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Lung nitroxidative stress in mechanically-ventilated septic patients: A pilot study

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ABSTRACT

Purpose: During sepsis and mechanical ventilation oxidative stress is generated by endothelial and inflammatory lung cells. Our main objective was to study pulmonary •NO (nitric oxide) production and nitroxidative stress in mechanically-ventilated septic patients.
Methods: We study 69 mechanically ventilated patients, 36 with sepsis and 33 without sepsis within the first 48 h of ICU admission compared with 33 mechanically ventilated patients without sepsis (MV) plus eight operating room patients without lung disease served as control healthy group (ORCG). Nitrite plus nitrate (NO_x^-), 3-nitrotyrosine and malondialdehyde (MDA) in bronchoalveolar lavage fluid (BALF) were analyzed.
Results: BALF NO_x^-, BALF 3-nitrotyrosine, BALF MDA, and plasma NO_x^- were higher in the Sepsis than in MV patients (all $p < .05$). Both SG and MV patients had higher BALF NO_x^- than the healthy control group ($p < .001$). In the Sepsis patients, the ICU non-survivors had higher levels of BALF NO_x^- than ICU survivors 80(70–127) μM versus 31(15–47) μM, $p < .001$.
Conclusions: We conclude that during early phases of sepsis there is an enhanced lung nitroxidative stress due to an increase of •NO production leading to secondary •NO-derived oxidants, which promote protein nitration and lipid peroxidation.

1. Introduction

The sepsis mortality rate in hospitals and intensive care units (ICUs) is within a range of 20–50% [1,2]. Lung involvement during sepsis is characterized by an inflammatory response accompanied by edema, cellular infiltration, and pulmonary hypertension [3]. Inflammatory cytokines such as tumor necrosis alpha, interferon gamma, interleukins and lipopolysaccharide activate immune cells for the production of reactive oxygen species, mainly superoxide ($O_2^{\bullet -}$), which in turn acts as a precursor of yet more powerful oxidants such as hydrogen peroxide and hydroxyl radical [4,5]. In addition, there is a systemic overproduction of •NO (nitric oxide), mostly derived from inducible nitric oxide synthase during sepsis leading to pathological peripheral vasodilation and systemic tissue injury [6,7]. The unusual high levels of •NO achieved in this pathological state will favor its reaction with $O_2^{\bullet -}$ (a diffusion-controlled reaction [8]) yielding peroxynitrite ($ONOO^-$) a powerful oxidizing and nitrating agent leading to nitroxidative stress [8,9]. Accumulation of nitrotyrosine reflects oxidative stress due to

Abbreviations: APACHE II, Acute Physiology and Chronic Health Evaluation syndrome; ARDS, acute respiratory distress syndrome; AUC, area under the curve; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; FIO_2, fraction of inspired oxygen; $CO_3^{\bullet -}$, carbonate radical; CVP, central venous pressure; CI, confidence interval; ELISA, enzyme linked immunoadsorbent assay; HPLC, high pressure liquid chromatography; ICU, intensive care unit; LISS, lung injury severity score; MODS, multiple organ dysfunction score; NO_x^-, nitrite plus nitrate; •NO, nitric oxide; $•NO_2$, nitrogen dioxide; NOS, nitric oxide synthase; MDA, malondialdehyde; MV, mechanical ventilation; nm, nanomolar; $O_2^{\bullet -}$, superoxide radical; •OH, hydroxyl radical; ONOO−, peroxynitrite; ORCG, operating room control group; PAR, pressure adjusted heart rate ratio; PaO_2/FiO_2, partial pressure of oxygen/fraction of inspired oxygen; PEEP, positive end expiratory pressure; ROC, receiver operator curve; SG, sepsis group.

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nitric oxide-derived oxidants, a process called nitroxidative stress [10]. Sepsis-induced lung injury is a clinically relevant finding in patients with or at risk of sepsis [11]. Increase production of •NO or its derived metabolites in pulmonary edema fluid [12] have been reported in acute respiratory distress syndrome (ARDS) acute lung injury and in the development of lung insult during sepsis [13]. Oxygen-derived free radical damage to lipids results in lipid peroxidation in the diseased lungs. In this regard, malondialdehyde (MDA) (a product of lipid peroxidation), has been used as biomarker of free radical-mediated damage in critically ill and/or pulmonary injured patients [14]. Most of the injurious effects of •NO have been attributed to $ONOO^-$, given that this oxidant can nitrate tyrosine residues and [15,16] nitrated proteins have been found in bronchoalveolar lavage fluid (BALF) and plasma of patients with ARDS [12]. •NO and its derived metabolites have been detected in the lung of experimental septic animals and human patients and proposed to act as biomarkers of sepsis diagnosis and prognosis [17,18].

It is known that •NO and superoxide production are stimulated by inflammatory mediators in sepsis and from various physical stimuli induced by mechanical ventilation [19]. It has been reported that ARDS is associated with nitrooxidative stress [12]. It is not known if early high levels of lung and plasma Nitroxidative stress could be associated with ICU evolution in septic mechanically-ventilated patients without severe lung injury. Nitroxidative stress may begin in the early phases of sepsis when there was not severe lung injury and gas exchange impairment. We hypothesized that this mechanism could begin very early in septic patients before severe lung injury develops. In addition, we also investigated whether lung nitroxidative stress is associated with the ICU mortality in mechanically ventilated patients.

2. Materials and methods

2.1. Design

A convenience sample of critically ill patients was included after ICU admission. Exposure was sepsis, our main outcome variable was ICU mortality. Blood and BALF samples were obtained after study patient inclusion.

