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Sputum Induction

Literature Review and Proposal for a Protocol

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
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College of Public Health
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Sputum Induction: Literature Review and Proposal for a Protocol

Indrek Melder, M.D.

ABSTRACT

Sputum induction by inhalation of hypertonic saline has been used for more than 15 years. It has become one of the most intriguing methods to study airway inflammation. It is the only direct, non-invasive method for measuring airway inflammation indices. Sputum induction has been used in the diagnosis of many respiratory illnesses including asthma, chronic pulmonary obstructive disease, tuberculosis, chronic cough, lung cancer and Pneumocystis Carinii on patients who are unable to produce sputum spontaneously. There are currently many different methods used worldwide to induce sputum, but there is a lack of one generally accepted “gold standard” method.

The proposed protocols for sputum induction proved to be safe, simple and produced satisfactory amount of expectorate. However, it did not contain enough cells from the lower respiratory tract and was contaminated by squamous cells when compared to another method based on the work of F. E. Hargreave.

Investigation demonstrated that the use of impulse oscillometry, which requires no effort from patients, needs further research with larger study samples before it could be used instead of spirometry to evaluate airway obstruction.

Initial methylene blue stain of the fresh expectorate smear was shown to be useful tool for identifying grossly contaminated sputum samples by squamous epithelial cells. Our first study group included 20 volunteers in good health. Sputum was induced by inhalation of 3% saline mist created by ultrasonic nebulizer at maximum output (4 ml/min). Sputum induction intervals lasted 4-5 minutes with cumulative duration of induction about 4-15 minutes which was tolerated well. Lung function was evaluated for obstruction at baseline and every 5 minutes with spirometry and impulse oscillometry. The whole expectorated sample was processed and slides were stained with HEMA 3 stain. With this method we were able to collect a mean of 6.1 ml expectorate. The mean total cell count was 804 000 with high proportion of squamous cells.

The second study group included 5 volunteers in good health. This method utilized 3%, 4% and 5% saline mist for inhalation, 7 minutes each. Ultrasonic nebulizer was set at low output of 0.9 ml/min. This procedure was also tolerated well without major adverse effects. Lung function was evaluated at baseline and every 7 minutes for obstruction. Only dense portions of expectorate were selected and processed. Slides were stained with Wright stain. This method produced much more total cells with a mean of 3 385 000 per gram of sputum which came from the lower airways and were not contaminated by squamous cells.

The second method was far superior producing adequate sputum sample with cells from the lower airways and minimal squamous cell contamination and will be used in our Breath Lab.

Introduction

The purpose of sputum induction is to obtain an adequate amount of secretions from lower airways in subjects who are unable to produce sputum spontaneously and study the airway inflammation in asthma and other respiratory disorders[2]. For more than 15 years, sputum induction by inhalation of hypertonic saline has become one of the most widely used methods for studying a number of airway inflammatory conditions[1]. The mechanism by which inhalation of hypertonic saline induces sputum is not clear, but it has been hypothesized by researchers that the increased osmolarity also increases vascular permeability in bronchial mucosa[2]. The study of cells and mediators in airway secretions from patients with pulmonary diseases has been performed in samples of spontaneously expectorated sputum on the fluid obtained by bronchoscopy and lavage[4]. These sampling methods are both limited in their applicability. Bronchoscopy can only be employed on patients who are well enough to tolerate the procedure. Bronchoscopy cannot easily be applied repeatedly to study inflammatory changes in airway secretions over short periods of time[9]. Sputum induction by inhalation of hypertonic saline has been suggested as the only direct, relatively non to invasive, and safe method for studying airway inflammation indices[6].

Spirometry involves measurements of a subject's lung flow rates through a forceful exhalation to evaluate obstructive lung deficits. However, many debilitated patients are unable to perform forceful exhalation maneuvers.

In contrast, impulse oscillometry uses a pseudorandom noise signal generated in a normal tidal breathing air column. Advanced digital signal analysis techniques are used to derive respiratory parameters from air column pressure and flow data. These techniques allow localization of airway obstruction to distal or proximal airways[40]. Advantages of impulse oscillometry are:

1. It allows determination of lung characteristics during one minute of normal tidal breathing.
2. It requires practically no patient effort, which makes it especially useful for geriatric, pediatric, and occupational medicine subjects.
3. It allows localization of airway obstruction to distal or proximal airways.

The purpose of initially using a methylene blue stain in this study is to identify grossly contaminated (by squamous cells) sputum samples. The advantage of initial methylene blue stain is its use as a quick and simple method for evaluation of sputum sample adequacy based on epithelial cell contamination before sending it to the laboratory.

The aims of this study were to:

1. Propose a protocol for sputum induction based on previous research and compare it with another method in use based on the work of F.E. Hargreave,
2. Obtain FEV1 values with conventional spirometry and compare those to values obtained with impulse oscillometry,
3. Evaluate sputum sample adequacy based on squamous cell contamination by using the initial methylene blue staining method.

Review of the Literature

Sputum (also phlegm) definition: expectorated lower respiratory secretions[49].

History

Hippocrates described sputum as one of the four essential "humours" of the body[1]. Bronchial secretions from the respiratory epithelium and submucosal glands, together with inflammatory cells that have migrated from the blood, are found in sputum[50]. Gollasch found eosinophilic leukocytes in sputum from asthmatic patients over a century ago[52]. Expectorate from asthmatic patients occasionally demonstrates clusters formed by eosinophiles: so to called Charcot to Leyden crystals[51]. Curschman demonstrated clusters of shed epithelial cells in acute asthma in 1885[53]. He also noted the presence of corkscrew to shaped twists of condensed mucus (Curschman`s spirals)[53]. The clusters of epithelial cells were later called Creola`s bodies[54]. From the 1950s to 1980s, sputum was analyzed microscopically, and fluid phase components were measured to diagnose asthma and chronic bronchitis, and to assess disease severity[1]. In the 1950s, sputum testing was developed in connection with the diagnosis of lung cancer and tuberculosis[1].

Because many patients were unable to provide sputum samples spontaneously, some were asked to inhale hypertonic saline to induce sputum. In relation to asthma, international interest in sputum testing arose after it was observed that epithelial damage and inflammatory changes are present even in early to stage asthma[55].

In 1964, Cleland identified dithiothreitol (DTT) which could reduce and split mucoprotein disulfide bonds[56].

In 1965, Shah and Dye showed that treatment with DTT allowed dispersion of sputum before processing of smears[57]. Since the 1980s, sputum induction by inhalation of hypertonic saline has been successfully used for diagnosing Pneumocystis Carinii pneumonia in patients infected with HIV[4].

In 1992, Pin et al. induced sputum production with the help of hypertonic saline to allow determination of eosinophilic inflammation in asthmatic patients[15].

Induced Sputum

Production of the sputum is induced with inhalation of hypertonic saline mist to collect adequate samples of secretions from the lower airways. The induced sputum allows researchers to study airway changes caused by diseases in patients who are unable to produce sputum spontaneously.

Studies have demonstrated that inhalation of isotonic or hypertonic saline solutions, via an ultrasonic nebulizer, can induce production of small amounts of secretions from the airways[2]. The mechanism of this process is not fully understood. It is hypothesized by researchers that increased osmolarity of bronchial lining fluid will

increase vascular permeability in the bronchial mucosa and production of mucus by submucosal glands[2].

Induced sputum advantages compared to bronchoscopy include the following:

1. its relatively non to invasive nature;
2. samples are obtained from several proximal airways;
3. it can be performed repeatedly;
4. it is safe in cases of severe disease;
5. there is no need for expensive equipment and;
6. large patient populations can be studied[1].

