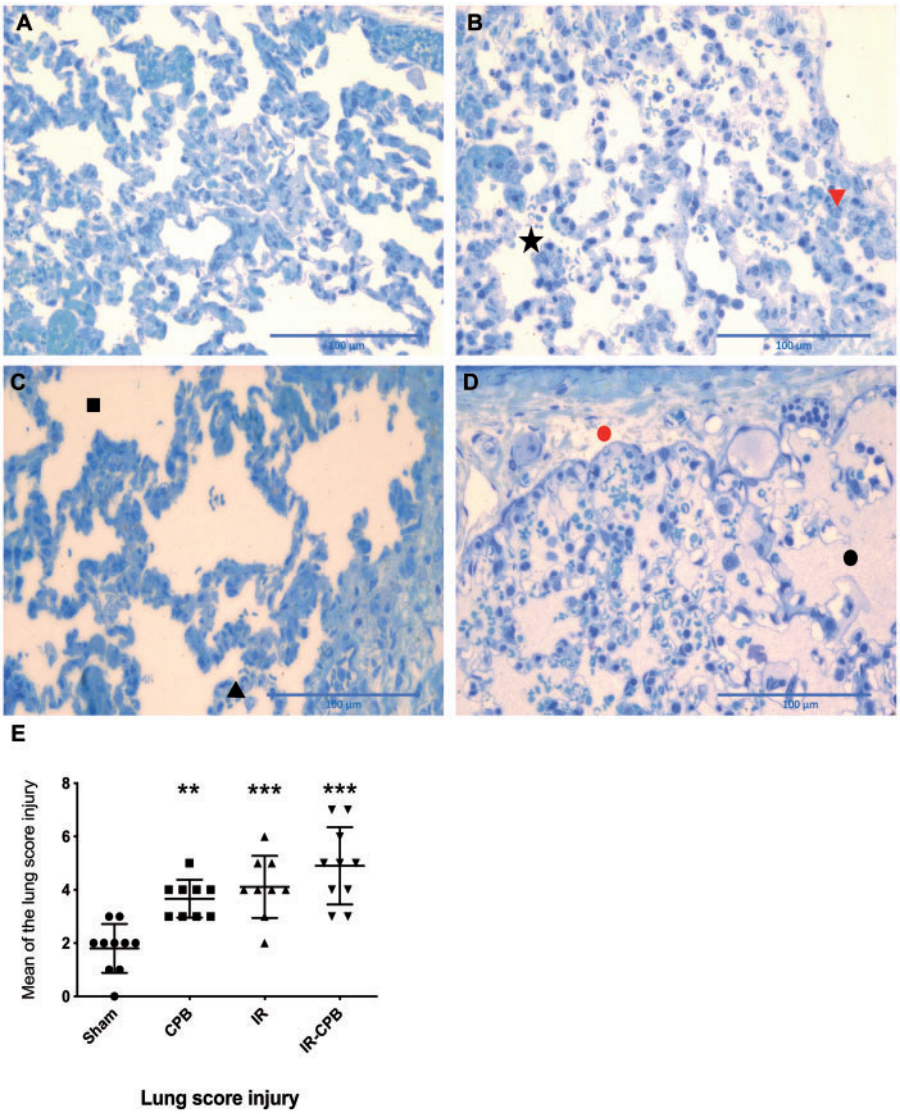

Figure 7: Lung parenchyma histology (toluidine blue, magnification ×40, scale bar = 100 µm). (**A**) Sham group. (**B**) CPB group. Microhemorrhages are represented with a black star and neutrophils in the pulmonary interstitium are represented with a red arrow head. (**C**) IR group. A neutrophil in the alveolar lumen is represented with a black arrow head and alveolar overdistension is represented with a black square. **(D)** IR-CPB group. Alveolar oedema is represented with a black circle and interstitial oedema is represented with a red circle. (**E**) Mean value of lung injury score. $^{**}P < 0.01$; $^{***}P < 0.001$ vs sham. For statistical analyses, ANOVA was used, followed by Tukey's Multiple comparison (post hoc) testing. A result with p <0.05 was considered to be statistically significant. Data are presented as mean ± SD. CPB: cardiopulmonary bypass; IR: ischaemia-reperfusion.

to the sham group. We also observed aggregation between glycocalyx and red blood cell which may be at the origin of microthrombi in the small pulmonary vessels. These elements suggest that glycocalyx damage is higher when CPB is associated with IR and may explain the pulmonary artery endothelial dysfunction in our model.

