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Angiotensin-converting enzyme 2 activation attenuates inflammation and oxidative stress in brain death donor followed by rat lung transplantation

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Brain death (BD) provides most of the donor organs destined for lung transplantation (LTx). However, the organs may be affected by inflammatory and oxidative processes. Based on this, we hypothesize that the angiotensin-converting enzyme 2 (ACE2) activation can reduce the lung injury associated with LTx. 3 h after BD induction, rats were injected with saline (*BD group*) or an ACE2 activator (*ACE2a group*; 15 mg/kg^{-1}) and kept on mechanical ventilation for additional 3 h. A third group included a control ventilation (*Control group*) prior to transplant. After BD protocol, left LTx were performed, followed by 2 h-reperfusion. ACE2 activation was associated with better oxygenation after BD management ($p = 0.01$), attenuating edema ($p = 0.05$) followed by the reduction in tissue resistance ($p = 0.01$) and increase of respiratory compliance ($p = 0.02$). Nrf2 expression was also upregulated in the ACE2a group ($p = 0.03$). After transplantation, ACE2a group showed lower levels of TNF-α ($p = 0.02$), IL-6 ($p = 0.001$), IL-1β ($p = 0.01$), ROS ($p = 0.004$) and MDA ($p = 0.002$), in addition to higher CAT activity ($p = 0.04$). In conclusion, our study suggests that ACE2 activation improves anti-inflammatory and antioxidant activity in a model of LTx.

Keywords Brain death, Donor management, Lung transplantation, Angiotensin-converting enzyme 2, Ischemia–reperfusion injury

Brain death (BD) is characterized by the irreversible loss of brain and brainstem function[1]. Despite many accompanying deleterious physiological processes, BD is responsible for providing most donor for lung transplantation[2,3].

Recently, we demonstrated that lungs from animals submitted to BD presents an inflammatory profile[4]. In the quest to repair and improve donor graft quality, new pathways, and strategies to manage potential BD donors have received important attention[5,6].

Modulation of the renin-angiotensin system (RAS) aiming to approach the different pathways involved in the establishment and progression of ischemia–reperfusion injury (IRI) have been suggested[7–9]. One of the key targets within this context is the angiotensin converting-enzyme 2 (ACE2). The conversion of angiotensin II (Ang II) into angiotensin-(1–7) [Ang-(1–7)] promoted by ACE2 activity counterbalances the deleterious effects of the classical RAS pathway and has been widely suggested as a lung protective strategy, given its therapeutic effect in several preclinical models of acute lung injury (ALI)[10–13].

Here, we hypothesized that the management of donors through ACE2 activation could counterbalance the deleterious effects associated with BD events.

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Results

Brain death-induced lung injury results in the deterioration of donor lung function

In order to evaluate the impact of the ACE2 activation during the management of the BD donors, a rodent BD sudden onset model was developed. 3 h after BD induction, the animals were randomized to ACE2 activation or not, and the left lung was subsequently transplanted. A third group evaluating control conditions was included. The experimental design is shown in Fig. 1. Donor animal weight was similar amongst the groups (354 ± 1 g; $p = 0.97$). To perform the injury, a catheter was inserted and inflated into the intracranial cavity, promoting an abrupt increase in intracranial pressure (ICP) followed by sudden transient increase in MAP (Time 0—BD: 169 ± 3.7; ACE2a: 170 ± 9.1 vs. control: 110 ± 0.5 mmHg; $^{*}p = 0.0004$; Fig. 2A). After BD establishment, the donor was stabilized and maintained at mechanical ventilation for a period of 6 h, with assessment being performed immediately before lung extraction. After balloon inflation, the abrupt increase in blood pressure levels was followed by a rapid decay of MAP (Time 0:30—BD: 62 ± 5; ACE2a: 60 ± 4; control: 101 ± 2 mmHg). Hypotension remained for approximately 150 min, when MAP levels returned to values close to normotension (Time 2:30—BD: 88 ± 10; ACE2a: 93 ± 13; control: 100 ± 7 mmHg). Despite the decrease in MAP levels after treatment, no differences were found when comparing donor average pressure values over the BD protocol (BD: 74 ± 7; ACE2a: 68 ± 9; vs. control: 100 ± 4.5 mmHg; $p = 0.13$; Fig. 2A). The donor P/F ratio in healthy subjects was 427 ± 21 mmHg, which dropped to 290 ± 28 mmHg after BD induction ($p = 0.001$). However, the BD donors managed through ACE2 activation showed significantly better oxygenation (ACE2a: 499 ± 69 mmHg) compared to BD group ($p = 0.01$; Fig. 2B).

ACE2 activation during the brain death donor's management improves graft quality

BD has been correlated with deleterious changes of the pulmonary parenchyma followed by mechanical and lung function compromise. Therefore, we evaluated 2 different mechanical parameters at the end of BD protocol. When compared to all other groups, ACE2a showed higher compliance of the respiratory system (control: 0.64 ± 0.01; BD: 0.61 ± 0.01; ACE2a: 0.76 ± 0.05 mL/cmH_2O; $p = 0.02$) and lower tissue damping (control: 0.40 ± 0.02; BD: 0.41 ± 0.01; ACE2a: 0.25 ± 0.03 cmH_2O/s/mL; $p = 0.01$; Fig. 3A).

