Received: 19 February 2020 | Revised: 24 June 2020 | Accepted: 28 June 2020

DOI: 10.1111/ajt.16187

BRIEF COMMUNICATION

AJT

Organ Care System Lung resulted in lower apoptosis and iNOS expression in donor lungs

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Funding information
Regione del Veneto

Ischemia-reperfusion (IR) injury after lung transplantation is still today an important complication in up to 25% of patients. The Organ Care System (OCS) Lung, an advanced normothermic ex vivo lung perfusion system, was found to be effective in reducing primary graft dysfunction compared to standard organ care (SOC) but studies on tissue/molecular pathways that could explain these more effective clinical results are lacking. This observational longitudinal study aimed to investigate IR injury in 68 tissue specimens collected before and after reperfusion from 17 OCS and 17 SOC preserved donor lungs. Several tissue analyses including apoptosis evaluation and inducible nitric oxide synthase (iNOS) expression (by immunohistochemistry and real-time reverse transcriptase-polymerase chain reaction) were performed. Lower iNOS expression and apoptotic index were distinctive of OCS preserved tissues at pre- and post-reperfusion times, independently from potential confounding factors. Moreover, OCS recipients had lower acute cellular rejection at the first 6-month follow-up. In conclusion, IR injury, in terms of apoptosis and iNOS expression, was less frequent in OCS- than in SOC-preserved lungs, which could eventually explain a better clinical outcome. Further studies are needed to validate our data and determine the role of iNOS expression as a predictive biomarker of the complex IR injury mechanism.

KEYWORDS

cell death: apoptosis, clinical research/practice, ischemia-reperfusion injury (IRI), lung transplantation: living donor, organ perfusion and preservation, pathology/histopathology

Abbreviations: ACR, Acute cellular rejection; AI, apoptotic index; BAL, bronchoalveolar lavage; CT, cycle threshold; DAD, diffuse alveolar damage; ECMO, extracorporeal membrane oxygenation; eNOS, endothelial NOS; EVLP, ex vivo lung perfusion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICU, intensive care unit; iNOS, inducible nitric oxide; IR, ischemia-reperfusion; LB, lymphocytic bronchiolitis; NO, nitric oxide; OCS, Organ Care System; OP, organizing pneumonia; OR, odds ratio; PaO_2/FiO_2, arterial oxygen partial pressure/fraction of inspired oxygen; PGD, primary graft dysfunction; SOC, standard organ care; TBB, transbronchial biopsy; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling.

1 | INTRODUCTION

Lung transplantation is the only curative treatment for several types of end-stage lung disease. Static cold storage, usually ranging between 4 and 8 hours, is the gold standard for donor lung preservation and should be kept as short as possible, as it represents one of the major influencing factors for ischemia-reperfusion (IR) injury (IRI). Indeed, during cold preservation a number of events may occur leading to the activation of several mediators that are ultimately deleterious to the preserved organ. Among IRI key mediators, nitric oxide (NO) metabolites are thought to play a crucial role in posttransplant pulmonary vascular dysfunction and alveolar epithelial injury.[1-3]

Over the past decades, several strategies have been introduced into clinical practice to optimize lung preservation. Normothermic ex vivo lung perfusion (EVLP) has been shown to be an important step forward in clinical lung transplantation.[4] Several clinical and experimental studies have shown that EVLP provides an opportunity for good organ preservation (less IR tissue injury) and reassessment before transplantation.[5-8] The portable Organ Care System (OCS) Lung is an advanced EVLP system, which allows the explanted lungs to be preserved in the OCS at the donor hospital, thus minimizing the cold ischemic time, and thereby enabling the assessment and improving the condition of standard and potential donor organs.[9] The prospective randomized phase 3 INSPIRE International Lung Trial with the Organ Care System Technology (OCS) has demonstrated the safety and efficacy of OCS in terms of reduced incidence of primary graft dysfunction (PGD) grade 3 within 72 hours after lung transplantation compared with cold storage.[10]

Currently, no reliable investigations exist that analyze the underlying biological pathways of OCS-preserved lungs associated with a better clinical outcome after lung transplantation.

Considering that preservation of lungs in OCS solution is more similar to physiological conditions (mainly concerning body temperature), the rationale of the study was that this method could have an impact on the donor lung tissue condition. Thus the main goal of this research, which includes a subset of the phase 3 INSPIRE trial population, was to assess the difference in ischemic tissue damage in cases of OCS vs standard organ care (SOC) preservation, before the lung transplantation surgical procedure. The secondary aim was to evaluate if any possible histological difference would have been detected also after reperfusion. Only as an exploratory goal, we aimed to compare follow-up of patients who received OCS-or SOC-preserved lungs (acute cellular rejection [ACR], infections).

