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Changes in sputum composition during 15 min of sputum induction in healthy subjects and patients with asthma and chronic obstructive pulmonary disease

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Summary
Introduction: The use of sputum induction by inhalation of hypertonic saline to study the cellular and biochemical composition of the airways allows noninvasive sampling of the airways content and identification of markers of airways inflammation.
Objective: The present study aimed to identify possible changes in the cellular composition of induced sputum between samples obtained sequentially (three periods of 5 min each) during one sputum induction. Moreover, difference between these samples and the mixed one (mixture of samples obtained after 5, 10 and 15 min of induction) was investigated.
Methods: Forty-six subjects (10 healthy volunteers, 12 patients with chronic obstructive pulmonary disease (COPD) and 24 patients with asthma) (mean age 53.0±14.0 yr, forced expiratory volume in one second (FEV_1) 71.8±19.0% pred) produced sputum after three consecutive 5 min periods of hypertonic (4.5%) saline inhalation. Stained cytospins from the three periods separately and from the mixed sample were produced and analyzed.
Results: The mean percentage of neutrophils, eosinophils, lymphocytes and epithelial cells did not change significantly in samples obtained consecutively after 5, 10 and 15 min

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of the induction procedure. There was no significant difference in the cellular composition of samples obtained after 5, 10 and 15 min of induction and the cellular composition of the mixed sample ($P = 0.06$).
Conclusion: The separate analysis of induced sputum from three consecutive sampling and the mixed sample did not demonstrate significant changes in their cellular composition. Fifteen minutes induction procedure with the fixed concentration of hypertonic saline and processing of the mixed sample can be recommended for clinical settings and clinical trials.

Introduction

The use of sputum induction by inhalation of hypertonic saline to study the cellular and biochemical composition of the airways has increased significantly since 1990s. The induced sputum technique allows sampling of the airways content in a noninvasive fashion and offers a unique opportunity to identify biomarkers of potential clinical utility in respiratory medicine.[1] Adequate sample of lower airway secretions can be collected during the induction procedure from patients who are not able to produce sputum spontaneously, and the features of airway inflammation in asthma, chronic obstructive pulmonary disease (COPD) and other respiratory disorders can be studied. The method of sputum induction has been demonstrated to be reproducible, sensitive and valid.[2–4]

At least three studies have reported the changes of cellular and biochemical constituents of induced sputum during the induction procedure.[5–7] One of these studies showed that percentages of neutrophils decreased and percentages of macrophages increased significantly in the induced sputum of asthmatics and healthy individuals obtained during three consecutive inhalation periods of 10 min each of one induction procedure.[5] The second study demonstrated pronounced differences in neutrophil counts in two consecutive samples of sputum derived from healthy subjects within 30 min of one induction procedure.[6] However, in the COPD group, percentages of neutrophils did not change between the two sputum inductions.[6] The third study collecting sputum samples at 4 min intervals during the 20 min induction procedure has also identified significant changes in the cellular composition.[7] In particular, the percentages of eosinophils and neutrophils decreased, while the percentages of macrophages increased.

Changes of the cellular composition during the induction procedure can introduce a bias in clinical settings and in clinical trials. Moreover, the standardized methodology of sputum induction and processing states that shorter inhalation times (e.g. 15–20 min) appear to have similar success rates and feasibility to longer inhalation times (30 min).[8] For most purposes, the consensus is to use a cumulative duration of nebulization of 15–20 min.[8] To the best of our knowledge, none of the published studies has analyzed possible significant changes in the cellular composition of different samples obtained sequentially during 15 min of inhalation procedure. Moreover, not all patients and healthy subjects are able to produce adequate samples of sputum during the whole induction procedure. This problem may be solved by mixing available samples and analyzing the mixed sample. To the best of our knowledge, none of the studies has evaluated possible changes between consecutive sputum samples obtained during one induction procedure and the mixed one.

Thus, the aim of the present study was to identify possible changes in the cellular composition of induced sputum between samples obtained sequentially (three periods of 5 min each) during one sputum induction. Moreover, difference between these samples and the mixed one (mixture of samples obtained after 5, 10 and 15 min of induction) was investigated.