Induced sputum disadvantages compared to bronchoscopy include:

1. the risk of bronchoconstriction which increases with saline concentration (0.9% saline concentration is the safest and is used for high risk patients, 7% saline is the most dangerous);
2. the success rate is only about 80%;
3. the processing methods are laborious and;
4. the results not available immediately[1].

Clinical Applications

Asthma

Asthma is considered a chronic inflammatory airway disease in which mast cells, eosinophiles and T lymphocytes play important roles[1]. The inflammation causes recurrent episodes of wheezing, shortness of breath, chest tightness, and cough, especially at night. The symptoms are associated with intermittent airflow limitation, which is reversible. Chronic airway inflammation also results in increased airway responsiveness to various stimuli (NHLBI/WHO 1995). This condition is commonly associated with sputum eosinophilia (>3%)[5]. There is some evidence that there is an increase of eosinophiles in sputum during workplace exposure in subjects with occupational asthma[4]. The validity of high sputum eosinophilia has shown to be better than pulmonary function tests variation and bronchodilator response at making the diagnosis of asthma[5].

The sputum fluid phase allows us to measure eosinophil cationic protein, some cytokines, and histamine[5]. The study of sputum inflammation indices would help us to understand better the complex relationships between inflammatory cells, mediators, cytokine mechanisms in asthma and enables us to monitor the condition and maybe clarify the diagnosis[4]. Assessing airway inflammation by studying sputum could also be used for evaluation of the effects of drugs on asthmatic airway inflammation[1].

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease is characterized by the presence of airflow obstruction which is associated with chronic bronchitis or emphysema[1]. The airway obstruction tends to be progressive and irreversible. Cigarette smoking is the main culprit in its etiology. The pathophysiology of chronic obstructive pulmonary disease is an

inflammatory disorder which is characterized by neutrophilic inflammation with the presence of macrophages and lymphocytes on airway tissue[4]. Marked sputum neutrophilia is very characteristic to chronic obstructive pulmonary disease[1]. Bronchoscopic procedures are often not possible due to the condition severity and sputum induction becomes useful tool for pathophysiology studies[4]. The sputum neutrophil count is usually high. It can be correlated with a reduction of forced expiratory volume in one second and the rate of decline in forced expiratory volume in one second[4]. Inhaled steroid treatment has been shown to reduce neutrophil count in induced sputum[4].
Sputum eosinophilia in some patients with chronic obstructive pulmonary disease may predict those patients who will benefit from oral steroid therapy[4].

Chronic cough

Sputum neutrophilia is demonstrated in patients with chronic cough, but up to 40% of subjects with chronic cough have sputum eosinophil counts of more than 3%[4]. Some patients with chronic cough and normal lung function demonstrate eosinophilic bronchial inflammation[1]. Patients with chronic cough and sputum eosinophilia demonstrate an objective response to corticosteroid treatment, which results in fall of the sputum eosinophil count[4]. Chronic cough patients who have responded to treatment with corticosteroids have also shown to be similar to patients with asthma in relation of gene expression of some cytokines[58].

Tuberculosis

The World Health Organization recommends the detection of acid to fast bacilli in the respiratory secretion samples as the initial approach to the diagnosis of tuberculosis[59].

Unfortunately, this method has low sensitivity and has no value in patients who are unable to produce sputum spontaneously[4]. The studies have shown that sputum induction proves to be a safe procedure with high diagnostic yield. Also, the procedure highly correlates with the results of fiber optic bronchoscopy for the diagnosis of TB in HIV to seronegative and HIV to seropositive patients[60]. Sputum induction is considered an alternative approach to the diagnosis of sputum smear to negative TB[60].

Cystic Fibrosis

Cystic fibrosis is an autosomal, recessive, hereditary disease which results in production of abnormal secretion from a variety of exocrine glands[4]. Lung involvement is manifested by recurrent infections with Pseudomonas Aeruginosa and Staphylococcus Aureus.

Sputum induction is a relatively safe, noninvasive method of obtaining airway secretions from subjects with cystic fibrosis, especially those who are unable to produce sputum spontaneously[4].

Induced sputum provides an accurate measure of infection and inflammation in the lungs and appears to be comparable to spontaneously expectorated samples[61].

Induced sputum has demonstrated a higher number of viable cells and less squamous cell contamination[4].

Lung cancer

Sputum sample cytological examination has been shown to lead to lung cancer detection at an earlier stage. Unfortunately, the yield of this test is low[4]. Recent studies have demonstrated that adequate sputum samples will allow genetic analysis to be performed, which could prove to be a useful tool for cancer screening[16].

Pneumocystis carinii

Pneumocystis carinii pneumonia is characterized by fever, shortness of breath, substernal tightness, and non to productive cough, and affects mainly immunosuppressed patients. Transbronchial biopsy and bronchoalveolar lavage are considered a "gold standard" in diagnosis of Pneumocystis carinii pneumonia, but they result in some morbidity and are expensive. Induced sputum analysis was found to be a safe, sensitive, specific, rapid and low to cost technique for the diagnosis of Pneumocystis carinii pneumonia[62].

Induced sputum fluid to phase analysis

Sputum fluid to phase has shown to contain many more mediators than broncoalveolar lavage[5]. Inflammatory mediators include cytokines, chemokines, granulocyte proteins, markers of vascular leakage, eicosanoids and proteases[45].

Markers of eosinophil activation include eosinophilic cationic protein (most cytotoxic), major basic protein, eosinophil protein X and eosinophil peroxidase[63].

Myeloperoxidase is a protein released from primary granules of neutrophils, and can serve as a marker of neutrophil activation. Human neutrophil lipokalin is another protein released from secondary neutrophil granules[64].

Fluid to phase mediators can be assessed by Immuno- , Bio- , or Enzyme Assays[4].

Methods

The study subjects included 25 healthy, non-smoking adults between the ages of 19 to 51 years old who were asked to volunteer for the study in the Breath Laboratory at the College of Public Health. IRB approval # 103292. Volunteer subjects were given information about the purpose of the study, elements of the procedure, possible risks, potential benefits, and alternatives to the study protocol. All pertinent questions were answered, and relevant issues were discussed. When subjects demonstrated a full understanding of the study protocol and indicated a desire to proceed, they were required to sign an informed consent.

After informed consent was obtained, subjects completed a Medical Screening Questionnaire and underwent a brief physical examination to assess their cardiopulmonary system. All aspects of the protocol were completed in the presence of an advanced cardiac life support (ACLS) certified physician. Following the questionnaire and physical exam, complete spirometry, including FEV1 was performed under ATS criteria[39]. Subjects were excluded from further study if their FEV1 was below 60% of predicted, which is indicative of moderately severe bronchial obstruction[38]. Following spirometry, subjects had their respiratory impedance measured with the Jaeger Impulse Oscillometer[40], which uses a pseudorandom noise signal generated in the normal tidal breathing air column. Advanced digital signal analysis techniques are used to derive respiratory parameters from air column pressure and flow data, allowing localization of airway obstruction to the distal or proximal airways.

The first study group of 20 subjects underwent sputum induction and processing by proposed protocol and the rest of the 5 subjects followed the sputum induction and processing protocol based on F.E. Hargreave work.

Proposed Sputum Induction Procedure and Processing Protocol:

1) The subject should rinse out his/her mouth thoroughly with water and place the nose clip on the nose[1,2].

2) Breathe tidally for 5 minutes while inhaling a 3% hypertonic saline mist generated by the (Devilbiss Ultra to Neb Large Volume Ultrasonic Nebulizer model 099HD) ultrasonic nebulizer (start with low out to put for about 10 seconds. Then gradually increase to maximum out to put of 4 ml/min to prevent cough)[2,25].