2 | MATERIALS AND METHODS

2.1 | Study design and population

This was an observational longitudinal study including a subset of the phase 3 INSPIRE trial population[10]: all the 54 patients of the INSPIRE trial in Padova and Hannover centers in the 21-month period defined by the Veneto Region grant (Prot. 60888, November 2012) were recruited for the study. However, the tissue evaluation of IRI was available in only 34 subjects (20 were excluded as they did not have lung biopsies) (Figure 1, Table 1-2). The study protocol included tissue sampling at 2 time points: (1) after OCS or SOC preservation and immediately before transplantation (pre-reperfusion sample); and (2) after in vivo reperfusion of the first implanted lung (post-reperfusion sample) (Figure 1). The research was done in accordance with the principles of the Helsinki Declaration of 1975 as revised in 1983 and the guidelines for Good Clinical Practice. The institutional ethics committee approved the study and all patients gave written informed consent.

2.2 | Morphological analyses

All fragments were sampled from the middle lobe or lingula, formalin-fixed paraffin-embedded, and analyzed in several ways.

Different morphological parameters (edema, blood extravasation, and leukocyte margination) were quantified and graded with the following scoring system: score 1 = mild, <30% of analyzed tissue; score 2 = moderate, 30%-50% of analyzed tissue; and score 3 = severe, >50% of analyzed tissue. Two pathologists (FC and FP) independently quantified the histological parameters and agreement between them was expressed by using Cohen's Kappa statistic (see supplement Data S1).

2.3 | Immunohistochemistry and TUNEL analysis

Serial sections from the same paraffin-embedded lung specimens were used for inducible nitric oxide synthetase (iNOS) immunohistochemistry and terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay. Immunohistochemistry was performed by using the primary monoclonal antibody anti-iNOS (CLONE SP126; Abcam, Cambridge, UK). At least 5 high power fields were analyzed in each sample, and the mean positive cell number/mm^2 of tissues was reported, distinguishing intra-alveolar (mainly macrophages: CD68 positive) and wall (mainly epithelial cells: MNF116 positive and more rarely endothelial cells: CD31 positive) compartments.

Molecular analysis for apoptosis detection by the TUNEL technique was carried out. At least 300 cells were counted in 3 high power fields. Apoptotic index (AI) was expressed as number of TUNEL-positive cells/total cell number ×100. Quantitative evaluation was performed by computer-assisted morphometric analysis (Image Pro-Plus version 5) (see supplement Data S1).

2.4 | Real-time PCR for iNOS expression

The analysis was performed in a subset of 24 frozen samples to ascertain the occurrence of iNOS expression at the messenger RNA (mRNA) level. Total RNA was extracted from frozen lung tissues,

