Experimental *ex vivo* lung perfusion with sevoflurane: effects on damaged donor lung grafts

Xingyu Wang[a], Roumen Parapanov[a,b], Cyril Francioli[a], Jean Yannis Perentes[a], Igor Letovanec[c], Michel Gonzalez[a], Christian Kern[d], Hans-Beat Ris[a], Lise Piquilloud[b], Carlo Marcucci[d], Thorsten Krueger[a], Lucas Liaudet[b] and Fabrizio Gronchi[d,*]

[a] Service of Thoracic Surgery, Faculty of Biology and Medicine, University Hospital Medical Center, Lausanne, Switzerland
[b] Service of Adult Intensive Care Medicine, Faculty of Biology and Medicine, University Hospital Medical Center, Lausanne, Switzerland
[c] Service of Pathology, Faculty of Biology and Medicine, University Hospital Medical Center, Lausanne, Switzerland
[d] Service of Anesthesiology, Faculty of Biology and Medicine, University Hospital Medical Center, Lausanne, Switzerland

* Corresponding author. Service of Anesthesiology, Faculty of Biology and Medicine, University Hospital of Lausanne (Centre Hospitalier Universitaire Vaudois), 1011 Lausanne, Switzerland. Tel: +41-079-5566761; e-mail: fabrizio.gronchi@chuv.ch (F. Gronchi).

Abstract

OBJECTIVES: Volatile anaesthetics can provide significant protection against reperfusion injury in various experimental settings. The aim of this study was to assess the potential of sevoflurane treatment, the most commonly used volatile anaesthetic in modern anaesthesia, in rat lungs donated after circulatory death and reconditioned in an *ex vivo* lung perfusion (EVLP) system.

METHODS: Fifteen rats were sacrificed and divided into 3 groups. In the control and sevoflurane groups, the heart–lung blocks were exposed to 1 h of warm ischaemia and 2 h of cold ischaemia and were mounted on an EVLP circuit for 3 h, in the absence or in the presence of 2% sevoflurane. In the baseline group, heart–lung blocks were harvested immediately after euthanasia. Physiological data, lung nitro-oxidative stress, lactate dehydrogenase (LDH), expression of cytokines, oedema and histopathological findings were assessed during or post-EVLP.

RESULTS: The sevoflurane group showed significantly reduced LDH (8.82 ± 3.58 arbitrary unit vs 3.80 ± 3.02 arbitrary unit, $P = 0.03$), protein carbonyl (1.17 ± 0.44 nmol·mg^{-1} vs 0.55 ± 0.11 nmol·mg^{-1}, $P = 0.006$), 3-nitrotyrosine (197.44 ± 18.47 pg·mg^{-1} vs 151.05 ± 23.54 pg·mg^{-1}, $P = 0.004$), cytokine-induced neutrophil chemoattractant factor 1 (1.17 ± 0.32 ng·mg^{-1} vs 0.66 ± 0.28 ng·mg^{-1}, $P = 0.03$) and tumour necrosis factor alpha (1.50 ± 0.59 vs 0.59 ± 0.38 ng·mg^{-1}, $P = 0.02$) when compared with the control group. In addition, sevoflurane lungs gained significantly less weight (0.72 ± 0.09 g vs 0.72 ± 0.09 g, $P = 0.044$), had less perivascular oedema (0.58 ± 0.09 vs 0.47 ± 0.07, $P = 0.036$), and improved static pulmonary compliance (+0.215 ml·cmH$_2$O^{-1}, $P = 0.003$) and peak airways pressure (–1.33 cmH$_2$O, $P = 0.04$) but similar oxygenation capacity (+1.61 mmHg, $P = 0.77$) and pulmonary vascular resistances (+0.078 mmHg·min·ml^{-1}, $P = 0.15$) when compared with the control group.

CONCLUSIONS: These findings suggest that the potential of sevoflurane in protecting the lungs donated after cardiac death and reconditioned using EVLP could improve the outcome of these lungs following subsequent transplantation.

Keywords: *Ex vivo* lung perfusion • Sevoflurane • Damaged donor lung grafts

INTRODUCTION

The low acceptance rate of donor lungs is a challenge in lung transplantation [1, 2]. Ways to increase the number of eligible organs have been evaluated and include donation after circulatory death (DCD) [3] and removing extra fluids from the lungs by using *ex vivo* lung perfusion (EVLP) to recondition damaged lungs [4, 5]. However, because of an unavoidable period of warm ischaemia (WI), DCD lungs are at increased risk of primary graft dysfunction [6]. Primary graft dysfunction is the main cause of short-term morbidity and mortality and may be associated with chronic allograft dysfunction [7]. The leading contributors to primary graft dysfunction are the duration of WI and the subsequent ischaemia–reperfusion/reoxygenation (IR) injury [8]. Theoretically, IR injury can be prevented or blunted by pharmacological reconditioning during EVLP [9], which would therefore rehabilitate a damaged lung and make it more suitable for subsequent transplantation.

Volatile anaesthetics, such as isoflurane, sevoflurane or desflurane, can provide significant protection against reperfusion injury in various experimental settings [10]. This led to the concept of anaesthetic preconditioning (before the period of ischaemia) and post-conditioning (after the period of ischaemia), in the context of heart transplantation, with non-conclusive results. In lung transplantation, the preconditioning of lungs with inhaled

sevoflurane has been associated with reduced IR injury in *ex vivo* models of isolated rat lungs [11] and *in vivo* autotransplanted pig lungs [12]. In contrast, post-conditioning with sevoflurane has only been evaluated in a rat lung transplantation model after cold ischaemia [13].

The aim of this study was to test the hypothesis that sevoflurane treatment during EVLP can attenuate IR injury of rat lungs damaged by WI.

MATERIALS AND METHODS

Animals

Fifteen male adult (9–11 weeks) Sprague–Dawley rats weighing 300–350 g (Charles River Laboratory, L'Arbresle, France) were divided into 3 groups: baseline (the BASELINE group, $n = 3$) group, the control group (CONT group, $n = 6$) and the sevoflurane group (SEVO group, $n = 6$). In the BASELINE group, rats were euthanized without any intervention. The CONT and SEVO groups underwent the complete surgical procedure and EVLP procedure. All the animal experiments were performed in accordance with the Animal Welfare Act and the National Institute of Health 'Guidelines for the Care and Use of Laboratory Animals' and were approved by our local ethics committee (Service de la Consommation et des Affaires Vétérinaire Cantonal de l'Etat de Vaud, Epalinges, Switzerland, Authorization No. 2637).