Setting. Ten bed Critical care unit at University Hospital.

Participants. Critical care patients admitted to intensive care unit.

The present study was performed with the approval by the Institutional Review Board and the Bioethics Committee of the Hospital de Clínicas, Facultad de Medicina, Universidad de la República and in accordance to the Helsinki declaration about ethical principles of medical investigation on human subjects. Family members, relatives or volunteers signed informed consent.

2.2. Septic patients

Septic patients ($n = 36$) were included during the first 48 h of ICU admission. Sepsis was defined according to the American European Consensus Criteria [20]. The severity of illness was evaluated by the APACHE II score [21]. Multiple organ failure (MOF) and Multiple organ dysfunction syndrome was evaluated by Multiple Organ Dysfunction Score (MODS) [2]. Lung injury was assessed by the American European Consensus Committee criteria [22], Berlin definition [23], and by the Murray Lung Injury severity score (LISS) [24]. All patients were ventilated and sedated.

Patients were excluded if they met one or more of the following criteria: pregnancy, long-lasting resuscitation, life-threatening arrhythmias, acute myocardial infarction with <48 h of evolution, bleeding disorder and refusal to participate. PaO_2/FiO_2 <100 was an exclusion criterion for the bronchoalveolar lavage (BAL) procedure.

2.3. Mechanical ventilated patients

Patients undergoing mechanical ventilation (MV group) ($n = 33$) for <70 h, without sepsis or ARDS were chosen as the mechanical ventilation control group. BAL procedure was performed after study inclusion. All ventilator parameters were recorded from ventilator readings.

2.4. Operating room control group

Blood and BALF samples were taken from 8 patients without pulmonary disease in the operating room after the induction of anesthesia.

2.5. Bronchoalveolar lavage protocol

BALF from septic patients was obtained within 48 h after ICU admission. All patients were sedated and pre-oxygenated for 10 min before the BAL procedure ($FIO_2 = 1.0$). Briefly, an indwelling catheter was inserted through the endotracheal tube until a wedged position was reached, afterwards 100 mL of normal saline solution divided in five aliquots were injected, after 20 s each aliquot was aspirated then collected and frozen for further analysis.

Variables. We measured clinical and physiologic variables; biochemical variables related to nitric oxide production were measured in plasma and BALF.

2.5.1. Sampling protocol

Blood sampling was contemporary to BALF sampling. BALF sampling was done within 48 h of ICU admission. Sampling time was 24 (12–43) hours in sepsis and 12(6–48) hours in mechanical ventilation group, p non-significant. There were no statistical difference in sampling time between survivors and non survivors, 24(12–44) hours versus 24(12–41) hours in sepsis group and 12(6–48) hours versus 12(7–30) hours in mechanical ventilation group.

2.6. Nitrite and nitrate assay

Nitrite and nitrate was measured by the Griess/vanadium chloride reduction method [25].

2.7. Malondialdehyde assay

MDA was measured by using a modified method adapted from [26]. MDA was detected by isocratic elution in reverse phase HPLC equipped with a fluorescent detector set at λ_{ex} 532 nm and λ_{em} 553 nm.

2.8. Protein 3-nitrotyrosine quantification

Protein bound 3-nitrotyrosine was measured by the ELISA method [27]. 3-nitrotyrosine concentration was normalized to protein concentration and expressed as pmoL/mg of protein.

2.9. Statistical analysis

Values were expressed as medians, 25th and 75th percentile. Mann-Whitney and Kruskal-Wallis test were used to compare variables. Categorical variables were compared with the Chi-square test. After BALF NO_x^- results were obtained the cutoff value for BALF NO_x^- at study admission was selected by constructing receiver operating curves (ROC) for ICU mortality. The value of BALF NO_x^- corresponding to the highest sensitivity plus specificity value was chosen as the cutoff point. Survival analysis was done by transforming BALF NO_x^- values into a dichotomous variable with BALF NO_x^- cutoff greater or equal to 53 μM.

The hazard ratio of ICU mortality was evaluated by using a logistic regression model including variables that were significant in the

univariate analysis. Data were analyzed with SPSS 15.0 version. A $p < .05$ was considered significant.

3. Results

3.1. Participants

Septic patients ($n = 36$) were included between September 2000 and June 2004. Flow diagram for inclusion and exclusion of patients is depicted in Fig. 1. Patient characteristics are depicted in Tables 1 and 2. Sepsis origin in SG was depicted in Table 1. Reasons for mechanical ventilation in sepsis group were: respiratory failure ($n = 11$), shock ($n = 9$), both respiratory failure and shock ($n = 8$), decrease of the level of consciousness ($n = 4$), multiorgan failure ($n = 1$), metabolic acidosis ($n = 1$). Diagnosis in MV group was: trauma ($n = 21$), postoperative ($n = 6$), stroke ($n = 4$), hypothyroidism ($n = 1$), status epilepticus ($n = 1$). ICU mortality was 44% and 18% in the SG and in the MV group, respectively.

