Inflammatory mediators in exhaled breath condensate of healthy donors and exacerbated COPD patients

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ABSTRACT

Samples of exhaled breath condensate (EBC) provide a convenient and non-invasive method to study inflammation in lung diseases. The aim of the present study was to evaluate and compare the inflammatory protein mediator levels in EBC from healthy donors (HD) and from patients with exacerbation of chronic obstructive pulmonary disease (COPD) using an EBC collection device with and without a coating of albumin as a carrier. We studied 13 HD and 26 patients with exacerbation of COPD. The concentrations of myeloperoxidase (MPO), IFNγ and secretory leukocyte protease inhibitor (SLPI) in EBC were measured by immunoassays. The EBC samples from HD and COPD patients showed higher concentrations of MPO when samples were recovered with an albumin-coated device. Furthermore, levels of MPO in COPD patients were significantly higher than in HD. An inverse correlation was observed between MPO and spirometric parameters (FVC and FEV1). Almost all samples collected with the albumin-coated device showed higher amounts of IFNγ and SLPI than those collected with the uncoated device. The levels of SLPI in COPD patients were significantly higher than in HD. A direct correlation was observed between FVC% and SLPI.

We concluded that coating the collection device with albumin increased the sensitivity of the technique, at least for measurements of MPO, SLPI and IFNγ. Furthermore, the higher levels of MPO and SLPI and lower levels of IFNγ in EBC from COPD patients could reflect the immunological status and the response of lung parenchyma to treatment during the exacerbation of the illness.

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1. Introduction

Collection of exhaled breath condensate (EBC) is a non-invasive method used to obtain samples from the respiratory tract [1,2]. EBC contains water vapor, various biomarkers, volatile substances, and aerosolized particles that may reflect biochemical changes in the respiratory tract. However, several methodological issues regarding sampling and analysis have been raised [2–8]. Among them, the low concentration and the instability of some mediators have been criticized as constraints for the reproducibility of the method [4]. Many of the inflammatory mediators are volatile, such as 8-isoprostane; others are proteins and have been detected in the EBC of patients and normal volunteers [9–13]. Many cytokines are detected in the EBC such as IL-1, TNFα, IL-4 [14], IL-6 [15], IL-8 [16], IFNγ [12] and IL-10 [16]. However, other inflammatory mediators, such as secretory leukocyte protease inhibitor (SLPI), have been detected in sputum [17] and bronchoalveolar lavage (BAL) [18,19] but not in EBC.

In general, custom-built collection devices used to obtain EBC have different tubing or collection chambers with different adhesive properties [20] ranging from glass to polystyrene, Teflon and polypropylene. Given the protean nature of many inflammatory mediators, and findings in other previous publications [21,22], we speculate that adding a carrier to block the binding of the peptides to a glass device would increase the amount of protein mediators recovered. Thus, one of the aims of the present study was to determine whether pre-soaking of the 12-inch-long, 2-cm-diameter glass device with albumin might improve the detection of inflammatory mediators such as IFNγ, MPO and SLPI in non-concentrated EBC samples. Albumin was chosen as a carrier because it is an anionic protein at a pH of 7.4 and it can bind cationic molecules such as MPO and SLPI. Furthermore, the relationship between EBC inflammatory mediators and COPD exacerbation was also explored. Our results show that significantly higher amounts of MPO, IFNγ and SLPI are recovered by pre treating the glass device with albumin. Furthermore, both an increase in...
Saliva of inflammatory mediators (MPO, IFNγ or ex-smoker) were included in the study.

2. Materials and methods

2.1. Subjects

The study protocol was approved by the Ethics Committee of Hospital de Clínicas “José de San Martín”.

The enrolled population consisted of 13 healthy donors (HD) (6 men and 7 women, age range 32–60) and 26 patients with exacer-
bated COPD (16 men and 10 women, age range 57–88). The inclusion criteria were as follows: FEV1/vital capacity <70%, FEV1 <80% predicted value and all recruited patients were in stage 3 or 4 of GOLD [25]. HD and COPD patients with a smoking history (current or ex-smoker) were included in the study.