Materials and methods

Study subjects

Forty-six subjects (10 healthy volunteers, 12 patients with moderate COPD and 24 patients with mild to severe asthma) were selected from already examined group consisting of individuals who easily produce sputum samples. All subjects produced easily all three samples of sputum during 15 min of the induction procedure. Demographic and clinical data for all subjects enrolled in the present study is presented in Table 1. Healthy subjects were aged-matched; they had no history of asthma, chronic bronchitis, wheezing or allergies. The diagnosis of COPD was based on the Global Strategy for the diagnosis, management and prevention of COPD (GOLD).[9] The diagnosis of asthma was based on Global Initiative for asthma (GINA).[10] Treatment of patients with asthma and COPD was performed according to GOLD and GINA standards, respectively.

The protocol was approved by the Local Committee of Fondazione Salvatore Maugeri and all subjects gave their informed written consent.

Study design

The subjects visited laboratory during one day. Written consent was obtained, subjects characteristics were documented, and spiromentry was performed followed by inhalation of 200 μg salbutamol and sputum induction for three consecutive periods of 5 min each (totally 15 min). The selected portions of sputum were analyzed.

Sputum induction

After baseline FEV_1 and forced vital capacity (FVC) measurements, subjects inhaled 200 μg salbutamol from a

Table 1 Demographic and clinical data of 46 subjects enrolled in the study.*

Group	*n*	Gender M/F	Age (yr)	FEV_1 (% pred)	FVC (%)
Healthy	10	1/9	53.3±16.5	100.1±6.8	100.6±5.3
COPD	12	1/11	53.2±6.5	53.2±6.5	79.9±6.0
Asthma	24	12/12	49.0±11.8	69.4±11.5	84.6±7.4
Total	46	14/32	53.0±14.0	71.8±19.0	86.9±10.0

M, male; F, female; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; COPD, chronic obstructive pulmonary disease.
*Data are expressed as mean±SD.

metered dose inhaler. Then, subjects inhaled hypertonic (4.5%) saline from an ultrasonic nebulizer (DeVilbiss 65, DeVilbiss Corporation, Somerset, PA, USA)[11] for three consecutive periods of 5 min each (totally 15 min). FEV_1 was measured 1 min after each inhalation period to detect any bronchoconstriction. Severe adverse effects and complications were absent in healthy volunteers and patients. The sputum samples produced after the first, the second and the third 5 min inhalation periods were processed separately. The fourth or the mixed sample was obtained by mixing together portions of samples derived after 5, 10 and 15 min of induction. Sputum samples were examined within 2 h after induction.

Sputum processing

Sputum was collected and processed as previously described.[11] In brief, selected portions of sputum sample originating from the lower respiratory tract were chosen using an inverted microscope and weighed. Dithiothreitol (DTT) (Sputolysin, Calbiochem Corp, San Diego, CA, USA) freshly prepared in a dilution of one in 10 with distilled water, was added to sputum sample. The volume (in μl) of DTT was equal to four times the weight of the sputum portion (in mg). The samples of selected sputum were placed in a shaking water bath at 37 °C for 20 min to ensure complete homogenization. The liquid resulted from selected portions was further diluted with phosphate buffered saline (PBS) in a volume equal to the sputum plus DTT. The suspensions were filtered through nylon gauze to remove mucus and were centrifuged at 1000 rpm for 5 min. The supernatants were aspirated and frozen at −70 °C for later analysis. The cell pellets were resuspended in PBS. Total cell count (TCC) and viability (Trypan blue exclusion method) were determined using a Burkers chamber haemocytometer. Cell suspensions were placed in a Shandon 3 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA) and cytospins were prepared at 450 rpm for 6 min. Cytospin slides were fixed by methanol and stained by May Grunwald Giemsa for overall differential cell count on 500 nucleated nonsquamous cells. Definition of an adequate selected sputum was one in which there were fewer than 20% squamous cells and viability >50%.[11]

Statistical analysis

Descriptive statistics were used to summarize clinical and demographic characteristic of the subjects. The results were expressed as mean and standard deviation for age, lung function values and cellular composition of induced sputum. The comparison of differential cell count between 5, 10, 15 min and the mixed samples and the comparison of differential cell count between patients with asthma, COPD and healthy volunteers were assessed by ANOVA. A value of $P<0.05$ was considered statistically significant.