3) Ask the subject to perform a maximal inhalation of 3% saline mist and to hold their breath for 5 seconds. Then exhale. This was performed a total of 3 times[12].

4) Following the final deep inhalation, the subject is to cover their mouth and cough deeply from the base of their chest[12].

5) Collect the expectorated mucus in a 50 ml conical tube, with the goal of collecting at least 5ml[2,12].

6) Perform the sputum collection procedure at least 3 times to maximize the quality and quantity of cells. Typically, this will require 6 minutes per procedure, or a total of 20 to 30 minutes until an adequate sample is obtained[1,2].

7) Check the FEV1 again after each attempt to induce and collect sputum. Then compare to the best FEV1 from the baseline evaluation. If the FEV1 drops more than 20%, the study should be stopped. In addition, if the subject has an adverse symptom such as chest tightness, shortness of breath, chest pain, or if they wish to stop, no further testing should be done[66,67].

8) Follow each spirometry by one maneuver of impulse oscillometry using the Jaeger IOS[40].

Sputum Processing and Analysis:

Initial (before adding DTT) sputum smear staining by methylene blue stain was performed using 1% methylene blue aqueous solution (cover the smear with 1 to 2 drops of stain) for one minute (time is not critical, 30 seconds to 2 minutes will give you acceptable stain) after heat fixation (pass the slide 3 times over the Bunsen burner flame)[36]. Samples with fewer than 50% squamous cells in the field of view is considered adequate (use100x magnification)[1].

1) Add an equal volume of freshly prepared DITHIOTHREITOL (DTT) 10% solution by adding 90ml of distilled, sterile water to 10ml of Sputolysin[41] to preweighed entire expectorate[28].

2) Aspirate the expectorate with a disposable pipette several times and then briefly agitate with a vortex mixer[47].

3) Place the conical tube in a shaking water bath (Bransonic model 2510R with 40 kHz output) set at 22 degrees Celsius (may range from 22 to 37 degrees Celsius)[28] bath for 15 minutes, or until the mucus has dissolved, and the expectorate is a thin fluid.

4) Filter the fluid through a 70 micron nylon mesh[28].

5) Perform a manual, total cell count using a hemacytometer. First, dilute 100 microliters of cell suspension with an equal volume of 0.4 % Trypan blue. Then, mix well and place a drop (~10 to 20 microliters) at each notch of the hemacytometer with a micropipette. First count squamous cells in large corner squares using 100 times magnification followed by total cell count. Count the total number of cells in the four, 1 mm corner squares using 400x magnification and get the average. Multiply the number of cells by 10 000 (the corner square has a volume of 0.0001 ml)[30] and then multiply by a dilution factor of 4 to determine the number of cells per milliliter .

6) Assess cell viability with the Trypan blue exclusion method. Trypan blue stain was added to the cell suspension in the previous step. Viable cells will not stain with Trypan blue, while dead cells will stain dark blue[37]. Centrifugation is recommended at this point if interested in fluid phase measurements. Suggested centrifugation speeds have ranged from 300 to 1500g with 5 to 10 durations[23].

7) Prepare the sample for the Cytospin[28]. Depending on the previous manual cell count, the sample may need to be diluted with phosphate buffered saline (add equal amount of PBS to reduce the cell count to one half in one ml) or concentrated (after centrifugation re to suspend the cell pellet with half of the previous sample volume of BPS). Optimum number of cells for cytospin is 40 000 to 60 000[28]. The recommended cell suspension dilution is around 0.5 million cells per ml. Transfer 0.1ml (appropriate dilution should produce monolayer of cells on the slide which will facilitate the identification of different cell types) of the cell suspension into a disposable cytochamber that plates the suspension onto a Thermo Shandon coated to slide. This preparation is centrifuged in the Cytospin at 440 RPM for 6 minutes[28]. Let the slides dry after centrifugation [34].

8) Stain using the HEMA 3 Stain set, which is comparable to Wright to Giemsa stain:

A) Dip the prepared slide into the HEMA 3 Fixative Solution for 1 second, five individual times. Allow the excess solution to drain prior to the next step.
B) Dip the slide 3 to 5 times for one second each time in the Hema 3 Solution One. Allow the excess solution to drain prior to the next step.
C) Dip the slide 3 to 5 times for one second each time in the Hema 3 Solution Two. Allow the excess solution to drain prior to the next step.
D) Rinse the slide with de to ionized water.
E) Allow the excess water to dry.

9) Perform a differential cell count on the prepared slide under an oil immersion lens. There should be a minimum of 400 non to squamous cells[32,35]. Count 400 cells in the field of view at 400x magnification and report the proportion of cells counted. Identify the following cell types [32].

Neutrophils: nucleus has 2 to 8 lobes, 10 to 12 micron diameter, nucleus stains purple, granules red to lilac and cytoplasm light pink.

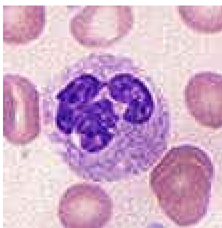

Figure 1: Neutrophil (35)

Eosinophils: nucleus bi to lobed, 12 to 15 micron diameter, nucleus stains dark blue, granules red to orange and the cytoplasm is medium blue.

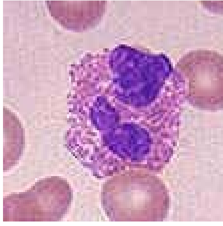
Figure 2: Eosinophil (35)

Basophils: nucleus is bi to or tri to lobed, 9 to 10 micron diameter, nucleus stains dark blue, granules dark purple and cytoplasm light blue.

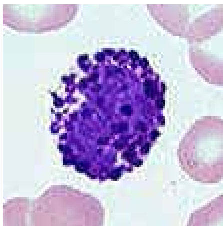
Figure 3: Basophil (35)

Monocytes: (these will become macrophages) indented nucleus, 20 micrometer diameter, nucleus stains violet, and the cytoplasm is light blue.

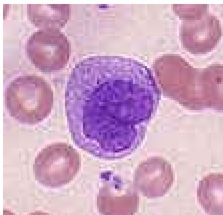
Figure 4: Monocyte (35)

Lymphocytes: nucleus spheroid or ovoid, 6 to 8 or 8 to 12 micrometer diameter, nucleus stain violet.

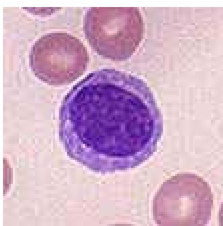
Figure 5: Lymphocyte (35)

Sputum Induction and Processing Protocol based on F.E. Hargreave work[69]:

1) The subject should rinse out his/her mouth thoroughly with water and place the nose clip on the nose[1,2].

2) Breathe tidally for 7 minutes while inhaling a 3% hypertonic saline mist generated by the (Devilbiss Ultra to Neb Large Volume Ultrasonic Nebulizer model 099HD) ultrasonic nebulizer at output of 0.9 ml/min.[68,70]

3) Measure FEV1 again using spirometry followed by three maneuvers of impulse oscillometry by the Jaeger IOS. If the FEV1 drops more than 20% compared to the best baseline value or troublesome symptoms such as chest tightness/pain, shortness of breath occur the procedure should be stopped. If fall in FEV1 is between 10% to 20% repeat previous inhalation. Also if the subject wishes to stop, no further testing should be done[66,69].

4) Blow nose, rinse mouth, swallow water and expectorate sputum into Petri dish[68,69].

5) Repeat this procedure with 4% and 5% saline[68,69].