Table 1
Sources of sepsis in Sepsis patients.

	n (%)
Peritoneal	13 (36.11)
Respiratory	13 (36.11)
Endovascular	3 (8.33)
Mediastinal	1 (2.78)
Pancreatic abscess	1 (2.78)
Enteric	1 (2.78)
Urinary	1 (2.78)
Soft tissue infection	1 (2.78)
Cholecystitis	1 (2.78)
Unknown source of infection	1 (2.78)

3.2. Clinical descriptive variables at admission to the ICU

The clinical variables at study admission on the SG and MV groups are shown in Table 1. APACHE II, MODS and LIS were higher in the SG

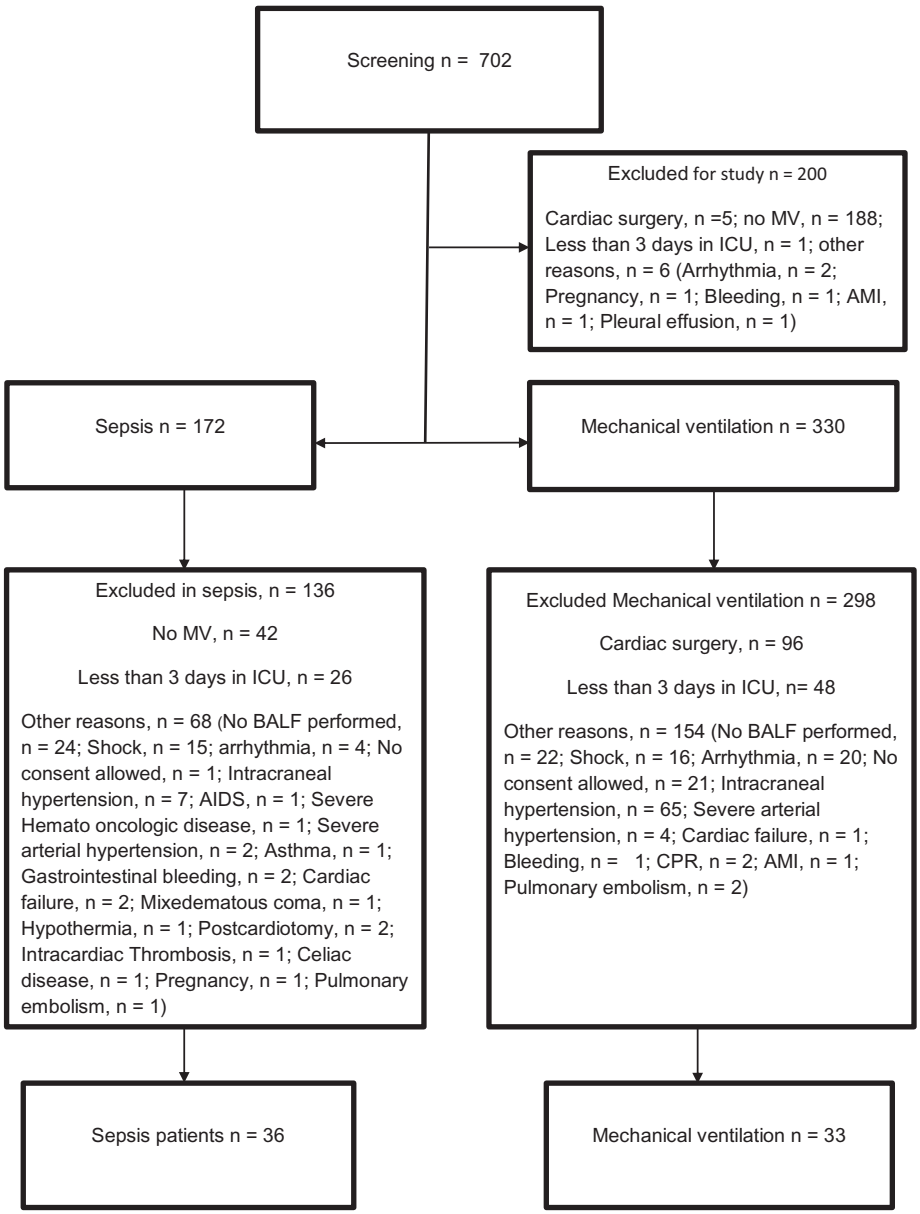

Fig. 1. Patient inclusion and exclusion flow diagram. AMI, acute myocardial infarction; AIDS, acquired immune deficiency syndrome; BALF, bronchoalveolar lavage fluid; CPR, cardiopulmonary resuscitation; ICU, intensive care unit; MV, mechanical ventilation

Table 2
Clinical variables.