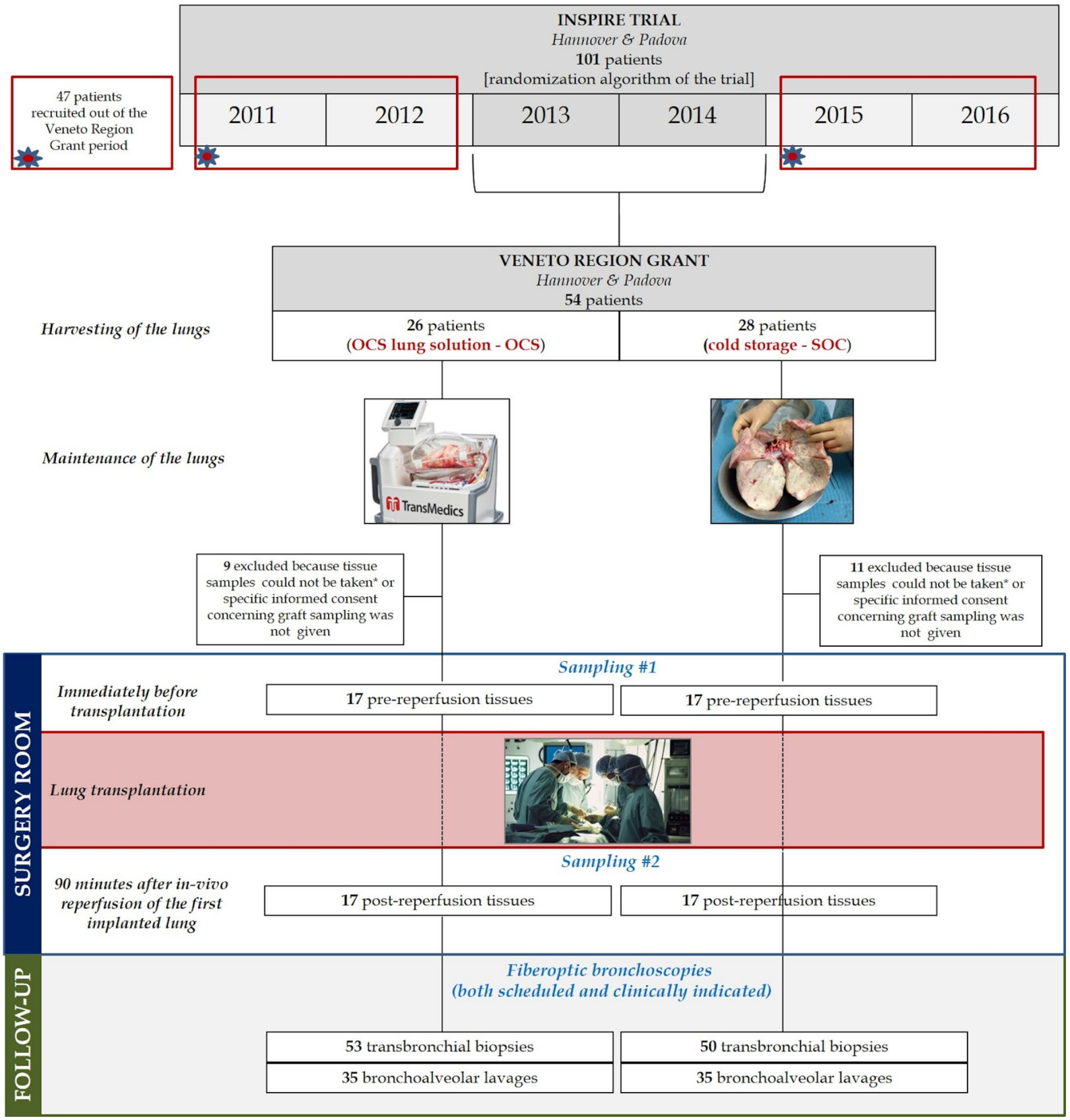

FIGURE 1 Flow chart describing the study population and design

and quantitative determination of mRNA levels of *iNOS* gene was performed in triplicate on a Light Cycler 480 II (Roche Applied Science, Mannheim, Germany) using SYBR Green-based quantification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize target genes. The average cycle threshold (CT) was determined and ΔCT was calculated by normalizing with the housekeeping gene. Different expressions were then evaluated with the ΔΔCT method[11] (see supplement Data S1).

2.5 | Clinical data

Cold ischemia time was reported as median of averages ischemia times of the 2 lungs. The use of preoperative and/or intraoperative extracorporeal membrane oxygenation (ECMO) was also recorded (Table 1). The PGD score, the length of intensive care unit (ICU) stay, and 30-day mortality rates of this subset of patients were collected from the INSPIRE registry[10] (see supplement Data S1).

TABLE 1 Study population

Variables	OCS group (17)	SOC group (17)	*P* values
Recipient			
Age, years, mean (SD)	40.4 (14.2)	43.5 (15.5)	.55
Gender (M: F, %)	7:10 (41:59)	9:8 (53:47)	.49
Weight, kg, mean (SD)	54.4 (13.4)	63.7 (16.2)	.05
Height, cm, mean (SD)	161.6 (11.1)	167.1 (9.8)	.13
Native disease			
CF/bronchiectasis, n (%)	10 (59)	8 (47)	.20
IPF, n (%)	5 (29)	6 (35)	
COPD, n (%)	0 (0)	3 (18)	
Others, n (%)	2 (12)	0 (0)	
PAH, n (%)	9 (53)	6 (35)	.30
PAP, mm Hg, mean (SD)	25.8 (11.8)	21.2 (7.6)	.39
Follow-up, months, mean (SD)	15.0 (12.0)	14.7 (13.2)	.86
Surgical parameters			
ECMO pre/intra, n (%)	11 (65)	4 (24)	**.02**
Inhaled NO, n (%)	6 (35)	5 (29)	.7
Total lung ischemia time, minutes, median, Q_1-Q_3	260.0, 247.0-306.0	315.0, 286.0-355.0	**.04**
OCS running time, minutes, median, Q_1-Q_3	280.0, 177.5-360.5	/	/

Abbreviations: OCS, Organ Care System; SOC, standard organ care; SD, standard deviation; M, male; F, female; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; COPD, chronic obstructive pulmonary disease; LTx, lung transplantation; PAH, pulmonary arterial hypertension; PAP, pulmonary artery pressure; ECMO, extra corporeal membrane oxygenation; NO, nitric oxide; Q_1, first quartile; Q_3, third quartile.

2.6 | Follow-up data

During a 6-month follow-up, scheduled transbronchial biopsies (TBBs) and bronchoalveolar lavages (BALs) (3-4 weeks, 9-12 weeks, and 6 months), together with additional samples when clinically indicated, were collected. In these patients, all TBBs and BALs were obtained during scheduled bronchoscopies, never for clinical suspicion. Histological signs of IRI were recorded and parenchymal ACR or lymphocytic bronchiolitis (LB) were evaluated in all TBBs according to the International Society for Heart and Lung Transplantation grading system.[12]

The ACR index was evaluated in each patient as number of TBB with ACR ≥A2/total TBB number X100. The LB index was also calculated as number of TBB with LB/total TBB number X100. Infections were investigated in BAL samples and indexes were evaluated in each patient as number of BAL with infection/total BAL number X100 (see supplement Data S1).

2.7 | Analysis

Descriptive statistics were used to summarize the characteristics of both groups. For quantitative variables, the normality of distribution was tested by the Shapiro-Wilk test and comparisons were performed by the Student *t* test or Mann-Whitney test. For categorical variables, comparisons were performed by chi-square test or Fisher exact test. The comparison of the lung morphological/molecular parameters between the 2 lung preservation strategies was obtained by applying the nonparametric analysis of variance (ANOVA) for repeated measures (Proc MIXED in SAS). The strength of the association between each dichotomized outcome at follow-up and the preservation technologies (independent variable: 1 = OCS, 0 = SOC) were obtained by simple logistic regression models. Adjusted odds ratios (ORs) were obtained, controlling for ischemic time. Nonparametric Spearman correlation coefficient was computed to explore linearity of association between ischemic time and morphological/molecular features. The relationship between AI and iNOS expression was determined by linear regression analysis. Statistical analyses were performed with the SAS statistical software release 9.3 (SAS Institute) and significance was set at 2-tailed 0.05 (see supplement Data S1).

