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ORIGINAL ARTICLE

Failure of IL-8 to assess early reperfusion injury following lung transplantation of cardiac death donor pigs

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Summary

Although interleukins (IL) 8 and 10 predict lung viability in lung transplantation from heart beating donors (HBD) and IL-1β is a marker of *ex vivo* performance from after cardiac death donors (ACDD), IL expression in the recipient remains unknown. This study assessed IL-1β, IL-8 and IL-10 as indicators of functional performance in single-lung transplantation from ACDD pigs. Animals were divided into: (i) HBD: immediate lung excision; (ii) ACDD: fibrillation, 30 min warm ischemia and 3 h topical cooling. Left lungs of both groups were then flushed with Perfadex and stored at 3–4 °C for 3 h. IL in bronchoalveolar lavage fluid (BAL) and hemodynamic and graft function were measured in the donor and during the 2 h reperfusion period in the recipient. Myeloperoxidase, nuclear factor kappa beta, wet/dry weight ratio and a histologic injury score were assessed from biopsies in basal conditions in the donor and at the end of reperfusion. Despite similar pulmonary function and histologic markers of injury in both groups and higher IL-1β in the donor of ACDD, IL-8 during reperfusion was significantly lower in ACDD (119 ± 33% of basal) than in HBD (306 ± 238%, $P < 0.05$) recipients. The paradoxical behavior of IL-8 makes it an unreliable predictor of ACDD early outcome in this transplantation model.

Introduction

Lung transplantation is limited by shortage of suitable donors. Among the various attempts to expand the pool of potential organs, after cardiac death donors (ACDD) emerge as an important alternative as sudden cardiac arrest is responsible for more than half of all deaths due to cardiovascular disease [1]. Moreover, unlike other solid organs, the lung is the only one whose parenchymal cells do not require blood perfusion for cellular respiration, suggesting suitable transplantation outcome even when considerable intervals after circulatory arrest have elapsed before organ recovery [2].

Following the initial experiments in dogs showing good gas exchange in transplanted animals with organ recovery 1 h after death [3], many attempts have been made to extend the time prior to organ excision in ACDD [4–6]. Recent animal studies have reported that warm ischemic lung tolerance after cardiac arrest seems to be limited only to 1 h [7], but further protection obtained with topical cooling extends the period before organ recovery up to 7 h with encouraging hemodynamic and lung function recuperation in isolated organ [8,9] and transplanted animal models [6,10,11]. However, incomplete knowledge of the mechanisms underlying primary lung graft dysfunction and its effective treatment lies at the base of the restricted use of ACDD lungs, despite some promising results in patients [12–14]. Therefore, it is relevant to investigate possible markers of injury to gain insight into the pathophysiology of primary graft failure in the recipient of lungs from ACDD.

It has been shown that the duration of the ischemic period is limited mainly by the activation of inflammatory mediators that are deleterious to the organ at the time of reperfusion. The potent interleukin-8 (IL-8) has been

reported to increase during reperfusion in single lung transplantation from heart-beating donor (HBD) pigs [15,16] and in patients with bilateral lung transplantation [17–19]. In contrast, the anti-inflammatory IL-10 has been recognized to counteract this deleterious process partly [20]. Consistent with these results, *ex vivo* assessment of lung viability in ACDD pigs has shown that the warm ischemic period-related graft dysfunction correlates with an increased expression of the proinflammatory IL-1β in bronchoalveolar lavage (BAL) fluid at the time of organ recovery [21]. However, the inflammatory response in the recipient of ACDD lungs remains unknown.

Thus, to assess the effect of donor circulatory arrest on the inflammatory response of the recipient, we analyzed IL-1β, IL-8, IL-10 and nuclear factor kappa beta (NFKB) as indicators of primary graft dysfunction in left single-lung transplantation from ACDD in pigs.

Materials and methods

Thirty-four male, castrated Landrace pigs weighing 38.6 ± 0.5 kg were used. The animals received humane care in compliance with our institutional Guide of Care and Welfare of Laboratory Animals (NIH-PHS approval A5556-01) and the Principles of Laboratory animal care, (NIH publication Vol 25, No 28 revised 1996). The animals were assigned to two groups: HBD and ACDD (Fig. 1).

