

Manuscript type: Original Article

DOI: 10.5152/TurkThoracJ.2018.180147

Title: IS INDUCED SPUTUM A USEFUL NONINVASIVE TOOL IN DIAGNOSIS OF PULMONARY SARCOIDOSIS?

Short title: Induced Sputum in Sarcoidosis

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Received: 10.09.2018

Accepted: 05.11.2018

Cite this article as: Baha A, Yıldırım F, Stark M, et al. IS INDUCED SPUTUM A USEFUL NONINVASIVE TOOL IN DIAGNOSIS OF PULMONARY SARCOIDOSIS? Turk Thorac J 2018; DOI: 10.5152/TurkThoracJ.2018.180147

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Abstract

OBJECTIVE: Provokation of sputum expectoration by inhalation of hypertonic saline (NaCl) has been investigated as an alternative diagnostic tool to invasive procedures in patients with pulmonary sarcoidosis. We aimed to investigate the diagnostic value of IS by looking at cell distribution of sputum in patients with confirmed histopathological diagnosis of sarcoidosis.

MATERIAL AND METHODS: In this prospective, cross sectional study, we compared IS results of twenty histopathologically confirmed pulmonary sarcoidosis patients with twenty-four healthy volunteers. Percentages of macrophages, lymphocytes, neutrophils, eosinophils in IS, and the CD4+/CD8+ ratio were compared.

RESULTS: The percentage of lymphocytes in IS was significantly higher in pulmonary sarcoidosis than the control group (41.6% vs 8.9%, $p < 0.001$). There were no significant differences in the other IS cell percentages and CD4/CD8 ratio between the groups. Sputum induction was well tolerated.

CONCLUSION: Induced sputum (IS) by inhalation of hypertonic saline is safe, inexpensive, less invasive, and easily repeated method can be a valuable diagnostic tool alternative to other invasive diagnostic methods in the diagnosis of pulmonary sarcoidosis.

Keywords: Clinical problems, diagnostic methods, interstitial lung disease

INTRODUCTION

Provokation of sputum expectoration had been first described in 1958 by H. Bickerman by inhalation of hypertonic saline (NaCl). Bickerman had used this technic for the diagnosis of lung cancer patients by obtaining cytological material from lower respiratory tract [1]. Then this induced sputum (IS) technic was used to diagnose various pulmonary conditions such as pulmonary infections and airway inflamations in different pulmonary stiuations [2-5].

Sarcoidosis is characterised with hilar lymphadenopathy, pulmonary infiltration, and ocular and cutaneous lesions. It is described as a systemic granulomatous disease and mostly affecting young adults. The causes of sarcoidosis are not known exactly. The diagnosis of pulmonary sarcoidosis is mainly based on radiologic futures and the detection of nonnecrotisan granulomas in histopathological materials [6]. Histopathological materials [parenchymal, nodal biopsies and

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bronchoalveolar lavage (BAL)] to diagnose pulmonary sarcoidosis can be taken with fiberoptic bronchoscopy (FOB), video assisted thoracoscopy (VATS), and open lung biopsy. All of these procedures are firstly invasive and are not guaranteed to show granulomas within the obtained sample. Among them, FOB is the least invasive one, but it is not free of complications. Diagnostic accuracy of FOB in the sarcoidosis diagnosis is near to 85 %. But it is not always possible to perform FOB in patients with suspected sarcoidosis. Some patients can refuse the procedure and to refer to FOB as an uncomfortable procedure [7-9]. In such situations to be sure for the pulmonary sarcoidosis diagnosis clinicians need to have noninvasive diagnostic methods such as IS.

Pathophysiology of pulmonary sarcoidosis is based on alveolitis caused by macrophages and T- cell lymphocytes [10]. Cell distribution in the BAL reflects the inflammation of the interstitium and analysis of cells within the BAL can be thought to be a diagnostic tool. In previous studies, lymphocytes, CD41 lymphocytes, and activated macrophages was found to be high in BAL of patients with pulmonary sarcoidosis [11,12]. In recent studies the use of IS alternative to BAL was investigated in sarcoidosis patients [13-16]. The results of studies in this field were conflicting and the efficiency of this method has not yet been clearly established.

We aimed to investigate the diagnostic value of IS by looking at cell distribution of sputum in patients with confirmed histopathological diagnosis of sarcoidosis.

MATERIAL AND METHODS

Study Population

This prospective, control group study was performed within a pulmonary clinic of a university hospital. The ethics committee of the institution had approved the study (University Research Ethics Committee of Medical Faculty, 19.01.2009 and No: 19). The study has been conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. An informed consent was obtained from both patients with sarcoidosis and control group.