3 | RESULTS

3.1 | Morphological and molecular findings

Both groups showed similar median scores of histological parameters in pre- and post-reperfusion fragments (Table 3). Interpathologist agreement for all the above-mentioned

TABLE 2 Donor characteristics

Variables	OCS group (17)	SOC group (17)	*P* values
Donor			
Age, years, mean (SD)	40.2 (13.5)	43.9 (10.9)	.39
Gender (M:F, %)	7:10 (41:59)	7:10 (41:59)	1.00
Weight, kg, mean (SD)	65.4 (14.0)	70.2 (11.4)	.27
Height, cm, mean (SD)	169.0 (6.8)	168.8 (7.3)	.92
Cause of death			
Vascular, n (%)	10 (59)	8 (47)	.49
Nonvascular, n (%)	7 (41)	9 (53)	
Final PaO_2/FiO_2 ratio, mean (SD)	427.5 (75.5)	468.9 (70.3)	.14
Eurotransplant donor score			
6, n (%)	9 (52)	5 (29)	.07
7, n (%)	3 (18)	10 (59)	
8, n (%)	3 (18)	2 (12)	
9, n (%)	2 (12)	0 (0)	
OTO donor score (median, Q_1-Q_3)	1.0, 0.0-5.0	2.0, 1.5-3.5	.42

Abbreviations: OCS, Organ Care System; SOC, standard organ care; SD, standard deviation; M, male; F, female; PaO_2/FiO_2, arterial oxygen partial pressure/fraction of inspired oxygen; Q_1, first quartile; Q_3, third quartile.

parameters was very good (Cohen kappa = 0.812). iNOS expression was strongly detected in the cytoplasm of both intra-alveolar (macrophages) and wall (mainly epithelial) cells, whereas it was never detected at the nuclear level. Staining sometimes showed some granular positivity.

In comparing OCS and SOC groups, a significantly lower intra-alveolar iNOS (mainly detected in macrophages) was found in the OCS group, both in pre- and post-reperfusion tissue samples (both $P = .04$; controlling for time $P = .001$; Table 3; Figures 2-3). A significant difference was also observed in the expression of total iNOS, which was lower in the OCS compared to the SOC group before the reperfusion ($P = .05$; controlling for time $P = .02$; Table 3). Wall iNOS (endothelial and pneumocyte related) expression values did not differ significantly in the 2 groups at any time (Table 3) and overall.

TUNEL analyses showed that apoptotic cell death equally involved pneumocytes and endothelial cells. A significantly lower AI was seen in the OCS compared to the SOC group, both in pre- and post-reperfusion fragments ($P = .04$ and $P = .02$, respectively; controlling for time $P = .0003$; Table 3; Figures 2-3).

Edema, blood extravasation, and leukocyte margination scores changed between pre- and post-perfusion time as indicated for time effect ($P = .06$, $P < .0001$ and $P = .003$, respectively), whereas molecular features did not, as shown by the nonsignificant *P* values (Table 3), with the exclusion of AI that showed a marginally significant effect for time ($P = .07$).

A direct relation was observed between iNOS expression (intra-alveolar value) and AI, especially when considering post-reperfusion time (RS = 0.47, $P = .007$) (Figure 2).

No association was found between ischemic time or the use of ECMO support and AI or iNOS expression values, both in pre- and post-reperfusion samples (data not shown). The small number of patients with poorer PGD (grade 2/3) at different times did not allow any reliable statistical correlation between PGD and morphological parameters, even if a higher AI and iNOS expression was seen in recipients with worse PGD grades.

Real-time PCR analysis confirmed iNOS expression at the mRNA level, with ΔCT (iNOS values normalized against GAPDH) ranging from 7.7 to 12.1 (mean value ± SD: 9.7 ± 1.5). In particular, at pre-reperfusion time, mean ΔCT ranged from 7.7 to 11.2 (9.4 ± 1.3), whereas at post-reperfusion time it ranged from 7.7 to 12.1 (10.1 ± 1.6). No significant differences in median ΔCT values were found between SOC and OCS patients; nor were they found at pre-reperfusion time (median, Q1-Q3: 8.6, 7.7-9.5 vs 10.3, 9.8-11) or at post-reperfusion time (median, Q1-Q3: 10.9, 9.8-12 vs 9.1, 7.8-10.6) (Figure S1). However, at the time of pre-reperfusion, the fold-change calculated with the $2^{-\Delta\Delta Ct}$ formula showed a 3-fold higher expression in SOC patients (Figure S1).