Within 24 h after admission, a spirometry (FEV1, FEV% predicted, FVC, FVC% predicted, FEV1/vital capacity), measurement of inflammatory mediators (MPO, IFNγ and SLPI) in EBC and symptom scores were performed.

2.2. Collection of EBC

A custom-built condensing device (Fig. 1) manufactured by Da-
gon (CABA, Argentina) was used to collect the EBC samples. The de-
vice consisted of an inner borosilicate glass cylinder (composed of 4% boron, 54% oxygen, 3% sodium, 1% aluminum, 38% silicon, and less than 1% potassium) (20 cm in length and 2 cm in diameter) inside a fixed condensing ice chamber (30 cm length, 4 cm in diam-
eter, 0 °C temperature). The condensate was collected at the open end of the condensing device directly into an exchangeable and disposable polyethylene tube held on ice. Then, samples were aliquoted and stored at −70 °C. The inner surfaces of the glass cyl-
der and the disposable polyethylene tube were coated before use with 1% bovine serum albumin solution (Sigma, MI, USA) for 15 min, or left uncoated. A saliva trap was used as well as a filter (applied between the mouthpiece and collection chamber), which filtered out salivary droplets. After each experiment, the device was rinsed repeatedly with twice-distilled water and subsequently left to air-dry.

Subjects underwent 12 h of fasting (no food or drink), then rinsed their mouth with water and performed tidal breathing for 15 min without a nose clip. For each subject, samples were collected twice during a period of 15 min within 2 h. One of the two collections was randomly performed with the glass device pre-soaked in a solution of albumin.

2.3. MPO detection

MPO concentrations were determined using a colorimetric ass-
say. In brief, MPO standards or EBC samples (50 μl) were added to BD Falcon™ 96-well clear ELISA plates. Then 50 μl of 3,3’,5,5’-tetramethylbenzidine (Abbott Laboratories, Argentina, Buenos Aires) substrate solution were added and incubated for 5–30 min. An acidic stop solution was then added to stop the reaction, and the absorbance was read at 450 nm. The detection limit of this assay was 7 ml/ml.

2.4. IFNγ and SLPI detection

SLPI was measured by sandwich ELISA. In brief, BD Falcon™ 96-
well clear ELISA plates were coated overnight at 4 °C in coating buffer with 1 μg/ml mouse anti-human SLPI monoclonal antibody (Clone 20,409 from R&D Systems, Minneapolis, MN, USA). Then the plates were washed and non-specific binding sites were blocked by incubating the wells with PBS-2% BSA in coating buffer for 30 min at 37 °C. Serially-diluted rhSLPI standard and samples were then added and incubated overnight at 4 °C. The wells were then washed, and polyclonal goat anti-human SLPI (Hb, Netherlands) was added. After 90 min incubation at 37 °C, the wells were washed and peroxidase anti-goat IgG (Sigma, St. Louis, USA) was added for 60 min at 37 °C. Then 50 μl of 3,3’,5,5’-tetramethylbenz-
idine (Abbott Diagnostics, Buenos Aires, Argentina) substrate solu-
tion were added and incubated for 5–30 min. The reaction was stopped with 50 μl of acidic stop solution and the plate was read at 450 nm. EBC IFN-γ levels were measured using a commercial ELISA kit (Pierce Endogen, Cambridge, MA, USA) as described by the manufacturer. The lower limits of detection for SLPI and IFNγ were 150 pg/ml and 15.2 ng/ml, respectively. All EBC samples were assayed without dilution.