Surgical preparation and lung harvesting

Animals were anaesthetized with intraperitoneal sodium pentobarbital (50 mg/kg) placed on a heating pad to maintain core temperature of 37°C. The trachea was cannulated, and mechanical ventilation was initiated using a rodent respirator (Model 683, Harvard Apparatus, Holliston, MA, USA), with a respiratory rate of 75 breaths·min^{-1}, a tidal volume of 7 ml·kg^{-1} and an FiO$_2$ of 0.21. After median thoracotomy, 600-IU heparin was administered into the right ventricle, and the pulmonary artery (PA) and left atrium were cannulated using metal catheters (Hugo Sachs Elektronik, Hugstetten, Germany). The animals were then sacrificed by exsanguination through a left ventricular puncture. In the CONT and SEVO groups, to mimic the DCD explantation procedure, the lungs were maintained *in situ* for 1 h at room temperature (WI time), in a deflated status, followed by the instillation of the cold Perfadex® (XvivoPerfusion, Göteborg, Sweden), through the PA cannula, and ventilation at a rate of 15 breaths·min^{-1} and a V_t of 7 ml/kg, at FiO$_2$ of 0.21. The heart–lung blocks were then harvested and stored at 4°C during 2 h (cold ischaemic time), in an inflated status with an FiO$_2$ of 0.5.

Ex vivo lung perfusion

After cold preservation, the heart–lung blocks were weighed, mounted in an isolated rat EVLP system (Harvard IL-2 System, Hugo Sachs Elektronik, Hugstetten, Germany) and primed with Steen® solution (Xvivo Perfusion, Göteborg, Sweden) (Fig. 1). In this system, a pump drives the perfusate from a reservoir through a gas exchanger and a heat exchanger before entering the PA. The pulmonary effluent from the left atrium drains back to the reservoir. During 30 min prior to the EVLP procedure, the circuit was run in closed loop at a flow of 10 ml/min to stabilize the perfusate
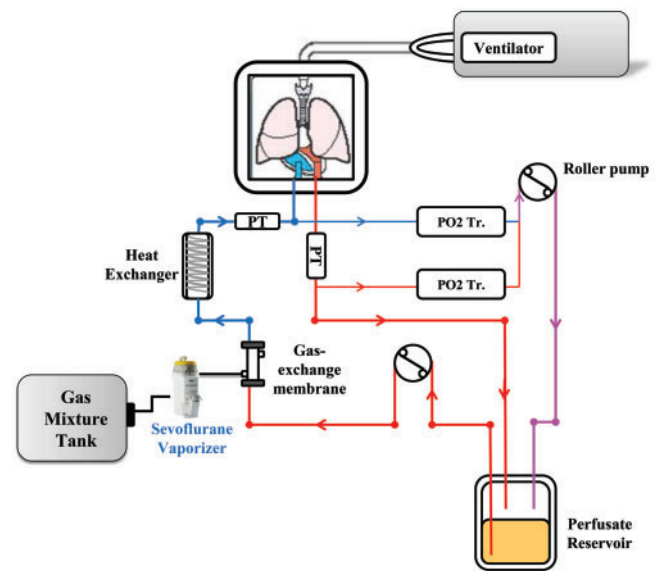

Figure 1: *Ex vivo* lung perfusion system. Blue line indicates affluent Steen® solution and red line indicates effluent Steen® solution. PO$_2$ Tr.: oxygen electrodes transducers; PT: pressure transducers.

temperature at 10°C by an external heater–cooler unit (Sarns TCMII, 3M, Saint Paul, MN, USA). During EVLP, the perfusate was deoxygenated using a gas mixture containing 6% O$_2$, 10% CO$_2$ and 84% N$_2$ delivered at a flow of a 1 l·min^{-1} over a gas-exchange membrane (Hemofilter D150, Medica S.P.A., Italy) connected to the affluent arm of the heart–lung block. The PO$_2$ in the affluent and effluent arms of the circuit was measured using O$_2$ electrodes (Hugo Sachs Elektronik, Hugstetten, Germany).

EVLP was initiated at a flow rate of 2 ml·min^{-1} at 10°C and was stepwise increased to the target flow defined as 7.5 ml·min^{-1}, corresponding to 7.5% of theoretical cardiac output [14] and rewarmed to 37°C (Alpha immersion thermostat 6, Laud-Brinkmann, Delran, NJ, USA) over 40 min. The left atrial pressure was set at 4 cm H$_2$O by adjusting the height of an outflow vessel. The pH of the perfusate was maintained in the range of 7.35–7.45 using THAM solution (Tham-Köhler 3M, köhler Pharma GmbH, Alsbach-Hähnlein, Germany). At 35°C, mechanical ventilation was initiated using a tidal volume of 3 ml·kg^{-1}, a respiratory rate of 7 min^{-1} and a FiO$_2$ of 0.21 (flexiVent FX3 ventilator, SCIREQ Inc., Montréal, Canada). After 40 min of EVLP, the perfusate reached 37°C, and the tidal volume was increased to 6 ml·kg^{-1}. A recruitment manoeuvre (inspiratory pressure of 15 cm H$_2$O during 20 s) was performed 60 min after the onset of EVLP and repeated every 30 min.

After 180 min of EVLP, the heart–lung block was withdrawn from the circuit and weighed. Two millilitres of sterile PBS, pH 7.4, was then instilled into the trachea to perform a bronchoalveolar lavage (BAL). The left lung was flash frozen and stored at -80°C, whereas the right lung was fixed in 4% paraformaldehyde for further histological analysis. This protocol is based on the strategy described for clinical EVLP by Cypel *et al.* [4] and adapted to our rat model of EVLP [15].

Experimental groups

In the CONT group, Steen solution alone was used throughout the EVLP procedure. In the SEVO group, a flow adaptable

sevoflurane vaporizer (Vapor2000 Sevoflurane, Drägerwerk AG, Lübeck, Germany) was connected in line with the gas-exchange membrane on the affluent arm of the perfusion circuit (Fig. 1). Based on previous studies [11, 12], a concentration of 2% sevoflurane was added into the gas mixture. To ensure a stable and relevant sevoflurane concentration in the Steen® solution, the circuit was primed with sevoflurane during the closed loop run. Sevoflurane was administered during the first 30 min of EVLP and stopped when mechanical ventilation was initiated.

In the BASELINE group, the heart–lung blocks were harvested immediately after euthanasia and were not subjected to EVLP. BAL and lung processing were done exactly as described earlier. This group was used to determine the normal values for the different biochemical measurements and normal lung histology in the absence of any intervention.