	All patients			Sepsis patients			MV patients			p[a]	p[b]	p[c]	p[d]
	All patients (n = 69)	Survivors (*n* = 47)	Non survivors (*n* = 22)	All sepsis (*n* = 36)	Survivors (*n* = 20)	Non survivors (*n* = 16)	All MV (*n* = 33)	Survivors (*n* = 28)	Non survivors (*n* = 5)				
Age (years)	53 (31–67)	48 (29–63)	58 (45–69)	57 (40–72)	55 (22–69)	62 (52–74)	45 (31–61)	43 (30–61)	51 (32–62)	NS	NS	NS	0. 027
APACHE II	14 (12–19)	12 (9–16)	18 (12–26)	16 (13–25)	15 (12–18)	23 (15–28)	11 (9–13)	10 (8–13)	12 (10–18)	<0.001	0.023	NS	0.001
MODS	5.00 (2.00–6.25)	2.00 (2.00–5.00)	5.00 (3.00–8.25)	5.00 (3.25–7.75)	5.00 (2.25–6. 75)	7.00 (5.00–9.75)	2.00 (1.00-3.00)	2.00 (1.00-3.00)	3.00 (2.50-5.00)	<0.001	NS	NS	<0.001
SOFA	4.00 (2.00–5.00)	2.00 (1.00–5.00)	5.00 (2.00–7.00)	5.00 (4.00–6.75)	5.00 (3.25–5.00)	5.00 (5.00–7.75)	1.00 (1.00-2.00)	1.00 (1.00-2.00)	1.50 (1.00-2.75)	<0.001	NS	NS	0.001
LISS	1.50 (1.00–2.00)	1.25 (0.75–1.75)	1.62 (1.00–2.06)	1.87 (1.50–2.25)	1.75 (1.30–2.20)	2.00 (1.50–2.25)	1.00 (0.62-1,25)	1.00 (0.50-1.25)	0.87 (0.69-1.06)	<0.001	NS	NS	NS
Shock (%)	33	23	55	47	62	35	18	33	15	0.011	NS	NS	0.011
ICU length of stay(days)	18 (9–24)	16 (9–24)	18 (6–25)	21 (14–29)	23 (15–35)	19 (8–26)	13 (7–21)	13 (8–21)	8 (5–26)	0.005	NS	NS	NS
VFD(days)	16 (8–21)	17 (9–21)	15 (7–22)	13 (6–20)	13 (6–20)	12 (7–21)	19 (11−23)	18 (11–23)	22 (5–23)	0.041	NS	NS	NS
PaO_2/FIO_2	366 (297–459)	383 (306–500)	340 (268–429)	340 (278–428)	375 (293–525)	297 (210–385)	420 (344–500)	400 (338–500)	429 (410–546)	0.001	NS	NS	NS
PEEP (cm of H_2O)	9 (6–11)	9 (6–10)	10 (5–12)	10 (9–15)	10 (9–16)	10 (9–15)	8 (5–10)	8 (5–9)	7 (5–10)	<0.001	NS	NS	NS
MAP (mm Hg)	85 (80–91)	87 (79–94)	82 (80–90)	86 (80–98)	89 (81–98)	82 (79–95)	84 (78–90)	84 (78–90)	86 (79–90)	NS	NS	NS	NS
CVP (mm Hg)	11 (10–14)	11 (10−13)	13 (10–16)	13 (10–16)	12 (10–14)	15 (10–18)	10 (9–12)	10 (8–12)	12 (9–13)	0.003	NS	NS	0.044
PAR	13 (10–16)	11 (9–13)	14 (13−23)	14 (12−20)	13 (10–16)	19 (13–24)	11 (9–13)	11 (8.5–12)	12 (10–13)	<0.001	0.007	NS	0.001
Serum creatinine (mg/mL)	0.84 (0.70–1.00)	0.80 (0.70–1.00)	1.00 (0.80–1.75)	1.00 (0.77–2.20)	0.98 (0.60–2.06)	1.15 (0.80–2.34)	0.80 (0.70-1.00)	0.80 (0.70-0.90)	0.92 (0.78-1.00)	0.007	NS	NS	0.025
Arterial pH	7.39 (7.35–7.42)	7.40 (7.37–7.43)	7.38 (7.30–7.42)	7.37 (7.30–7.40)	7.37 (7.34–7.40)	7.37 (7.29–7.42)	7.40 (7.38-7.44)	7.40 (7.38-7.45)	7.40 (7.34-7.47)	0.002	NS	NS	NS

Values are given as median, 25th and 75th percentiles. Abbreviations and definitions: CVP, central venous pressure, ICU, intensive care unit; I and V, inotropics and vasopressors use; LISS, lung injury severity score; MAP, mean arterial pressure; MODS, multiple organ dysfunction score; MV, mechanical ventilation; PAR, pressure adjusted heart rate; SOFA, score of sequential organ failure assessment score;; V_T/Kg, expired tidal volume divided by body weight; VFD, ventilator free days. All patients included sepsis and mechanical ventilation groups.

[a] For the comparison between sepsis group and MV group.

[b] Or the comparison between non survivors and survivors in sepsis group.

[c] For the comparison between non survivors and survivors in MV group.

[d] For the comparison between non survivors and survivors in all patients.

Table 3
Biochemical variables in relation to Intensive Care Unit mortality.

	All patients			Sepsis			Mechanical ventilation			ORCG	p^a	p^b	p^c	p^d	p^e
	All patients (n = 69)	Survivors (n = 47)	Non survivors (n = 22)	All patients (n = 36)	Survivors (n = 20)	Non survivors (n = 16)	All patients (n = 33)	Survivors (n = 28)	Non survivors (n = 5)	(n = 8)					
BALF NO_X^- (μM)	30 (17–70)	30 (15–35)	70 (22–91)	62 (21–88)	31 (15–47)	80 (70–127)	27 (15–34)	28 (18–35)	15 (4–29)	4 (3–5)	0.001	<0.001	0.08	0.003	<0.001
BALF NT (pmol/mg protein)	104 (32–240)	80 (30–200)	136 (36–282)	165 (33–660)	173 (32–787)	149 (33–329)	60 (29–175)	60 (29–174)	92 (33–286)		0.049	0.626	0.768	0.629	
BALF MDA (nM)	90 (26–191)	84 (16–138)	129 (53–208)	144 (31–212)	114 (11–212)	174 (85–232)	78 (26–100)	79 (26–108)	73 (17–86)		0.014	0.312	0.388	0.169	
Plasma NO_X^- (μM)	97 (50–226)	98 (45–226)	94 (60–238)	150 (64–242)	130 (51–240)	156 (71–250)	54 (30–119)	52 (29–145)	60 (39–74)		0.001	0.962	0.737	0.331	

ORCG, operating room control group; MV, mechanical ventilation group. Values are given as median, 25th and 75th percentiles. All patients included sepsis and mechanical ventilation groups.
[a] for the comparison between sepsis group and MV group.
[b] for the comparison between non survivors and survivors in sepsis group.
[c] for the comparison between non survivors and survivors in MV group.
[d] comparison between non survivors and survivors in all patients.
[e] for the comparison with ORCG.