Inflammation response

The cytokine profile confirms the functional observations. Indeed, the proinflammatory cytokine IL-1β increased in the CPB groups compared to the sham group. We did not show any differences between the IR and sham groups, which is surprising in view of what is described in IR of the lung [25]. This difference can be explained by the aggressiveness of surgery in the sham group (artery and vein cannulation and thoracotomy) and by the too short ischaemic and reperfusion periods. However, the association of CPB and pulmonary IR increases systemic inflammation. In parallel, TNF-α which is mainly produced by macrophages and T lymphocytes in pulmonary IR increased in all groups compared to the sham group. However, the time chosen for the determination of plasma concentration of TNF-α (45 min after CPB) is probably too early. Various studies in humans showed that kinetic responses of TNF-α following CPB are in 2 phases (a first peak 2h30 and a second 48 h after CPB) [26]. Interestingly, we observed that the anti-inflammatory cytokine IL-10 evolved in the same direction as IL-1β [27]. We noted that T-lymphocyte levels were similar in all groups. Such results could be attributed to short reperfusion periods, as increasing rates of lymphocyte mobilization are usually seen after several hours of reperfusion [28]. We observed a greater sequestration of macrophages in the pulmonary parenchyma (interstial and alveolar) in

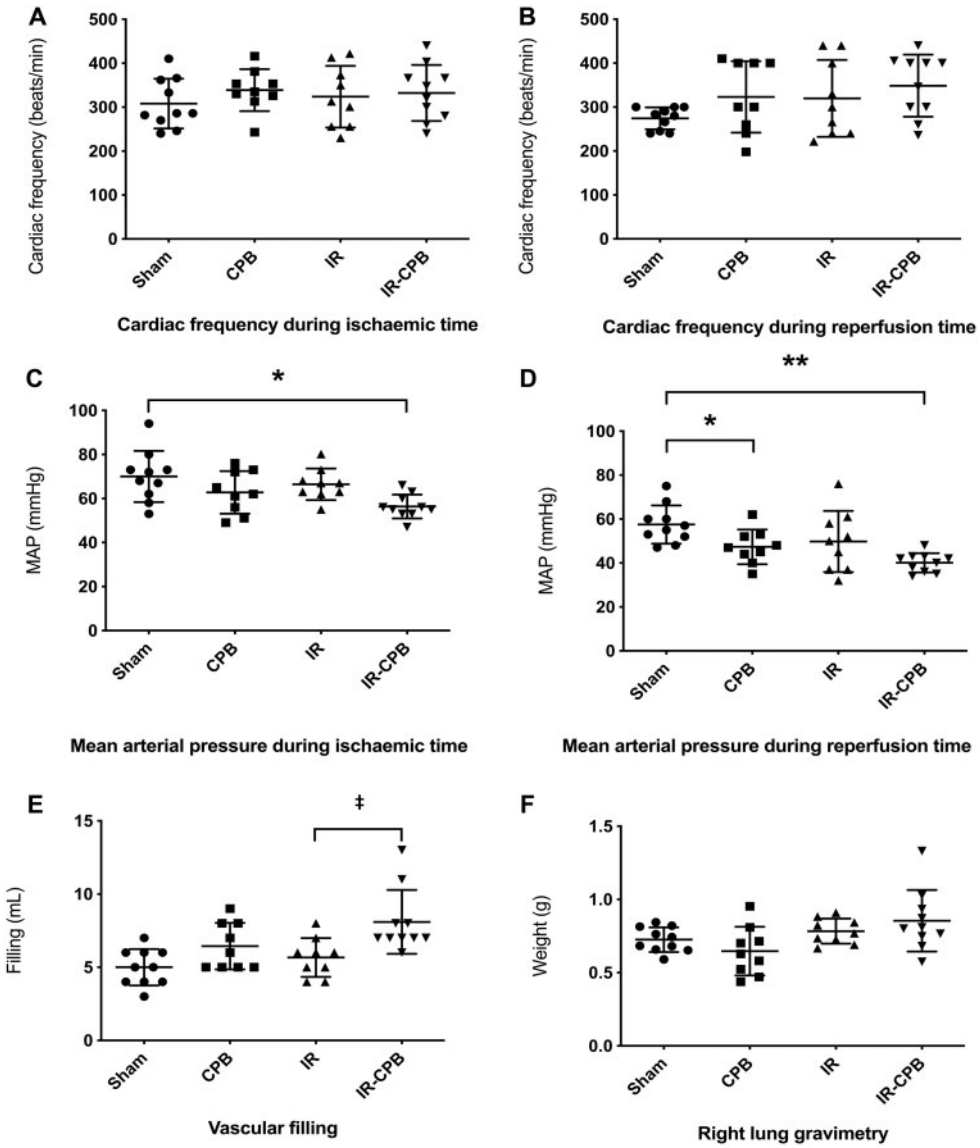

Figure 8: Clinical parameters. (**A**) Heart rate during ischaemia. (**B**) Heart rate during reperfusion. (**C**) Mean arterial pressure during ischaemia. $^{*}P < 0.05$ vs sham. (**D**) Mean arterial pressure during reperfusion. $^{*}P < 0.05$; $^{**}P < 0.01$ vs sham. (**E**) Vascular filling. $^{\dagger}P < 0.05$ vs IR. (**F**) Right lung gravimetry. For statistical analyses, ANOVA was used, followed by Tukey's Multiple comparison (post hoc) testing. A result with $p < 0.05$ was considered to be statistically significant. Data are presented as mean ± SD. IR: ischaemia-reperfusion.