HBD procedure

Organ recovery was performed as previously described [16]. Briefly, a BAL was performed for IL-1β, IL-8, IL-10 and polymorphonuclear leukocyte (PMN) measurements in basal conditions, and a femoral arterial line and Swan Ganz catheter were placed for pressure monitoring and blood sampling. A biopsy of the right medial lobe was taken for control NFKB and myeloperoxidase (MPO) assays, wet/dry weight (W/D) assessment, and histological examination. Thirty minutes later another BAL was performed. During this time, the lungs were prepared for organ recovery. Then the left lung was first perfused with 750 ml cold Perfadex preservation solution to cleanse it from blood, and after the heart lung block was excised, the left lung was perfused with another 750 ml Perfadex and stored inflated in the preservation solution at 4 °C. The duration of cold ischemia was 3 h, from clamping in the donor until reperfusion in the recipient.

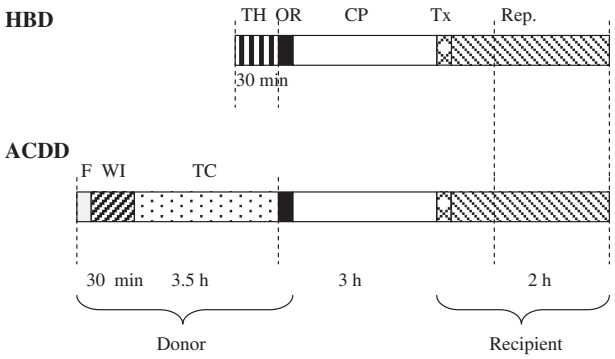

Figure 1 Experimental protocol. Dotted vertical lines indicate bronchoalveolar lavage fluid (BAL) procedures. HBD, heart beating donor; ACDD, after cardiac death donor; TH, time before organ recovery; F, fibrillation; WI, warm ischemia (30 min); TC, topical cooling (3.5 h); OR, organ recovery; CP, cold storage with preservation solution (Perfadex) (3h); Tx, transplantation; Rep, reperfusion (2 h). During reperfusion BAL was taken at 30 and 120 min reperfusion.

After ACDD procedure

Following basal measurements, a right medial lobe lung biopsy was taken through a small right thoracotomy. Death was then induced by ventricular fibrillation followed by ventilator disconnection. Ten minutes later, heparin was infused through the central venous catheter and 10 cardiac massage compressions were made. The animal was left untouched to complete 30 min warm ischemia after which in situ topical cooling was performed [10]. Briefly, this procedure consisted in placing two drains in each pleural space, connected to two 2 l bags of cold saline (4 °C). For each pleural space, one of the bags was positioned above the animal and emptied through one of the drains filling the pleural cavity space and the other bag was placed in ice slush at floor level. A temperature probe was placed in the trachea and in each pleural cavity to control a core temperature between 6–10 °C. When the temperature increased to 10 °C, the cooled bags were swapped with the empty ones, allowing the simultaneous drainage and filling of the pleural space. This procedure was repeated for 3.5 h, following which a BAL was performed, and the heart-lung block was excised and perfused with cold Perfadex solution, and stored as in the HBD group.

Recipient procedure

In both groups, another pig was prepared as previously described [16]. After single-lung allotransplantation, the lung was reperfused, the sternotomy was closed and the animal was followed up for a 2-h period.

At 30 and 120 min reperfusion, a BAL was performed. A biopsy of the transplanted left lung was taken before sacrificing the animal with an overdose of sodium thiopental.

BAL

Bronchoalveolar lavage was performed according to a standard procedure previously described [16]. Each BAL was performed five times, the first for IL-1β, IL-8, IL-10, and PMN measurements, the second for bacteriological assay, and the last three were frozen, and stored as reserve. Care was taken to perform the BAL always in the same region in the consecutive procedures in the donor and recipient.

IL-1β, IL-8, IL-10, PMN, NFKB, and MPO

Interleukins and PMN were measured as previously described [16]. Quantification of NF-kB p65 DNA-binding activity was performed using the NF-kB TransAM kit (Active Motif, Buenos Aires, Argentina) according to the manufacturer's instructions. NFKB and MPO samples were obtained from the donor right upper lobe, and from the grafted left middle lobe, and frozen until assay [22,23].

Histologic study

After sample removal for MPO, the whole lung was fixed in 10% formaldehyde and two samples from each lobe involving whole organ thickness were analyzed under light microscopy. A semi-quantitative morphologic study was made using four levels (0–3) of intensity for the presence of intraalveolar edema, polymorphonuclear cells and atelectasis in histologic samples. The total histologic injury score for each experiment was obtained adding the level of each histologic feature [16].