3.2 | Follow-up data

A total number of 103 TBBs and 70 BALs were considered (Table 4). In multivariate analysis, we found that the probability to have at least one TBB with ACR ≥2 was significantly influenced only by preservation technology; ischemia time was uninfluential. When considering the ACR index, median values were higher in SOC than OCS groups ($P = .03$, Table 4). The bacterial infection index was also significantly higher in SOC than OCS patients ($P = .03$, Table 4). No other differences were found. (More details are reported in the "Data S1" word file.)

4 | DISCUSSION

This prospective study, including a subset of the phase 3 INSPIRE trial patients, demonstrated for the first time that donor lungs preserved using the OCS device are characterized by significantly lower tissue damage in terms of apoptotic cell death and iNOS expression, both at pre- and post-reperfusion time.

Currently, there is no histological IRI scoring system available to quantify morphological parameters (edema, blood extravasation, and leukocyte margination) during preservation and reperfusion of transplanted lungs. This emphasizes the need for a more standardized method to evaluate traditional histological changes, and overall the importance of using different parameters for more sensitive detection of cellular damage and critically related pathways, such as apoptotic cell damage.

TABLE 3 Lung morphological and molecular findings at pre- and post-perfusion time by type of lung preservation strategy

	Pre-reperfusion			Post-reperfusion			Strategy effect	Time effect	Interaction
Variables	OCS (17)	SOC (17)	P^a	OCS (17)	SOC (17)	P^a	P^b	P^c	P^d
Edema score, n (%)									
0	12 (80)	14 (87)	.79	12 (75)	8 (53)	.57	.48	.06	.14
1	2 (13)	2 (13)		3 (19)	4 (27)				
2	1 (7)	0 (0)		1 (6)	2 (13)				
3	0 (0)	0 (0)		0 (0)	1 (7)				
Congestion score, n (%)									
0	9 (60)	8 (50)	.83	0 (0)	0 (0)	.72	.79	**<.0001**	.63
1	3 (20)	5 (31)		5 (31)	7 (47)				
2	2 (13)	3 (19)		8 (50)	5 (33)				
3	1 (7)	0 (0)		3 (19)	3 (20)				
Leukocyte margination score, n (%)									
0	3 (20)	8 (50)	.35	1 (6)	5 (33)	.21	.06	**.003**	.75
1	10 (66)	6 (38)		6 (37)	3 (20)				
2	1 (7)	1 (6)		3 (19)	4 (27)				
3	1 (7)	1 (6)		6 (38)	3 (20)				
Apoptotic index,[e] median, Q_1-Q_3	1.2, 0.4-1.8	1.6, 0.9-2.5	**.04**	1.4, 0.8-1.9	2.6, 1.3-5.0	**.02**	**.0003**	.07	.61
Intra-Alveolar iNOS,[f] median, Q_1-Q_3	0.4, 0.1-2.8	4.4, 0.9-12.1	**.04**	1.3, 0.2-3.9	7.3, 1.8-16.7	**.04**	**.001**	.39	.92
Wall iNOS,[f] median, Q_1-Q_3	20.3, 11.9-29.1	24.5, 10.7-47.6	.33	20.8, 10.7-30.8	20.8, 12.4-49.1	.92	.43	.97	.60
Total iNOS,[f] median, Q_1-Q_3	21.5, 14.5-30.8	35.3, 19.7-49.9	**.05**	23.3, 16.4-33.2	26.3, 22.6-64.7	.22	**.02**	.51	.61

Abbreviations: OCS, Organ Care System™; SOC, standard organ care; iNOS, inducible nitric oxide synthase; Q_1, first quartile; Q_3, third quartile.

[a]*P* level for the comparison between OCS and SOC, separately at pre- and post-perfusion (time).

[b]*P* level for the comparison between OCS and SOC, controlling for time.

[c]*P* level for the effect of time.

[d]*P* level for the interaction between strategy and time.

[e]Data are expressed as apoptotic cells/total cell number x100.

[f]Data are expressed as positive cell number/mm^2 of tissue.

Several clinical and experimental studies have demonstrated that apoptosis is the principal cell death occurring after IRI.[13] Experimental studies have shown that long periods of cold ischemia (>12 hours) before reperfusion are associated with more extensive tissue injury and cell death, primarily of the necrotic type.[14] Necrosis was never detected in our samples, as the maximum ischemia time was 10 hours; however, we cannot rule out that microscopic necrotic areas could have been present but not detected in our biopsies. For the analyzed time points, in our study, the OCS group showed significantly lower apoptotic cell deaths compared to the SOC group. Different mediators released during IR may cause increased vascular permeability, microvascular injury, and alveolar epithelial cell damage. During IR time, high concentrations of NO are produced, due to the activation of several types of NO synthases, but the role of this molecule is debated in the literature. Some studies in experimental and clinical lung transplantation have shown benefits, [15,16] whereas others have demonstrated detrimental or neutral effects.[17,18] Some authors suggest that NO can be either protective or toxic to lung grafts depending on dose, timing, and duration of exposure, and that NO has a narrow therapeutic window.[19] It is now widely accepted by several authors that NO has a detrimental effect after lung transplantation when released at high concentrations. Combined with reactive oxygen species, it produces peroxynitrite and other reactive nitrogen species[20,21] resulting in severe cellular damage. Two different principal NO synthases are involved in regulating pulmonary vascular function: endothelial NOS (eNOS) and iNOS, principally expressed by inflammatory cells.[22,23] iNOS is calcium independent, and can produce a large, continuous flux of NO that is 1000-fold higher than that of eNOS, lasting for hours to days in response to conditions of stress and inflammation.[24] Several experimental studies have