2.5. Statistical analysis

Statistical analysis was performed using a Bland–Altman plot to determine the bias and agreement between measurements of EBC obtained using the glass tube device with or without an albumin coating. Also, normally and not normally distributed data were expressed as mean ± SE and median, respectively. To estimate vari-
ance within a single method of measurement (albumin coating), CV was used as described previous by Rosias et al. [26]. To avoid unnecessary inconvenience to COPD patients, variability measure-
ments were performed only with HD. For this, within a 2-h inter-
val, two samples were obtained from the same individual and collection condition, i.e. 1st and 2nd sample recovered without or with albumin coated device. Furthermore, inter-subject variabil-
ity and sequence variability were evaluated by analyzing differ-
ences between samples obtained from different individual but under the same condition (with or without albumin coated device) and different sequences of time; i.e. mediators found in the 1st sample from an HD was compared with mediators found in the 2nd sample from a different HD, both obtained under the same condition (with or without albumin coated device).

Comparisons between two groups were made using the Wilco-
xon signed-ranks test. All statistical tests performed were two-sided and p < 0.05 was accepted as significant. The corre-
lation between inflammatory and spirometric parameters was based on Spearman’s rank correlation. All statistical tests per-
formed were two-sided and p < 0.05 was accepted as significant. Condensate samples with a biomarker concentration below the

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lower detection limit were given an arbitrary value between zero and the lower detection limit as follows: 5 mU/ml for MPO, 120 pg/ml for SLPI and 12 ng/ml for IFNγ.

3. Results

3.1. Effect of albumin on the detection of inflammatory mediators

As mentioned above, EBC samples were collected using the glass tubing device randomly coated with albumin or left uncoated. Within a 2-h interval, two samples (collection device coated with albumin and uncoated) were collected from the same patient or volunteer. The clinical characteristics of the studied population are shown in Table 1. Absence of saliva in the EBC samples was confirmed by measuring salivary amylase activity with a colorimetric assay with a detection limit of 2 U/ml (Wiener Laboratories, Rosario, Argentina) (data not shown). The levels of MPO, SLPI and IFNγ were examined in the EBC samples recovered with or without albumin from healthy volunteers. Fig. 2(A) shows that the level of MPO in these samples was close to or below the detection limit of the assay when samples were recovered without albumin. However, seven out of the 13 samples from healthy volunteers turned out to be detectable. Moreover, the mean concentration was statistically significantly greater when samples were recovered with the glass tube device pre-treated with albumin compared to samples recovered with an uncoated device (p = 0.0078, Wilcoxon matched-pairs signed-ranks test). Evaluation of variability was assessed in EBC samples obtained from the same HD when both samples were taken in the absence of albumin or in the presence of it within a 2-h interval. This analysis shows that the reproducibility (expressed as CV%) did not significantly differ between samples obtained first and second without albumin (CV = 20.9% vs. 25.8%, p = 0.2345) vs. first and second with albumin (CV = 43% vs. 42%, p = 0.4309). This observation allowed us to rule out differences in MPO due to timing of the samplings.

Even though the level of MPO detected with the albumin-coated device was greater, the amount of MPO found in EBC samples from HD was still very low. Therefore, in order to increase the chances of detecting a higher amount of MPO, we next analyzed 13 EBC samples from COPD patients. Fig. 2(B) shows that EBC samples of nine COPD patients were below the detection limit of the assay (7 mU/ml) when samples were recovered without albumin. In contrast, 10 out of 13 EBC samples from the same patients using an albumin-coated device showed MPO concentrations above the detection limit of the assay. This difference between the level of mediator found in the EBC samples from COPD patients recovered with or without the albumin-coated device was statistically significant (p = 0.0182, Mann–Whitney test). Moreover, differences between HD and COPD patients were statistically significant only when samples were recovered with albumin (HD: 15.9 ± 7.86; COPD: 112.2 ± 41.21; p = 0.0182, Mann–Whitney test).