Processing:

1) Select dense portions (plugs) of expectorate which appear macroscopically free of salivary contamination (up to 1000 mg) using a straight blunted forceps and place them into 50 ml conical tube (if necessary using inverted microscope)[68,69].

2) Weigh the selected sputum and add with pipette four times of the volume of freshly prepared DITHIOTHREITOL (DTT) 10% solution by adding 90ml of distilled, sterile water to 10ml of Sputolysin[41,68,69].

2) Aspirate the expectorate with a disposable pipette several times. Then briefly agitate with a vortex mixer for 15 seconds[47,69].

3) Rock on bench rocker for 15 minutes[68,69].

4) Mix with equal volume to DTT of Dulbecco`s phosphate buffered saline (D to PBS)[68] and continue on bench rocker for another 5 minutes.

5) Filter the fluid through a 70 micron nylon mesh[28,68,69].

6) Centrifuge at 790g for 10 minutes if interested in fluid phase components measurements. Aspirated supernatant should be stored at to 70° of Celsius for later assays[68,69].

7) Re to suspend cell pellet in a volume of D to PBS[68,69].

8) Perform a manual, total cell count using a hemacytometer. First, dilute 100 microliters of cell suspension with an equal volume of 0.4 % Trypan blue using pipette. Then, mix well and place a drop (~10 to 20 microliters) at each notch of the hemacytometer with a micropipette. Begin counting squamous cells in large corner squares using 100 times magnification, followed by total cell count. Count the total number of cells in the four, 1 mm, corner squares using 400x magnification and get the average. Multiply the number of cells by 10,000 (the corner square has a volume of 0.0001 ml)[30]. Finally, multiply by the dilution factor to determine the number of cells per milliliter.

9) Assess cell viability with the Trypan blue exclusion method. Trypan blue stain was added to the cell suspension in the previous step. Viable cells will not stain with Trypan blue, while dead cells will stain dark blue[37].

10) Prepare the sample for the Cytospin[28]. Depending on the previous manual cell count, the sample may need to be diluted with phosphate buffered saline (add equal amount of PBS with pipette to reduce the cell count to one half in one ml) or concentrated (after centrifugation re to suspend the cell pellet with half of the previous sample volume of BPS). Optimum number of cells for cytospin is 40 000 to 75 000[28,68]. The recommended cell suspension dilution is around 1 million cells per ml. Transfer 75 microliters (appropriate dilution should produce monolayer of cells on the slide which will facilitate the identification of different cell types) of the cell suspension with pipette into a disposable cytochamber that plates the suspension onto a Thermo Shandon coated to slide. This preparation is centrifuged in the Cytospin at 450 RPM for 6 minutes[28,68,69]. Let the slides dry after centrifugation [34].

11) Stain using the Wright`s stain[69]:

A) Dip slides in stain for 10 to 15 seconds.
B) Dip slides in de to ionized water for 15 to 30 seconds.
C) Rinse slides by dipping in de to ionized water for a few seconds and air dry.

12) Perform a differential cell count on the prepared slide under an oil immersion lens. There should be a minimum of 400 non to squamous cells[32,35]. Count 400 cells in the field of view at 400x magnification and report the proportion of cells counted.

Results

The first study population consisted of twenty, healthy, non-smoking, adult subjects (ten male and ten female). The age of the population ranged from 19 to 51 years with a mean age of 30.15 years. All subjects denied any major cardiopulmonary conditions. Health problems that were reported included Crohn disease, kidney stone, goiter, kidney transplant, high blood pressure, gout, mononucleosis, migraines and GERD.

Thirteen people underwent a single, four-minute, sputum induction interval, which produced a mean of 6.07 ml expectorate with range from 4.8 to 10 ml (extreme value of 30 ml was excluded).

Two subjects underwent two, four-minute, sputum induction intervals, which produced a mean of 6.15 ml of expectorate (6 and 6.3 ml).

The last five subjects underwent three, five-minute, sputum induction intervals, which produced a mean of 6.19 ml of expectorate with range from 5 to 7.65 ml.

For the whole study population, the mean total cell count was 804 100 cells per ml, with range from 310 000 to 3 690 000 cells per ml. The mean proportion of squamous cells was 38% ranging from 3% to 81%.

Thirteen subjects who underwent one, four-minute, sputum induction interval had a mean total cell count of 946 000 cells per ml with range from 360 000 to 3 690 000 cells per ml and mean proportion of squamous cells of 37% (3% to 67%).

Two subjects who underwent two, four-minute, sputum induction intervals had a mean total cell count of 705 000 cells per ml (310 000 and 1 100 00) with the mean proportion of squamous cells of 57% (81% and 33%).

Five subjects who underwent three, five-minute, sputum induction intervals had a mean total cell count of 694 400 per ml (360 000 to 1 100 000) with the mean proportion of squamous cells of 34% (25% to 57%).

Subject 37 who underwent three, five-minute, intervals of sputum induction produced 1.5 ml of expectorate after the first interval which contained 400 000 cells per ml (15% squamous cells), 3.5 ml of expectorate after the second session, which contained 440 000 cells per ml (82% squamous cells), and 2.5 ml of expectorate after the third session containing 240 000 cells per milliliter (75% squamous cells).

Based on FEV1 and impulse oscillometry measurements, a correlation coefficient was calculated for the thirteen subjects who underwent one, four-minute, sputum induction interval which demonstrated value of to 0.196 at p=0.52. Two subjects who underwent two, four-minute, intervals of sputum induction had correlation coefficient of to 1.000. A p value was not reported by the SAS software due to small sample size of this group. The last five subjects who underwent three, five-minute, intervals of sputum induction demonstrated correlation coefficient of to 0.622 at p=0.26.

Freshly expectorated sputum smears were prepared on the slides and stained by methylene blue stain, which demonstrated more than 50% squamous cell predominance in the field of view for subjects 23,25,28,37 and 26. Accordingly, their counted squamous cells proportions were 81%, 67%, 58%, 79% and 54%. It is very subjective and hard to

evaluate, but subjects 29.24,25,31,35,22,21,36 and 38 seem to have squamous cell proportions seen on slides in the field of view around 50%. Their calculated squamous cell percentages were: 42%,42%,40%,38%,35%,33%,33%,32% and 31%. The rest of the subjects 40,33,39,34,30 and 32 seem to have less than 50% of squamous cells in the field of view. According calculated percentages: 26%,26%,25%,23%,21% and 3%.

Cytospin slides demonstrated mostly squamous epithelial cells, which made it impossible to perform differential cell count.

The second study population consisted of 5 healthy, non-smoking, adult males. The ages ranged from 20 to 46 years with a mean age of 34.4 years. The mean total cell count was 3 385 000 cells per ml with range from 1 150 00 to 6 250 000 cells per ml. The mean proportion of squamous cells was 11.2% ranging from 8% to 19%.

Cells viability ranged from 53% to 73% with a mean of 66.2%. Differential cell counts demonstrated a mean of 0.4% of Eosinophils, 19.2% Neutrophiles, 79.1% of Macrophages and 1.3% of Lymphocytes.

Based on FEV1 and impulse oscillometry measurements a correlation coefficient was calculated, which demonstrated no correlation.

The data was analyzed using SAS version 9.1 statistical software.