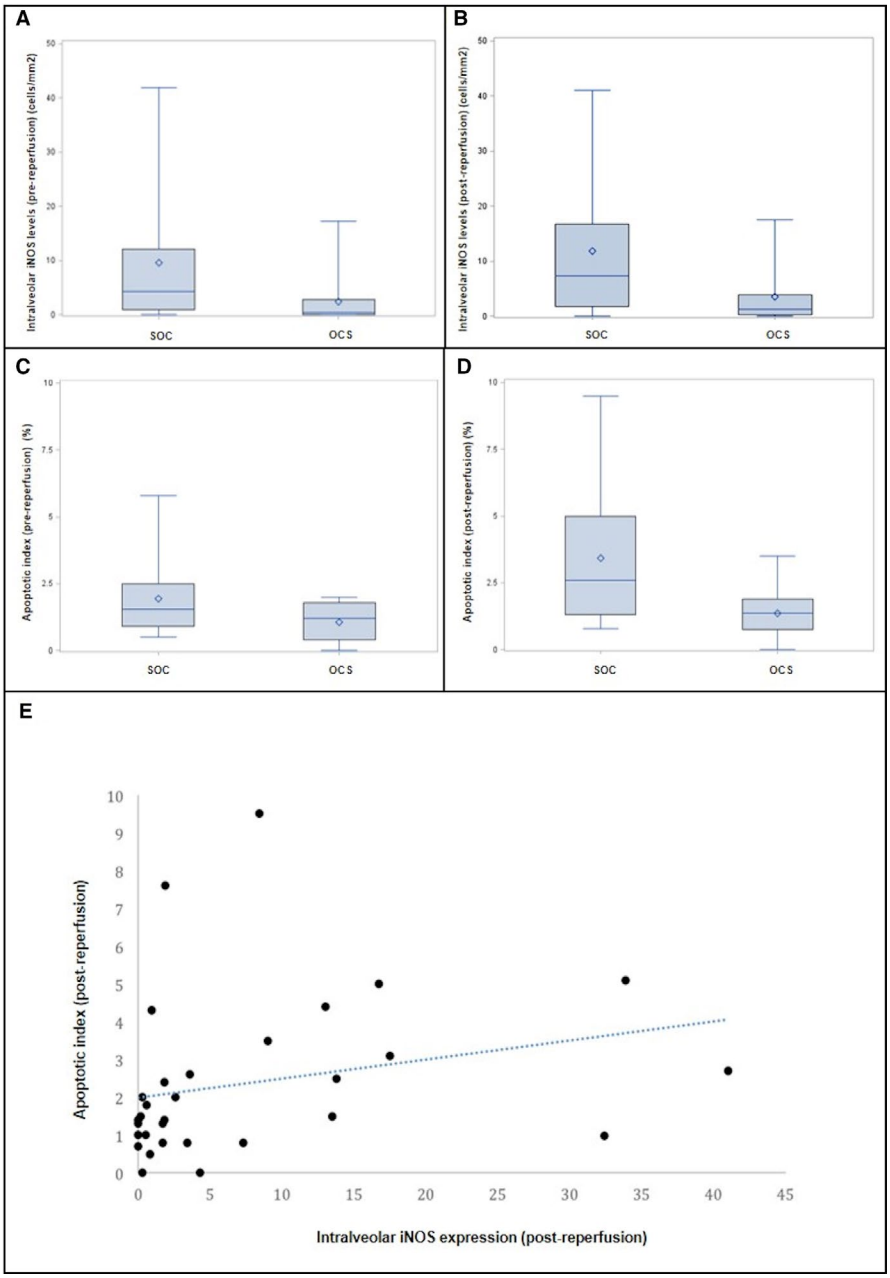

FIGURE 2 Box plots are representative of inducible nitric oxide synthase (iNOS)-positive cells (A, B) and terminal deoxynucleotidyl transferase-mediated dUTP-digoxygenin nick end labeling (TUNEL) positive cells (C, D) from 17 Organ Care System (OCS) and 17 standard organ care (SOC) preserved donor lungs, showing minimum (min), maximum (max), median value, and first (Q1) and third (Q3) quartiles. A statistically significant difference was detected comparing OCS iNOS and TUNEL-positive cells with SOC iNOS and TUNEL-positive cells, both at the time of pre- (both $P = .04$) and post-reperfusion ($P = .04$ and $P = .02$, respectively). iNOS expression (intra-alveolar value) and apoptotic index (AI) (E): A direct relation was observed when considering post-reperfusion time (RS = 0.47, $P = .007$)

demonstrated that inhibition of iNOS with iNOS inhibitors or short interfering RNA lowers the hypoxia-induced rise of NO production, lipid peroxidation, LTB4 levels, caspase-3 activity, and apoptosis.[25,26] In our study, a significantly lower expression of iNOS was detected in the fragments of OCS-preserved lungs, with a mild relation between the expression of iNOS and the AI (mainly at post-reperfusion time), suggesting a possible role of NO in the complex apoptotic cell death induction. Multiple logistic regression model analyses confirmed lower IR tissue injury (particularly in terms of AI and iNOS expression) in the OCS than SOC group. Moreover, the lower variability of these parameters between pre- and post-reperfusion time seems to support the hypothesis of better and more uniform preservation of lung tissue using this technology.