Next, SLPI was analyzed in the same EBC samples of HD and COPD patients described above. The concentrations of SLPI from EBC samples obtained using an uncoated device from most HD and COPD patients were close to or below the detection limit of the assay (Fig. 3A and B). However, when samples were recovered with albumin, the SLPI levels were higher for both groups of subjects studied (HD: p = 0.0327, COPD: patients p = 0.0005; Wilcoxon matched-pairs signed-ranks test). Moreover, samples recovered using an albumin-coated glass tube device were statistically different between HD and COPD patients (HD: 2.82 ± 0.58; COPD: 4.06 ± 0.71; p = 0.048, Mann–Whitney test), while samples collected using an uncoated device were not.

Table 1
Healthy subjects and COPD patients characteristics.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>HD</th>
<th>COPD</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
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</tr>
<tr>
<td>Gender</td>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Median ± SD</td>
<td>53.9 ± 10.1</td>
<td>69.9 ± 8.2</td>
</tr>
<tr>
<td>Range</td>
<td>32–60</td>
<td>50–88</td>
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<tr>
<td>Smoking status (%)</td>
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<td></td>
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<tr>
<td>Current smoker</td>
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<td>19.3</td>
</tr>
<tr>
<td>Former smoker</td>
<td>78.5</td>
<td>80.7</td>
</tr>
<tr>
<td>Spirometric parameters median ± SD</td>
<td></td>
<td></td>
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<tr>
<td>FEV1</td>
<td>0.87 ± 0.24</td>
<td>37.5 ± 2.5</td>
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<tr>
<td>FEV1/FVC (%)</td>
<td>46.7 ± 5.15</td>
<td>1.99 ± 0.55</td>
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<tr>
<td>FVC</td>
<td>63.3 ± 14.5</td>
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<td>FVC% predicted</td>
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Like for MPO, we did not find differences for SLPI data due to timing of the samplings among HD analyzed. Thus, the CV for samples obtained without albumin, first and second time was 29% and 39% ($p$ = 0.2099), respectively. While samples recovered the first and second time with albumin were 35% and 41% ($p$ = 0.3888), respectively.

We next analysed IFN$_c$, which has been shown to be present in small amounts in the EBC samples of children [12,27]. The concentrations of IFN$_c$ from EBC samples of 12 HD were very low but increased if samples were recovered with an albumin-coated device (Fig. 4(A); $p$ = 0.0005, Wilcoxon matched-pairs signed-ranks test). In contrast, most samples from COPD patients were below the detection limit of the assay. Only in six samples did the IFN$_c$ levels increase when the device was coated with albumin (Fig. 4B).

Furthermore, intrasubject variability of MPO and SLPI was also examined by performing the analysis of two samples obtained with a difference of time of at least 1 month. Samples obtained from HD did not show statistically differences for MPO concentration in the condensate obtained at two different time points, regardless the way of the type of device used, i.e. with or without albumin (CV = 19% and 22% ($p$ = 0.3720 for samples obtained without albumin; CV = 49% and 44% ($p$ = 0.4032 for samples obtained with albumin).

The analysis of the IFN$_c$ data for reproducibility (expressed as CV%) in HD, showed us the lack of statistically differences between samples obtained 1st and 2nd time without albumin (CV = 28% and 32% for 1st and 2nd time, respectively; $p$ = 0.2596). The same is observed with samples obtained both time with albumin (CV = 36% and 31% for 1st and 2nd time, respectively; $p$ = 0.4032).

The limit of detection of the ELISA assay is shown as a dotted line in A and B. (C) Bland–Altman plots for IFN$_c$ differences of values obtained with coated and uncoated device. The mean and the SD for difference are shown in the graph as dotted lines.
CV = 35% and 42%, p = 0.2714 for samples obtained without albumin; and CV = 53% and 51%, p = 0.1959 for samples recovered with albumin.

In order to assess agreement between the two methods of EBC recovery, we finally analysed the data using the method described by Bland and Altman [28]. Figs. 2-4(C) show a clear lack of agreement between the two methods of EBC samples for recovery of MPO, SLPI or IFNγ suggesting that the data obtained with an albumin-coated device improved the recovery of mediators from EBC samples.