Results

The cellular composition of samples obtained after 5, 10 and 15 min of induction procedure and the mixed sample

The weight, TCC and the cellular composition of samples from 46 subjects (healthy volunteers and patients with asthma and COPD) obtained after 5, 10 and 15 min of one induction procedure and the mixed sample are presented in Table 2 and Fig. 1. No statistical difference in TCC and the mean percentage of neutrophils, eosinophils, lymphocytes and epithelial cells was observed between samples obtained after 5, 10 and 15 min of induction. The mean percentage of macrophages was significantly different ($P<0.05$) in samples obtained consecutively after 5, 10 and 15 min of the induction procedure. However, there was no significant difference in the cellular composition of samples obtained after 5, 10 and 15 min of induction and the cellular composition of the mixed sample ($P=0.06$) (Fig. 1). When analyzing three different groups of subjects (healthy volunteers and patients with asthma and COPD) separately, no significant difference was found in the mean percentages of cells in samples obtained after 5, 10 and 15 min of induction and the mixed sample ($P>0.05$). The significant increase of macrophages in three samples (5, 10 and 15 min) was characteristic for patients with asthma ($P<0.05$). When analyzing the difference in the cellular composition in absolute numbers, no statistical difference was observed between samples obtained after 5, 10 and 15 min of induction and between the three samples and the mixed one (Fig. 1).

The cellular composition of induced sputum obtained from healthy volunteers and patients with asthma and COPD

Sample obtained 5 min after induction from patients with asthma and COPD were characterized by decreased

Table 2 Weight and TCC in 5, 10, 15 min and the mixed samples obtained from healthy volunteers and patients with asthma and COPD.*

Group	Healthy	Asthma	COPD	Total
Weight mg				
5 min sample	65±50	93±71	112±100	92±76
10 min sample	76±65	91±64	72±57	83±61
15 min sample	91±83	76±74	58±50	75±69
Mixed sample	211±167	252±136	229±157	237±146
TCC × 10^6 cells mL^{-1}				
5 min sample	1.0±1.2	1.2±1.4	1.3±1.2	1.2±1.3
10 min sample	0.8±1.2	0.8±1.1	1.2±1.4	0.9±1.2
15 min sample	0.8±0.8	0.7±0.9	1.5±1.6	0.9±1.1
Mixed sample	0.8±0.8	0.9±1.1	1.3±1.2	1.0±1.0

COPD, chronic obstructive pulmonary disease.
*Data are expressed as mean±SD.

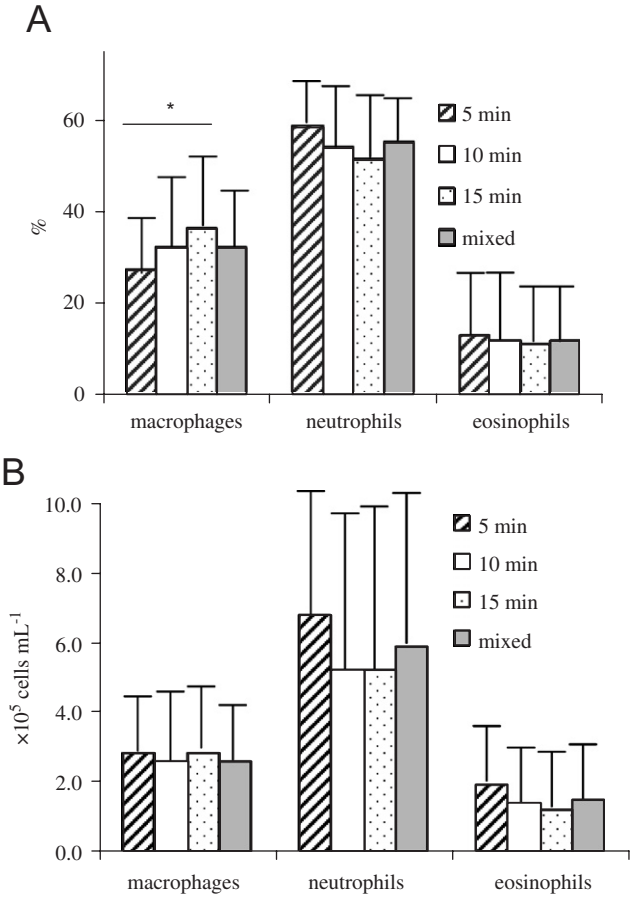

Figure 1 The comparison of differential cell count in percentages (A) and absolute numbers (B) between 5, 10, 15 min and the mixed samples for 46 subjects involved in the present study; *$P<0.05$.