Measurements

Physiological variables. PA and left atrium pressures were continuously recorded using pressure transducers (Hugo Sachs Elektronik, Hugstetten, Germany). Pulmonary vascular resistances (PVRs) were calculated according to the standard formula: PVR = (mean pulmonary artery pressure-left atrial pressure)/flow. Peak airway pressure (PAWP) was continuously recorded. Static pulmonary compliance (SPC) was determined after 60 min and repeated every 30 min, by computing the change in lung volume elicited by an automated stepwise increase of inspiratory pressure up to 15 cmH$_2$O. Oxygenation capacity (DppO$_2$) was calculated as the difference between effluent and affluent PO$_2$. Finally, the difference in lung weight before and after EVLP was used as a measure of lung oedema.

Inflammatory cytokines in lung tissue. The lung tissue was grounded in liquid nitrogen using a mortar and a pestle, then homogenized in lysis buffer (TrisHCl 10 mM, NP40 0.5%, NaCl 0.15 M, Na$_3$VO$_4$ 1 mM, NaF 10 mM, PMSF 1 mM, ethylenediaminetetraacetic acid 1 mM, aprotinin 10 µg/ml, leupeptin 10 µg/ml and pepstatin 1 µg/ml), sonicated and incubated for 20 min on ice. After centrifugation, cytokines were measured in the supernatant. Protein concentration was measured with the BCA assay (Thermo Scientific Pierce, Rockford, IL, USA) and expressed in mg·ml^{-1} BAL fluid. The concentrations of tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and cytokine-induced neutrophil chemoattractant factor 1 (CINC-1) were measured using specifically designed ELISA kits (R&D System, Minneapolis, MN, USA) and were normalized to the concentration of proteins (ng·mg^{-1} protein).

Protein carbonyls. Protein carbonyls were quantified as an index of oxidative modifications in lung tissue proteins using an ELISA-based assay (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc., San Diego, CA, USA) and expressed in nanomol·mg^{-1} protein.

Nitro-oxidative stress. 3-Nitrotyrosine was determined in lung's tissue as a marker of peroxynitrite generation using an ELISA kit (Rat 3-Nitrotyrosine ELISA kit; Amsbio, Abingdon, UK) and expressed in pg/mg tissue.

Protein concentration and lactate dehydrogenase (LDH) levels in bronchoalveolar lavage. Total protein concentration in BAL was determined using the BCA assay and expressed in mg·ml^{-1} BAL fluid. LDH in BAL was measured using the Cytotoxicity Detection Kit^{PLUS} (Roche Molecular Biochemicals, Basel, Switzerland) and was expressed in arbitrary units (AUs).

Histological evaluation. The right lungs were fixed with OCT and 4% paraformaldehyde, embedded in paraffin, sliced longitudinally in 5-µm slices and stained with haematoxylin and eosin. All slides were digitalized using Hamamatsu NanoZoomer HT Digital slide scanner (Hamatsu Photonics, K.K., Japan) and visualized by uploading to an image analysis programme (Slidepath, Leica Biosystems) for morphometric determination. Pulmonary perivascular oedema was quantified by the ratio of perivascular oedema thickness to the inner diameter of the vessel. Twenty symmetrically cross-sectioned vessels per slide were independently evaluated by 2 investigators who were blinded for the experimental groups.

Statistical analysis

All the data are presented as mean ± standard deviation. Data analysis was performed by the Stata 14.2 (StataCorp LLC, TX, USA). The Kolmogorov–Smirnov test was performed for testing normality of the distribution. For repeated physiological measurements during EVLP, data were analysed using multilevel mixed-effects linear regression to assess for the effects of time and treatment group. In case of significant time–group interaction, further pairwise comparisons were done to assess differences between treatment groups at selected time points using the Sidak's test. In the absence of significant time–group interaction, we evaluated differences between groups by computing the overall effects collapsed over time, expressed as the difference in means (95% confidence interval) and using marginal testing with Delta-method adjustment. The unpaired *t*-test was used to compare the lung weight gain between the CONT and SEVO groups. For all the other comparisons, 1-way analysis of variance (ANOVA) followed by the Tukey's correction was used. A *P*-value <0.05 was considered statistically significant.

Sample size was chosen on the basis of previous studies [15, 16].

RESULTS

Physiological data during *ex vivo* lung perfusion

Physiological data obtained during EVLP in the CONT and SEVO groups are presented in Fig. 2.

