Original article

Comparison between exhaled and bronchoalveolar lavage levels of hydrogen peroxide in patients with diffuse interstitial lung diseases

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Abstract. The aim of the present study was to compare hydrogen peroxide (H_2O_2) levels resulting from oxidative stress in exhaled breath condensate (EBC) and bronchoalveolar lavage (BAL) supernatants of subjects with different diffuse interstitial lung diseases (DILDs). Twenty-one patients who underwent BAL procedure for various DILDs were studied. EBC, which was collected the day before bronchoscopy, was obtained by cooling exhaled breath; BAL was processed for differential cellular count and supernatant was stored. H_2O_2 in both fluids was measured using a commercial fluorimetric kit with a limit of detection of 0.01 μM. No difference in H_2O_2 levels in EBC and in BAL was observed [median (range), 0.07 μM (0.01-0.6) and 0.08 μM (0.01-0.8), respectively]. No correlation was observed between BAL and EBC levels. H_2O_2 in BAL did not correlate with differential cellular count, whereas H_2O_2 EBC did correlate positively with the percentage of epithelial cells (r=0.5, p=0.007) and negatively with the number of macrophages (r=-0.4, p=0.03). No correlation was observed between H_2O_2 levels either in EBC or BAL and lung function data. The data show that in DILDs H_2O_2 can be detected in both EBC and supernatants of BAL and that their relative concentrations are similar but not correlated with each other. The positive correlation between H_2O_2 levels in EBC and percentage of epithelial cells leads to the speculation that airway epithelia may play a relevant contribution in H_2O_2 production in the airway lumen. (www.actabiomedica.it)

Key words: Exhaled breath condensate, oxidative stress, hydrogen peroxide, bronchoalveolar lavage, interstitial lung diseases

Introduction

Oxidative stress (OS) processes underlie the pathogenesis of various disorders affecting the respiratory system. OS is characterised by the increased production of reactive oxygen species (ROS) and their release into the affected tissues. Superoxide anions and hydrogen peroxide (H_2O_2) are examples of ROS, i.e. compounds with the ability to form covalent bonds with nucleophilic sites of macromolecules (e.g., enzymes) with various consequences on their biochemical properties (e.g., inactivation), leading to cell malfunction and death (1, 2).

Diffuse interstitial lung disorders (DILDs) represent an heterogeneous group of diseases affecting primarily the lung interstitium (3-5). Although different for etiologic factors, for their clinical presentation and outcome, they share a pathogenetic link with OS. OS is in fact considered as a key factor in the pathogenesis of many DILDs as it seems relevant in the derangement of interstitium and in the activity of the inflammatory process (6, 7).

Assessment of OS can be achieved in a biological matrix by measuring the levels of ROS. In this context, in DILDs increased concentrations of ROS, including H_2O_2, have been found in many biological matrices, as airways, lung tissue, bronchoalveolar lavage (BAL), induced sputum (IS) and blood (8-11).

Assessment of pathogenetic processes in DILD has been accomplished mainly by studying BAL components (12-15). BAL is a methodology allowing in fact recovery from the lower respiratory tract (LRT) of cells and a-cellular components. BAL is considered useful to study the different patterns of cells and their subpopulations driving the inflammatory processes into the LRT. Among the a-cellular components, cytokines, enzymes and ROS have been measured (10-15).

Exhaled breath condensate (EBC) analysis is a new non invasive methodology to obtain information about biological matrices from the respiratory tract. It allows the measurement of many different chemical compounds dispersed in water obtained by condensation of exhaled breath. EBC has been applied to a variety of pulmonary diseases and conditions, including DILDs (15, 16).

In the context of DILDS, it is unclear to what extent the analysis of EBC mirrors the OS ongoing in the lung interstitium, as reflected by the analysis of BAL. An answer to this question could be obtained from the comparison between H_2O_2 levels resulting from oxidative stress in EBC and BAL supernatant of subjects with different DILDs.