To confirm the effectiveness of the intervention, we evaluated ACE2 activity in lung tissue. As expected, administration of diminazene aceturate, a pharmacological tool commonly used to activate ACE2, enhanced the ACE2 activity in the ACE2a group compared to the control (6.2-fold) and BD group (9.2-fold) (control: 0.1 ± 0.01, BD: 0.07 ± 0.01, ACE2a: 0.6 ± 0.14 pmol/min/mg; p = 0.0003; Fig. 3B). Besides this, W/D ratio analysis revealed that ACE2a group presented lower edema compared with the BD group (ACE2a: 1.7 ± 0.05 vs BD: 2 ± 0.07; $p = 0.05$; Fig. 3C). Histological analysis of donor lung tissue revealed that lungs under BD conditions showed significantly higher signs of acute lung injury compared with the control group (control: 2.2 ± 0.1; BD: 3.2 ± 0.3;

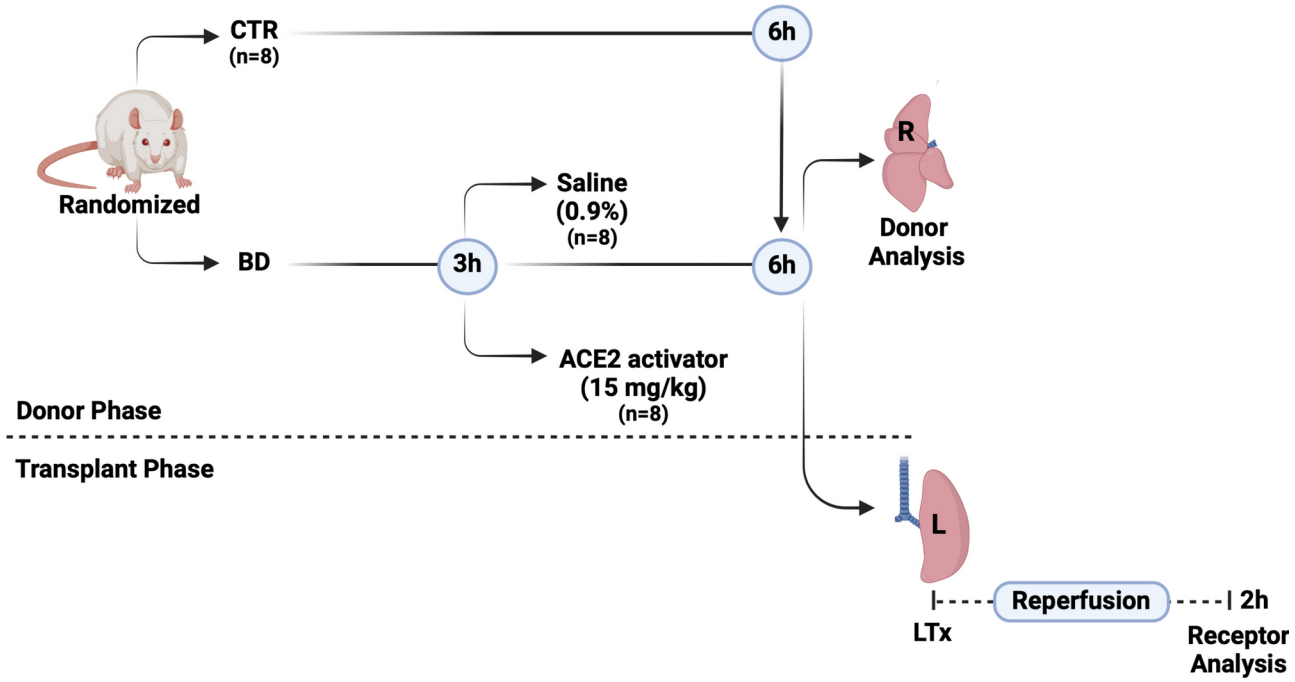


Fig. 1. Graphic schema of experimental design. (***Donor Phase***) BD was established model through an intracranial balloon inflation using a reproducible method. 3 h after BD induction, animals were randomized into 2 different groups. Rats were injected i.p. with 0.9% saline (*BD group;* 0.1 mL/100 g) or a single dose of ACE2 activator diminazene aceturate (*ACE2a group*; 15 mg/kg^{-1}) and kept on mechanical ventilation for additional 3 h. A third group included a control ventilation (CTR—*Control group*) prior to transplant. (***Transplant phase***) After each BD protocol, left lung transplants were performed, followed by 2 h reperfusion.

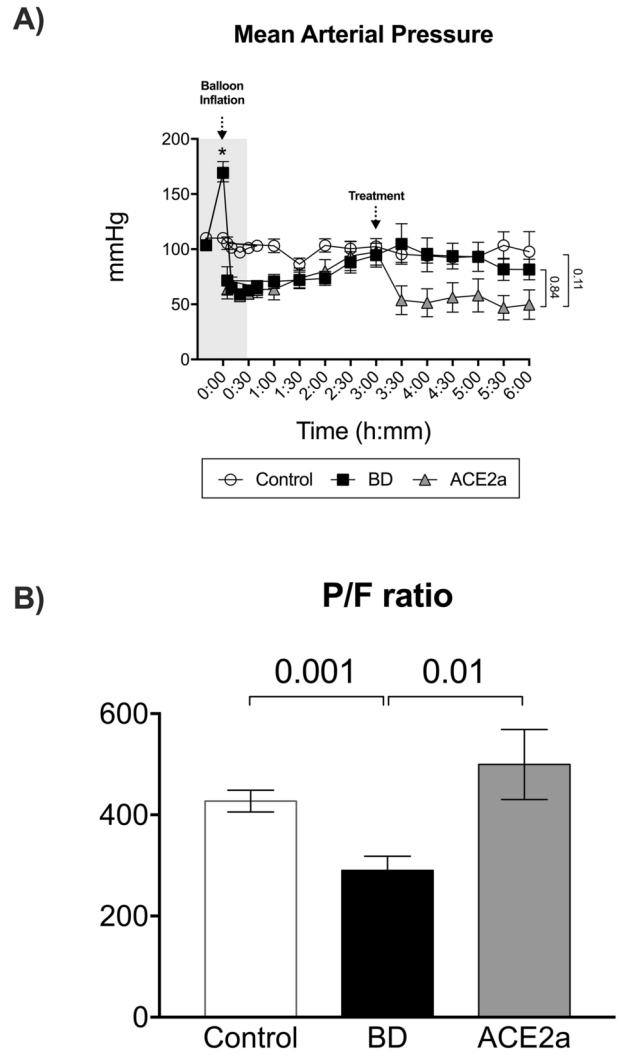


Fig. 2. Brain death-induced lung injury results in the deterioration of donor lung function. **(A)** Mean arterial pressure (MAP) recorded at different time points (baseline to 6 h) in rats submitted to brain death (BD). * means the significant difference between Control and BD groups during hypertensive response to balloon inflation (Time 0—Gray area). Two-way ANOVA testing was performed followed by Student–Newman–Keuls method of multiple comparisons. Brackets on the right represents the p-value comparing the overall average pressure between the groups measured through one-way ANOVA. **(B)** P/F ratio (as an oxygenation index). The values for all of the measurements are expressed as the means ± SEM (n = 7–8 per group). One-way ANOVA testing was performed followed by Tukey method of multiple comparisons.