3.2. Correlation with spirometry (FEV1, FVC, FEV1/vital capacity) parameters

Finally, the levels of inflammatory mediators assessed in ECB samples obtained using an albumin-coated device from COPD patients were correlated with spirometry parameters, such as FEV1, FVC and FEV1/vital capacity and % predicted values. It is important to point out that for this analysis we decided to incorporate more subjects to the study. Since, the albumin coated device allowed us to detect more amount of the mediators, samples were obtained only with albumin coated device. Fig. 5 shows an inverse correlation between FVC and FEV1 with MPO (r = −0.707, p = 0.0005; and r = −0.4513, p = 0.0262, respectively; nonparametric Spearman correlation), and a direct correlation between FVC% predicted value with SLPI (r = 0.4449, p = 0.0114; nonparametric Spearman correlation) (Fig. 5).

4. Discussion

Concentrations of solutes in EBC are often extremely low. Herein, we described a method that improves the recovery and detection of some inflammatory mediators of a peptide nature in COPD patients. In addition, we show for the first time, detection of SLPI in ECB samples.

Physicians have several methods to detect and monitor airway inflammation in respiratory diseases, such as BAL, induced sputum and EBC samples. The latter is preferred over the others due to its simplicity and non-invasiveness. Nevertheless, the usefulness of EBC in the detection of several substances has been questioned due to lack of reproducibility, low sensitivity and droplet dilution, among other issues [3,29,30]. To improve the analysis of EBC sample data, several dilutional markers have been used [31]. However, it is unclear whether a dilutional marker aids in interpretation of EBC analysis [32,33]. Therefore, we have not used a dilutional marker in this study.

Many proteins have been described in lung-derived fluid, such as MPO, lactoferrin, elastase, alpha 1-proteinase inhibitor, alpha 2-macroglobulin and even albumin [5,34]. However, the level of albumin present in lung-derived fluids can be variable depending on the degree of tissue and/or organ damage. For example, sputum albumin concentration rises across the spectrum of asthma severity [35]. Thus, variable levels of albumin in EBC could account in some way for the inconsistency of peptide detection in some samples. We believe that by binding albumin to the glass device, the binding of the putative free EBC inflammatory-derived peptides to the glass is minimized. This allows the recovery and detection of more MPO, SLPI and IFNγ in the EBC samples, and this is in concordance with our previous results and those described by Tufvesson and Bjerm [21,22]. It is important to point out that Tufvesson and Bjerm concentrated their samples and added albumin + Tween20 in order to increase the sensitivity of the method [22]. We avoided using detergent such as Tween20 because it could interfere with the detection of MPO [36,37]. Also, we did not concentrate the samples. Perhaps avoiding detergents was crucial to recover detectable mediators, which was even further improved by the addition of albumin to the glass device as well as to the collecting tube. The reliability of the recovery of the samples was another important issue. In this regard, it is important to point out that measurements of MPO, SLPI and IFNγ from the same subject were not significantly different whether samples were obtained at the beginning (time 0 h) or end of the protocol (2 h later; data not shown). Thus, the differences found in mediator concentrations were not ascribed to the sampling order but rather to the albumin coating of the device.

MPO, expressed in polymorphonuclear neutrophils (PMNs), plays an important role in innate immunity. The measurement of its activity is used as an index of inflammation, because MPO levels
are elevated in inflammatory tissue, largely secondary to PMN extravasation. It is well known that activated neutrophils develop in severe COPD, while IFNγ is increased in mild/moderate disease [38]. This is in agreement with our results since we were working with severe COPD patients (GOLD stages 3 and 4) at the time of exacerbation. Furthermore, we found differences between HD and COPD patients in MPO and SLPI concentrations, but not in IFNγ. The latter could be due to the degree of the COPD [38], and the fact that most of the patients tested for IFNγ, did not show detectable values of the cytokine, even if the samples were recovered with an albumin-coated device. This is not a surprise considering that T-lymphocyte subpopulations of COPD patients at the onset of mild exacerbations, such as the patients studied herein, show a decrease in CD4 + IFNγ/CD4 + IL-4 sputum cell ratios [39].