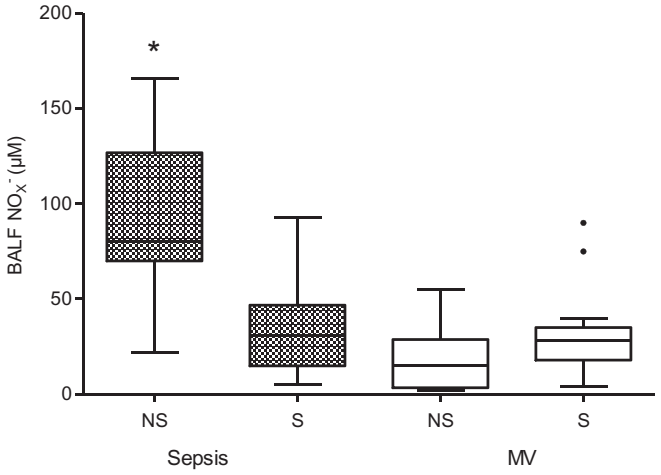

Fig. 2. BALF NO_x^- (bronchoalveolar lavage fluid nitrite plus nitrate) according to Intensive Care Unit mortality. MV, Mechanical ventilation; NS, non-survivors; S, survivors. *, $p < .05$ versus survivors in sepsis group. Shaded bars denote patients with sepsis.

compared to the MV group (Table 2). CVP and PAR were higher in the SG than in MV group whereas arterial pH was lower in SG than in MV group. Ventilator variables are shown in Table 1. PaO_2/FIO_2 ratio was lower in SG. Serum creatinine, PEEP and plateau pressure were higher in SG compared to MV. PAR score was found to be elevated in non-survivors compared to survivors on the SG.

In the sepsis group we found bilateral infiltrates that were used for lung injury score. Accordingly, they have 2 (1–3) chest radiographs quadrants occupied.

3.3. Protein concentration in bronchoalveolar lavage fluid

BAL fluid protein concentration was 0.50 (0.17–1.28) mg/mL in sepsis patients versus 0.22 (0.07–1.16) mg/mL in mechanical ventilation group.

3.4. Evaluation of nitrooxidative damage to the injured lung

Nitrite plus nitrate (NO_x^-) are end products of •NO and •NO-derived oxidants such as $ONOO^-$ that can be measured as surrogate markers in the BALF. We found higher levels of NO_x^- in both groups of critical ill patients (SG and MV) than the control group (ORCG). Analysis of patients admitted to the ICU revealed that the SG had higher BALF NO_x^- levels

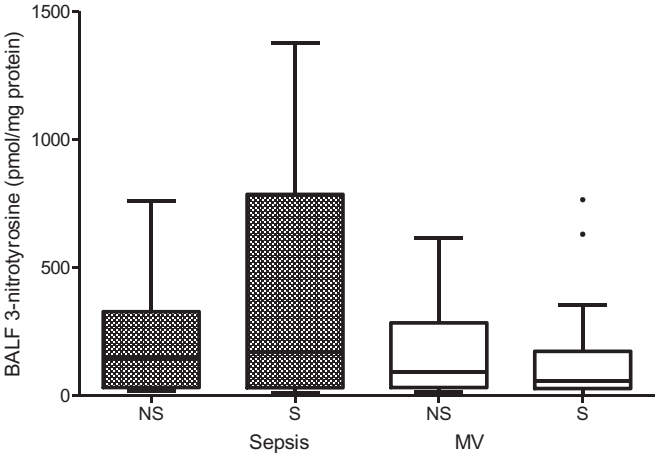

Fig. 3. BALF 3-nitrotyrosine (bronchoalveolar lavage fluid 3-nitrotyrosine) according to Intensive Care Unit mortality. MV, Mechanical ventilation; NS, non-survivors; S, survivors. Shaded bars denote patients with sepsis.

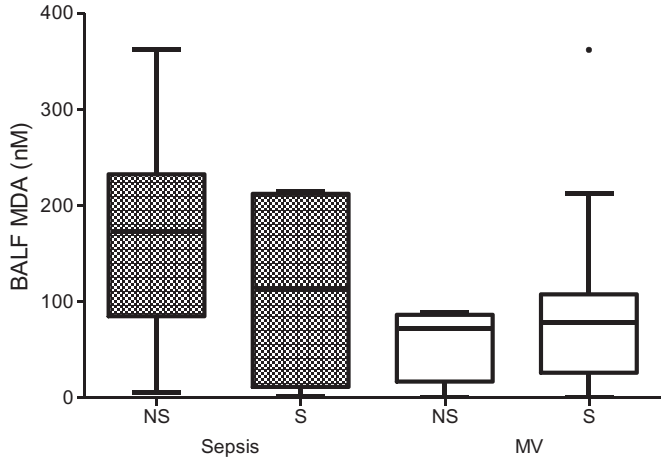

Fig. 4. BALF MDA according to ICU mortality. MV, Mechanical ventilation; NS, non-survivors; S, survivors. Shaded bars denote patients with sepsis.

than the MV group 62 (21–88) μM versus 27(15–34) μM respectively, $p < .001$.This result is in line with the expected increased on •NO production observed during sepsis. Moreover, BALF NO_x^- levels were higher in septic non-survivors than in survivors, underscoring the association between nitrooxidative stress and the severity of the disease 80 (70–127) μM versus 31(15–47) μM, respectively, $p < .001$; Table 3 and Fig. 2). •NO is not a potent oxidant itself but in the presence of $O_2•^-$ will give rise to $ONOO^-$, a principal cause of nitroxidative damage. $ONOO^-$ derived radicals such as $•NO_2$, •OH and carbonate radical ($CO_3•^-$), can initiate lipid peroxidation and nitration of protein bound and free tyrosine. We therefore evaluated MDA and 3-nitrotyrosine in BALF to evaluated lung nitroxidative damage.