Experimental protocol

Systolic, diastolic, mean systemic, pulmonary arterial, wedge and central venous pressures, heart rate, and arterial and venous blood samples for gas and blood analysis were acquired in basal conditions in the donor and at basal, 10, 30, 60, 90, and 120 min reperfusion in the recipient.

IL-1β, IL-8, IL 10 (pg/ml), and PMN (% of total cells) were measured in the BAL in basal conditions and before organ recovery in the donor and at 30 and 120 min in the recipient. NFKB, MPO (ΔOD ml/mg), W/D weight ratio and the histologic injury score were assessed from biopsies of the right lung in the donor and the transplanted lung at 120 min reperfusion in the recipient.

Statistical analysis

Results were expressed as mean ± SE. One-way analysis of variance was used to assess hemodynamic and lung functional performance throughout reperfusion. If F was significant, *post hoc* analyses were made using Scheffé test for multiple comparisons. Comparisons between two groups or situations within the same group were performed using Student's *t*-test. Histological injury score was analyzed by the non-parametric Mann–Whitney test. A $P < 0.05$ was considered statistically significant.

Results

Table 1 shows that there were no differences between basal donor and recipient systemic mean arterial pressure, mean arterial pulmonary pressure, pulmonary vascular resistance (PVR), arterial oxygen pressure/fraction of inspired oxygen (PaO_2/FiO_2), and static compliance (StC) of HBD and ACDD. On the other hand, systemic mean arterial pressure was significantly lower than donor baseline pressure throughout reperfusion in the recipient of both groups, PVR was increased, and PaO_2/FiO_2 decreased at the beginning of reperfusion in the ACDD group, whereas StC was significantly decreased only in the HBD group during the whole reperfusion period (Table 2). However, there were no differences between both groups at any measured time point.

Table 3 shows that MPO and histologic injury score were higher than basal values at 2 h of reperfusion both in HBD and ACDD, whereas this increase was not significant for NFKB nor W/D weight ratio. Nevertheless, no significant increase was found between groups for any parameter (Figs 2a,b and 3a,b).

Table 1. Hemodynamic and pulmonary function in basal conditions.

	Donor		Recipient	
	HBD (n = 7)	ACDD (n = 10)	HBD (n = 7)	ACDD (n = 10)
Systemic mean arterial pressure (mmHg)	106.1 ± 7.8	121.7 ± 6.7	97.4 ± 6.6	106.5 ± 8.6
Mean arterial pulmonary pressure (mmHg)	24.9 ± 1.4	24.6 ± 1.9	24.9 ± 1.5	23.8 ± 0.9
Pulmonary vascular resistance (dines·s/cm^5)	144.8 ± 15.8	164.8 ± 24.8	134.3 ± 11.2	148.1 ± 21.8
PaO_2/FiO_2 (mmHg)	433.4 ± 30.1	456.2 ± 10.4	453 ± 10	433.9 ± 33.3
Static compliance (ml/cm H_2O)	22.3 ± 1.6	25.5 ± 1.3	24.6 ± 1.7	36.6 ± 7.5

PaO_2/FiO_2, arterial oxygen pressure/fraction of inspired oxygen; HBD, heart beating donors; ACDD, after cardiac death donors. Mean ± SE.

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Table 2. Percent hemodynamic and pulmonary function in the recipient.

		Donor baseline	10 min Rep.	30 min Rep.	60 min Rep.	90 min Rep.	120 min Rep.
Systemic	HBD	100	81.1 ± 7.7*	75.2 ± 7.7†	70.2 ± 5.9*	67.8 ± 6.1*	68.2 ± 5.4†
MEAN arterial pressure	ACDD	100	69.7 ± 3.3†	65.5 ± 3.5†	63.8 ± 2.1†	62.3 ± 2.6†	63.3 ± 3.1†
Pulmonary	HBD	100	106.6 ± 7.3	109.0 ± 8.7	106.3 ± 7.3	100.6 ± 7.9	100.1 ± 7.3
MEAN arterial pressure	ACDD	100	122.4 ± 12.6	113.2 ± 11.5	117.4 ± 13.3	114.1 ± 12.5	113.2 ± 12.6
Pulmonary	HBD	100	138.8 ± 34.2	117.9 ± 24	127.6 ± 24.6	160.6 ± 50.7	165.9 ± 38.3
VASCULAR resistance	ACDD	100	208.7 ± 40*	238.6 ± 56*	188.8 ± 58	185.7 ± 48	187.2 ± 47.3
PaO_2/FiO_2	HBD	100	99.6 ± 8.6	96.9 ± 9.6	100.7 ± 8.3	102.5 ± 9.4	100.6 ± 10.2
	ACDD	100	77.3 ± 7.7*	88.7 ± 10.4	86.1 ± 6.7	84.8 ± 9.4	93.6 ± 11.2
Static	HBD	100	83.5 ± 8.6*	81.1 ± 4.4†	80.0 ± 3.4†	80.2 ± 3.9†	80.9 ± 3.8†
Compliance	ACDD	100	90.8 ± 6.3	90.2 ± 7.4	86.2 ± 6.8	86.4 ± 6.7	91.5 ± 8.7