the group associating CPB and pulmonary IR, in agreement with previous data [29]. In fact, macrophages are involved in early reperfusion lesions (30 min after reperfusion) leading to activation and sequestration of neutrophils that amplify the inflammatory response in the lung causing major IR lesions [30].

Limitations

Our work has a number of limitations. Our model is a warm IR model and concerns only one lung. In addition, a longer duration of both ischaemia and reperfusion would have been preferable but the aggressiveness of the model forced us to limit the duration of the surgery. Moreover, we were not able to explore all pathways of endothelial dysfunction other than NO, because the 10-h duration of the procedure does not allow it. Also, the analysis of the degradation of glycocalyx in electron microscopy was only qualitative and not quantitative. Regarding the results on systemic inflammation, the absence of difference between the IR-CPB and CPB groups does not allow us to suggest that CPB increases the effects of IR on inflammation. A lung transplant model in rats would be ideal and would allow us to increase ischaemic times and improve our clinical approach.

CONCLUSION

In conclusion, we have demonstrated in this rat model that CPB significantly increased the effects of pulmonary IR on pulmonary vascular dysfunction. In patients, this questions the routine use of CPB during LTx, which could be deleterious in endothelial function. Our results should be confirmed by future larger studies.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *EJCTS* online.

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Author contributions

Jean Selim: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing—original draft; Surgery, Pulmonary vascular studies, ELISA analysis. **Mouad Hamzaoui:** Pulmonary vascular study. **Inès Boukhalfa**: Pulmonary vascular study. **Zoubir Djerada:** Formal analysis. **Laurence Chevalier:** Transmission electron microscopy. **Nicolas Piton:** Transmission electron microscopy and histology. **Damien Genty:** Transmission electron microscopy. **Emmanuel Besnier:** Writing—review & editing. **Thomas Clavier:** Writing—review & editing. **Anaïs Dumesnil**: Histology and immunostaining studies. **Sylvanie Renet**: ELISA analysis. **Paul Mulder:** Resources; Software. **Fabien Doguet:** Conceptualization; Methodology. **Fabienne Tamion:** Conceptualization; Methodology. **Benoît Veber**: Supervision; Writing—review & editing. **Vincent Richard:** Conceptualization; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing—review & editing. **Jean-Marc Baste**: Conceptualization; Methodology; Supervision; Validation; Visualization; Writing—review & editing.