Throughout EVLP, SPC increased and was higher in the SEVO group when compared with the CONT group (Fig. 2A). At 90 min, SPC was 0.70 ± 0.21 ml·cmH$_2$O^{-1} in the SEVO group vs 0.41 ± 0.08 ml·cmH$_2$O^{-1} in the CONT group (*P* = 0.015); at 120 min, 0.70 ± 0.19 ml·cmH$_2$O^{-1} in the SEVO group vs 0.44 ± 0.09 ml·cmH$_2$O^{-1} in the CONT group (*P* = 0.001); at 150 min, 00.69 ± 0.2 ml·cmH$_2$O^{-1} in the SEVO group vs 0.45 ± 0.09 ml·cmH$_2$O^{-1} in the CONT group (*P* < 0.001) and at 180 min, 0.69 ± 0.22 in the SEVO group vs 0.45 ± 0.09 in the CONT group (*P* < 0.001). The sevoflurane treatment overall effect collapsed over time was +0.215 ml·cmH$_2$O^{-1} (95% confidence interval 0.040–0.389; *P* = 0.003).
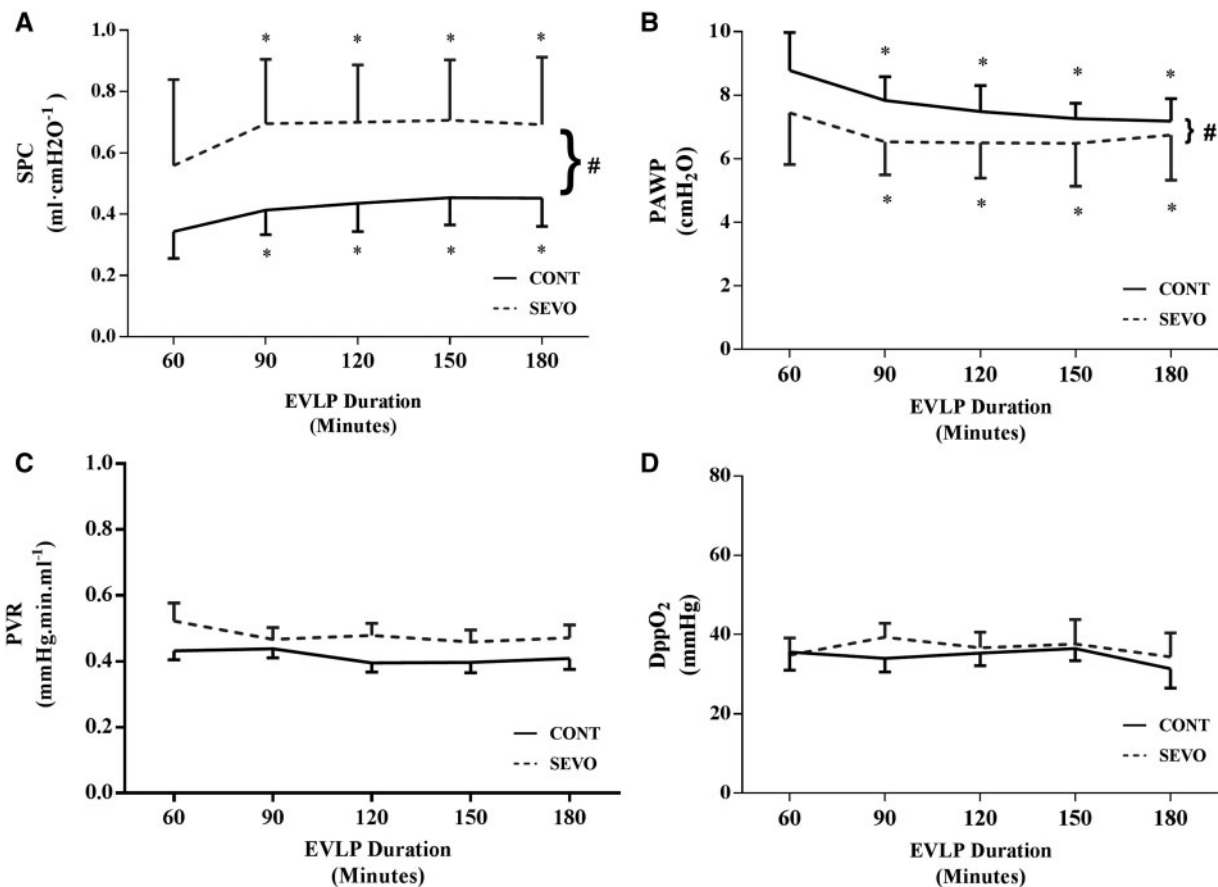
Figure 2: Pulmonary physiological variables during EVLP. (**A**) SPC, (**B**) PAWP, (**C**) PVR and (**D**) DppO$_2$. Data are presented as mean ± standard deviation. *P-value <0.05 between SEVO and CONT at the same time points. #P-value <0.05 overall effects collapsed over time. CONT: control group; DppO$_2$: oxygenation capacity; EVLP: *ex vivo* lung perfusion; PAWP: peak airway pressure; PVR: pulmonary vascular resistances; SEVO: sevoflurane group; SPC: static pulmonary compliance.

At all time points, PAWP was lower in the SEVO group than that in the CONT group (Fig. 2B). At 90 min, PAWP was 6.53 ± 1.04 cmH$_2$O in the SEVO group vs 7.83 ± 0.75 cmH$_2$O in CONT group (P = 0.014); at 120 min, 6.50 ± 1.11 cmH$_2$O in the SEVO group vs 7.48 ± 0.82 cmH$_2$O in the CONT group (P = 0.001); at 150 min, 6.48 ± 1.3 cmH$_2$O in the SEVO group vs 7.27 ± 0.48 cmH$_2$O in the CONT group (P < 0.001) and at 180 min, 6.75 ± 1.43 in the SEVO group vs 7.18 ± 0.71 cmH$_2$O in the CONT group (P < 0.001). The sevoflurane treatment overall effect collapsed over time was −1.33 cmH$_2$O (95% confidence interval -2.47 to -0.18; P = 0.04).

Finally, no significant changes regarding PVR (Fig. 2C) and DppO$_2$ (Fig. 2D) were observed.

Biochemical data

Lung nitro-oxidative stress and LDH release in bronchoalveolar lavage during *ex vivo* lung perfusion. The protein carbonyl content of lungs (Fig. 3A) in the CONT group (1.17 ± 0.44 nmol·mg^{-1}) was significantly higher when compared with both the BASELINE and the SEVO groups (0.48 ± 0.18 nmol·mg^{-1}, P = 0.02 and 0.55 ± 0.11 nmol·mg^{-1}, P = 0.006, respectively). No significant difference between the BASELINE and SEVO groups (P = 0.94) was observed.

The 3-nitrotyrosine content of lungs (Fig. 3B) was higher in the CONT group (197.44 ± 18.47 pg·mg^{-1}) when compared with both

BASELINE and SEVO groups (47.99 ± 23.75 pg·mg^{-1}, P < 0.0001 and 151.05 ± 23.54 pg·mg^{-1}, P = 0.004, respectively). Also, the difference in 3-nitrotyrosine between BASELINE and SEVO groups was highly significant (P < 0.0001).

BAL LDH content (Fig. 3C) in the CONT group was 8.82 ± 3.58 AU and significantly higher than that in the BASELINE group (0.33 ± 0.13 AU, P = 0.005) and the SEVO group (3.80 ± 3.02 AU, P = 0.035). LDH in the SEVO group was comparable with that in the BASELINE group (P = 0.2733).

Expression of cytokines during *ex vivo lung perfusion*. We measured TNF-α (Fig. 4A), IL-6 (Fig. 4B) and CINC-1 (Fig. 4C) as markers of inflammation in the pulmonary parenchyma. In the BASELINE group, the levels were very low (TNF-α 0.07 ± 0.02 ng·mg^{-1}; IL-6 0.04 ± 0.02 ng·mg^{-1} and CINC-1 0.08 ± 0.03 ng·mg^{-1}). In the CONT group, all the cytokines were increased with respect to the BASELINE group (TNF-α 1.50 ± 0.59, P = 0.003; IL-6 2.63 ± 1.06, P = 0.002 and CINC-1 1.17 ± 0.32, P = 0.0003).