PaO_2/FiO_2, arterial oxygen pressure/fraction of inspired oxygen; HBD, heart beating donors; ACDD, after cardiac death donors.
HBD (n = 7), ACDD (n = 10). Mean ± SE.
*$P < 0.05$.
†$P < 0.01$, t-test against 100.

Table 3. Lung injury parameters.

	HBD (n = 7)		ACDD (n = 10)	
	Basal	120 min Rep.	Basal	120 min Rep.
MPO (ΔOD/ml/mg)	0.056 ± 0.003	0.079 ± 0.006**	0.06 ± 0.005	0.094 ± 0.009*
NFKB p65 activation (ng/ml)	3.81 ± 0.06	3.98 ± 0.22	3.84 ± 0.17	4.25 ± 0.13
W/D	5.14 ± 0.067	6.85 ± 0.47	2.88 ± 0.43	4.27 ± 0.47
Histologic injury score	0.1 ± 0.1	3.2 ± 0.4**	0.4 ± 0.2	3.9 ± 0.8**

MPO, myeloperoxidase; NFKB, nuclear factor kappa beta; W/D, wet/dry weight ratio; HBD, heart beating donors; ACDD, after cardiac death donors.
The histologic injury score was calculated as the sum of the level of intensity for the presence of intraalveolar edema, polymorphonuclear cells and atelectasis. Mean ± SE.
*$P < 0.05$.
**$P < 0.01$ versus basal.

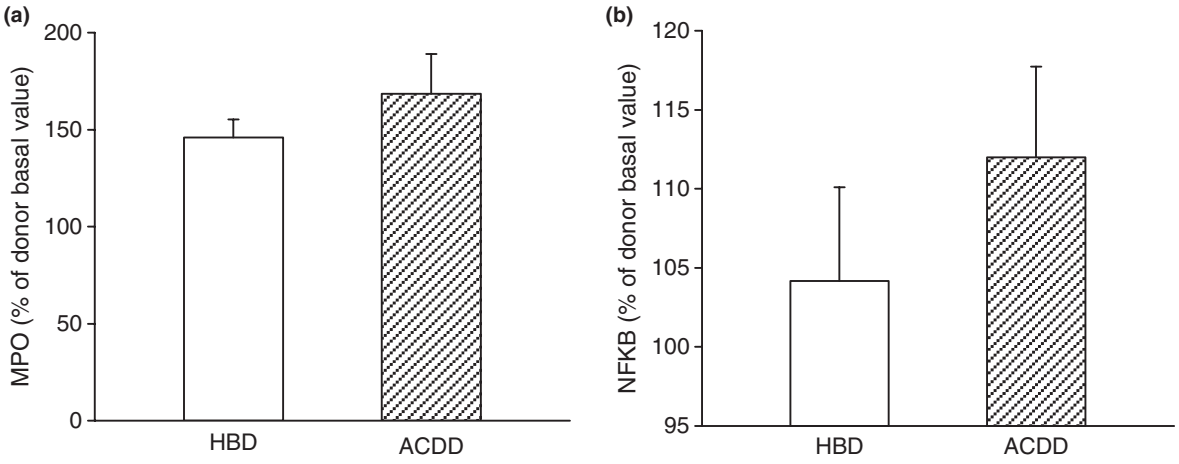

Figure 2 Myeloperoxidase (MPO) (a) and nuclear factor kappa beta (NFKB) (b) in the lung at 120 min reperfusion in heart beating donors (HBD) and after cardiac death donor (ACDD). Data are expressed as % of donor basal value.