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REFERENCES

[1] Lee JC, Christie JD, Keshavjee S. Primary graft dysfunction: definition, risk factors, short- and long-term outcomes. Semin Respir Crit Care Med 2010;31:161–71.
[2] Christie JD, Van Raemdonck D, de Perrot M, Barr M, Keshavjee S, Arcasoy S *et al.* Report of the ISHLT Working Group on Primary Lung Graft Dysfunction part I: introduction and methods. J Heart Lung Transplant 2005;24:1451–3.
[3] Porteous MK, Lee JC. Primary graft dysfunction after lung transplantation. Clin Chest Med 2017;38:641–54.
[4] Sellers D, Cassar-Demajo W, Keshavjee S, Slinger P. The evolution of anesthesia for lung transplantation. J Cardiothorac Vasc Anesth 2017;31:1071–9.
[5] Kachulis B, Mitrev L, Jordan D. Intraoperative anesthetic management of lung transplantation patients. Best Pract Res Clin Anaesthesiol 2017;31:261–72.
[6] Nagendran M, Maruthappu M, Sugand K. Should double lung transplant be performed with or without cardiopulmonary bypass? Interact CardioVasc Thorac Surg 2011;12:799–804.
[7] Hoechter DJ, von Dossow V, Winter H, Müller H-H, Meiser B, Neurohr C *et al.* The Munich Lung Transplant Group: intraoperative extracorporeal circulation in lung transplantation. Thorac Cardiovasc Surg 2015;63:706–14.
[8] Lick SD, Brown PS, Kurusz M, Vertrees RA, McQuitty CK, Johnston WE. Technique of controlled reperfusion of the transplanted lung in humans. Ann Thorac Surg 2000;69:910–12.
[9] Marczin N, Royston D, Yacoub M. Pro: lung transplantation should be routinely performed with cardiopulmonary bypass. J Cardiothorac Vasc Anesth 2000;14:739–45.
[10] Nazarnia S, Subramaniam K. Pro: Veno-arterial Extracorporeal Membrane Oxygenation (ECMO) Should Be Used Routinely for Bilateral Lung Transplantation. J Cardiothorac Vasc Anesth 2017;31:1505–8.
[11] Levy JH, Tanaka KA. Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 2003;75:S715–20.
[12] Weber D, Cottini SR, Locher P, Wenger U, Stehberger PA, Fasshauer M *et al.* Association of intraoperative transfusion of blood products with mortality in lung transplant recipients. Perioper Med 2013;2:20.
[13] McRae K. Con: lung transplantation should not be routinely performed with cardiopulmonary bypass. J Cardiothorac Vasc Anesth 2000;14:746–50.
[14] Tong MZ-Y. Con: extracorporeal membrane oxygenation should not routinely replace cardiopulmonary bypass as the preferred method of support during lung transplantation. J Cardiothorac Vasc Anesth 2017;31:1509–10.
[15] Lebreton G, Tamion F, Bessou J-P, Doguet F. Cardiopulmonary bypass model in the rat: a new minimal invasive model with a low flow. Interact CardioVasc Thorac Surg 2012;14:642–4.
[16] Barrier M, Bégorre M-A, Baudrimont I, Dubois M, Freund-Michel V, Marthan R *et al.* Involvement of Heme Oxygenase-1 in particulate matter-induced impairment of NO-dependent relaxation in rat intralobar pulmonary arteries. Toxicol in Vitro 2016;32:205–11.
[17] Doguet F, Litzler P-Y, Tamion F, Richard V, Hellot M-F, Thuillez C *et al.* Changes in mesenteric vascular reactivity and inflammatory response after cardiopulmonary bypass in a rat model. Ann Thorac Surg 2004;77:2130–7.
[18] Le Guillou V, Tamion F, Jouet I, Richard V, Mulder P, Bessou JP *et al.* Mesenteric Endothelial Dysfunction in a Cardiopulmonary Bypass Rat Model: The Effect of Diabetes. Diab Vasc Dis Res 2012;9:270.
[19] Chevalier L, Selim J, Genty D, Baste JM, Piton N, Boukhalfa I *et al.* Electron microscopy approach for the visualization of the epithelial and endothelial glycocalyx. Morphologie 2017;101:55.
[20] Kiziltug H, Falter F. Circulatory support during lung transplantation. Curr Opin Anaesthesiol 2020;33:37–42.
[21] Rodman DM, Yamaguchi T, Hasunuma K, O'Brien RF, McMurtry IF. Effects of hypoxia on endothelium-dependent relaxation of rat pulmonary artery. Am J Physiol 1990;258:L207–14.
[22] Gao L, Lipowsky HH. Composition of the endothelial glycocalyx and its relation to its thickness and diffusion of small solutes. Microvasc Res 2010;80:394–401.
[23] Brettner F, von Dossow V, Chappell D. The endothelial glycocalyx and perioperative lung injury. Curr Opin Anaesthesiol 2017;30:36–41.
[24] Kirshbom PM, Jacobs MT, Tsui SSL, DiBernardo LR, Schwinn DA, Ungerleider RM *et al.* Effects of cardiopulmonary bypass and circulatory arrest on endothelium-dependent vasodilation in the lung. J Thorac Cardiovasc Surg 1996;111:1248–56.
[25] Chang DM, Hsu K, Ding YA, Chiang CH. Interleukin-1 in ischemia-reperfusion acute lung injury. Am J Respir Crit Care Med 1997;156:1230–4.
[26] Mitchell JD, Grocott HP, Phillips-Bute B, Mathew JP, Newman MF, Bar-Yosef S. Cytokine secretion after cardiac surgery and its relationship to postoperative fever. Cytokine 2007;38:37–42.
[27] Eppinger MJ, Ward PA, Bolling SF, Deeb GM. Regulatory effects of interleukin-10 on lung ischemia-reperfusion injury. J Thorac Cardiovasc Surg 1996;112:1301–5; discussion 1305–6.
[28] Gielis JF, Jungraithmayr W, Boulet GA, Bogers JP, Weder W, Cos P *et al.* A murine model of lung ischemia and reperfusion injury: tricks of the trade. J Surg Res 2015;194:659–66.
[29] Doguet F, Tamion F, Le Guillou V, Bubenheim M, Thuillez C, Richard V *et al.* Albumin limits mesenteric endothelial dysfunction and inflammatory response in cardiopulmonary bypass. Artif Organs 2012;36:962–71.
[30] Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res 1999;43:860–78.