In the SEVO group, only IL-6 (1.58 ± 029 ng·mg^{-1}) was increased when compared with the BASELINE group (P = 0.002). No difference was observed for TNF-α (0.59 ± 0.38 ng·mg^{-1}; P = 0.3) and CINC-1 (0.66 ± 0.28 ng·mg^{-1}; P = 0.066). The increase in cytokines in the CONT group when compared with the SEVO group was statistically significant for TNF-α (P = 0.0185) and CINC-1 (P = 0.028) but not for IL-6 (P = 0.11).
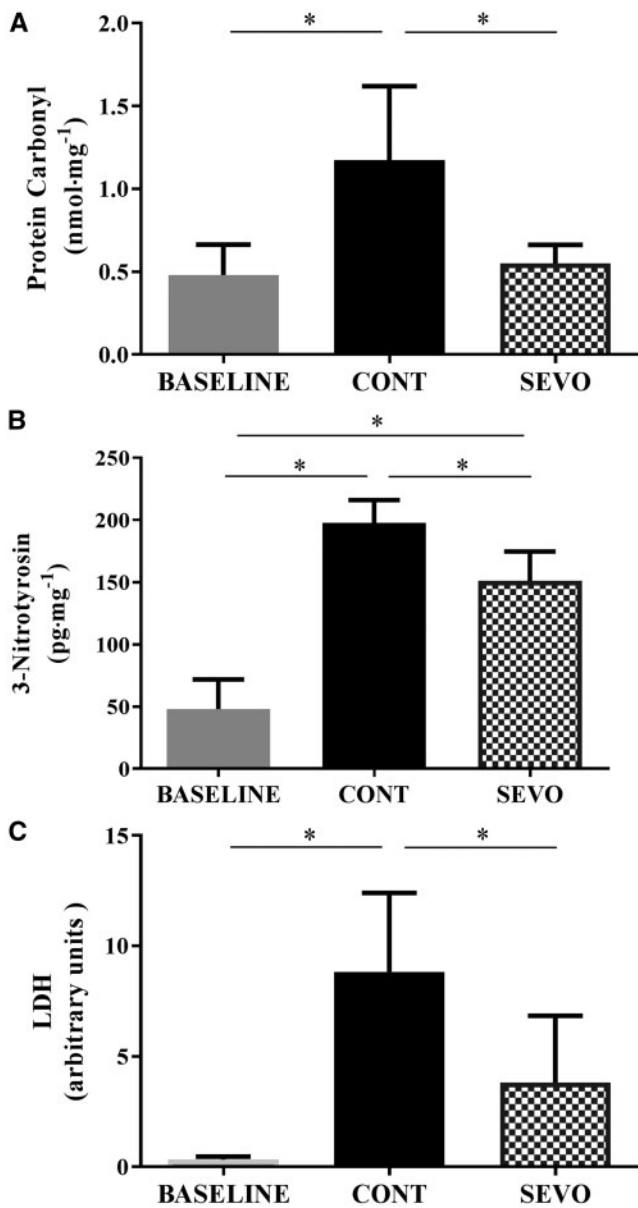
Figure 3: Lung nitro-oxidative stress and LDH release in bronchoalveolar lavage. (**A**) Tissue protein carbonyl content. (**B**) Tissue 3-nitrotyrosin content. (**C**) LDH bronchoalveolar lavage content. Data are presented as mean ± standard deviation. *P-value <0.05. BASELINE: baseline group; CONT: control group; LDH: lactate dehydrogenase; SEVO: sevoflurane group.

Figure 4: Expression of cytokines after *ex vivo* lung perfusion. (**A**) TNF-α, (**B**) IL-6 and (**C**) CINC-1. Data are presented as mean ± standard deviation. *P-value <0.05. BASELINE: baseline group; CINC-1: cytokine-induced neutrophil chemo-attractant factor 1; CONT: control group; IL-6: interleukin-6; SEVO: sevoflurane group; TNF-α: tumour necrosis factor alpha.

Pulmonary oedema during* ex vivo *lung perfusion. The protein content in BAL was measured as a marker of alveolar epithelial lesions. As illustrated in Fig. 5G, it was highly pronounced in both CONT (4.41 ± 0.90 mg·ml^{-1}) and SEVO (3.61 ± 0.30 mg·ml^{-1}) groups when compared with the BASELINE group (0.76 ± 0.15 mg·ml^{-1}; $P = 0.001$ and $P = 0.01$, respectively). However, weight gain due to pulmonary oedema (Fig. 5H) was significantly lower in the SEVO group (0.52 ± 0.06 g) than that in the CONT group (0.72 ± 0.09 g; $P = 0.044$).

Histopathological findings. Perivascular oedema was present in the CONT and SEVO groups (Fig. 5C–F) but not in the BASELINE group, which showed normal pulmonary macrostructure (Fig. 5A, B). It was strongly increased in the CONT group
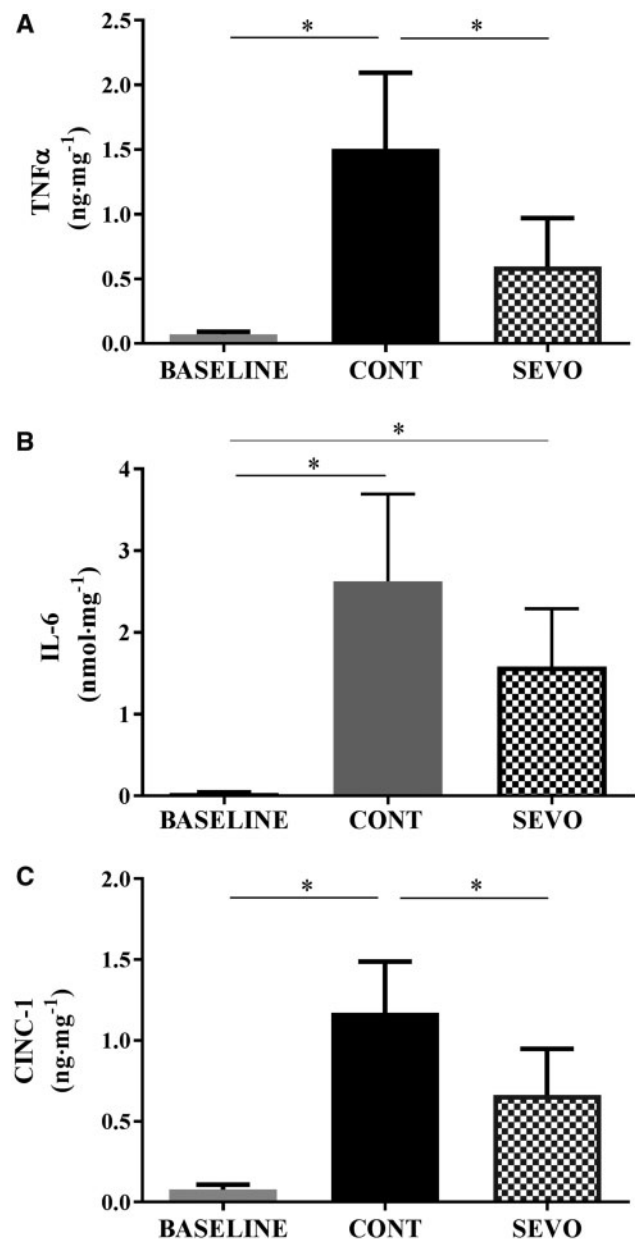
when compared with the BASELINE group (0.58 ± 0.09 vs 0.05 ± 0.04; $P \leq 0.001$) (Fig. 5I). Perivascular oedema was significantly less important in sevoflurane-treated lungs when compared with the control (0.58 ± 0.09 vs 0.47 ± 0.07, $P = 0.036$).

