Mechanical ventilation induces changes in exhaled breath condensate of patients without lung injury

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Summary
Introduction: Measurement of biomarkers in exhaled breath condensate (EBC) may be useful for monitoring lung inflammation and injury in mechanically ventilated patients. The aim of this study was to analyze changes in biomarkers of inflammation in EBC associated with prolonged mechanical ventilation.

Methods: EBC samples were collected from critically ill patients weaning from mechanical ventilation without lung disease and from healthy nonsmokers. The following parameters were measured: pH after helium deaeration, nitrogen oxide and 8-isoprostane concentrations.

Results: EBC was obtained from 10 patients and 20 controls. Ventilation time before the start of sample collection was 250 (85–714) h. The post-deaeration pH of EBC samples was significantly lower in ventilated patients than controls (7.50 [7.28–7.70] vs 8.07 [7.60–8.40]; P = 0.008). Ventilation time before sample collection inversely correlated with pH (r = −0.636; P = 0.048). A significantly higher concentration of nitrogen oxide (μM) was seen in ventilated patients vs controls (66.22 [22.26–83.13] vs 15.06 [10.73–23.30]; P = 0.002), whereas levels of 8-isoprostane (pg/mL) were not significantly different between both groups (5.73 [4.0–11.4] vs 9.09 [6.63–11.43]; P = 0.169). The nitrogen oxide concentration correlated negatively with dynamic compliance (r = −0.952; P < 0.001) and positively with respiratory rate (r = 0.683; P = 0.029).
Introduction

Mechanical ventilation provides essential support for patients with respiratory failure, but may itself cause lung injury (ventilator-associated lung injury: VALI). The main biophysical mechanisms are alveolar over-distension and repeated alveolar collapse and expansion (atelectrauma). In addition to propagating lung injury, deleterious ventilation strategies can result in the release of inflammatory mediators into the systemic circulation (biotrauma). Injurious mechanical ventilation is a risk factor for subsequent lung injury in patients with healthy lungs at the time of tracheal intubation.

The use of protective ventilation with low tidal volumes and moderate levels of positive end-expiratory pressure (PEEP) improved the prognosis of patients with acute respiratory distress syndrome, and was associated with more rapid attenuation of the inflammatory response. Thus, the current objectives of mechanical ventilation are to provide patients with adequate gas exchange, whilst minimizing the risk of developing VALI.

Inflammatory phenomena play an important role in the pathophysiology of a large variety of lung diseases. Quantifying inflammation is complex, however, but traditionally requires the use of invasive techniques such as bronchoscopy to collect samples from the lower respiratory tract. EBC analysis is an attractive option for monitoring the evolution of respiratory disease, particularly in mechanically ventilated patients, because sample collection is non-invasive and can be repeated with almost limitless frequency. Nonetheless, the effect of prolonged mechanical ventilation on EBC composition in critically ill patients with no evidence of previous lung injury is unknown.

The aims of this study were to evaluate EBC characteristics of mechanically ventilated critically ill patients without evidence of acute lung injury (ALI), and compare the acidity and concentrations of several biomarkers in EBC of these patients with those of a healthy reference population.

Materials and methods

Study population and clinical data

EBC composition of a series of critically ill mechanically ventilated (MV) patients with no acute lung injury was compared with a reference population of healthy controls (HCs). Exclusion criteria to participate in this study for the mechanically ventilated patients were: age less than 18 years, presence of ALI according to established criteria, active smoking habit, a history of chronic obstructive pulmonary disease or asthma, a diagnosis of pneumonia, surgical procedure required within 24 h before enrollment, immunosuppressive therapy (steroids greater than the equivalent of prednisolone 5 mg/day, chemotherapy, or other immunosuppressive agents within 2 weeks), pregnancy, or participation in other interventional trials 30 days prior to enrollment. The data compiled before beginning EBC collection included ventilatory parameters (respiratory rate [RR], tidal volume [VT], peak inspiratory pressure [PIP], PEEP), pulmonary gas exchange, Sequential Organ Failure Assessment (SOFA) score, heart rate, blood pressure, and temperature. Electrocardiographic, hemodynamic, and respiratory parameters were monitored during EBC collection.