The significantly lower tissue injury in OCS-preserved donor lungs could depend on several factors. The most important factor could have been that the OCS device, by allowing contemporary perfusion and ventilation under physiological conditions and by reducing ischemia time, significantly influenced the release of several toxic mediators and consequently apoptotic cell death. Our statistical analysis computed to explore linearity of association between ischemic time and morphological/molecular features suggests that the lower ischemia time of OCS technology has less influence than normothermic perfusion/ventilation. However, the contribution of lower ischemia time should be studied in depth on larger case series and overall using experimental models. These could be particularly useful to better address the impact of both ischemia time and normothermic

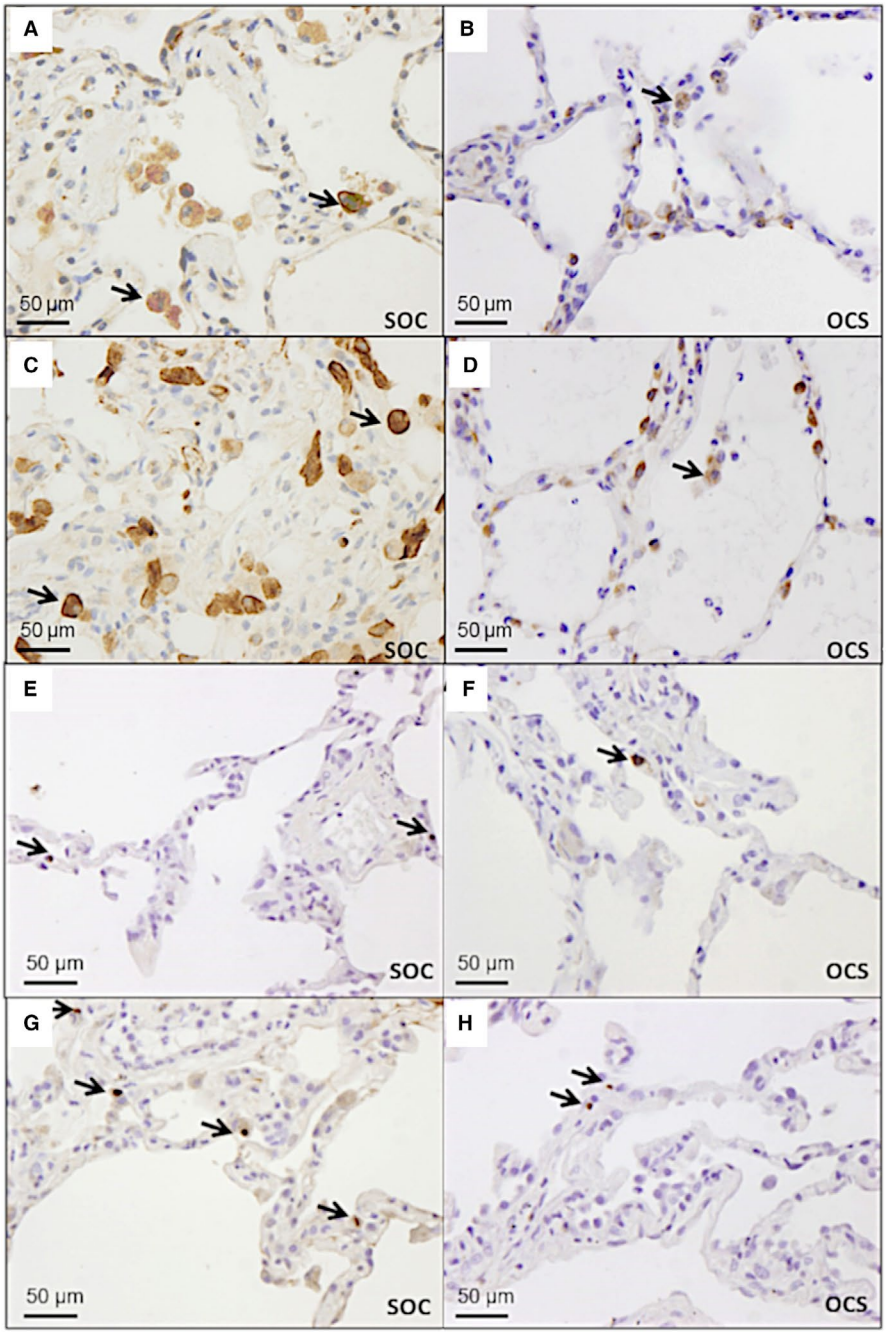

FIGURE 3 Inducible nitric oxide synthase (iNOS) immunohistochemistry in 17 standard organ care (SOC)- and 17 Organ Care System (OCS)-preserved donor lungs; higher iNOS expression seen mainly in intra-alveolar macrophages (arrows) of the SOC compared to the OCS group at pre- and post-reperfusion time (A-D). Scale bar: 50 µm. Terminal deoxynucleotidyl transferase-mediated dUTP-digoxygenin nick end labeling (TUNEL) analysis in SOC and OCS groups; more apoptotic cells (dark nuclei, arrows) are seen in the SOC than OCS group at pre- and post-reperfusion time (E-H). Scale bar: 50 µm

perfusion/ventilation on tissue preservation, individually or in combination.