DISCUSSION

EVLP, first developed as a tool for the evaluation and preservation of marginal donor lungs, has been studied in recent years as a modality to treat injuries of donor lungs such as oedema, inflammation, embolism or atelectasis. It opens a platform for the administration of various agents such as anti-inflammatory
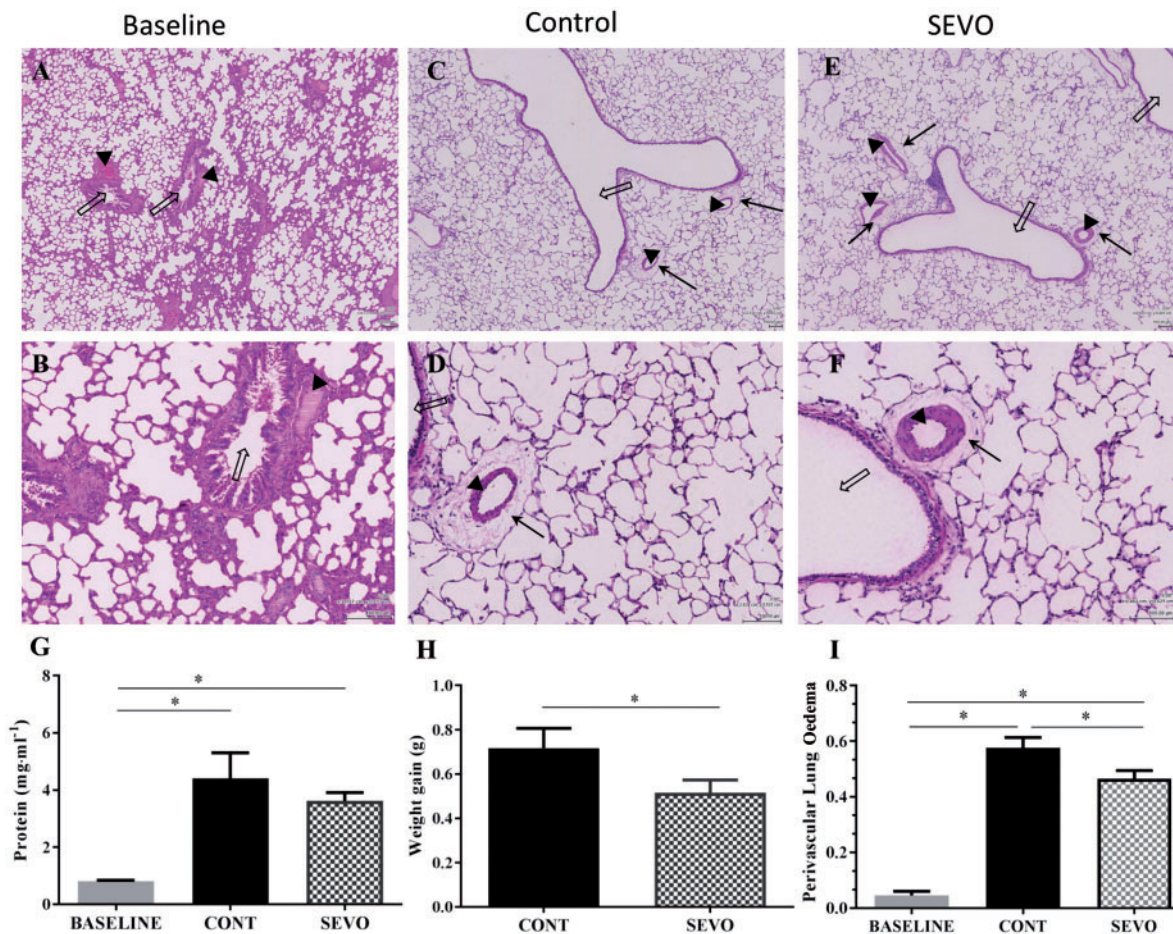
EXPERIMENTAL

Figure 5: Pulmonary oedema after ex vivo lung perfusion and histopathological changes. Histopathological sections (HE staining, magnification ×4 in upper and ×10 in lower pictures). (**A**, **B**) The BASELINE group, (**C**, **D**) CONT group and (**E**, **F**) SEVO group. Black arrows indicate perivascular oedema, arrowheads indicate vessels and open arrows indicate bronchial structures. (**G**) Protein content in bronchoalveolar lavage. (**H**) Weight gain. (**I**) Quantification of perivascular lung oedema in each group. Data are presented as mean ± standard deviation. *P-value <0.05. BASELINE: baseline; CONT: control; HE: haematoxylin and eosin; SEVO: sevoflurane.

drugs [14], antioxidants [17], vasodilators [18], bronchodilators [19] and fibrinolytics [20]. Our study is the first evaluating sevoflurane treatment during EVLP of damaged DCD lungs.

Sevoflurane is a volatile anaesthetic widely used for induction and maintenance of general anaesthesia. Sevoflurane has been reported to have protective effects against IR injury in solid organs such as the heart, liver and brain. Its use is associated with a decrease in reactive oxygen species, an inhibition of cell death cascades and reduced neutrophil/platelet adhesion to the endothelial wall. In the lung, immune modulation may also represent an important aspect of sevoflurane effects, as indicated by reduced production of TNF-α and Nitric oxide by sevoflurane preconditioning [11, 12]. Such modulation of inflammation is, however, controversial. In intact porcine lung tissue, sevoflurane reduced lung TNF-α and interleukin-1β gene expression [21], yet it increased the levels of various inflammatory mediators (such as leukotrienes and nitrogen oxides) in BAL.