The HC subjects comprising the reference population were nonsmokers who were not mechanically ventilated and were in the same age range as the patient group. Individuals aged 50–65 years were recruited from the staff of our department and the population aged 65–75 years was a group of retired individuals. The HC population was not adjusted for sex because no gender-dependent differences in acidity or biomarkers have been reported. In all subjects, the clinical examination, spirometry, and radiographic findings were normal. Subjects were excluded from the study if they had a history of asthma, allergy, chronic obstructive pulmonary disease or other lung diseases, and if they had experienced a respiratory tract infection during the month prior to the study, physician-diagnosed gastroesophageal reflux disease, or any acute or chronic systemic illness. None of the participants were taking any medication. The study was approved by the local Ethics Committee.

Exhaled breath condensate collection

In the patient group, EBC was collected using a commercially available condenser (EcoScreen; Jaeger, Würzburg, Germany) fitted with an adapter for mechanically ventilated patients (VentAdapter, FILT Lung and Chest Diagnostic GmbH, Berlin, Germany). The heat and moisture exchanger was removed one minute before starting EBC collection. The EBC condenser cooled exhaled breath at −10 °C. The collector temperature was measured at the beginning of collection.

In the HC population, EBC was collected during tidal breathing, as described, using the same condenser as in the patient group. Individuals breathed through a mouthpiece connected to the condenser while wearing nose clips. Subjects were instructed to refrain from food intake during the 2 h prior to sample collection. In both groups, EBC collection was performed to obtain approximately 2 mL of sample.

After collection, each EBC sample was divided into 500-μL aliquots in 2–4 polypropylene tubes (Biosigma, Venice, Italy). The aliquots, used for measuring nitrogen concentration, were stored at −80 °C until analyses were performed. The pH measurements of the breath condensate were conducted within 24 h of collection.

Conclusions: EBC analysis is a non-invasive technique that can be used to monitor ventilated patients. Mechanically ventilated patients had higher EBC acidity and nitrogen oxide concentrations. Duration of ventilation correlated with breath condensate pH.

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oxides (NO$_2$/NO$_3$) and 8-isoprostane (8-isopGF$_{2x}$) were immediately stored at −70 °C, and analyzed within 1 month of collection. Another aliquot was used to measure the pH after deaeration.

**pH measurement**

Immediately after deaeration with helium (350 mL/min for 10 min), pH was measured in one of the aliquots, using a model GLP 21 calibrated pH meter (Crisson Instruments SA, Barcelona, Spain) with an accuracy of ±0.01 pH, and a probe for small volumes (Crisson 50 28). The probe was calibrated daily with standard pH 7.02 and 4.00 buffers.\(^{12}\)

**Exhaled breath condensate NO$_2$/NO$_3$ and 8-isopGF$_{2x}$ analysis**

Concentrations of NO$_2$/NO$_3$ were determined by a colorimetric assay based on the Griess reaction in which sample duplicates were reacted with Griess reagent (Cayman Chemical, Ann Arbor, MI) and measured at 540 nm absorbance with a microplate reader. Assay sensitivity was 2.5 μM. Within-run and between-run coefficients of variation (CVs) were 4% and 5%, respectively.

The EBC 8-isopGF$_{2x}$ concentration was determined by a competitive enzyme immunoassay using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Assay sensitivity was 4 pg/mL. Within-run and between-run CVs were 11% and 15%, respectively.\(^{12}\)

**Measurement of α-amylase activity**

Amylase concentrations in the EBC were measured with an EnzChek Ultra Amylase Assay Kit (E-33651) from Molecular Probes (Eugene, OR). It detects activity down to a final concentration of $2 \times 10^{-3}$ U/mL.