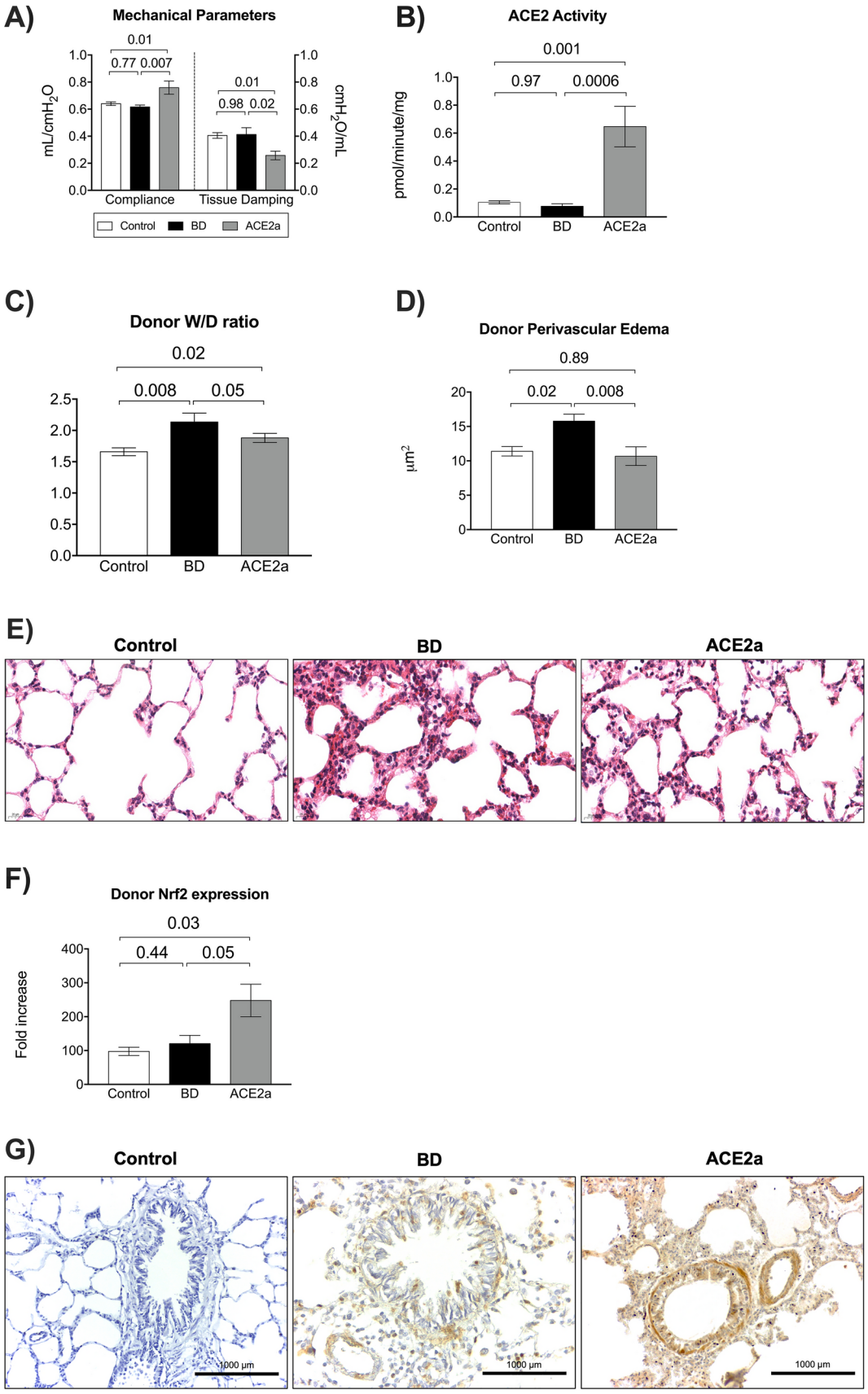
ACE2a: 3 ± 0.2; $p = 0.008$; Table 1). Despite not reducing the total injury score and corroborating with W/D ratio findings, ACE2a group showed reduced alveolar hemorrhage (Table 1) and lung perivascular edema (Control: 11.4 ± 0.7; BD: 15.7 ± 1; ACE2a: 10.7 ± 1.3 μm^2; p = 0.006) (Fig. 3D). A representative image of histology lung sections is shown in Fig. 3E.

Finally, we evaluated the Nrf2 expression since their key role to induces the antioxidants response elements (ARE) which are able to protect against ROS. Analysis of lung tissue revealed that Nrf2 expression was upregulated in the ACE2a group compared to the others (control: 97 ± 12; BD: 120 ± 24; ACE2a: 248 ± 48 fold increase; $p = 0.03$; Fig. 3F,G).

Previous ACE2 activation during BD management did not translate to better early post-transplant graft function

After animals were allocated to a dedicated donor management condition, left lung transplant was performed into a recipient animal. Baseline characteristics of recipient animals and operative factors were similar amongst the 3 groups (Table 2).

Following implantation, the recipient animal was monitored for 2 h of graft reperfusion. This time point is critical for the assessment of IRI[14]. At the end of reperfusion period, we did not observe differences neither the P/F ratio through selective PV gases (control: 239 ± 67; BD: 163 ± 51; ACE2a: 206 ± 44; $p = 0.82$) nor in peak inspiratory pressure (PIP) between the groups (control: 27 ± 1; BD: 26 ± 2.6; ACE2a: 28 ± 0.3 cmH_2O; $p = 0.51$).