In our rodent DCD model, sevoflurane was administered intravascularly early during EVLP to target the cellular signalling cascades involved in IR. A major consequence of IR injury is the development of pulmonary oedema. EVLP lungs in the SEVO group showed a reduced weight gain and PAWP, and improved SPC. As increased endothelial permeability is one of the principal mechanisms in the development of pulmonary oedema [22], this

implies that sevoflurane better preserved the functional integrity of the alveolocapillary membrane. This is further supported by the reduction in perivascular oedema. However, we did not notice a significant reduction in the release of proteins in BAL in our model.

We measured an important reduction in oxidative stress in the SEVO group, as indicated by the lack of increase in protein carbonyls. Although we did not explore the underlying mechanisms, they may be comparable with those reported in the heart, including the inhibition of extracellular signal-regulated kinase, glycogen synthase kinase-3b [23] and nitric oxide synthase [24]. Indeed, with respect to the latter, we found sevoflurane to be associated with a significant reduction in 3-nitrotyrosine release during EVLP, a sensitive marker of NO-derived peroxynitrite generation and nitro-oxidative stress [25]. Oxidative stress is a major mechanism of reperfusion injury, and the reduction may explain the significant attenuation of LDH release, a sensitive marker of cellular injury. Also, sevoflurane inhibits several cellular pathways implicated in necrotic cell death, such as the opening of the mitochondrial permeability transition pore, which leads to the release of mitochondrial proteases into the cytoplasm. The resulting rupture of the cell membrane promotes the release of cytoplasmic proteins within the extracellular milieu, including LDH [26].

It is well established that innate immune activation and expression of inflammatory cytokines upon reperfusion is triggered by the release of various 'danger signals' by necrotic cells. Accordingly, the significant cytoprotective effects of sevoflurane noted in this study mitigated the activation of inflammatory cascades, as shown by decreased expression of inflammatory cytokines. These anti-inflammatory effects may be highly relevant in the setting of lung reperfusion and transplantation. TNF-α promotes the sequestration of neutrophils within the lungs [27], which are key actors in the development of lung injury during reperfusion. CINC-1, a CXC chemokine acting as the rodent homolog of human IL-8 [28], plays a critical role in lung inflammation by orchestrating the accumulation of activated neutrophils. The significant reduction observed in CINC-1 supports the fact that sevoflurane may silence a major mechanism of lung neutrophils recruitment. Of note, neutrophils recruitment could not be investigated in this study due to the acellular fluid used for *ex vivo* perfusion. Finally, sevoflurane also tended to reduce the expression of IL-6, another important mediator of inflammatory injury during ischaemia and reperfusion [29].

No difference in $DppO_2$ was observed between the 2 experimental groups. As hypoxemia is related to intrapulmonary shunts caused by pulmonary oedema, the lack of an effect of sevoflurane seems contradictory. It may be explained by the fact that we used an FiO_2 of 0.21 throughout the procedure, where in clinical EVLP protocols, $DppO_2$ is generally evaluated using an FiO2 of 1.0 [4]. In addition, the use of $DppO_2$ as a parameter for the evaluation of lung function during acellular EVLP has been challenged. Yeung *et al.* [30] observed that the effect of shunt on PO_2 during EVLP could only be evidenced following the addition of red blood cells to the perfusate but not in the presence of an acellular perfusate, the likely reason being the linear relationship between oxygen content and PO_2 in acellular fluids.

PVR was not statistically different between both experimental groups throughout EVLP, a result which agrees with our previous work [15]. A likely explanation may be the heparinization of the heart–lung block before harvesting. It has been shown that thrombotic vascular obstruction is a major cause of increased PVR during EVLP [22].

Limitations

There are several limitations in this study. Sevoflurane was only administered during the first 30 min of EVLP, which may have limited its protective effects against IR injury. During this period of time, the perfusate was progressively rewarmed and flow progressively increased, whereas the lungs were not ventilated. Under such conditions, regional variations in lung perfusion cannot be ruled out, with possible inhomogeneous distribution of the perfusate, and hence of the treatment. Extending the duration of sevoflurane administration should be explored in future studies to address this issue. Also, in our model, static pulmonary compliance was measured by delivering a fixed level of positive pressure. This strategy may have induced some lung over-distention, particularly in lungs with well-preserved compliance. The possible resulting volotrauma could have blunted some of the beneficial effects of sevoflurane. Determination of compliance using preset volumes instead of pressures should be evaluated in future experiments. Further, in this study, *P*-value <0.05 was considered statistically significant despite multiple hypothesis testing. Thus some results may well be false positives. Another

limitation of our study is that we did not include a group of normal lungs subjected to EVLP to assess the intrinsic effects of EVLP in the absence of WI. However, we previously reported that in such conditions, no significant lung damage, oedema or physiological deterioration occurred [15]. Finally, although our data indicate that EVLP with sevoflurane significantly improved the status of lungs explanted after WI, it remains to be established whether this strategy adequately reconditions the damaged lungs for subsequent transplantation. Future *in vivo* studies will be necessary to answer this central clinical issue.

CONCLUSION

In conclusion, our study indicates that 2% sevoflurane, administered intravascularly as a post-conditioning strategy during EVLP, is associated with reduced oxidative stress, attenuated inflammatory response and tissue damage, as well as improved pulmonary physiological parameters in DCD rat lungs obtained after a period of WI. These findings argue in favour of the concept of pharmacological reconditioning using EVLP as a technique to rehabilitate damaged lungs for subsequent transplantation and support the use of sevoflurane in such indication.

Funding

Conflict of interest: None of the authors have any conflicts of interest or financial ties to disclose.

The experimental model and the method have been partially published as a thesis ('Experimental Ex-Vivo Lung Perfusion for Reconditioning of Lung Grafts') by Xingyu Wang in 2016 at the University of Lausanne and is available at https://serval.unil.ch/resource/serval:BIB_65F2BE7EA248.P001/REF.pdf.

REFERENCES

[1] Yeung JC, Cypel M, Waddell TK, van Raemdonck D, Keshavjee S. Update on donor assessment, resuscitation, and acceptance criteria, including novel techniques–non-heart-beating donor lung retrieval and ex vivo donor lung perfusion. Thorac Surg Clin 2009;19:261–74.

[2] Van Raemdonck D, Neyrinck A, Verleden GM, Dupont L, Coosemans W, Decaluwe H. Lung donor selection and management. Proc Am Thorac Soc 2009;6:28–38.