**Statistical analysis**

Descriptive statistics were expressed as the median (interquartile range [IQR]). Differences between groups were analyzed by the Mann–Whitney test or Fisher exact test. The Spearman rank correlation coefficient was applied to determine correlations between the various parameters studied. Undetectable 8-isopGF$_{2x}$ concentrations were assigned the value of the detection limit of the method (4 pg/mL). Significance was set at a P-value of <0.05 (two-sided). SPSS 13.0 for Windows (SPSS, INC, Chicago, IL) was used for the statistical analyses.

**Results**

**General characteristics of the study population**

Ten patients (6 men) receiving MV and 20 HC subjects (9 men) were included in the study (Table 1). Median age of the MV patients was 66 years (57–75) and median age of the HC group was 60 years (52–73). There were no significant differences in age or sex between the groups ($P = 0.502$ and $P = 0.700$, respectively). Before EBC collection, patients had been mechanically ventilated for 250 (85–714) h. At the time of EBC collection, arterial pH of patients included was 7.44 (7.38–7.48) and no inhaled nitric oxide was used. All patients were in the weaning phase of MV and the conditions that precipitated MV had resolved. No evidence of active infection or colonization of the airway was present at the time of EBC collection, although three patients had received antibiotic treatment for a previous episode of tracheobronchitis. There were no adverse events related to EBC collection.

**Characteristics of the EBC samples**

Analysis of EBC composition demonstrated detectable NO$_2$/NO$_3$ in the total population of both groups, whereas 8-isopGF$_{2x}$ was detectable in 6 (60%) MV patients and 18 (90%) HC subjects. No significant difference was observed in the

<table>
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<th>Table 1</th>
<th>Main characteristics of mechanically ventilated patients before the start of exhaled breath condensate collection.</th>
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<tr>
<td>General variables</td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>66 (57–75)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (60%)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (40%)</td>
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<tr>
<td>SOFA</td>
<td>4 (2–5.8)</td>
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<tr>
<td>MODS</td>
<td>2 (20%)</td>
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<tr>
<td>Duration of MV before EBC collection (h)</td>
<td>250 (85–714)</td>
</tr>
<tr>
<td>Treated with proton pump inhibitor</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Tracheostomy</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Diagnoses leading for the indication of MV</td>
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</table>
detection rate of EBC 8-isoPGE2 between MV and HC groups \(P = 0.141\). Amylase activity was undetectable in all samples analyzed.

Exhaled breath concentrate samples showed a lower median pH [range] in the MV group (7.49 [7.28–7.70]) than the HC group (8.06 [7.60–8.40]) \(P = 0.008\) (Fig. 1a) and a significantly higher NO2/NO3 concentration (66.22 \(\mu M\) [22.26–83.13] vs 15.06 \(\mu M\) [10.73–23.30]) \(P = 0.002\) (Fig. 1b). In contrast, there were no significant differences in 8-isoPGF2\(\alpha\) concentrations (5.73 pg/mL [4.00–11.40] vs 9.09 pg/mL [6.63–11.43]) \(P = 0.169\) (Fig. 1c).

Several correlations were found between EBC biomarkers or pH and the parameters studied. First, deaerated pH inversely correlated with the duration of mechanical ventilation before EBC collection \(r = -0.636; P = 0.048\) (Fig. 2). Second, a negative correlation was found between dynamic compliance and EBC NO2/NO3 \(r = -0.952; P < 0.001\) (Fig. 3a). Finally, RR was also correlated with EBC NO2/NO3 \(r = 0.683; P = 0.029\) (Fig. 3b). In contrast, neither tidal volume (Vt) nor PaO2/FIO2 ratio showed any correlations with EBC pH or biomarkers. Likewise, no correlation was observed between arterial pH and EBC pH \(r = -0.055, p = 0.881\).

**Discussion**

In this study the changes that occur in the composition of EBC samples were investigated in critically ill patients weaning from prolonged mechanical ventilation without evidence of lung injury. A significantly lower deaerated EBC pH, as well as a higher NO2/NO3 concentration was observed in comparison to the HC reference group.