Although SLPI has been described in sputum [36,40] and BAL fluid [41], to our knowledge there is no report showing SLPI in EBC samples. In fact, Bayley et al. [42] using a commercially available ELISA kit (R&D Systems Europe Ltd.) with a limit of quantification of 100 pg/ml, were unable to detect significant amounts of the mediator in EBC samples from HD and COPD bronchiectasis patients [42]. The ELISA assay used in our studies was set up in our laboratory and the limit of detection was close to that of the commercial kit. The higher amounts of SLPI detected in our HD samples (using an uncoated device), compared to the Bayley et al. study, may be ascribed to differences in the collection device rather than the ELISA assay, since the monoclonal antibody used to capture the protein was purchased from R&D Systems. Another explanation for the differences with the Bayley et al. study may be the type of COPD patients recruited [42]; while we studied samples from patients with GOLD 3 and 4 stages, they examined the mediator in patients with GOLD 2 and 3 stages. Nevertheless, it is clear that albumin-coated devices helped to improve the detection of the protein, both in HD and COPD patients.

The higher amount mediators detected in the samples recovered using the albumin-coated device allowed us to perform correlation studies with spirometry parameters. Both the indirect correlation found with MPO and the spirometric parameters FVC and VEF1 suggest that lower lung function is correlated with higher degree of inflammation and mediator levels. If we consider SLPI as an alarm protein, the same observation can be assumed with this mediator. However, in this case, a direct correlation has been found because the spirometric parameter analyzed was the FVC% predicted value. In fact, Hill et al. reported that COPD patients with alpha(1)-antitrypsin deficiency at the start of the exacerbation had lower sputum levels of SLPI and increased MPO levels, but that after treatment SLPI levels increased while MPO levels decreased [40]. Therefore, the correlations observed in our study follow the same trend found in the Hill et al. study; i.e., poorer spirometric parameters correlate with a decrease in SLPI and an increase in MPO levels. Whether treatment of the COPD patients may influence these results, is not yet clear. Several studies have addressed the effect of β2 agonists and corticoids, with conflicting results. For example, the concentration of exhaled hydrogen peroxide in COPD patients admitted to hospital because of exacerbations was not reduced during treatment [43]. However, it is known that inhaled corticosteroid treatment decreased lipid peroxidation in COPD [44], and nitric oxide [45]. In our case, we did not carry out a study of the effect of therapy. Indeed, all our patients received β2-agonist, corticosteroid and antibiotic as needed to treat their exacerbation symptoms.

In the present study, we describe a method that improves the detection of inflammatory mediators. Several of these mediators may play a role in lung diseases such as COPD, but also could be useful markers for the outcome of lung transplantation rejection and infection diseases associated to the immune suppression [46,47]. In fact, several cytokines have been detected in the EBC of lung transplant patients [48]. Antus et al. showed that nine cytokines out of 120 measured exhibited more than two fold increase in patients with and without clinical evidence of bronchiolitis obliterans syndrome [48]. We believe that recovering the EBC samples with albumin would increase the chance to detect these types of mediators in this type of studies. However, improving the detection of a mediator, such as a cytokine, does not mean decreasing the coefficient of variation for the mediator. In fact, the coefficient of variation of MPO, SLPI and IFNγ in the samples recovered with albumin was higher than those recovered without albumin (data not shown). Therefore, to define the variability of a mediator in a sample, it is necessary to perform a study with a higher number of patients recruited, which can be performed under the condition that it is described in this study, i.e. with albumin coated device. Overall, these results suggest that albumin-coated glass tubes can be used as carriers to reduce the loss of biomarkers in EBC samples and, thus, increase the detection of MPO, SLPI and IFNγ, allowing detection and correlation with clinical parameters.

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