Previous ACE2 activation during BD management tended to prevent lung edema after reperfusion (BD: 2.5 ± 0.1 vs. ACE2a: 2 ± 0.1; $p = 0.06$; Fig. 4A). No differences in lung injury scoring ($p = 0.95$; *Supplemental Table*) neither in perivascular edema of tissue histology ($p = 0.43$) were seen amongst the groups. A representative image of the histology lung sections and lung grafts post-reperfusion is shown in Fig. 4B and C.

◀**Fig. 3**. ACE2 activation during the brain death donor's management improves graft quality. **(A)** Left, compliance of the respiratory system; Right, tissue damping. **(B)** Angiotensin-converting enzyme 2 (ACE2) activity in the control, BD and ACE2a group. **(C)** Donor wet-to-dry weight ratio. **(D)** Donor perivascular edema. **(E)** Photomicrographs of lung sections stained with hematoxylin and eosin (×40 magnification; scale bar 20 μm) in the control, BD and ACE2a group. **(F,G)** Fold increase in Nuclear factor erythroid 2-related factor 2 (Nrf2) expression (×40 magnification; scale bar 1000 μm). The values for all of the measurements are expressed as the means ± SEM (n = 5—8 per group). One-way ANOVA testing was performed followed by Tukey's multiple comparison or Kruskal–Wallis' test method of multiple comparisons, when appropriate.

Group/Parameters	Control	BD	ACE2a	p-Value
Total injury score	2.2 ± 0.1	3.2 ± 0.3*	3 ± 0.2*	0.008
Alveolar hemorrhage	0.2 ± 0.04	0.8 ± 0.2*	0.55 ± 0.1*#	0.01
Vascular congestion	0.3 ± 0.1	1 ± 0.2	0.8 ± 0.2	0.11
Fibrinous exudates	1 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.15
WBC infiltration	0.9 ± 0.2	0.8 ± 0.1	1.1 ± 0.1	0.50

Table 1. Histologic scoring of lung in brain death animals. WBC, white blood cells infiltration. *Compared to control group; # compared to BD group.

Group/Parameters	Control	BD	ACE2a	p-Value
Weight, g	366 ± 11	365 ± 12	377 ± 9	0.69
Block preparation, min	26 ± 3	29 ± 3	28 ± 3	0.7
Cold ischemic time, min*	72 ± 5	54 ± 3	62 ± 6	0.13
MAP, mmHg	104 ± 5	93 ± 6.5	92 ± 7	0.38

Table 2. Operative factors did not differ significantly between groups. MAP, mean arterial pressure. *Cold ischemic time = block preparation plus implantation time (on ice).

ACE2 activation attenuates proinflammatory responses associated with post-reperfusion injury

To assess the level of inflammation after reperfusion, cytokines in lung tissue were measured. TNF-α levels increased ~ 3.8 folds in the BD group (0.6 ± 0.08 ng/mg ptn) compared to the control group (0.15 ± 0.03 ng/mg ptn; $p = 0.002$). ACE2a group showed ~ 1.88 folds reduction in the levels of TNF-α (0.3 ± 0.05 ng/mg ptn; $p = 0.02$). IL-6 levels were ~ 4.4 folds higher in the BD group (0.7 ± 0.1 ng/mg ptn) compared to the control group (0.2 ± 0.04 ng/mg ptn; $p = 0.002$). ACE2 activation was able to prevent post-transplant upregulation of IL-6 (ACE2a: 0.15 ± 0.03 ng/mg ptn; $p = 0.001$). IL-1β levels were significantly increased (~ 2.8x) when compared to the control group (control: 1.6 ± 0.4 vs. BD: 4.5 ± 0.4 ng/mg ptn; $p = 0.01$). Previous administration of ACE2 activator ameliorated the IL-1β levels (ACE2a: 2.8 ± 0.6 ng/mg ptn; $p = 0.01$) (Fig. 5A).

Reperfusion significantly increased the levels of Ang II in the BD lung tissues (BD: 3.4 ± 0.2 nmol/mg ptn) compared to the control group (Control: 2.3 ± 0.2 nmol/mg ptn). ACE2 activation was able to prevent this increase (ACE2a: 2.6 ± 0.1 nmol/mg ptn) (Fig. 5B; $p = 0.003$).

Previous ACE2 activation during BD management modulates redox imbalance after reperfusion

Since the excessive production of pro-inflammatory cytokines can exacerbate the redox imbalance and oxidative damage, we measured the ROS levels in the lung post-transplant tissue. ROS concentrations were significantly lower in the ACE2a group (control: 4326 ± 588; BD: 5509 ± 762; ACE2a: 2340 ± 352 μg formazan/ptn; $p = 0.004$; Fig. 6A). Besides this, MDA levels, a cytotoxic product of lipid peroxidation, were reduced in the ACE2a group compared to the other groups (control: 545 ± 25; BD: 577 ± 25; ACE2a: 388 ± 31 nmol/mg ptn; $p = 0.002$; Fig. 6B). CAT antioxidant activity was increased in the ACE2a group (19 ± 7.5 U/mg ptn) compared to the BD group (5 ± 0.8 U/mg ptn; $p = 0.04$; Fig. 6C). No differences in SOD activity were seen amongst the groups ($p = 0.39$; Fig. 6D).

Discussion

This is the first study to demonstrate the role of ACE2 activation in modulating BD donors and its anti-inflammatory and antioxidant effects in a rodent lung transplantation model.