Bronchoalveolar lavage results showed that PMN content expressed as percent of total cells was not significantly different between groups either at 30 min (0.6 ± 0.4% HBD versus 4.8 ± 4.6% ACDD) or at 120 min reperfusion (12.6 ± 3.6% HBD versus 17.5 ± 7.4% ACDD). However, examination of IL within each

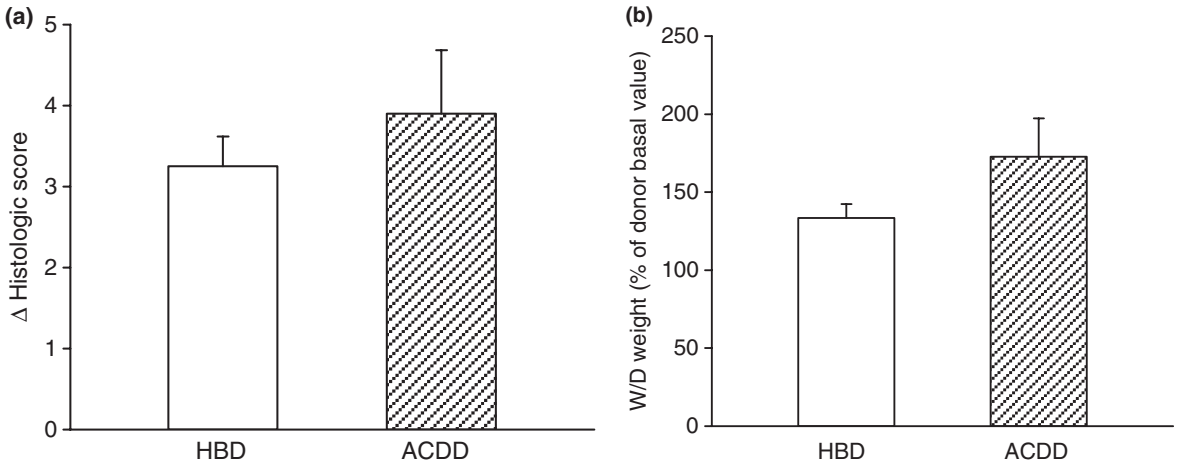

Figure 3 Histologic lung score (edema, presence of polymorphonuclear cells and atelectasis) and W/D weight ratio in heart beating donors (HBD) and after cardiac death donor (ACDD). (a) Data are expressed as the difference between the score at 120 min reperfusion minus basal score in the donor. (b) Data are expressed as % of basal donor.

Table 4. Interleukin profile in BAL throughout the experiment.

		Donor		Recipient	
		Baseline	Before organ recovery	30 min reperfusion	120 min reperfusion
IL-1β (pg/ml)	HBD	9.2 ± 0.9	15.0 ± 2.9	12.2 ± 2.9	10.7 ± 3.1
	ACDD	11.4 ± 1.3	33.8 ± 5.8*†	25.8 ± 4.2	18.0 ± 2.2
IL-8 (pg/ml)	HBD	13.1 ± 1.1	20.3 ± 2.9	31.5 ± 7.7	72.7 ± 21.8†
	ACDD	18.1 ± 2.8	31.3 ± 7.4	24.7 ± 4.5	20.1 ± 3.5
IL-10 (pg/ml)	HBD	5.4 ± 1.2	8.6 ± 1.9	9.0 ± 2.0	11.9 ± 2.5
	ACDD	4.5 ± 0.4	4.8 ± 0.6	4.1 ± 0.2	4.2 ± 0.6

HBD, heart beating donors; ACDD, after cardiac death donors.
Mean ± SE.
*$P < 0.05$ versus 120 min reperfusion.
†$P < 0.01$ versus basal.

group revealed a significant increase of IL-1β prior to organ recovery in ACDD, a prominent rise of IL-8 at the end of reperfusion in HBD, and a fairly constant IL-10 in both groups (Table 4). Moreover, comparison between groups (Fig. 4) showed contradictory results. IL-1β was higher in ACDD than in HBD animals prior to organ recovery with a decreasing difference during reperfusion that became nonsignificant at 120 min reperfusion, IL-10, as anticipated in lungs exposed to less ischemic injury, evidenced a significant rise prior to organ retrieval and during reperfusion in HBD compared to ACDD, but conversely, IL-8 was higher in HBD compared with ACDD at 120 min reperfusion. The unexpected behavior of IL-8, however, did not correlate with PaO_2/FiO_2, PVR or StC in HBD and ACDD in the recipient (Fig. 5).