Patient characteristics

Inclusion criteria: 20 newly diagnosed sarcoidosis patients without taking any treatment for sarcoidosis and having these criteria:

- Older than 18 years old
- Don't have any other respiratory diseases
- Non-smokers
- Had no symptoms of respiratory tract infection for the last four weeks

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- Don't receive oral or inhaled corticosteroids or antibiotics during the last 3 months prior the study.
- Having forced expiratory volume at 1 st (FEV1) were above 60%
- Don't have hypoxaemia and oxygen saturation above 90% at rest

Control group included 24 healthy volunteers having the same inclusion criteria [14].

Sarcoidosis Diagnosis and Staging

The diagnosis of pulmonary sarcoidosis had been established in accordance with the recommendations adopted by an international panel of experts [17]. All of the sarcoidosis diagnoses were confirmed histopathologically. Staging of patients' sarcoidosis was made by radiologically with chest X-ray.

Induced Sputum and Processing

Sputum was induced through inhalation of an aerosol of hypertonic saline as described by [18]. The sputum samples were processed by selecting the mucus plugs, mixing with four parts 0.1% dithiothreitol (DTT) and Dulbecco PBS, filtered through a 48-mm nylon mesh, and then centrifuged at 3000 rpm for 10 min at 4 °C. The cell pellet was resuspended in phosphate-buffered saline. The filtered cells were diluted by RPMI 1640 medium and cytopsin slides were prepared by cytocentrifuge (Shandon, Thermofisher Scientific) for 5 min at 1000 cycles. The cytopsin slides were stained with Giemsa. Samples containing less than 20% squamous cell were considered eligible for the study [19].

Pulmonary function tests

Pulmonary function tests (PFT) (Sensormedics Vmax Series 20C Respiratory Analyzer, Yorba Linda, California, USA) were enforced considering American Thoracic Society's guidelines [20]. Forced Expiratory Volume in 1 second (FEV1), Forced Vital Capacity (FVC), FEV1/FVC, carbonmonoxide diffusion capacity (DLCO), Vital Capacity (VC), Total Lung Capacity (TLC) of the participants were recorded.

Total cell count and T-cell phenotyping in IS

Total cell count per μ l of the processed sample was then assessed by flow cytometry (Cell Dyn 3200, Abbott, USA), and two cytopsin slides were prepared using 300 ml of the solution for each of them (Cytospin 3, Shandon Southern Instruments, USA). Cytospin slides were spotted with May-Grünwald-

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Giemsa and analysed by an experienced microbiologist, who received have no information regarding the participants. Differential cell count was performed combining all the cells contained in the cytopsin slides, including at least 400 non-squamous cells. Then count of macrophages (M), eosinophils (E), lymphocytes (L), neutrophils (S) was reported [21,22].

T-cell subpopulation analysis was carried out by flow cytometry using the FACSCalibur™ system (Becton Dickinson, San Jose, CA, USA) and the TriTEST CD4/CD8 monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instructions. The monoclonal antibody panel allowed us to assess the following T-cell subpopulations: CD4 (helper T cells) and CD8 (cytotoxic T cells). The anti-CD4, and anti-CD8 antibodies were conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) [23].

Statistical analysis

Datas were analyzed using SPSS (Version 21.0, Chicago, SPSS Inc). Age was presented as mean±standard deviation (SD). Data were expressed with frequency distribution and percentages. The Mann–Whitney U tests were used for comparing the differences between the groups. P values of <0.05 were considered statistically significant.

RESULTS

Forty-four patients were enrolled in the study (20 patient group, 24 control group). Seventy-two percent of them were female and their mean age was 41±12 years. Characteristics of patients and controls are seen in Table 1.

All of the sarcoidosis cases had the histopathological sarcoidosis diagnosis (15 patients with FOB, 3 patients with mediastinoscopy, 1 patients with VATS, 1 patient with supraclavikular lymph node biopsy). According to staging; 4 (20%) patients had stage I disease and 16 (80%) patients had stage II disease.

Control group included 24 healthy volunteers (18 women; age range, 21 to 72 years old).

Patients with sarcoidosis were older than control subjects and had lower FVC, VC and TLC, but these differences were no statistically significant. FEV1 (88.6% vs 101.2%, p=0.020), FEV1/FVC (79.8% vs 87.8%, p=0.001) and DLCO (68.0% vs 138.0%, p=0.04) values of sarcoidosis patients were statistically lower than control group. All patients and controls tolerated sputum induction well, without adverse events. The sputum samples were adequate in all subjects.