Firstly, we established a BD model through an intracranial balloon inflation. BD was confirmed by an abrupt and transient increase in the MAP levels, since the BD sudden onset model converges to a rapid and important hemodynamic change[15,16]. 3 h after BD-induction, a period in which significant signs of inflammation and lung injury are presumably already established[17,18], we administered the diminazene aceturate, a recognized ACE2

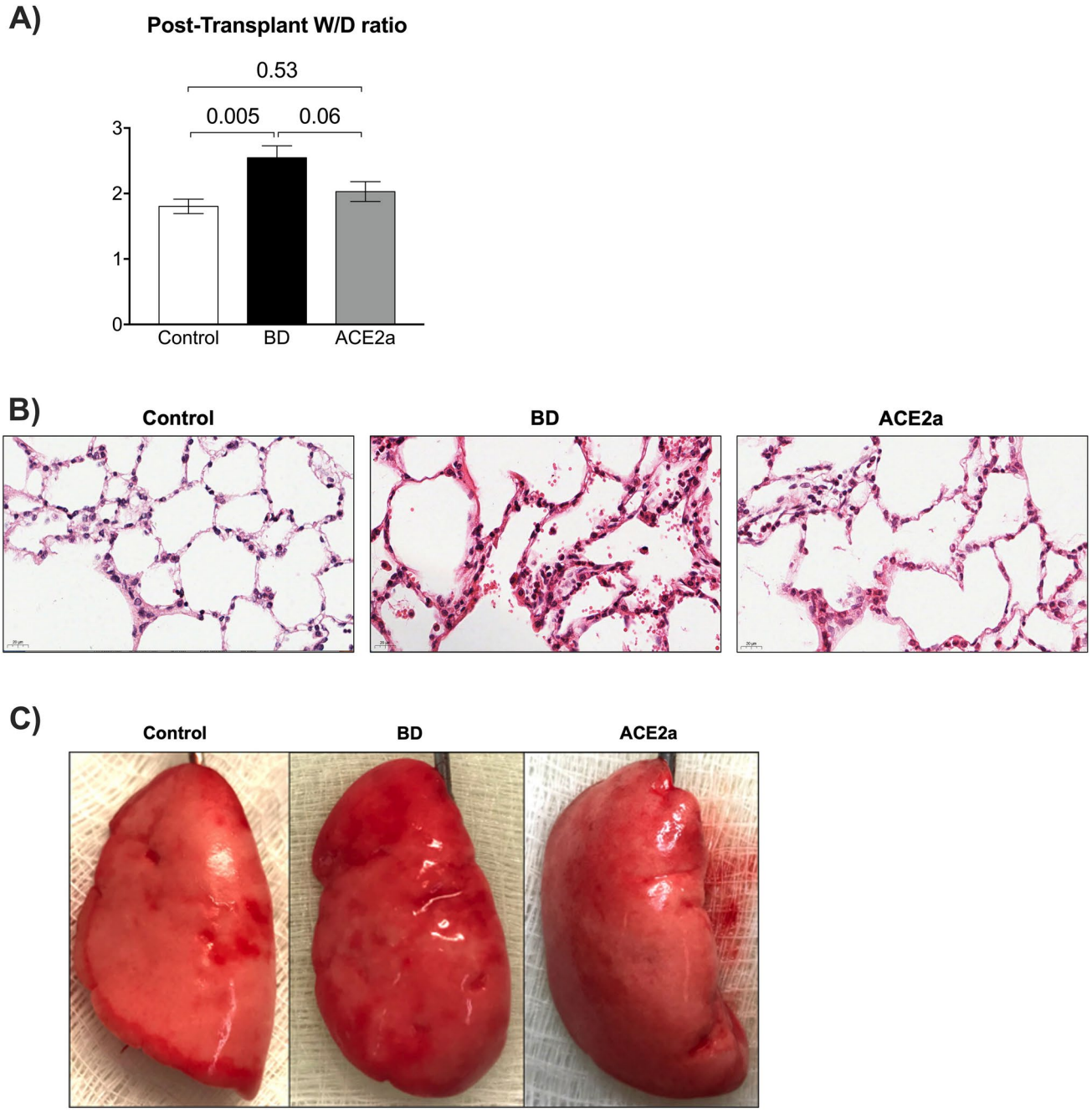
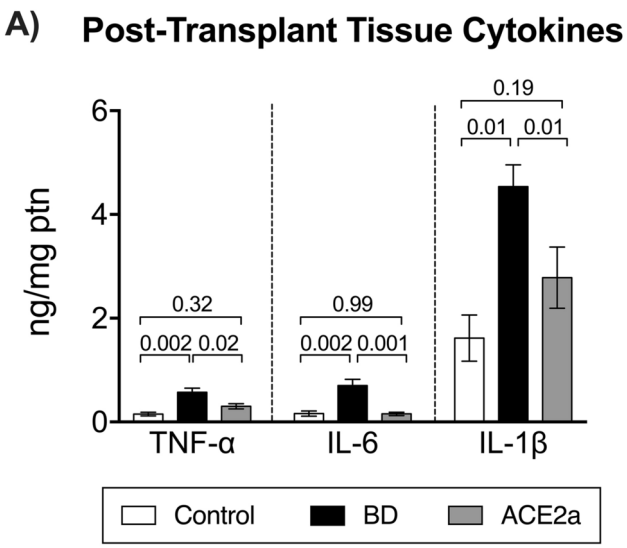


Fig. 4. Previous ACE2 activation during BD management did not translate to better post-transplant graft function. **(A)** Post-transplant wet-to-dry weight ratio. **(B)** Photomicrographs of lung sections stained with hematoxylin and eosin (×40 magnification; scale bar 20 μm) in the control, BD and ACE2a group after reperfusion. **(C)** Macroscopic view of lung grafts after reperfusion. The values for all of the measurements are expressed as the means ± SEM (n = 4–8 per group). One-way ANOVA testing was performed followed by Tukey's multiple comparison or Kruskal–Wallis' test method of multiple comparisons, when appropriate.

activator[10,19–21], and which has demonstrated good results in the treatment/prevention of cardiopulmonary injuries at the same dosage used in the present study[22–25]. Then, we followed up the animals for more 3 h.