Materials and methods

Subjects

Twenty-one patients with various DILDs entered the study. They were enrolled as consecutive patients undergoing fiberoptic bronchoscopy (FBS) and BAL for diagnostic purposes. They all gave written informed consent to the performed procedures. This study is part of a protocol centred on assessment of lung inflammation by various biological tools, including BAL and EBC, approved by the Local Internal Review Board.

At the end of the diagnostic process, the final diagnosis of the studied patients was interstitial pneumonia (UIP) form of idiopathic pulmonary fibrosis (IPF) in 8 patients, while in the other 13 subjects the diagnosis was bronchiolitis obliterans – organising pneumonia (BOOP) of various causes in 4 patients, sarcoidosis and non-specific interstitial pneumonia in 2 patients each, asbestosis, histiocytosis X, desquamative interstitial pneumonia, progressive systemic sclerosis and acute interstitial lung disease in 1 patient each (Tab. 1).

Collection and analysis of samples

FBS and BAL were performed as previously described and following international guidelines (12, 13, 17). Three 50 mL aliquots of sterile saline solution each were infused through the operation channel of the fiberoptic bronchoschope wedged in a subsegmental bronchus and, almost immediately after each infusion, BAL fluid was recovered by gentle suction. The recovered fluid from the first aliquot was processed separately from the other two aliquots. Each subsequent analysis, including the determination of ROS into the supernatants, was performed in this second "alveolar" part of BAL. BAL was processed for differential cellular count, as from international guidelines (12, 13) to obtain information useful for diagnostic purposes. BAL supernatants were stored at -70C° until its analysis for $H_2O_2$was performed.

All patients underwent EBC collection the day before BAL by cooling exhaled breath as from international guidelines and as previously described (16, 18). EBC samples were stored at -70C° until analysis was performed.

H_2O_2 in both fluids, i.e. BAL supernatants and EBC, was measured using a commercial fluorimetric kit (Amplex Red hydrogen peroxide assay kit; Molecular Probes, Eugene, OR, USA) with a limit of detection of 0.01 μM, as previously described (19). Samples were run no longer than 3 months after collection.

Statistical analysis

Data are expressed as median (range). Non-parametric statistical tests were used to analyse data. Correlations were assessed using the Spearman's test. A $p < 0.05$ was considered significant.

Table 1. Demographic and physiological characteristics of the studied patients with various DILDs

Patient n.	Diagnosis	Gender	FEV1 %	FVC %	DLCO%	P/y smoked	Steroid
1	NSIP	F	63	53	43	0	y
2	SAR	F	85	88.6	75	0	n
3	UIP	M	100	75	44.1	0	n
4	NSIP	F	100	0.82	NA	0	n
5	UIP	M	73.4	64.8	58.1	0	y
6	UIP	M	56.4	49	58.6	30	y
7	UIP	M	84	75	61.8	0	n
8	SAR	M	107	96	107	12	n
9	PSS	M	84	65	48.2	24	n
10	IST X	M	95	103	90.8	8	n
11	UIP	M	50	50	24	75	y
12	BOOP	F	56.6	55.8	60.9	10	y
13	BOOP	F	53.1	63.8	40	0	y
14	UIP	M	67.7	53.5	44.6	108	n
15	BOOP	M	64.6	69.2	39.8	60	n
16	AR	F	46.8	39	NA	0	y
17	ASB	M	89.2	69.7	115	5	n
18	UIP	F	80	62.5	NA	0	n
19	BOOP	F	NA	NA	NA	15	n
20	DIP	M	82.4	86.7	89	0	n
21	UIP	M	87	72	46.3	10	y

NSIP=Non-aspecific interstitial pneumonia, SAR=sarcoidosis, UIP=Usual interstitial pneumonia, PSS=Progressive systemic sclerosis, IST X=Histiocytosis X, BOOP=Bronchiolitis obliterans organizing pneumonia, AR=Acute interstial lung disease, ASB=asbestosis, DIP=desquamative interstitial pneumonia, P/y smoked=history of tabacco smoking expressed as pack/year, steroid=systemic steroid therapy ongoing at time of the study evaluation, y=yes, n=no, NA=data not available

Results

Both the endoscopic manoeuvres (FBS and BAL) and the collection of EBC were not associated with important side effects, a part from the usual discomfort patients experience during and immediately after FBS.