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The cell counts in IS from patients and normal subjects are reported in Table 2. Accordingly, the lymphocyte ratio of the patient group in the IS was statistically significantly higher than the control group (41.8% vs 8.90%, $p < 0.001$). But there were no difference between groups in terms of the CD4/CD8 ratio (4.5% vs %31 , $p=0.058$) (Table 2 and Figure 1).

DISCUSSION

In this study, we showed that IS samples from newly diagnosed, untreated patients with pulmonary sarcoidosis contain significantly more lymphocytes than healthy volunteers. As far as we know, this is the first report from our country about the comparison of cellular distribution together with T-cell subtyping in the IS of sarcoidosis patients.

Broncho-alveolar lavage (BAL) is a useful diagnostic tool to collect the cells and secretions from the especially lower respiratory tract [24]. BAL specimens, showing the inflammatory response in interstitial lung diseases has been used as a diagnostic tool in many specific diseases including sarcoidosis [25]. Although clinicians have the opportunity to obtain cells from the peripheral part of the lung it is a relatively invasive tool, it should be applied in proper conditions. The only currently available noninvasive method to directly study cellular inflammatory processes in the lower respiratory tract is the examination of sputum. IS provide cellular sample from the central region of the bronchial tree. Therefore, it can be considered as an unsuitable tool for the diagnosis of pulmonary sarcoidosis. However, it has been proven that the 20-min IS procedure can be used to obtain samples from the distal part of the lung [26]. Furthermore, lymphocytic infiltration in sarcoidosis can be involved not only in the interstitium but also in the bronchial epithelium [27,28]. This relatively cheap, less invasive, and easily repeatable procedure can be a precious diagnostic tool alternative to FOB and BAL.

Firstly, IS was studied as new diagnostic method for sarcoidosis diagnosis in the study of D'Ippolito et al. [13]. They examined IS of 15 newly diagnosed, untreated sarcoidosis cases and 12 healthy volunteers. They found that patients with sarcoidosis had a significantly more number of total cells (65.1% vs 30.1%, $p=0.01$), lymphocytes (9.4% vs 3.8%, $p=0.05$) in IS compared with healthy volunteers, while the number of macrophages was statistically significantly lower (60.4% vs 69%, $p=0.05$) [13]. After this study many studies have been conducted to investigate the value of IS in the diagnosis and clinical follow-up of sarcoidosis cases.

Lymphocyte count in IS in the sarcoidosis group was 41.6% in our study. The number of lymphocytes count in IS of our patients with newly diagnosed pulmonary sarcoidosis is comparable to that

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obtained in other studies ranging from 0.4 to 67.1% [13-15]. The evaluation of the lymphocyte counts and CD4/CD8 in BAL specimens is still in use and recommended in the clinical assessment of patients with pulmonary sarcoidosis [29]. Although, lymphocyte count of sarcoidosis cases were higher than control group, we couldn't find any difference in CD4/CD8 ratio between groups. We attributed this result to low number of our study population. Also our study population was homogeneous, both of the sarcoidosis and control groups were never-smoked, and none of them have another additional lung diseases. Previous studies that found higher CD4/CD8 ratio in IS of sarcoidosis patients mostly compared this ratio with non-sarcoid interstitial lung diseases. (NS-ILD) [23,30,31]. In our study we compared CD4/CD8 ratio in IS of sarcoidosis with healthy subjects and we didn't find any difference between CD4/CD8 ratios of groups. We attributed this indifference to high level of CD4/CD8 ratio of our control subjects.

Similar to our results; researchers couldn't find any difference in the lymphocyte counts between sarcoidosis and control groups in another study. The authors attributed that the lower lymphocyte count was because of the higher number of the patients with non-active sarcoidosis than active cases [14]. In our study, we had 16 patients in active stage.

In Fireman's study [30] they compared the differential cell counts in the IS specimens of the pulmonary sarcoidosis patients and NS-ILD patients. Two thirds (62.5%) of their 67 patients were in Stage 0-II and one third (37.3%) were in Stage III-IV. They showed that pulmonary sarcoidosis patients' IS had a statistically significantly higher percentage of the number lymphocytes (19.7% vs 15.0%, $p=0.04$), macrophages (36.4% vs 29.0%, $p=0.017$) and a lower percentage of neutrophils (38.3% vs 48.5%, $p=0.017$) and eosinophils (2.8% vs 7.2%, $p=0.041$) than NS-ILD group. Besides these results also they showed that CD4/CD8 ratio of sarcoidosis patients was significantly higher than NS-ILD group (5.7 vs 2.1 $p<0.0001$) (30). In our study, although we compared IS results of sarcoidosis patients with healthy control group, we detected the higher lymphocyte count in the sarcoidosis group, we didn't find differences in the neutrophil and eosinophil counts of groups.