Discussion

This study shows that although functional recovery and histological injury of ACDD recipients was similar to HBD at 2 h reperfusion, IL-8 levels were lower in ACDD compared with HBD. Lack of correspondence between cytokine expression and functional and histologic behavior in ACDD casts doubt on the efficacy of IL-8 assessment as an indicator of primary graft dysfunction in these experimental conditions.

Despite improvement in surgical techniques, immunosuppression and organ preservation, primary lung graft dysfunction occurs in 10–35% of patients with lung transplantation [24], leading to increased post-operative mortality and poor long-term outcome of the transplanted organ. Although the exact mechanism of primary graft

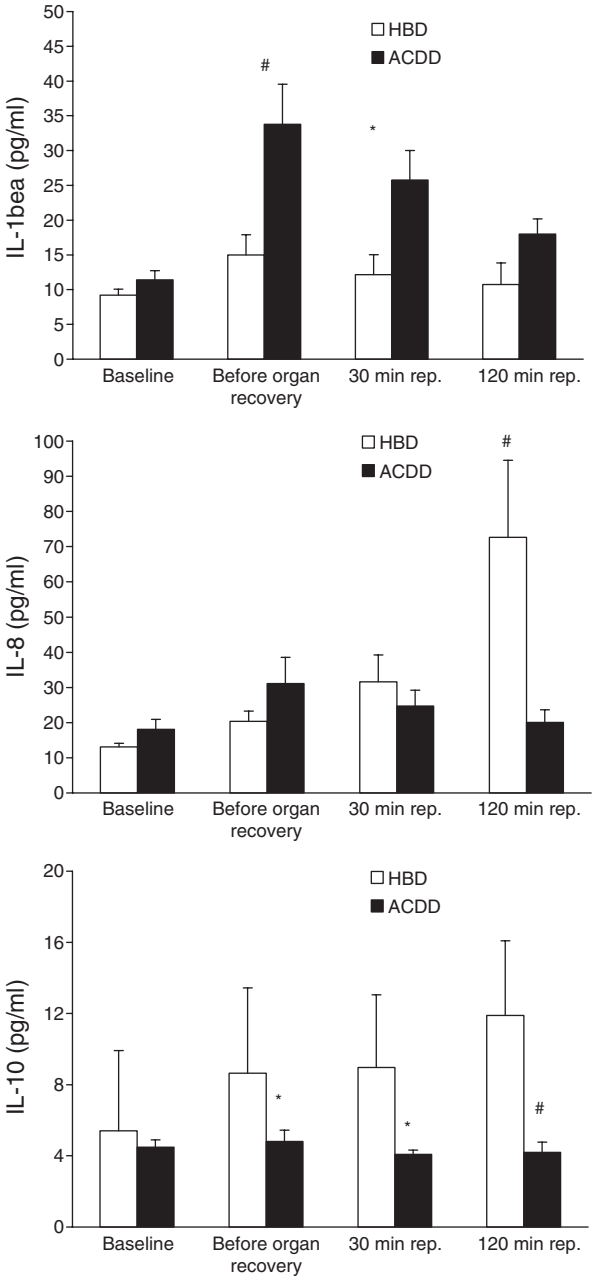

Figure 4 IL-1β, IL-8, and IL-10 at the start of the experiment (baseline), before organ recovery in the donor, and at 30 and 120 min reperfusion in the recipient of heart beating donors (HBD) and after cardiac death donor (ACDD). *$P < 0.05$, #$P < 0.01$ HBD versus ACDD.