**Figure 1** Differences in exhaled breath concentrate biomarkers of mechanically ventilated patients and healthy controls. a) Deaerated pH. b) Nitrogen oxides (NO2/NO3). c) 8-isoprostane (8-isoPGF2\(\alpha\)). MV: Mechanically ventilated patients. HC: Healthy control subjects.

Furthermore, a significant inverse correlation was found between deaerated EBC pH and the duration of mechanical ventilation before EBC collection.

The pH of airway lining fluid is the product of a balance between several buffer systems and release of acids in the airways.\(^{12}\) Airway acidity is a marker of lung inflammation in several lung conditions such as chronic obstructive pulmonary disease,\(^ {25}\) and asthma exacerbations,\(^ {25,26}\) cystic fibrosis,\(^ {27}\) bronchiectasis,\(^ {25}\) and ALI.\(^ {14–21}\) To our knowledge, the present study is the first to provide data on EBC composition in critically ill patients without ALI receiving mechanical ventilation for more than 96h. The results showed a significant decrease in EBC deaerated pH in MV patients compared with the HC. This pH decrease was even more pronounced than that seen in patients with ALI,
examined with the same methods. The differences in ventilation duration prior to EBC collection between the two study populations may partially explain these findings. In fact, an inverse correlation between EBC pH and the duration of mechanical ventilation prior to EBC collection was observed. The reason why mechanical ventilation results in EBC pH decreases is unknown. One hypothesis is that mechanical stimulation of the alveoli is proinflammatory, even during short-term ventilation and when a lung-protective strategy is used. The mechanical forces occurring during conventional mechanical ventilation may damage the lung through two processes that are distinct but inter-related. Positive-pressure ventilation may damage the lung through two processes that are forces occurring during conventional mechanical ventilation. Finally, necrosis of macrophages and neutrophils recruited by cytokine stimulation disrupts tissues and cells. In fact, some processes that induce pulmonary inflammation and produce physical disruption of tissues and cells. In fact, some processes that lead to airway acidification can be activated by inflammatory phenomena. Firstly, inflammation associated with mechanical ventilation may trigger nicotamide adenine dinucleotide phosphate (NADPH) oxidase-like activity producing superoxide. Secondly, cytokine stimulation inhibits glutaminase activity which contributes to alkalization of the proximal airway and may lead to EBC acidity in asthmatic patients. Finally, necrosis of macrophages and neutrophils recruited by cytokine stimulation decreases EBC pH. On the other hand, it is unknown whether the mechanisms that cause EBC acidification in other conditions, such as increased lactate production in patients with ALI, also have a role in EBC acidification in mechanically ventilated patients. Moreover, in the present series, a possible contribution of other factors to airway acidification is highly improbable. No evidence of active infection or airway colonization was observed at the time of EBC collection, all the patients were hemodynamically stable, there was no evidence of acidemia and all had a low SOFA score. In addition, no correlation was observed between arterial pH and EBC acidity. Finally, all MV patients were treated with proton pump inhibitors (PPI). Even though one study showed no effect of PPI on EBC pH when it was collected with the same device that we used, however the fact that PPI correct chronic cough in those patients who had a low EBC pH suggests that proton pump inhibition may increase airway pH. Hence, it is quite likely that EBC acidification in this population would be due to airway inflammation probably associated with prolonged use of mechanical ventilation.