3.5. Protein 3-nitrotyrosine in bronchoalveolar lavage fluid

Both study groups (SG and MV) showed evidence of protein nitration in BALF (Table 3). BALF 3-nitrotyrosine was higher in SG than in MV group. There was no statistical difference between survivors and non-survivors in any of the groups analyzed (Table 3 and Fig. 3). 3-nitrotyrosine from ORCG was not available.

3.6. Malondialdehyde in bronchoalveolar lavage fluid

MDA in BALF was determined in SG and in MV group. Both sepsis and MV groups shown evidence of lipid peroxidation (Table 3). BALF MDA was higher in SG compared to MV. There was no difference on MDA levels between survivors and non-survivors in both groups (Table 3 and Fig. 4).

3.7. Clinical course of patients in sepsis group and nitroxidative stress

Regarding patients evolution in sepsis group we found that: 20 patients survived and 16 patients died, 9 of out 16 died with MOF and 7 of out 16 died with ARDS. Reasons of death were classified according to the presence of organ failure or ARDS as main reason of death. We found that in sepsis group 9 patients died with organ failure and 7 patients died with ARDS. Furthermore, although BALF NO_x^- was high in non survivors we found no difference in BALF NO_x^- concentration. NO_x^- in BALF was 80 (70–142) μM in patients that die with organ failure and 72 (56–90) μM in patients that die with ARDS, $p = 0{,}408$. Patients that died with organ failure and patients that died with ARDS had high levels of pulmonary NO_x^-. Nevertheless, there were no differences in lung NO_x^- production between ARDS and organ failure patients.

In our study population high nitroxidative stress was not significantly related to lung injury worsening. We use the Berlin definition of ARDS (no ARDS, mild ARDS, moderate ARDS, and severe ARDS) to characterize severity of lung involvement in sepsis group. Then we compared the initial Berlin score with the last value of Berlin score obtained in each septic patient. By comparing initial against last value of Berlin definition severity, we classified patient evolution as improvement, worsening or no change in Berlin definition severity status. We separate them based on initial BALF NO_x^- levels (high or low). We found that in patients with no change in Berlin status ($n = 25$) there were 14 and 11 patients with low and high levels of BALF NO_x^-; in patients with improvement in Berlin status ($n = 7$) there were 5 and 2 patients with low and high levels of BALF NO_x^-; in patients with worsening in Berlin status ($n = 4$) there were 1 and 3 patients with low and high levels of BALF NO_x^-; respectively. As a result no clear differences in nitroxidative stress levels between Berlin severity status evolutions were observed.

3.8. Survival analysis according to BALF NOx^-

ICU mortality was 32%, whereas ICU mortality was 44% in sepsis and 18% in mechanical ventilation without sepsis.

We performed survival analysis in all patients (sepsis plus mechanical ventilation, $n = 69$) AUC for ROC analysis was 0.72 (95% CI: 0.57 to 0.87) for BALF NO_x^-. We found that at the sensitivity of BALF $^-NO_x^-$ in all patients for ICU mortality was 73% (95% CI: 50% to 89%), the specificity was 8% (95% CI: 74% to 95%), the positive likelihood ratio was 6 (95% CI: 3 to 13), the negative likelihood ratio was 0.31 (95% CI: 0.16 to 0.62), positive predictive value was 73% (95% CI: 50% to 89%), negative predictive value was 87% (95% CI: 74% to 95%). In the multivariate analysis after correcting for APACHE II, BALF $NO_x^- \geq 53$ μM remained as significant prognostic variable (Table 4).

In sepsis patients AUC for ROC analysis was 0.84 (95% CI: 0.71 to 0.99) for BALF NO_x^-. Septic patients with BALF NO_x^- concentration equal or higher than 53 μM had an ICU mortality of 79%, whereas patients with BALF NO_x^- <53 μM had an ICU mortality of 6% ($p < .05$). At this cutoff the sensitivity of BALF NO_x^- in sepsis patients for ICU mortality was 87% (95% CI: 62% to 98%), the specificity was 80% (95% CI: 56% to 94%), the positive likelihood ratio was 4 (95% CI: 2 to 11), the negative likelihood ratio was 0.16 (95% CI: 0.04 to 0.58), positive predictive value was 78% (95% CI: 52% to 93%), and negative predictive value was 89% (95% CI: 65% to 98%).

3.9. Nitrite and nitrate in plasma

Nitrite plus nitrate (NO_x^-) concentration in plasma was determined in SG ($n = 36$), MV group ($n = 24$) and ORCG patients. Plasma NO_x^- on both SG and MV groups were elevated when compared to ORCG (Table 3). Plasma NO_x^- was higher in SG compared to MV. There were no differences in plasma NO_x^- concentration between non-survivors and survivors in any of the groups.

Table 4
Logistic Regression for ICU mortality.