dysfunction is still unknown, recent studies have indicated that aside from ischemia-reperfusion injury and ineffective preservation, cytokine release might play a critical role in its pathophysiology. During ischemia, activation of donor lung macrophages [25] induces the release of proiinflammatory cytokines such as IL-1β, which in turn stimulates release of IL-8 from macrophages and endothelial cells. IL-8 is a potent chemotactic factor which promotes neutrophil migration and adhesion [26], neutrophil accumulation being responsible for pulmonary fibrosis [27], asbestosis [28], and acute respiratory distress syndrome [29]. Accordingly, IL-8 has been shown to be higher in patients presenting these diseases [30–32]. In lung transplantation, IL-8 has also been found to play an important role in primary graft dysfunction. In patients, the progressive increase of IL-8 in biopsies obtained before and after transplantation from brain-dead donors correlated positively with postoperative mean airway pressure and negatively with PaO_2/FiO_2 after 2 h of reperfusion [18]. Similar negative correlation results between IL-8 and PaO_2/FiO_2 were attained with IL measurement in BAL of brain dead donors [17] and also in patients transplanted with lungs from HBD, increased IL-6, IL-8, and IL-10 levels were consistent with post-lung transplant reperfusion edema and primary graft failure [19]. Likewise, a positive linear correlation between *ex vivo* PVR, mean airway pressure, and W/D weight ratio with IL-1β from BAL performed immediately after organ recovery was achieved with longer warm ischemic periods in ACDD pigs [21], IL-1β rise being later confirmed in donor lungs submitted to 3 h warm ischemia and 1 h topical cooling [33]. Consistent with these studies, IL-1β was seen to be higher prior to organ recovery in ACDD than in HBD animals and progressively decrease during reperfusion. An increase of IL-8 in response to IL-1β would then have been expected to occur during reperfusion in ACDD [18], especially as NFKB, MPO, W/D weight ratio and histologic score indicated tendency of greater injury, whereas in HBD, more elevated values of IL-10 suggested protection of this group. However, this cytokine was elevated in HBD instead of in ACDD animals at the end of reperfusion. Furthermore, unlike reported findings in HBD and ACDD [17,18,21], no correlation of IL-8 with pulmonary function during reperfusion was found either in HBD or in ACDD. This discordant result does not seem to be because of defective BAL in HBD yielding comparably higher IL-8 levels, as the mean value at 2 h reperfusion was 35% lower than that previously obtained in an equally treated HBD group [16]. Therefore, protocol differences such as *ex vivo* evaluation and different preservation solution (use of Eurocollins and University of Wisconsin in patients), dissimilar inflammatory response in humans and animals and cause of death in the donor [17,18] in HBD might justify contradictory functional-IL-8 correlation outcomes.

Another paradoxical finding was that although MPO increased significantly compared to basal values in both groups, it did not accompany the difference in IL-8 levels between HBD and ACDD. This response could probably be attributed to the biphasic pattern of ischemia-reperfusion injury. The early phase during the first