All BAL samples were suitable for a cytological analysis and their different cellular compositions were useful to establish final diagnosis, together with other relevant clinical and radiological data (Tab. 2).

Concerning H_2O_2, its level in EBC was [median (range)] 0.07 (0.01-0.6) μM, whilst in BAL supernatants it was 0.08 (0.01-0.8) μM. No difference was seen between the two matrices for the levels of H_2O_2. In addition, we observed no correlation between the two matrices levels.

H_2O_2 levels in BAL did not correlate with differential cellular count, whereas H_2O_2 levels in EBC positively correlated with % of BAL bronchial epithelial cells (r=0.5, p=0.007) (Fig. 1).

Moreover, H_2O_2 levels in EBC, correlated slightly negatively with the number of alveolar macrophages (AM)(r=-0.4, p=0.03) (not shown).

Finally, no correlation was observed between H_2O_2 levels in both EBC and BAL and lung function data.

Discussion

This study demonstrates that OS, as evaluated by the determination of H_2O_2, seems to be similarly present into the whole respiratory system, but the source of H_2O_2 in EBC seems more linked to the airways as opposed to the parenchymal compartment.

BAL is considered the golden standard to evaluate the biological processes ongoing into the LRT, including lung interstitium, i.e. the pulmonary parenchyma (3-5, 12-15). This is particularly true in patients with DILDs, in which the data from BAL are also useful in the clinical scenario (3-5). In contrast,

Table 2 - BAL cellular components in the studied patients

Patient n.	Total cells 10^6	Macrophages %	Neutrophils %	Eosinophils %	Limphocytes %	Epithelial cells %
1	13.1	91.2	2.6	1.6	4.2	0.4
2	15	63.7	2	0.8	33.5	0
3	14.2	78.3	10.2	10	0.8	0.6
4	12.9	75.7	7.6	2	14.5	0
5	6.2	81.7	10.9	1.7	5.2	0.5
6	3.6	48.8	34.7	6.6	8.6	1.3
7	37.9	77.6	1.8	2.6	17.6	0.4
8	40.1	92.6	1.5	0.2	5.5	0.2
9	19.5	58.2	5.2	12.3	23.7	0.4
10	43.8	91.4	4.2	2.4	2	0
11	4.4	52	43.3	2.2	1.9	0.5
12	NA	11.9	34.1	0	4.5	49.2
13	48.3	8.8	86.1	0.6	4.6	0
14	11.25	85.7	8.9	4.1	1.2	0
15	10.2	90.3	4.3	0	3.5	1.9
16	3.7	82.2	1.4	0	14.4	2
17	10.1	89.4	1.6	0.2	8.2	0.6
18	30.5	36.7	49.5	6.8	5.8	1
19	39.9	57.7	23.7	0	19.2	0
20	2	66.3	6.7	5.7	8	13
21	14.6	93.8	3.5	0	2.4	0.2

NA=data not available

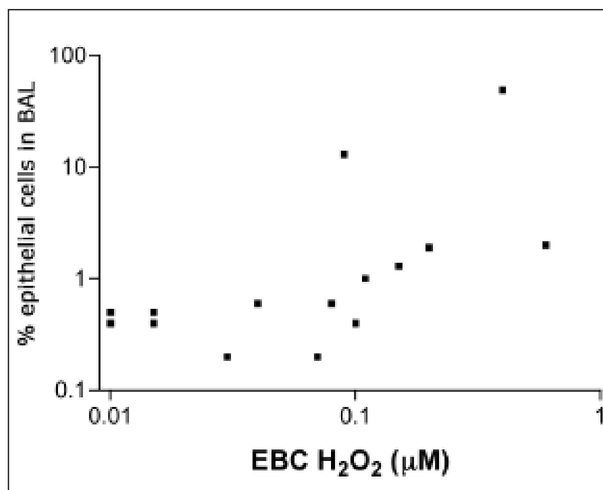

Figure 1. Correlation between the percentages of bronchial epithelial cells in BAL and the levels of H_2O_2 in EBC (r=0.5, p=0.007)