Variable	B	E.T.	Wald	gl	Sig.	OR (95% C.I.)
APACHE II	0.105	0.055	3.585	1	0.058	1.11 (0.99 – 1.24
BALF NO_x^-	2.424	0.685	12.531	1	0.0001	11.30 (2.95 – 43.21)
Constant	-3.326	0.913	13.270	1	0.0001	0.036

4. Discussion

In mechanically ventilated septic patients we observed very early high nitroxidative stress production and a relationship between pulmonary •NO-derived oxidants, and patient outcome. We found that nitroxidative stress begins early before severe lung injury develops. Besides, we found that early BALF NO_x^-concentration is elevated both in patients who get worse lung injury and in patients who did not get worse lung injury. Patients that die with ARDS or with organ failure had

elevated BALF NO_x^- concentration; this was related to lung and whole-body persistent nitroxidative stimulus. Sepsis-induced lung inflammation and injury are associated with overproduction of •NO in a pro-inflammatory environment such as the lungs, where an increase in formation of reactive oxygen species, in particular $O_2^{\bullet-}$ is expected [28]. •NO production and oxidative stress have been described in various experimental animal models of sepsis [29] and acute lung injury/ARDS [7]. In humans, increased pulmonary •NO production has been reported during sepsis [30], severe sepsis [31], septic shock [32] and acute respiratory distress syndrome [33]. An inflammatory and oxidative mechanism triggered by cytokines has been proposed to explain the increased lung and systemic •NO concentration in those patients [34]. Zhu and colleges reported elevated NO_x^- in pulmonary edema fluid from 34 patients with acute lung injury/ARDS (38% with sepsis) versus 20 patients with hydrostatic pulmonary edema. A persistent elevation on BALF NO_x^- in ARDS patients of multiple origins has been reported in previous work, in which BALF NO_x^- levels were higher in non survivors evaluated at days 7 and 21 [12]. We found that the increase on NO_x^- in the BALF occurs early in ventilated septic patients independent of the presence of primary lung injury. We found a predictive value of BALF NO_x^- in a homogeneous population of septic patients.

Since mechanical ventilation can induce biotrauma, we could speculate that NOS activity would be elevated in sepsis and in mechanical ventilation groups. Inflammatory stimulus from sepsis increases NOS expression further, as a result nitrite and nitrate concentrations in plasma and in BAL samples are higher in sepsis than in mechanical ventilation group. Sepsis-induced lung NOS expression together inflammatory cytokines have been described in the lungs of ARDS patients following sepsis [18].

Oxidative stress is an imbalance between oxidants and antioxidants on a cellular or individual level. The entire basis of the biomarker phenomenon is the measurement of a compound that directly reflects certain biological events related to the pathogenesis of a disease, in the case of MDA, lipid oxidation is the biological event. Several hypotheses describing the formation of MDA in vivo have been proposed. The mechanism is thought to involve formation of prostaglandin-like endoperoxides from polyunsaturated fatty acids with two or more double bonds [35] or alternatively trough hydroperoxide formation and cleavage of polyunsaturated fatty acids [36]. Other minor sources exist such as by-products of free radical formation. The use of the Thiobarbituric acid assay to measure MDA can give confounding results due to the lack of specificity in complex biological mixtures, as several products other than MDA can yield adducts with similar optical properties. In our study we used an HPLC based method with fluorescence detection, which significantly improves the specificity of the MDA measurement as previously reported [37]. MDA in BALF has been reported as a biomarker of increased lung oxidative injury and lipid peroxidation [38]. In our study we found that septic patients have higher MDA production than the MV group. Lung nitroxidative stress during sepsis is demonstrated by the augmented production of NO_x^-, 3-nitrotyrosine and MDA in the septic group. Although some degree of nitroxidative damage was also observed in the MV group, we showed that the septic state itself is a stronger inducer of ·NO and its byproducts, which are associated with patient outcome.

Disparities in mortality between SG and MV are due to organ failure. In the SG, an increase on APACHE II, PAR score and BALF NO_x^- was found in ICU non-survivors. When we analyzed those variables in a multivariate model that included BALF NO_x^- as dichotomous variable, we found that BALF NO_x^- remained as a significant predictive variable for ICU mortality. This finding reflects pulmonary high inflammatory state that occurs in sepsis patients. We cannot elucidate if this is the expression of local production or if it is the result of systemic nitric oxide production. Moreover, it does not imply causality since it is a lung biomarker expression.

The combination of additional biomarkers may increase its predictive accuracy. However, in the univariate analysis for mortality, BALF nitrotyrosine, BALF malondialdehyde and plasma malondialdehyde were not associated with poor prognosis in sepsis patients. In the ROC analysis BALF NOx had an area under the curve for mortality of 0.82, whereas nitrotryosine and malondialdehyde and plasma NO_x^- were close to the threshold of chance. Therefore, we included only BALF NO_x^- together with APACHE II score in the regression analysis. As we pointed out above this is probably due to the small sample size of this pilot study. From a pathophysiological point of view it is expected that nitroxidative stress would be associated with elevated nitrotyrosine (protein nitration) and MDA (lipidperoxidation). On the other hand, it would be possible to have a type I error due to small sample size. To confirm that combination of biomarkers may increase predictive accuracy we will need a population sample calculated for each biomarker in order to avoid errors. The association of $NO_x$and mortality should be analyzed in a larger population sample, taken into account different predictive variables and related covariates.