Table 1: First Study Population Characteristics

Subject	Gender	Age	Expectorate Volume After 4 minutes (ml)	Expectorate Volume After 8 minutes (ml)	Expectorate Volume After 15 minutes (ml)	Total Cell Count per Mililiter	% of Squamous cells
21	M	33	.	6.00	.	1100000	33
22	F	51	7.50	.	.	1320000	33
23	F	51	.	6.30	.	310000	81
24	M	27	10.00	.	.	360000	42
25	M	19	7.00	.	.	720000	40
26	M	19	5.00	.	.	980000	54
27	M	34	5.00	.	.	450000	67
28	M	20	4.80	.	.	1090000	58
29	M	22	4.90	.	.	690000	42
30	F	41	9.00	.	.	610000	21
31	M	20	5.00	.	.	800000	38
32	F	23	4.90	.	.	3690000	3
33	M	46	30.00	.	.	380000	26
34	F	24	4.80	.	.	440000	23
35	M	46	4.90	.	.	770000	35
36	F	34	.	.	5.00	530000	32
37	F	23	.	.	7.50	360000	57
38	F	23	.	.	5.00	1050000	31
39	F	22	.	.	5.80	432000	25
40	F	25	.	.	7.70	1100000	26

Table 2: Spirometry Measurements (FEV1 in Liters)

Subject	Predicted	Baseline	1st Induction	2nd Induction	3rd Induction
21	4.51	5.04	4.87	5.06	.
22	2.67	3.23	2.89	.	.
23	2.75	3.73	3.79	3.84	.
24	4.69	3.62	3.82	.	.
25	4.58	4.36	4.34	.	.
26	4.58	4.22	4.62	.	.
27	4.32	4	3.43	.	.
28	3.85	3.68	3.31	.	.
29	4.38	3.37	2.75	.	.
30	3.26	2.73	3	.	.
31	3.99	4.1	3.86	.	.
32	3.96	3	2.98	.	.
33	3.97	4.74	4.73	.	.
34	2.9	2.93	2.87	.	.
35	3.97	3.93	4.18	.	.
36	3.19	3.11	3.05	3.01	3.05
37	3.39	3.39	3.07	3.07	3.04
38	3.23	3.58	3.44	3.51	3.39
39	3.33	3.07	2.9	2.92	2.9
40	3.67	2.95	2.9	2.73	2.81

Table 3: Spirometry Measurements to Percentages of Predicted Values

Subject	% of Predicted Baseline	% of Predicted 1st Induction	% of Predicted 2nd Induction	% of Predicted 3rd Induction
21	111.6	107.9	112.1	.
22	121	108.1	.	.
23	135.8	137.7	139.6	.
24	77.1	81.5	.	.
25	95.3	94.7	.	.
26	92.1	100.9	.	.
27	92.6	79.4	.	.
28	95.5	85.8	.	.
29	76.9	62.8	.	.
30	83.8	91.8	.	.
31	102.8	96.8	.	.
32	75.8	75.3	.	.
33	119.6	119.2	.	.
34	101.1	99.1	.	.
35	99.2	105.3	.	.
36	97.5	95.5	94.3	95.7
37	100.1	90.6	90.6	89.8
38	110.8	106.4	108.4	104.9
39	92	86.9	87.6	87.2
40	80.4	79.2	74.6	76.7

Table 4: Impulse Oscillometry Measurements (R5)

Subject	Predicted	Baseline	1st Induction	2nd Induction	3rd Induction
21	2.75	3.36	3.84	4.14	.
22	3.87	4.32	4.3	.	.
23	3.87	5.28	4.44	4.48	.
24	2.69	2.71	3.47	.	.
25	2.6	3.62	3.64	.	.
26	2.6	4	3.52	.	.
27	2.76	2.46	2.15	.	.
28	2.62	2.79	3.01	.	.
29	2.63	2.85	2.82	.	.
30	3.7	8.67	9.16	.	.
31	2.61	3.46	4.37	.	.
32	2.96	4.47	3.69	.	.
33	2.9	3.66	3.94	.	.
34	3.41	5.34	4.35	.	.
35	2.9	4.07	3.73	.	.
36	3.57	5.17	4.67	4.79	5.26
37	3.4	3.02	3.1	3.19	2.9
38	3.4	2.73	3.16	2.82	3.16
39	3.38	4.73	4.97	4.86	4.97
40	3.43	4.56	4.93	5.28	4.84

Table 5: Impulse Oscillometry Measurements to Percentages of Predicted Values (R5)

Subject	% of Predicted Baseline	% of Predicted 1st Induction	% of Predicted 2nd Induction	% of Predicted 3rd Induction
21	122.2	139.4	150.4	.
22	111.7	111	.	.
23	136.4	114.8	115.6	.
24	101.1	129.3	.	.
25	139.4	140.2	.	.
26	154.2	136	.	.
27	89	78	.	.
28	106.8	115.2	.	.
29	108.4	107.1	.	.
30	234.2	247.5	.	.
31	132.6	167.6	.	.
32	150.9	124.6	.	.
33	126.4	136	.	.
34	156.4	127.5	.	.
35	140.6	128.8	.	.
36	144.9	131.1	134.4	147.7
37	89.1	91.2	93.8	85.4
38	80.3	93.2	83	93.5
39	139.9	147	143.8	147.3
40	133.1	143.8	154	141

Table 6: Spirometry Measurements to Percentages and Differences of Change from Baseline Values (FEV1 in Liters)

Subject	Differences			Percentages		
	1st Induction to Baseline	2nd Induction to Baseline	3rd Induction to Baseline	1st Induction to Baseline	2nd Induction to Baseline	3rd Induction to Baseline
21	-0.17	0.02	.	-3.373	0.39683	.
22	-0.34	.	.	-10.5263	.	.
23	0.06	0.11	.	1.6086	2.94906	.
24	0.2	.	.	5.5249	.	.
25	-0.02	.	.	-0.4587	.	.
26	0.4	.	.	9.4787	.	.
27	-0.57	.	.	-14.25	.	.
28	-0.37	.	.	-10.0543	.	.
29	-0.62	.	.	-18.3976	.	.
30	0.27	.	.	9.8901	.	.
31	-0.24	.	.	-5.8537	.	.
32	-0.02	.	.	-0.6667	.	.
33	-0.01	.	.	-0.211	.	.
34	-0.06	.	.	-2.0478	.	.
35	0.25	.	.	6.3613	.	.
36	-0.06	-0.1	-0.06	-1.9293	-3.21543	-1.9293
37	-0.32	-0.32	-0.35	-9.4395	-9.43953	-10.3245
38	-0.14	-0.07	-0.19	-3.9106	-1.95531	-5.3073
39	-0.17	-0.15	-0.17	-5.5375	-4.88599	-5.5375
40	-0.05	-0.22	-0.14	-1.6949	-7.45763	-4.7458

Table 7: Impulse Oscillometry Measurements to Percentages and Differences of Change from Baseline Values

Subject	Differences			Percentages		
	1st Induction to Baseline	2nd Induction to Baseline	3rd Induction to Baseline	1st Induction to Baseline	2nd Induction to Baseline	3rd Induction to Baseline
21	0.48	0.78	.	14.2857	23.2143	.
22	-0.02	.	.	-0.463	.	.
23	-0.84	-0.8	.	-15.9091	-15.1515	.
24	0.76	.	.	28.0443	.	.
25	0.02	.	.	0.5525	.	.
26	-0.48	.	.	to 12	.	.
27	-0.31	.	.	-12.6016	.	.
28	0.22	.	.	7.8853	.	.
29	-0.03	.	.	-1.0526	.	.
30	0.49	.	.	5.6517	.	.
31	0.91	.	.	26.3006	.	.
32	-0.78	.	.	-17.4497	.	.
33	0.28	.	.	7.6503	.	.
34	-0.99	.	.	-18.5393	.	.
35	-0.34	.	.	-8.3538	.	.
36	-0.5	-0.38	0.09	-9.6712	-7.3501	1.7408
37	0.08	0.17	-0.12	2.649	5.6291	-3.9735
38	0.43	0.09	0.43	15.7509	3.2967	15.7509
39	0.24	0.13	0.24	5.074	2.7484	5.074
40	0.37	0.72	0.28	8.114	15.7895	6.1404