[3] Van Raemdonck DE, Rega FR, Neyrinck AP, Jannis N, Verleden GM, Lerut TE. Non-heart-beating donors. Semin Thorac Cardiovasc Surg 2004;16:309–21.

[4] Cypel M, Yeung JC, Liu M, Anraku M, Chen F, Karolak W *et al*. Normothermic ex vivo lung perfusion in clinical lung transplantation. N Engl J Med 2011;364:1431–40.

[5] Ingemansson R, Eyjolfsson A, Mared L, Pierre L, Algotsson L, Ekmehag B *et al*. Clinical transplantation of initially rejected donor lungs after reconditioning ex vivo. Ann Thorac Surg 2009;87:255–60.

[6] Botha P, Trivedi D, Weir CJ, Searl CP, Corris PA, Dark JH *et al*. Extended donor criteria in lung transplantation: impact on organ allocation. J Thorac Cardiovasc Surg 2006;131:1154–60.

[7] Bharat A, Kuo E, Steward N, Aloush A, Hachem R, Trulock EP et al. Immunological link between primary graft dysfunction and chronic lung allograft rejection. Ann Thorac Surg 2008;86:189–95; discussion 96–7.

[8] Suzuki Y, Cantu E, Christie JD. Primary graft dysfunction. Semin Respir Crit Care Med 2013;34:305–19.

[9] Cypel M, Keshavjee S. Extending the donor pool: rehabilitation of poor organs. Thorac Surg Clin 2015;25:27–33.

[10] Erturk E. Ischemia-reperfusion injury and volatile anesthetics. Biomed Res Int 2014;2014:526301.

[11] Liu R, Ishibe Y, Ueda M. Isoflurane-sevoflurane administration before ischemia attenuates ischemia-reperfusion-induced injury in isolated rat lungs. Anesthesiology 2000;92:833–40.

[12] Casanova J, Garutti I, Simon C, Giraldez A, Martin B, Gonzalez G et al. The effects of anesthetic preconditioning with sevoflurane in an experimental lung autotransplant model in pigs. Anesth Analg 2011;113:742–8.

[13] Ohsumi A, Marseu K, Slinger P, McRae K, Kim H, Guan Z et al. Sevoflurane attenuates ischemia-reperfusion injury in a rat lung transplantation model. Ann Thorac Surg 2017;103:1578–86.

[14] Meers CM, Wauters S, Verbeken E, Scheers H, Vanaudenaerde B, Verleden GM et al. Preemptive therapy with steroids but not macrolides improves gas exchange in caustic-injured donor lungs. J Surg Res 2011; 170:e141–8.

[15] Wang X, Wang Y, Parapanov R, Abdelnour E, Gronchi F, Perentes JY et al. Pharmacological reconditioning of marginal donor rat lungs using inhibitors of peroxynitrite and poly (ADP-ribose) polymerase during ex vivo lung perfusion. Transplantation 2016;100:1465–73.

[16] Kakishita T, Oto T, Hori S, Miyoshi K, Otani S, Yamamoto S et al. Suppression of inflammatory cytokines during ex vivo lung perfusion with an adsorbent membrane. Ann Thorac Surg 2010;89:1773–9.

[17] Rega FR, Wuyts WA, Vanaudenaerde BM, Jannis NC, Neyrinck AP, Verleden GM et al. Nebulized n-acetyl cysteine protects the pulmonary graft inside the non-heart-beating donor. J Heart Lung Transplant 2005; 24:1369–77.

[18] Nakajima D, Chen F, Yamada T, Sakamoto J, Ohsumi A, Bando T et al. Reconditioning of lungs donated after circulatory death with normothermic ex vivo lung perfusion. J Heart Lung Transplant 2012;31:187–93.

[19] Valenza F, Rosso L, Coppola S, Froio S, Colombo J, Dossi R et al. Beta-adrenergic agonist infusion during extracorporeal lung perfusion: effects on glucose concentration in the perfusion fluid and on lung function. J Heart Lung Transplant 2012;31:524–30.

[20] Inci I, Zhai W, Arni S, Inci D, Hillinger S, Lardinois D et al. Fibrinolytic treatment improves the quality of lungs retrieved from non-heart-beating donors. J Heart Lung Transplant 2007;26:1054–60.

[21] Takala RS, Soukka H, Salo MS, Kirvela O, Kaapa P, Aantaa R. Gene expression of pulmonary cytokines after sevoflurane or thiopentone anaesthesia in pigs. Acta Anaesthesiol Scand 2006;50:163–7.

[22] Sanchez PG, Bittle GJ, Williams K, Pasrija C, Xu K, Wei X et al. Ex vivo lung evaluation of prearrest heparinization in donation after cardiac death. Ann Surg 2013;257:534–41.

[23] Pagel PS, Krolikowski JG, Neff DA, Weihrauch D, Bienengraeber M, Kersten JR et al. Inhibition of glycogen synthase kinase enhances isoflurane-induced protection against myocardial infarction during early reperfusion in vivo. Anesth Analg 2006;102:1348–54.

[24] Krolikowski JG, Weihrauch D, Bienengraeber M, Kersten JR, Warltier DC, Pagel PS. Role of Erk1/2, p70s6K, and eNOS in isoflurane-induced cardioprotection during early reperfusion in vivo. Can J Anesth 2006;53:174–82.

[25] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424.

[26] Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods 2008;44:205–21.

[27] Tassiopoulos AK, Hakim TS, Finck CM, Pedoto A, Hodell MG, Landas SK et al. Neutrophil sequestration in the lung following acute aortic occlusion starts during ischaemia and can be attenuated by tumour necrosis factor and nitric oxide blockade. Eur J Vasc Endovasc Surg 1998;16:36–42.

[28] Takaishi K, Ohtsuka T, Tsuneyoshi S, Maehara N, Harada M, Yoshida H et al. Inhibition of the production of rat cytokine-induced neutrophil chemoattractant (CINC)-1, a member of the interleukin-8 family, by adenovirus-mediated overexpression of IkappaBalpha. J Biochem 2000; 127:511–16.

[29] Tan J, Liu D, Lv X, Wang L, Zhao C, Che Y et al. MAPK mediates inflammatory response and cell death in rat pulmonary microvascular endothelial cells in an ischemia-reperfusion model of lung transplantation. J Heart Lung Transplant 2013;32:823–31.

[30] Yeung JC, Cypel M, Machuca TN, Koike T, Cook DJ, Bonato R et al. Physiologic assessment of the ex vivo donor lung for transplantation. J Heart Lung Transplant 2012;31:1120–6.