It is well documented that the catecholamine storm and systemic vasoconstriction commonly observed in BD may result to increased pulmonary hydrostatic pressure and endothelial damage followed by increased pulmonary capillary permeability and edema[26,27]. In fact, what we observed was an increased histologic signs of lung injury (perivascular edema and alveolar hemorrhage). Despite the low relative W/D ratio values for all groups, the overall pulmonary edema after BD protocol was reduced after ACE2 activation. Previous studies also have demonstrated different values for this edema index[4,28,29], and our findings are consistent with previously



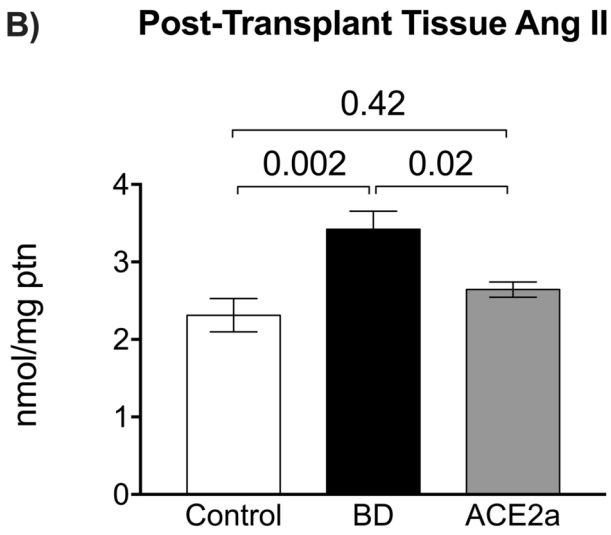


Fig. 5. ACE2 activation attenuates proinflammatory responses associated with post-reperfusion lung injury. **(A)** Left, TNF-α levels; Middle, IL-6 levels; Right, IL-1β levels after reperfusion. **(B)** Post-transplant Tissue Ang II. The values for all of the measurements are expressed as the means ± SEM (n = 4—6 per group). One-way ANOVA testing was performed followed by Tukey's multiple comparison.

published literatures that show the ACE2 protective effects on endothelial adhesion capacity and vascular hemostasis[30,31].

BD changes in the pulmonary parenchyma are usually accompanied by compromised mechanical and lung function[32,33]. However, we did not observe changes in the mechanical parameters evaluated after the BD protocol. This may have been related to the standardized lung volume used to ventilate BD animals, the low variability in rodent lung function compared to clinical situations, requiring chronic lung injury protocols for significant changes in the elastic components and consequently in lung mechanics[33–35]. Surprisingly, when evaluating tissue damping, a parameter that reflects the tissue resistance and contains a contribution related to the resistance to air flow in the peripheral airways[36], we observed a reduction after ACE2 activation. Moreover, this finding is supported by the offset effects of Ang-(1–7) administration in an airway hyperresponsiveness animal model[37]. These modulations could increase the compliance of the respiratory system after ACE2 activation, resulting in better systemic P/F ratio.

In addition to regulating the physiological processes described above, ACE2 activation promoted nuclear factor erythroid 2-related factor 2 (Nrf2) upregulation. Nrf2 is an important transcription factor for the ARE expression such as antioxidant enzymes, playing a critical role in redox balance[38]. Accordingly, Fang et al. highlight the role of ACE2 activation in the modulation of Nrf2 pathway and the subsequent upregulation of antioxidant enzymes in a hyperoxic lung injury model[12]. Overall, ACE2 activation appears to establish a precondition of the graft for oxidative events that follow reperfusion injury.

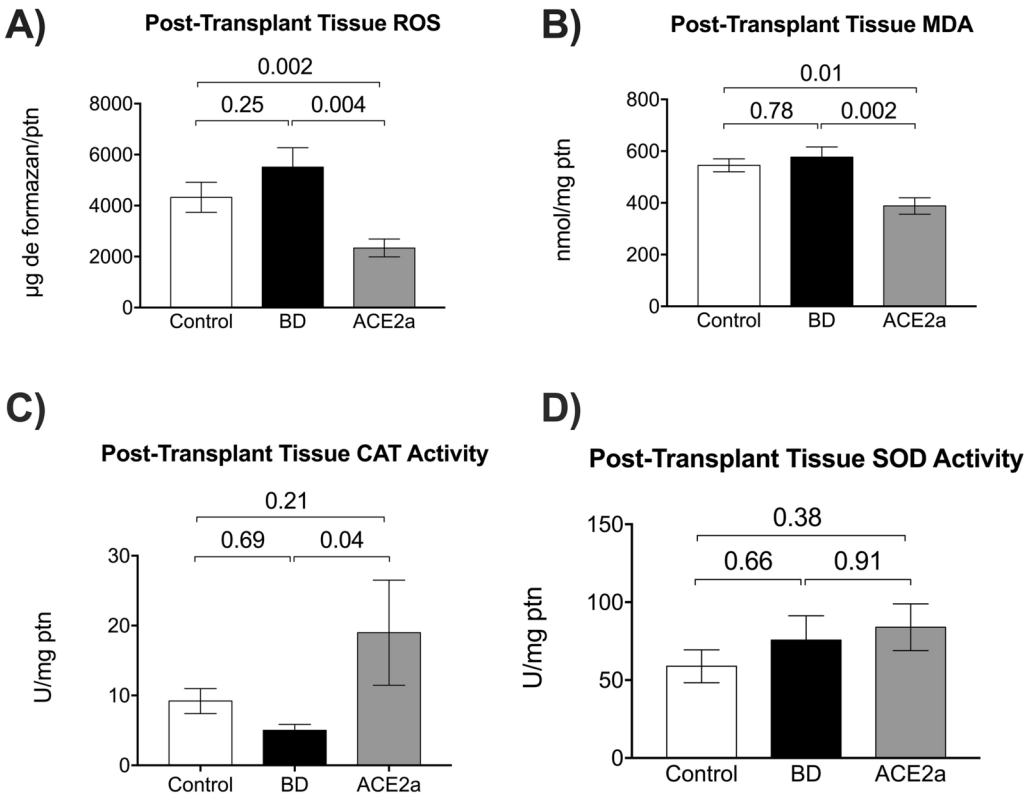


Fig. 6. Previous ACE2 during BD management modulates redox imbalance after reperfusion. **(A)** Post-transplant tissue reactive oxygen species (ROS). **(B)** Post-transplant tissue malondialdehyde (MDA). **(C)** Post-transplant tissue catalase (CAT) activity. **(D)** Post-transplant tissue superoxide dismutase (SOD) activity.