the origin and source of the different mediators and chemical compounds, including H_2O_2, measured in EBC is still elusive (8-11, 15,16). In some studies, EBC levels of ROS or of different metabolites of the nitrosative stress pathways have been considered as biomarkers of airway inflammation and derangement (18). However, EBC and its biomarkers have also been considered as usefully reflecting inflammation/disease processes in other type of disorders, such as cancer, infections and DILDs (8, 9).

We evaluated the levels of H_2O_2 in BAL and, as comparison, in EBC from a cohort of consecutive patients with different DILDs undergoing FBS for clinical purposes. Our aim was to evaluate the levels of H_2O_2, as a biomarker of OS in both matrices rather than their absolute values. This, in turn, with the aim to understand to what extent OS is ongoing into the two compartments explored by the two methodologies.

We observed similar H_2O_2 levels in the supernatant of BAL and in EBC. This may mean that a similar burden of OS is unveiled by the analysis of the two matrices, i.e. both the LRT and the respiratory system as a whole are involved in OS in patients with DILDs and this may be unveiled by both BAL and EBC analysis.

However, the H_2O_2 levels in BAL and in EBC were not correlated with each other. This may suggest that indeed the analyses of the two matrices are reflecting pathogenetic processes ongoing in different,

or only partially overlapping, compartments of the respiratory system.

This is in agreement with the study of Jackson et al. (20) which showed that markers of inflammation and oxidative stress are measurable in EBC and BAL using standard laboratory techniques but do not correlate.

The correlations between H_2O_2 levels in EBC and % of BAL bronchial epithelial cells and alveolar macrophages seems to indicate that, at least in our patients with DILDs, EBC is reflecting more the OS ongoing in the airways than that of the parenchyma.

Bronchial epithelial cells are considered a by-product of BAL. In analysing the cellular components of BAL, many authors consider that great proportions of epithelial cells may indicate a "bronchial contamination" of the BAL samples, thus suggesting not to consider that particular sample useful for subsequent analysis, as it would not reflect the "alveolar" compartment (12-15). We evaluated the recovered fluid from the infusion of the second and third 50 mL aliquots of BAL. This is considered a good step to avoid contamination of BAL from bronchial cells and to indicate that a BAL sample is really representative prevalently of the alveolar compartment. In addition, the vast majority of our BAL cell counts showed a percentage of epithelial cells < 2.5%, i.e. a proportion showing the fact that the BAL sample under analysis is more reflecting the alveolar compartment than the bronchial one.

Nevertheless, any BAL sample is also containing fluids and solutes, including reactive species as H_2O_2, related to the airway tree, albeit in its lower part (14). For thee reasons we believe that our data are indicating that OS ongoing and unveiled by EBC analysis in DILDs is more related to the pathogenetic processes taking place into the airways than to those occurring into the parenchyma.

Finally, this work may be considered part of the efforts aimed at understanding the contribution that biomarkers can give in the evaluation of pathogenetic processes ongoing into the respiratory system. In this context, the comparison of the same biomarkers, in our case a marker of OS, in different biological sources, BAL and EBC, can give precious information not only on the pathogenesis of a group of disorders, but it can be useful to understand the different compartment evaluated by different methodologies. This will be of paramount importance to study each disease process with the appropriate methodology, in order to gain clearer information that, hopefully, will be useful also in the clinical scenario.

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