The results of the present study also showed significantly higher concentrations of NO2/NO3 in EBC of patients in the MV group compared to the healthy reference group. Moreover, EBC NO2/NO3 was inversely correlated with dynamic compliance and positive correlated with RR. Nitrogen oxide release is stimulated by mechanical forces; hence, the elevated concentration of nitrates and nitrates we observed in MV patients could be related to the mechanical stress associated with positive-pressure mechanical ventilation. The same fact could explain the correlations observed with dynamic compliance and RR. In this sense, previous studies in MV patients have detected significant correlations between the EBC NO2/Vt ratio and the Lung Injury Score and PaO2/FIO2 ratio, suggesting that increases in the EBC NO2/Vt ratio may be explained by greater mechanical stress. In the present study, however, this correlation was not found, possibly because of the small sample size and the fact that only patients without ALI were included. In ALI patients, concentrations of nitrates and nitrates were elevated in pulmonary edema fluid and plasma, associated with nitrated surfactant protein A (SP-A). Nitration of SP-A may decrease its protective role against small airway collapse; therefore, reductions in NO2/NO3 concentrations could be a potential target of therapeutic interventions in these patients. Nitrite and nitrate concentrations in EBC in the present study were higher than the levels reported in ALI patients. Although the source of NO may not be the same, this observation may relate to the recordings of exhaled NO in patients with acute respiratory distress syndrome, which are lower than those in matched ventilated controls. Recently, a strong association between higher levels of endogenous nitric oxide (NO) and better outcomes, including better survival and more ventilator-free days, in those patients has been described suggesting the possibility of a protective role of nitrogen oxides in ALI. This finding could be related to the fact that NO is produced by alveolar epithelial and endothelial cells, as well as alveolar macrophages. Therefore, destruction of alveolae that occurs in ALI may lead to a decrease in EBC NO and higher levels of NO could reflect a greater percentage of intact lung.

No differences in 8-isoPGF2α concentrations were observed between MV patients and the controls. Isoprostanes are prostaglandin-like compounds produced by free radical-induced peroxidation of arachidonic acid. Isoprostanes are markers of oxidative stress and may act as
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mediators of oxidant injury in the lung 14,15,38 as suggested in experimental studies showing that these compounds cause airway obstruction and plasma exudation. 39 In injured lung, activated neutrophils release oxygen free radicals, which may lead to peroxidation of cell membrane phospholipids, thereby increasing alveolar capillary permeability. 15 Accordingly, higher levels of 8-isoPGF2α and hydrogen peroxide have been found in patients with ALI or acute respiratory distress syndrome compared with healthy controls ventilated for surgical procedures. 15,38 More recently, a trend towards decreased 8-isoPGF2α was observed following a single dose of salbutamol in ALI patients, 20 suggesting that beta-adrenergic stimulation may play a role in preventing lipid peroxidation. Nevertheless in the present study, no significant differences were observed in 8-isoPGF2α concentrations in MV patients as compared to the HC group. This fact could be explained by different reasons. First, only patients without lung injury were included and mechanical ventilation may not, in itself, produce airway oxidative stress. Second, the low 8-iso-PGF2α concentration detected in most of EBC samples made the study resulted in insufficient power to detect any significant difference in 8-isoPGF2α concentration.

Exhaled breath condensate can be safely collected in mechanically ventilated patients; nevertheless, the technique is not exempt from limitations. First, the concentration of some biomarkers may be at the low end of the sensitivity of available assays. Second, the exact origin of EBC is uncertain, and the relative contribution of each component of the respiratory tract to the final composition of the sample obtained is unknown. Third, there is a potential for contamination of the sample by saliva, although this factor is minimized with the use of certain commercially available condensors in spontaneously breathing patients 40 and with endotracheal tubes in MV patients. The absence of α-amylase activity in the EBC samples suggests that contamination with saliva was not significant. However, most of the NH₃ in EBC is derived from saliva and it could diffuse as NH₃ gas into the EBC. Although, recent data revealed that oral ammonia is not an important determinant of EBC pH. 41 Last, the retrospective observational design of the study might not be the best means of analyzing the effect that prolonged mechanical ventilation on EBC biomarkers. However, these results demonstrate that EBC may be a useful tool in assessing lung inflammation in MV patients.

In conclusion, EBC collection and analysis is a non-invasive technique that can be safely used in MV patients to detect inflammatory markers even when there is no clinical evidence of lung injury. In our series, the duration of mechanical ventilation correlated with EBC acidity and MV patients showed increased EBC nitrogen oxides and decreased pH in comparison with healthy reference subjects.

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Conflict of interest statement

The authors of this study declare that they have no competing interests.

References