We then proceeded to evaluate these lungs through graft reperfusion, using a syngeneic model of lung transplantation. The reestablishment of perfusion in the lung ischemic graft have been associated with high levels of Ang II, a pro-inflammatory RAS peptide[9,39]. We believe that previous ACE2 activation in the BD donor model was responsible for the modulation in this peptide after lung transplantation, since ACE2 activity is responsible for greater degradation of Ang II[10]. Post-transplant lung tissue also showed an increase in pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, being prevented by previous BD donor management. In this context, previous studies have shown the anti-inflammatory effects achieved by ACE2 activation. In general, these studies demonstrate the participation of ACE2 activators in the inhibition of the NF-kB pathway[7,9,12].

During reperfusion, the inflammatory process is followed by an oxidative response in the pulmonary territory[40]. We assessed redox imbalance by measuring ROS and MDA concentrations, as well as the antioxidant enzymatic activity of SOD and CAT. Post-transplant lung tissue showed a drastic increase in ROS levels, followed by an increase of MDA concentration. The ACE2 activation significantly blocked the increase in ROS, suggesting that this compound has an antioxidant effect and, therefore, reduces the oxidative damage in lung tissue. ACE2 activation proved oxidant/antioxidant balance, promoting an increase in CAT activity and, consequently, leading to a greater conversion of H_2O_2into hydroxyl radical and water. This likely reduced lipid peroxidation resulting in lower levels of MDA. In short, these results corroborate with Nrf2 expression in BD grafts which had ACE2 activated pre-reperfusion, suggesting that ACE2 activation improved the enzymatic antioxidant defense after reestablishment of blood flow in accordance with previous studies[9,12]. Those are very promising results since the ischemia/reperfusion process is often associated with oxidant-antioxidant imbalance and could predispose recipients to primary graft dysfunction after transplantation[41].

Our study has limitations. We focused on acute phases changes after reperfusion as largely disseminated in the literature[42–44]. Despite having demonstrated stable rat lung function during 2 h reperfusion, we did not observe changes in post-transplant lung function with the previous ACE2 management. However, graft function assessed by P/F ratio is a constantly changing variable in the first 24 h after lung transplantation[45]. Furthermore, injury score may not fully capture the inflammatory and oxidative markers improvements observed with ACE2 activation. Among the possible causes for the lack of improvement in lung function and injury score are the short observation time after reperfusion and the acute reaction to graft reperfusion, often making it difficult to read the histological slides, causing a high variability of the rat lung transplant histology. Additionally, despite the use of low tidal volume during the lung transplantation phase we observed a high PIP. The values found are not uncommon in the literature and may be related to the factors mentioned above[46,47].

To study if the anti-inflammatory and antioxidant effects of ACE2 activation would persist for longer and translate into improved lung function and consequently better outcomes, further work must use longer-term

survival models. Finally, future studies should be performed to detect the concentration of RAS components in plasma, donor lungs and in the post-transplant tissue for further mechanistic information.

In conclusion, this study suggests that ACE2 activation improves anti-inflammatory and antioxidant activity post-reperfusion. Therefore, BD donor management through administration of ACE2 activators is a promising pharmacological tool for protection during ischemia and reperfusion events after transplantation.

Methods

Ethics

All procedures were approved by the Ethics Committee for Experimental Animals Use and Care—University of Sao Paulo (protocol number—1630/2021). The protocol was based on ethical principles and was established in accordance with the current standards of the Brazilian College of Animal Experimentation (COBEA). All animals were treated according to Brazilian regulations for the use of animals in scientific research and received care in accordance with international standards of animal care and experimentation in compliance with the ARRIVE guidelines.

Study design

In order to evaluate the potential physiological and biological benefits of ACE2 activation in the management of BD donors, we conducted retrieval and transplant experiments using male Lewis rats. 3 h after BD induction, animals were randomized into 2 different groups. Rats were injected i.p. with 0.9% saline (*BD group;* 0.1 mL/100 g) or a single dose of ACE2 activator diminazene aceturate (*ACE2a group*; Cat#D7770, Sigma-Aldrich; 15 mg/kg^{-1}) and kept on mechanical ventilation for additional 3 h. A third group included a control ventilation (*Control group*) prior to transplant. After each BD protocol, left lung transplants were performed, followed by 2 h reperfusion. Both organ retrieval and transplant procedures followed the methods previously described[42].

Brain death induction and procurement

The BD model used in this study had been previously described[16]. Briefly, the animals were anesthetized with 5% isoflurane. The animals were intubated (14G angiocath) and kept in mechanical ventilation (Harvard Apparatus, model 683; tidal volume, 10 mL/kg; frequency of 60 breaths/min; inspired oxygen fraction (FiO_2) of 0.21; positive end-expiratory pressure (PEEP) of 3 cmH_2O). The femoral vein was cannulated for continuous infusion of saline (0.9%; 2 mL/h), the same being done to the artery for blood pressure monitoring (DIXTAL®, DX 2021, Brazil).

Through a frontolateral hole drilled (Midetronic, Brazil) in the skull, a no. 4 Fogarty catheter (Edwards Lifesciences, USA) was placed in the intracranial cavity. Fast induction of BD was achieved by inflating the balloon with 0.5 mL/min[15,16,48]. BD was confirmed by absence of corneal reflexes, loss of spontaneous breathing with positive apnea testing, and a transient increase in the mean arterial pressure (MAP). After 6 h of BD induction, a median laparosternotomy was performed, 500 UI/mL of heparin was injected into the inferior vena cava (IVC) and the lungs were flushed with 20 mL of low-potassium dextran-glucose solution (Perfadex, XVIVO Perfusion, Sweden). Immediately after flushing, the trachea was clamped at the end of inspiration to preserve the lungs in an inflated state, followed by heart–lung block harvest. The right lung was utilized for analyses related to the donor model and the left lung was prepared just before recipient operation by placing a 16-gauge cuff to the pulmonary artery and vein. The trachea was intubated with a 14-gauge metal cannula for independent ventilation of allograft. Lung grafts were stored on ice until implantation.