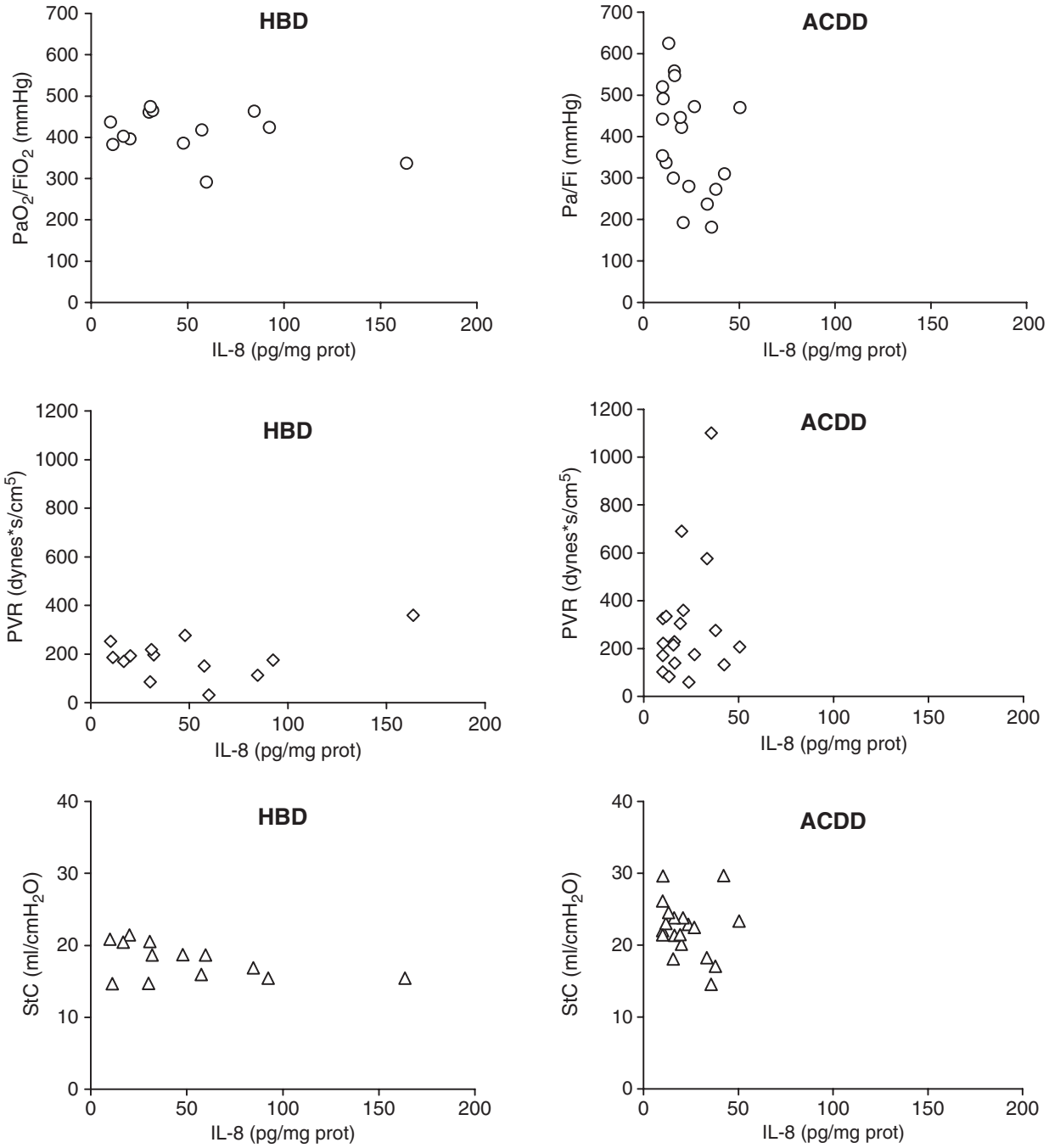

Figure 5 Relationship between PaO_2/FiO_2, pulmonary vascular resistance and static compliance and IL-8 in heart beating donors (HBD) and after cardiac death donor (ACDD). Each animal contributed with measurements at 30 and 120 min reperfusion. No significant linear correlation was observed in either group.

few hours of reperfusion consists of neutrophil-independent events and the late phase, after 4 h of reperfusion, is mediated by neutrophil-dependent events [34]. Thus, IL-8 is produced by macrophages during the early phase of reperfusion, and by recruited neutrophils during the late phase [35]. Lack of correspondence between MPO released upon neutrophil degranulation and IL-8 could then be explained by the short reperfusion period of this experimental model during which cytokine expression alone might have a more important role, and variations in its level would predominate over the concurrent release of MPO. Moreover, in support of this hypothesis,

similar neutrophil BAL content in both groups indicates lack of correlation between IL-8 and neutrophil activation.

Considering that it is currently accepted that 1 h warm ischemia and 6 h topical cooling postmortem preserve pulmonary graft function [6,9,10], the similar performance and histologic outcome of the recipient in HBD and ACDD could be ascribed to the short (30 min) warm ischemic interval used in this study. However, this brief period was chosen because duration of warm ischemia raises concerns regarding bronchial healing. In ACDD transplantation in pigs, 1 h warm ischemia resulted in impaired bronchial healing 3 weeks after transplantation [36]. Consequently, in this and in other rat lung transplantation studies [37,38], 30 min is the acceptable limit of lung procurement for successful bronchial anastomoses healing. It could also be argued that limitation of reperfusion to 2 h is too brief a period to reveal functional and cytokine differences. Nevertheless, 2-h reperfusion exhibits peak apoptotic pneumocytes in rats [39] and patients [40] and has been found to put in evidence lung functional changes in human transplantation from brain dead donors [18]. Moreover, several works point to an increase of IL-8 levels within the first 2 h of reperfusion. In a model of lipopolysaccharide induced lung injury, IL-8 peaked at 2 h of reperfusion [41] and a cold/rewarming protocol of alveolar epithelial cells showed increased IL-8 mRNA as early as 1 h after rewarming [42]. Similarly, during a 2-h reperfusion period, IL-8 increased in the recipient from HBD in pigs [16] and predicted primary graft dysfunction after human lung transplantation from brain dead donors [18].

In conclusion, the present results suggest that although the functional and histologic behavior of ACDD was similar to HBD, IL-8 expression did not accompany this performance, suggesting that it is not a reliable predictor of primary graft dysfunction in single lung transplantation from ACDD.

Authorship

EC: analyzed data; wrote the paper. A: designed research; performed surgery. C: controlled anaesthesic procedure; collected data. J: performed bronchoalveloar lavage; collected data. JA: collected lung performance data; performed statistical analysis. L: performed interleukin analysis; analyzed data. GG: performed interleukin analysis. LE: performed surgery. R: performed histological analysis. R: performed critical analysis of the paper.

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