($P<0.05$) percentage of macrophages compared with samples from healthy volunteers (Fig. 2). The percentage of neutrophils in all samples obtained from patients with asthma and COPD was increased significantly ($P<0.05$, $P<0.001$) in comparison with samples from healthy volunteers. The percentage of eosinophils in all samples obtained from patients with COPD and asthma were significantly higher ($P<0.001$) than those in samples from healthy volunteers.

Discussion

According to the aim of our study, we identified changes in the percentage of macrophages in samples obtained sequentially (three periods of 5 min each) during one induction procedure. However, the difference in macrophages expressed in absolute numbers was not significant. Moreover, we compared the cellular composition of samples derived after 5, 10 and 15 min of one induction procedure with the cellular composition of the mixed sample. No significant changes were identified. In addition, specific differences of the cellular composition of induced sputum samples obtained from healthy volunteers and patients with asthma and COPD were evident. The sputum of patients with asthma was characterized by eosinophilia. Neutrophilia was characteristic for sputum obtained from patients with COPD.

Previous studies with long (30 min)[5,6] and short (20 min)[7] induction procedure have documented substantial changes in the cellular composition of induced sputum. In our study, only the increasing trend of macrophages in 5, 10 and 15 min samples was found significant, which was specifically characteristic for patients with asthma. Technical factors including the duration of inhalation procedure, the concentration of saline solution, analysis of entire or selected parts of sputum and pretreatment with bronchodilator might have influenced the procedure and the cellular characteristics of induced sputum. The concentration of saline solution used for sputum induction was different in all studies. The concentration of saline in the study of Holz et al.[5] was changed during the procedure, starting with 3% and subsequently increasing to 4% and 5%, while the concentration of saline in the study of Richter et al.[6] (3%), Gershman et al.[7] (3%) and our study (4.5%) was constant. All patients enrolled in these studies received pretreatment with 200 μg