Table 8: Impulse Oscillometry Measurements (R20)

Subject	Predicted	Baseline	1st Induction	2nd Induction	3rd Induction
21	2.34	2.73	3.26	3.25	.
22	3.26	3.67	3.63	.	.
23	3.26	4.63	3.97	3.88	.
24	2.28	2.25	2.89	.	.
25	2.19	2.71	2.64	.	.
26	2.19	3.56	3.25	.	.
27	.	.	.	.	.
28	.	.	.	.	.
29	.	.	.	.	.
30	3.08	5.54	5.36	.	.
31	2.2	2.96	3.75	.	.
32	2.55	4.11	3.37	.	.
33	2.49	2.98	3.3	.	.
34	2.8	4	3.42	.	.
35	2.49	3.91	3.44	.	.
36	2.95	4.11	3.74	3.92	4.25
37	2.78	2.55	2.34	2.67	2.58
38	2.78	2.83	3.07	2.96	3.01
39	2.76	4.59	4.72	4.68	4.86
40	2.81	4.01	4.23	4.29	4.23

Table 9: Correlation Coefficients for Spirometry (FEV1) and Impulse Oscillometry (R5) Measurements of Percentages of Change of 1st Induction from Baseline Values (SAS Output)

Pearson Correlation Coefficients, N = 20 Prob > |r| under H0: Rho=0		
	FEV1 % change 1st Induction to Baseline	R5 % change 1st Induction to Baseline
FEV1 % change 1st Induction to Baseline	1.00000	-0.02410 0.9197
R5 % change 1st Induction to Baseline	-0.02410 0.9197	1.00000

Table 10: Correlation Coefficients for Spirometry (FEV1) and Impulse Oscillometry (R5) Measurements of Percentages of Change of 2nd Induction from Baseline Values (SAS Output)

Pearson Correlation Coefficients, N = 7 Prob > |r| under H0: Rho=0		
	FEV1 % change 2nd Induction to Baseline	R5 % change 2nd Induction to Baseline
FEV1 % change 2nd Induction to Baseline	1.00000	-0.32009 0.4840
R5 % change 2nd Induction to Baseline	-0.32009 0.4840	1.00000

Table 11: Correlation Coefficients for Spirometry (FEV1) and Impulse Oscillometry (R5) Measurements of Percentages of Change of 3rd Induction from Baseline Values (SAS Output)

Pearson Correlation Coefficients, N = 5 Prob > |r| under H0: Rho=0		
	FEV1 % change 3rd Induction to Baseline	R5 % change 3rd Induction to Baseline
FEV1 % change 3rd Induction to Baseline	1.00000	0.39609 0.5092
R5 % change 3rd Induction to Baseline	0.39609 0.5092	1.00000

Table 12: Slide Estimates and Counted Percentage of Squamous Cells

	Slide Estimation			Counted Percentage of Squamous Cells %
Subject	< 50%	Around 50% (Undetermined)	>50%	
23	.	.	y	81
27	.	.	y	67
28	.	.	y	58
37	.	.	y	57
26	.	.	y	54
29	.	y	.	42
24	.	y	.	42
25	.	y	.	40
31	.	y	.	38
35	.	y	.	35
22	.	y	.	33
21	.	y	.	33
36	.	y	.	32
38	.	y	.	31
40	y	.	.	26
33	y	.	.	26
39	y	.	.	25
34	y	.	.	23
30	y	.	.	21
32	y	.	.	3

Table 13: Second Study Group Population Characteristics

Subject	Gender	Age	Total Cell Count/ gram of sputum	% of Squamous Cells	% of Viability
41	M	34	1,150,000	11	65
42	M	41	1,625,000	19	53
43	M	46	5,600,000	5	71
44	M	31	2,300,000	13	69
45	M	20	6,250,000	8	73

Table 14: Spirometry and Impulse Oscillometry Measurements – Percentages of Predicted Values

	Spirometry				IOS to R5/R20			
Subject	Baseline	% of Predicted 1st Induction	% of Predicted 2nd Induction	% of Predicted 3rd Induction	Baseline	% of Predicted 1st Induction	% of Predicted 2nd Induction	% of Predicted 3rd Induction
41	131.1	129.8	130	124.7	93.0/98.9	103.3/103.7	92.6/101.1	89.1/93.3
42	68.7	60.4	53.1	55.2	203.6/168.6	203.3/144.4	228.8/162.2	186.8/148.1
43	107.9	105	94.8	96.7	161.4/187.0	140.7/155.0	141.0/154.0	117.6/134.1
44	104.8	103.8	102.9	102.5	187.9/185.8	251.3/259.7	199.3/197.9	169.1/179.3
45	94.8	93.1	91.5	89.2	112.8/113.9	135.1/134.8	137.9/140.6	131.2/125.0

Table 15: Impulse Oscillometry and Spirometry Measurements to Percentages Differences of Change from Baseline Values

	IOS to R5			Spirometry		
Subject	% of Predicted 1st Induction	% of Predicted 2nd Induction	%of Predicted 3rd Induction	% of Predicted 1st Induction	% of Predicted 2nd Induction	%of Predicted 3rd Induction
41	10.3	-0.4	-3.9	-1.3	-1.1	-6.4
42	-0.3	25.2	-17.4	-8.3	-15.6	-13.5
43	-20.7	-20.1	-43.8	-2.9	-13.1	-11.2
44	63.4	11.4	-18.8	-1	-1.9	-2.3
45	22.3	25.1	18.4	-1.7	-3.3	-5.6

Table 16: Differential Cell Counts (Percentage of Total Nucleated Cells)

Cell Type	Subject 41	Subject 42	Subject 43	Subject 44	Subject 45	Mean
Eosinophils	0.5	0.25	0.25	0.75	0.5	0.45
Neutrophils	18	22	16	24	26	21.2
Macrophages	80	76.5	82.25	73.25	72.5	76.9
Lymphocytes	1.5	1.25	1.5	2.0	1.0	1.45

Table 17: Correlation Coefficients for Spirometry (FEV1) and Impulse Oscillometry (R5) Measurements of Percentages of Change from Baseline Values

Pearson Correlation Coefficients, N = 5
Prob > |r| under H0: Rho=0

I1	I2	I3	S1	S2	S3
1.00000	0.44647 0.4510	0.37620 0.5326	0.46334 0.4319	0.69739 0.1905	0.85817 0.0627
0.44647 0.4510	1.00000	0.70794 0.1809	- 0.34220 0.5730	0.10476 0.8669	0.12224 0.8447
0.37620 0.5326	0.70794 0.1809	1.00000	0.23557 0.7029	0.59032 0.2947	0.43859 0.4600
0.46334 0.4319	- 0.34220 0.5730	0.23557 0.7029	1.00000	0.84842 0.0692	0.84551 0.0712
0.69739 0.1905	0.10476 0.8669	0.59032 0.2947	0.84842 0.0692	1.00000	0.93070 0.0217
0.85817 0.0627	0.12224 0.8447	0.43859 0.4600	0.84551 0.0712	0.93070 0.0217	1.00000

Discussion

The Proposed method was designed with patient safety in mind, followed by efficiency of sputum sample production.