Another important factor that should be considered is the washout of apoptotic cells carried out by the perfusate flow. Even though the "washout effect" cannot be excluded, its role in passively removing apoptotic cells would be more likely if only endothelial cells were involved. Our TUNEL analysis showed that other cell types different from endothelial cells, such as pneumocytes, undergo apoptotic cell death; thus the mechanistic washout effect of the perfusate flow could be only minimal.

Regarding the follow-up parameters, an interesting finding of our work was the lower ACR in TBB and infection index in BAL detected in OCS recipients, both with and without adjusting the ACR index for ischemic time. At the moment, we do not have any concrete explanation for that. We can hypothesize that a lower ACR index may be related to the anti-inflammatory property of the OCS perfusion ("washout effect") and to less severe tissue IRI (less apoptotic cell death). Several clinical and experimental studies have demonstrated an important influence of IR damage in both early and late immunological disorders, including ACR.[27-29] The same mechanism of "washout effect" with clearance of microorganisms in donor lungs could be due to the large spectrum of antibiotics present in the perfusate, which could explain this additional positive outcome.[30] In conclusion, IRI assessed in this preliminary study in terms of apoptosis and iNOS expression was less frequent in OCS than in SOC preserved lungs, which could possibly explain a better clinical outcome.

TABLE 4 Morphological outcomes during follow-up

Morphological outcomes (follow-up)		OCS group (17)	SOC group (17)	*P* values
TBBs	Number of TBBs (median, Q_1-Q_3)	4.0, 3.0-4.0	4.0, 2.0-4.0	.74
	IR signs (DAD and/or OP) % (n/N)[a]	47% (7/15)	60% (9/15)	.58
	ACR index (A ≥ 2)[b] (median, Q1-Q3)	0 (0-12.5)	25 (0-50)	.03
	LB index[b] (median, Q1-Q3)	0 (0-0)	0 (0-16.5)	.75
BALs	Number of BALs (median, Q_1-Q_3)	3.0, 2.0-3.0	3.0, 1.0-3.0	.88
	Viral infection index[c] (median, Q_1-Q_3)	0 (0-0)	0 (0-33)	.43
	Bacterial infection index[c] (median, Q_1-Q_3)	0 (0-0)	0 (0-33)	.03

Abbreviations: OCS, Organ Care System™; SOC, standard organ care; TBBs, transbronchial biopsies; Q_1, first quartile; Q_3, third quartile; ACR, acute cellular rejection; LB, lymphocytic bronchiolitis; IR, ischemia-reperfusion; DAD, diffuse alveolar damage; OP, organizing pneumonia; BALs, bronchoalveolar lavages. All TBBs and BALs were obtained during scheduled follow-up bronchoscopies.

[a]Follow-up of 2 patients in the OCS group and 2 patients in the SOC group was not available as these patients died soon after surgery.

[b]Indexes were calculated for each patient as follows: number of rejections/total TBB number ×100.

[c]Indexes were calculated for each patient as follows: number of infections/total BAL number ×100.

4.1 | Strengths and limitations

The study was a double center experience with a small cohort of a subset of the phase 3 INSPIRE trial. Thus, the statistical power of our analysis may be limited due to the low number of cases, and a larger study population is certainly needed to more strongly support our data. Regrettably, lung tissue fragments were not available in all patients (only 63%) because we could not perform tissue sampling due to the lack of written informed consent and to the overall clinical condition. Nonetheless, this study may provide an important steppingstone in increasing the awareness about pathological substrates of lungs preserved with this advanced EVLP system.

It would have been interesting to have baseline data of iNOS expression and AI before preservation when the lungs were obtained. Notwithstanding, because all lungs met the criteria of being suitable for transplantation, we assumed a comparability of their baseline characteristics, or at least an equal distribution between the 2 groups.

Molecular analysis for iNOS mRNA was also performed on a small number of cases, thus limiting the power of statistical tests. In a future multicenter study, we plan to use a larger number of patients, take baseline samples before perfusion with OCS and SOC, and take small samples to be frozen for future molecular investigations.

ACKNOWLEDGMENTS

This research was funded by the Veneto Region, "Ricerca Sanitaria Finalizzata 2012" (Prot. 60888, November 2012), but this sponsor did not have any role in the study design, collection, analysis, or interpretation of data. The authors thank Dr Judith Wilson for English revision.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

All relevant data were reported within the article. Further supporting data will be provided upon written request addressed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Calabrese F, Schiavon M, Perissinotto E, et al. Organ Care System Lung resulted in lower apoptosis and iNOS expression in donor lungs. *Am J Transplant*. 2020;20:3639–3648. https://doi.org/10.1111/ajt.16187