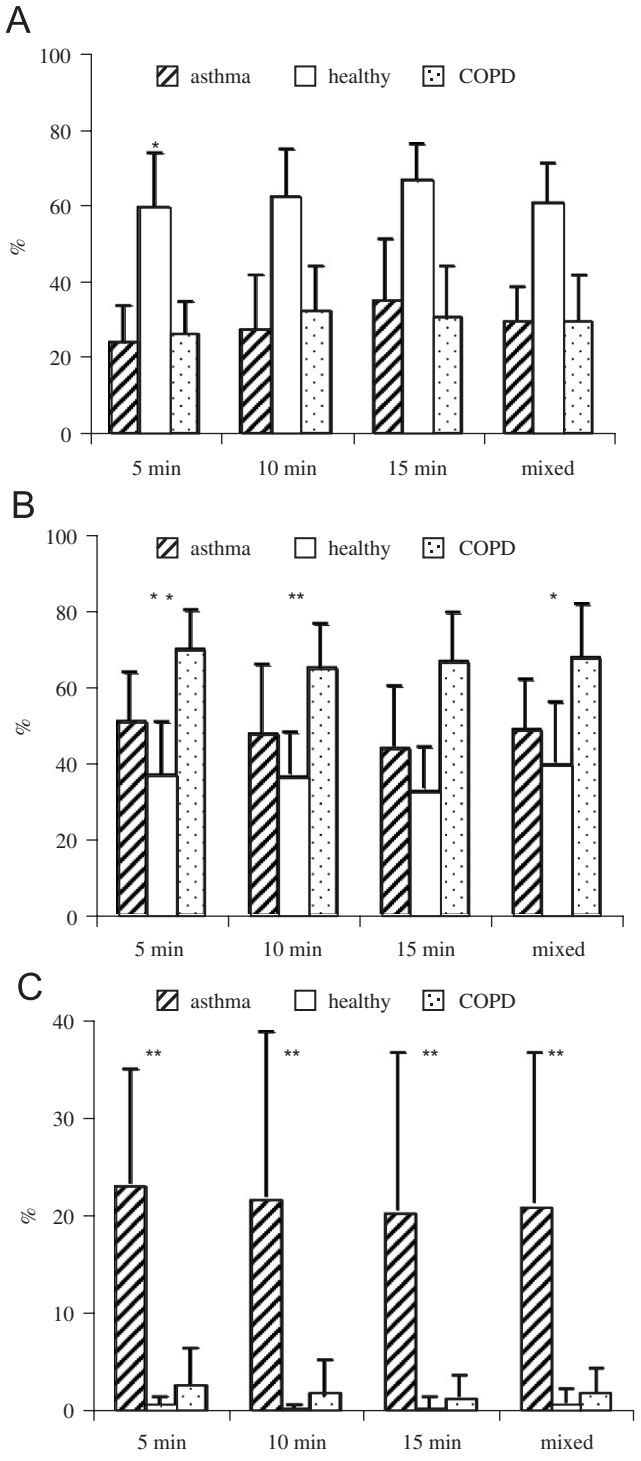

Figure 2 The comparison of differential cell count (A—macrophages, B—neutrophils and C—eosinophils) between patients with asthma, COPD and healthy volunteers. COPD: chronic obstructive pulmonary disease; $^{*}P<0.05$; $^{**}P<0.001$.

salbutamol (Holz,[5] Ritcher[6] and our study) or 360 μg albuterol (Gershman et al.[7] study). It is possible to suggest that saline concentration and the dose of bronchodilator might have influenced the procedure and different results. However, evidence has been provided that different saline concentrations and pretreatment with bronchodilator do not affect TCC and differential cell count.[12,13] Three of the studies (Holz et al.,[5] Ritcher et al.[6] and our study) used selected plugs of sputum for analysis, while Gershman et al.[7] preferred to analyze the entire expectorate. Conflicting data exists whether or not differential cell count differs between the two methods (selected sputum and entire sputum).[14] Thus, using different techniques in sputum processing and, in addition, different criteria for subject selection in the studies might have influenced the results.

The originality of our study includes comparison of the cellular composition of three consecutive samples obtained during 15 min of one inhalation procedure with the cellular composition of the mixed sample. The cellular composition of samples obtained after 5, 10 and 15 min of induction was similar to the cellular composition of the mixed sample. Thus, the mixed sample can represent the real picture of the induced sputum cellular composition in healthy subjects and patients with COPD.

Considering absence of significant difference in the cellular composition between the three samples obtained during 15 min induction procedure, each individual sample (5, 10 or 15 min) can be utilized for analysis. This can be applied in particular to subjects who are not able to produce all three adequate sputum samples of secretions from lower airways during induction. Moreover, the tendency of increasing of macrophages suggests that the optimal procedure should include the analysis of the mixed sample in order to avoid any bias during interpretation of the results. The analysis of the mixed sample can be also relevant for the situation when a patient has produced only two adequate samples of sputum. Current recommendations of the European Respiratory Society for sputum induction and processing highlights the need to standardize the duration of sputum induction.[8] Moreover, the standardized methodology of sputum induction and processing states that shorter inhalation times (e.g. 15–20 min) appear to have similar success rates and feasibility to longer inhalation times (30 min).[8] The duration of the inhalation procedure in our study was constant (15 min), which is in agreement with the consensus to use cumulative duration of nebulization of 15–20 min.[8] Thus, 15 min induction procedure and processing of sputum using the mixed sample can be recommended.

The analysis of differential cell count revealed specific patterns relevant for patients with asthma and COPD. The percentage of neutrophils is usually increased in sputum of COPD patients.[15] In our study, the neutrophil count in all samples obtained from COPD patients was significantly higher than that from healthy volunteers. We observed significantly increased percentage of eosinophils in all samples obtained from patients with asthma in comparison with samples from healthy volunteers and patients with COPD. All samples from patients with COPD were characterized by the significantly higher percentage of eosinophils than the samples obtained from healthy volunteers. This is in agreement with already existing knowledge that subjects with severe acute asthma and up to 40% of subjects with COPD usually exhibit marked sputum eosinophilia.[15]

In conclusion, the separate analysis of induced sputum from three consecutive sampling and the mixed sample did not demonstrate significant changes in their cellular

composition in healthy volunteers and patients with COPD and asthma. Fifteen minutes induction procedure with the fixed concentration of hypertonic saline and processing of the mixed sample can be recommended for clinical settings and clinical trials.

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