The first study population included ten male and ten female adult subjects. All subjects were healthy (no major cardio to pulmonary conditions), non-smoking and between the ages of 19 to 51 years old. Participation was entirely voluntary, and subjects signed a informed consent before the study began. The study was undertaken in the Breath Laboratory at the College of Public Health. The study was approved by IRB on 2/17/05 # 103292.

Subjects filled out a Medical Questionnaire and underwent brief physical exams, which included chest auscultation. Health problems that were reported included Crohn disease, kidney stones, goiter, migraines, mononucleosis, gout, GERD, kidney transplant and high blood pressure. There was no wheezing noted during chest auscultations.

Pulmonary function monitoring during sputum induction is necessary, or safety reasons, to assess possible excessive bronchoconstriction due to inhalation of hypertonic saline[43]. There is no standardized protocol for pulmonary function monitoring during sputum induction, but many authors measure pulmonary function every 5 to 10 minutes, and every time symptoms occur[1,2,4,66]. Working Group One recommends measuring FEV1 at the end of each 5 minute induction interval and stop induction if there is a drop in FEV1 of more than 20% compared with the post to bronchodilator value or when adverse effects occur[2]. Ten minute intervals seem too long for detection of possible hypertonic saline inhalation caused bronchoconstriction. Long intervals might endanger the subject's health. We decided to use 5 minute intervals (after each induction) for pulmonary function monitoring, which seems to be the safest and most rational solution.

Baseline FEV1 (for the first 8 subjects one measurement was obtained) and impulse oscillometry values which were obtained for safety reason were all in acceptable range. No EFV1 value was close to exclusion criteria of 60% of predicted (indicative of moderately severe bronchial obstruction)[66]. The lowest baseline FEV1 value measured in our study was 75.8% of predicted with subject.

A few subjects initially had problems performing spirometry maneuvers properly, but with some training they improved. Later they were able to perform well.

Hypertonic saline inhalation causes bronchoconstriction in asthmatic subjects[18]. The actual mechanism is not well known, but may involve activation of airway mast cells or sensory nerve endings[42]. Pretreatment with short-acting beeta-2 agonist (200 microgram of Salbutamol) is usually recommended as the standard procedure to avoid excessive bronchoconstriction when studying asthmatic patients[15]. Before and 10 minutes after the bronchodilator administration, FEV1 should be measured[66,67]. Since we excluded asthmatic subjects and studied only healthy people, pretreatment with short- acting beeta-2 agonist was not indicated for our subjects.

Different concentrations of saline have been used for sputum induction, which have ranged from 0.9% to 7%[65,66] with higher concentrations being related to increased side effects and reduced tolerability[2,67]. There seems to be no difference in the cellular

composition of sputum induced with either isotonic or hypertonic saline; and different saline concentrations do not affect total and differential cell counts[25]. Working Group One recommends to use either fixed concentration of sterile saline solution (3% or 4%) or incremental concentrations (3%, 4% and 5%)[2]. We decided to utilize 3% saline in our method for sputum induction because of its relatively low saline concentration, which helps to reduce adverse effects and has shown to be effective[1,2,12]. Alternatively, for high risk subjects, the sputum induction should start with 0.9% saline solution and last for 30 seconds, 1 minute and 5 minutes, measuring FEV1 after each induction for safety reason[2].

Nebulizers output varies between 0.21 to 6 ml minutes in different studies[1,2,4]. Some authors report greater sputum induction with higher nebulizer output[4]. Due to higher output of saline mist, ultrasonic nebulizers are recommended over jet nebulizers[25]. The higher success rate reported with high output ultrasonic nebulizers prompted us to select DeVilbiss ultrasonic nebulizer with maximum output[2,4,12]. We started induction with low output, which was gradually increased to maximum output (6 ml per min)

Studies have reported that the cellular and biochemical constituents of induced sputum change with the duration of inhalation of hypertonic saline mist[44]. It was noted that neutrophiles and eosinophiles are prominent in early collected samples (during first 4 minutes) and lymphocytes and macrophages predominate in later collected samples (16 to 20 minutes)[44]. For most purposes, the consensus is to use a cumulative duration of nebulization of 15 to 20 minutes[1,2]. We used 4 minutes nebulization for thirteen subjects, 8 (2x 4 min.) minutes nebulization for two subjects, and 15 (3x5 min) minutes of nebulization for 5 subjects.

Expectoration techniques and methods of subject preparation for sputum induction vary in different protocols[1,2]. Some have recommended that subjects use nose clips and rinse their mouth with water before induction which seems reasonable. We decided to adopt these precautions for our method[1,2]. Some subjects had difficulties keeping the nose clip on and complained that nose clip is uncomfortable. But after a while they got use to it and were able to use the proper technique. We also implemented three deep breath/cough procedure for sputum production, which has been effectively been used for some time and worked well with our subjects[12]. At the end of 5 minute sputum induction interval (continuous saline inhalation), the subject was asked to take deep breath of saline mist and hold it for 5 seconds followed by slow exhalation. Repeat this a total of three times, cover the mouth, cough deeply and spit the specimen into collection container. This technique seemed to facilitate sputum volume production for our subjects.

Some protocols utilize short sputum induction intervals (3 minutes) with cumulative nebulization duration of 15 to 20 minutes and report adequate sputum sample production[12]. In order to minimize possible adverse effects from hypertonic saline inhalation, we decided initially to induce sputum with 4 minute induction intervals. Nebulizer output was kept low (1 to 2 ml per min) for the first 10 seconds to prevent cough. Then gradually increased to maximum output (6 ml minute). A few patients experienced some minimal heaviness in the chest at the start of inhalation which resolved during the next 30 seconds. No subject complained adverse effects nor wanted to stop for any reason. The procedure was tolerated well by all subjects. FEV1 and impulse oscillometry measurements were performed after each induction interval which demonstrated no FEV1 drop exceeding 20% of baseline value.

The first fifteen subjects underwent sputum induction with 4 minute induction intervals. Twelve subjects were able to produce a mean of 6.07 (4.8 to 10) ml of expectorate after first induction interval which contained a mean of 993 333 (360 000 to 3 690 000) total cells per milliliter of which a mean 38% (3% to 67%) were squamous cells. This percentage of squamous cells is high compared to one study which had 6.3% (0 to 67%) of squamous cells in their healthy persons sputum sample[1]. One reason for that could be that we are studying only healthy subjects who are trying very hard to produce a sputum sample which may increase the proportion of squamous cells. Also, they do not perform the deep cough maneuver properly. One subject produced 30 ml of expectorate in 4 minutes which contained 380 000 total cells of which 26% were squamous cells. Two subjects were unable to produce adequate sputum sample in 4 minutes which forced us to continue for another 4 minutes. They produced a mean of 6.15 ml of expectorate which contained a mean of 750 000 total cells of which 57% were squamous cells. Subject 23, who had the highest percentage of squamous cells, had a very difficult time producing the sputum sample and performing spirometry.

For the last five patients, we increased the induction duration to 5 minutes (continuous saline inhalation) with routine three sessions (15 min. of cumulative inhalation duration) hoping to improve cellular quality and quantity. They all tolerated the increased inhalation duration well without any adverse effects or complaints of discomfort. The volume of the sputum sample seems to increase little with the increased induction time. They produced a mean of 6.19 ml (5 to 7.65 ml) of expectorate which contained a mean of 694 400 (360 000 to 1 100 000) total cells of which 34.3% (25% to 57.3%) were squamous cells. In our study, the total cell count and the proportion of squamous cells decreased slightly with increased induction duration. Subject 37 produced 1.5 ml of expectorate during the first 5 minutes of induction with 400 000 total cells of which 15% were squamous cells, 3.5 ml of expectorate during the second 5 minute induction with 440 000 total cells of which 82% were squamous cells, and 2.5 ml of expectorate during the third 5 minute induction with 240 000 total cells of which 75% were squamous cells. Subject 37 actually demonstrated increased proportion of squamous cells in her sample after 10 minutes of induction (82%) and 15 minutes (75%) with reduction of total cell count. Total cell counts seem to be similar to total counts reported by other authors (40 000 to 4 000 000)[1,6]. In other studies investigators have obtained 10 to 16.4 ml of expectorate with 4.5% saline induction for 20 minutes from patients with mild to moderate asthma[4].