Previous studies have shown that alveolar •NO and therefore, NO_x^- levels were higher in lung fluid than in plasma of patients with lung edema [33]. In the present study the measured levels of NO_x^- in lung were lower in the BALF than in plasma. BALF was used as the source for lung NO_x^- determination and the procedure leads to a dilution factor of ≈ 100-fold [39]. Since we did not measure NO_x^- in other regions we could not argue that all BALF NO_x^- measured is produced at the lung. Increased •NO production and/or derived oxidants in the lungs hampers the clearance of pulmonary edema, inflammatory solutes and is accompanied by the presence of nitrated proteins [33].

BAL fluid protein concentration was 0.50 (0.17–1.28) mg/mL in sepsis versus 0.22 (0.07–1.16) mg/mL in mechanical ventilation, p non-significant. Cederfur et al. reported that BALF protein concentration in healthy subjects was 0.04 mg/mL [40]. In our study BALF protein concentration in both sepsis and mechanical ventilation was higher than reported BALF protein concentration in normal subjects which is consistent with pulmonary inflammatory response in both groups. It has been reported that mechanical ventilation may induce a proinflammatory cytokine release that triggers lung injury [41]. We did not measure other specific proinflammatory markers that could add very important information that links NO production with lung inflammation. Kobayashi et al. reported high levels of NOS expression and NO derived products and proinflammatory cytokines in BALF from ARDS patients following sepsis [18].

There are limitations to this work. First, delayed analysis after sampling time in relation with nitrotyrosine analysis might induce analytical pitfalls. Therefore, biological samples were frozen after collection and analyzed quickly. Thus, the actual data was obtained right after sample collection. Covalent Protein-Nitrotyrosine binding is stable after sample collection. Analytical methods for nitrotyrosine detection in biological samples have been reviewed. We used an immunochemical analysis for nitrotyrosine that could be semi-quantitative, this method has been validated by our group and others [27]. Since we used the same method for both groups there were no analytical bias. We could speculate that absolute values of nitrotyrosine may be different with other method, for example mass spectrometry-based. This biochemical analytical issue is not discussed in this paper, but we recognize that is still matter of debate, recently reviewed by our group [42]. Second, nitrotyrosine and malondialdehyde in ORCG BALF was not measured. Ozaras et al. reported BALF MDA concentration of 6 nanomolar in healthy control subjects measured with Richard et al. method [43]. Jafari et al. using the method of Ohkawa found 0.63 ± 0.067 nmol/mg protein concentration of MDA in BALF from healthy subjects [44,45]. On the other hand, De Andrade et al. reported ELISA measured Protein nitrotyrosine levels in BALF from healthy subjects of 28 (26–33) pmol/mg protein [46]. As a result we found higher levels of both nitrotyrosine and MDA in BALF than normal values reported elsewhere. NO_x^- values were found to be higher than in previous studies [33]. This could be due in part to the method used to analyze BALF NO_x^-, which utilizes the chemical reduction of nitrate to nitrite increasing the sensitivity of

the Griess method. The values of NO_x^- found in the ORCG recapitulate well to previous reports in control patients [33,39] where the formation of $ONOO^-$ is expected to be low. Third, the present clinical study was performed in the ICU; the role of NO_x^- at early stages of sepsis prior to admission deserves further investigation. Since we did not measure biomarkers of lung injury we could not assume that lung nitroxidative stress is associated with lung injury. Fourth, similarly, the contribution of neutrophil-derived myeloperoxidase in lung protein tyrosine nitration should be studied in future work. We used ELISA method for nitrotyrosine determination, a method with low specificity due to others mechanisms instead of peroxynitrite for protein nitration. We consider our results to be preliminary and hypothesis generating. Finally, this pilot study is not a large sample size population study and confounding factors that contribute to mortality, •NO production and oxidative stress could be concealed.

Nitroxidative stress following sepsis suggests that nitric oxide and related oxidants and proinflammatory cytokines might play a pivotal role in the pathogenesis of acute lung injury and be useful for monitoring sepsis-related organ failure. If predictive value of lung nitroxidative stress is confirmed in larger studies it may be useful at very early phases of sepsis to assess prognosis. Biomarkers also have the potential to guide ventilator management, and could be used to assess for ongoing ventilator-induced lung injury and to guide ventilator management in order to minimize lung strain.

5. Conclusions

In summary, our findings support the conclusion that pulmonary •NO production and •NO-derived oxidants are elevated very early before severe lung develops in sepsis and in mechanically-ventilated patients. Early NO_x^- production is associated with ICU outcome in patients with sepsis who received mechanical ventilation, reinforcing the pathophysiological deleterious effect of nitrooxidative stress in the lungs. Further research about pulmonary nitrooxidative stress during early stages of human sepsis is warranted.

Key messages

- Sepsis and mechanical ventilation in patients without severe lung injury are associated with early lung nitric oxide overproduction leading to nitroxidative stress
- BALF NO_X^- at admission may be related to ICU mortality

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JG, contributed to study design, data collection, data analysis, drafting and revision of the manuscript, and approval of the final manuscript and served as principal author. GP, contributed to data collection, data analysis, drafting and revision of the manuscript, and approval of the final manuscript. HB, contributed to data collection, revision of the manuscript, and approval of the final manuscript. CB, contributed to data collection, revision of the manuscript, and approval of the final manuscript. MN, contributed to data collection, revision of the manuscript, and approval of the final manuscript. EB, contributed data analysis, revision of the manuscript, and approval of the final manuscript. HC, contributed to study design, data analysis, revision of the manuscript and approved the final version of the manuscript. RR, contributed to study design, data analysis, revision of the manuscript and approved the final version of the manuscript.All authors read and approved the final manuscript.

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