Lung transplantation procedure

Lung transplantation was performed through an independent ventilation technique to assess allograft mechanics parameters as previously described[46,49].

Recipients were anesthetized and ventilated in the same fashion as commented above (FiO_2, 1.0). After a left thoracotomy, pneumonectomy was completed by clamping the left main bronchus, pulmonary artery and vein. The tracheal catheter (left allograft) was connected to a second volume-cycled ventilator (tidal volume, 4 mL/kg; frequency of 80 breaths/min; PEEP of 3 cmH_2O; FiO_2 of 1.0) and the lung was reinflated *(Figure S1)*. The transplanted lung was first ventilated, and then gradually reperfused. Recipient rats were ventilated and reperfused for 2 h.

Lung function analysis

The tracheal catheter was connected to a specific ventilator to measure respiratory mechanics (FlexiVent, Scireq, Canada). During transplant follow-up, only the trachea of the left lung was connected to this ventilator for graft specific functional assessments.

The data was collected using specific flexiWare7 software. Compliance was obtained by a single frequency forced oscillation (snapshot), which consists of imposing a volume-driven perturbation that match the animal's tidal volume and breathing frequency. Tissue damping, a parameter that reflects the tissue resistance[36], was assessed applying a quick-prime perturbation, which consists of imposing an air flow with an amplitude corresponding to the sum of sinusoids with frequencies between 1.00 and 20.5 Hz. The parameter exclusion criterion was COD < 0.9.

Sample collection protocol

Blood samples were taken from donors' femoral artery. For the recipients, blood was collected from left pulmonary vein (PV) with a 27-gauge needle directed toward the transplanted lungs after 2 h-reperfusion. Subsequently, tissue samples were taken and separated to be either snap-frozen and stored at − 80°C or formalin-fixed, paraffin-embedded, and sectioned for histological analysis.

Wet-to-dry weight ratio

After the end of each protocol, the lower lobe of the right lung (*BD donors*) and the lower part of the left lung (*recipients*) were harvested and then weighed for wet weight. The tissue was dried (80°C/48 h) and weighed for dry weight. The wet weight was divided by the dry one to obtain the wet-to-dry (W/D) weight ratio.

Biochemical analyses

Tissue lysates were used for biochemical assays. We measured reactive oxygen species (ROS)[50], Superoxide dismutase (SOD) activity[51]and Catalase (CAT) activity[52]. We used the thiobarbituric acid reactive substances method to analyze malondialdehyde (MDA) products at 532 nm[53]. IL-6, IL-1β, TNF-α (Sigma-Aldrich, St Louis, USA) and Ang II (BTLAB, Shangai, China) were measured using an ELISA kit following the manufacturer's instructions. ACE2 activity assay was carried out by means of a commercial kit (Sigma-Aldrich, St Louis, USA), according to the manufacturer's instructions.

Tissue analysis of acute lung injury scores and antioxidant markers

The medium lobe of the right lung (*BD donors*) and the respective part of the left lung (*recipients*) were fixed for 48 h before being embedded in paraffin. Sagittal 4-µm serial sections of the lungs were stained with hematoxylin and eosin for histological analyses. The degree of lung injury was determined using a lung injury score and by the evaluation of the perivascular edema area. The main criteria used to score the lung injury were: white blood cell infiltration, edema, alveolar hemorrhage and capillary congestion. The severity was scored as 0, absent; 1, mild; 2, moderate; and 3, severe. The combined score of all 4 parameters was taken for each animal, as previously described[54]. We calculated the perivascular edema area/vessel area ratio to normalize variations due to vessel size as previously described[55].

In order to assess nuclear factor erythroid 2-related factor 2 (Nrf2) expression, immunohistochemistry was performed on the paraffin Sect. (4-µm) of tissue samples. In short, all the sections were paraffin-embedded, deparaffinized and rehydrated. Sections were incubated with anti-Nrf2 primary antibody (1:100) 4°C overnight. Immunohistochemical staining was performed by means of HRP-labled secondary antibody (Spring) and the streptavidin–biotin complex method. DAB color developing reagent (Spring) was used as a chromogen. Hematoxylin was performed for the counterstain.

The result of IHC was analyzed by means of Image Pro-Plus 6.0 software. The fold increase of Nrf2 expression in different groups to Control was calculated following the formula: Fold increase in Group X (%) = Color value in Group X/Color value in Control*100%.

Statistics

GraphPad Prism 7 software was used for the statistical analysis (San Diego, CA, USA). Quantitative results were presented as mean ± standard error of means (SEM). One-way ANOVA was applied for comparison between the groups, followed by Tukey, Student–Newman–Keuls or Kruskal–Wallis test method of multiple comparisons, when appropriate. $p < 0.05$ was considered statistically significant.

Conference presentation

The 44th Annual Meeting and Scientific Sessions of the International Society for Heart and Lung Transplantation, Prague, Czech Republic, Apr 10-13, 2024.

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Received: 2 May 2024; Accepted: 1 October 2024
Published online: 09 October 2024

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Acknowledgements
This study was supported by a grant from CAPES (Grant number: 88887.524323/2020-00), CNPq (Grant number: 200171/2022-4) and FAPESP (Grant number: 2022/07610-8). Biorender – Confirmation of publication and licensing rights (Agreement number: MA25XGER1R).

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Funding
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Grant no. 88887.524323/2020-00); Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant no. 200171/2022-4); Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant no. 2022/07610-8).

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
MC is shareholder of Traferox Technologies Inc and consultant for Lung Bioengineering.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-75043-7.

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