In many previous studies the number of neutrophils in IS has been reported over 40% [31,32]. Although not statistically significant ($p=0.175$), in our study the rate of neutrophils in the sarcoidosis group was higher than control group (34.7% vs 15.5%). But it was still below 40%.

Recently in Porzezinska et al.'s study [14] neutrophil counts were found similar in both active and non-active sarcoidosis patients and healthy group similar to our results. Researchers explained this result with the methodological difference between studies. Also it was thought that the reason of

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lower number of neutrophils in their study was due to exaggerated number of mouth-cleaning cycle during the procedure [14].

There are some limitations of our study. Firstly, the number of enrolled patients was relatively small. Previous studies regarding IS in sarcoidosis patients included also small number of patients, also most of them were retrospective studies. Although small size of our study, we recruited all the patients prospectively, and we applied all IS at the time of pulmonary sarcoidosis diagnosis. Furthermore our control group was recruited from our historical subjects [21].

In conclusion, in our study similar to the previous studies, patients with newly diagnosed sarcoidosis had a higher count of lymphocytes than the healthy control group. IS may be a useful tool to diagnose sarcoidosis non-invasively. Future large-scale studies are warranted to confirm and expand on our findings.

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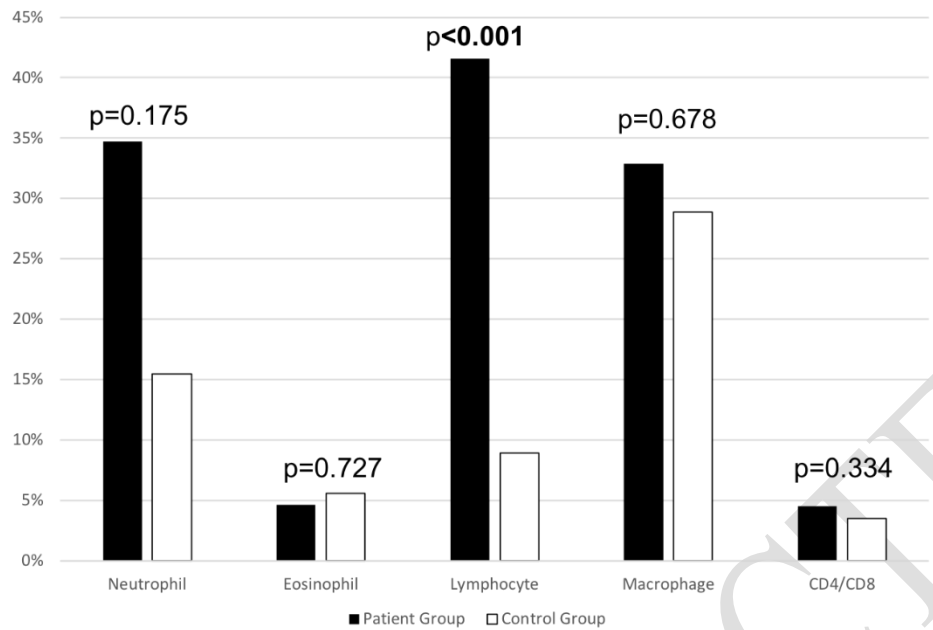


Figure 1: Differential cell counts of groups in IS

Table 1. The characteristics of examined groups

Parameters	Patient group n=20	Control group n=24	p
Age (mean)	42.00±11.47	39.45±17.39	0.579

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Sex (%)	Female	14 (70)	18 (75)	0.818
	Male	6 (30)	6 (25)	
PFT	FEV1 (%)	88.55±18.55	101.17±15.27	0.02
	FVC (%)	93.35±17.82	99.97±14.96	0.195
	FEV1/FVC	79.75±5.33	87.84±8.78	0.001
	DLCO (%)	68.05±23.43	132.17±38.78	0.04
	VC (%)	89.58±30.19	97.30±14.66	0.342
	TLC (%)	94.23±22.60	106.17±11.53	0.058

Abbreviations: PFT; pulmonary function test, FEV1; forced expiratory volume in 1 second, FVC; forced vital capacity, DLCO; carbonmonoxide diffusion capacity, VC; vital capacity, TLC; total lung capacity.

Table 2. The absolute cell counts and differential cell counts of IS in examined groups. The values are mean ± SD

	Patient group	Control group	p
	n=20	n=24	

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Macrophage (%)	32.9±24.5	28.9±25.3	0.678
Eosinophil (%)	4.6±2.8	5.6±3.4	0.727
Lymphocyte (%)	41.6±11.8	8.9±8.7	<0.001
Neutrophil (%)	34.7±12.8	15.5±10.2	0.175
CD4/CD8 ratio	4.5±4.4	3.1±2.2	0.334

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