Some authors discard the first sample in order to reduce squamous cells contamination[2,67]. In order to reduce squamous cell contamination proper technique should be strictly used with enough time given for slow expectoration. Longer cumulative induction times (15 to 20 minutes) seem to produce less contaminated samples. Subjects may also try to empty the mouth of saliva first by spitting, then performing deep cough exercise. Selecting only dense sputum portions for processing would reduce salivary contamination.

We decided to use 5 minutes sputum induction intervals for at least three times minimum or until getting optimal sputum sample (5 ml) with cumulative induction duration of 15 to 30 minutes to increase cellularity. In our study, we were able to obtain about 5 to 6 ml of expectorate during initial 4 minute induction (13 subjects) which was later increased to 15 minutes (5 subjects with three 5 minute induction intervals) in order

to improve cellularity. There was about 8 to 10 minutes between the induction intervals (continuous saline inhalation for expectoration, spirometry and impulse oscillometry measurements.)

All subjects underwent post to induction chest auscultation, which demonstrated no wheezing, and had no complaints of adverse effects such as chest tightness, shortness of breath or chest pain. Subjects were sent home in good condition, and they were reminded to call us if any problems should arise.

Preparing a sputum smear on the slide for methylene blue staining in order to evaluate squamous cells contamination creates some challenges. Due to the viscosity of the fresh expectorate, it is quite difficult to transfer some of the sample onto the slide. It is close to impossible to get a homogenous layer of expectorate on the slide for methylene blue staining, which makes the later evaluation difficult. A sample with fewer than 50% of squamous cells in the field of view was considered adequate, which presents a quite subjective estimate and complicates the evaluation[1]. Only very contaminated samples (81% squamous cells) were easy to identify, but slides with less (around 50%) squamous cell contamination (intermediate cases) were difficult to categorize to adequate or not adequate sample groups. We found that five samples (23, 27, 28, 37 and 26), which had a median of 69.5% (54% to 81%) of counted squamous cells proportions, demonstrated more than 50% of squamous cells per field of view after methylene staining and were categorized as contaminated samples. Nine samples (29, 24, 25, 31, 35, 22, 21, 36, and 38) with a mean of counted squamous cells proportion of 36.2% (31% to 42%) were very hard to categorize into contaminated or not contaminated category. The squamous cells proportion on those slides in the field of view was around 50% and were categorized as undetermined. Six samples had counted squamous cells proportion mean of 20.7% (3% to 26%). These slides demonstrated less than 50% of squamous cells in the field of view and were categorized as not contaminated or adequate samples. This evaluation is quite subjective, but it allows us to identify grossly contaminated samples and is useful tool for future studies.

We always processed our sputum samples in less than two hours of induction, as recommended for optimal cell counting and staining[23]. Dithiothreitol solution (10%) was always freshly prepared by adding 90 ml of distilled water to 10 ml of Sputolysin[41] concentrate. Equal volume of 10% Sputalysin solution was added to preweighed entire expectorate for homogenization and placed in a shaking water bath for 15 minutes at 22 degrees of Celsius periodically aspirating the sample with disposable pipette[15]. Mucus usually dissolved in about 10 to 15 minutes after which the expectorate was filtered through a 70 micron nylon mesh which is strongly recommended to remove debris[31]. Manual total cell counting was performed using a hemocytometer. Cell viability was determined by the trypan blue exclusion method[45]. Centrifugation is required to separate sputum cells from the fluid phase which could be stored at -20 to -70 degrees Celsius and studied later[23]. Centrifugation time of 5 to 10 minutes at 300 to 1500g is recommended[45,23]. In our study, we did not perform fluid phase measurements and did not centrifuge the expectorate.

Initially, we followed Shandon cytocentrifuge instructions for cytospin preparations, which produced too thick layer of cells on the slide surface making it impossible to evaluate different cells. We also experimented with different centrifugation speeds and cell concentration until we selected optimal speed of 440 rpm for 6 minutes with 40 000

to 60 000 cells in cytochamber (used for the last three subjects) which gave us the optimal cell spread into monolayer on the slide surface[23,47].

Cytospin staining was uncomplicated and fast with HEMA 3 Stain producing good visualization. Cytospin slides demonstrated mostly squamous cells and macrophages (originate from monocytes) with occasional neutrophiles and lymphocytes.

Correlation coefficient was calculated using SAS version 9.1 statistical software based on percent differences of forced expiratory volume in one second and impulse oscillometry values. No correlation exists between 1st induction to baseline % of change with r= to 0.0241 at p=0.9197, n=20. No correlation exists between 2d induction to baseline % of change with r= to 0.32009 at p=0.484, n=7. No correlation exist between 3d induction to baseline % of change with r=0.39609 at p=0.5092, n=5.

The results may be due to improper impulse oscillometry technique and small sample sizes. Future research should involve larger sample sizes, and impulse oscillometry measurements should be increased from one to three, selecting the best attempt. Proper technique should be followed. This area needs additional research in order to replace conventional spirometry with impulse oscillometry to evaluate pulmonary function.

The second study group consisting of five healthy, non-smoking, male adults underwent sputum induction and processing based on F.E. Hargreave protocol which included inhalation of 3%, 4% and 5% saline mist for 7 minutes each. All patients tolerated this procedure well, except subject number 42 who had multiple episodes of cough, and his FEV1 dropped 13.1% after inhalation of 4% saline mist. This subject had his third induction done with 3% saline solution which he tolerated well and had no further respiratory complaints. With this protocol, we were able to obtain much higher total cell counts, which ranged from 1 150 000 to 6 250 000 with a mean of 3 385 000 compared to the proposed method. Squamous cells contamination was also much less ranging from 5% to 19% with a mean of 11.2% which made differential cell counting possible and is probably due to the fact that only dense portions of sputum were selected for processing.

This method also produced good number and quality of cells from the lower respiratory tract, probably due to higher saline concentrations and longer induction time.

The mean proportion of Eosinophils was 0.4%, Neutrophils 19.2%, Macrophages 79.1% and Lymphocytes 1.3% which correlates well with the data published by other researchers.

Correlation coefficient was calculated for the second study group using SAS version statistical software based on percent differences of forced expiratory volume in one second and impulse oscillometry values. There was no correlation found.

The second method proved to be far superior compared to the proposed method in obtaining quality sputum sample.

Two methods for processing of the expectorate have evolved[28]. The first method involves selecting all denser portions from the sample with the aid of inverted microscope[23]. Using only a selected viscid sputum makes cell counting easier to perform. The total cell count can be expressed per gram of lower airway secretion and concentrations of chemicals in the fluid phase are unaffected by the influence of saliva. Disadvantages of his method include the need for inverted microscope and longer processing time due to sputum selection[28].

The second method involves processing the entire expectorate including sputum and saliva[45]. We decided to select this method to process our samples because it is quicker to perform. Disadvantages include the fact that the expectorate contains variable mixture of sputum and saliva which may dilute the sputum and confound its analysis[28]. Also the reproducibility of cell counts has been lower if squamous cell contamination exceeds 20%[46]. Both methods have the same ability to distinguish asthmatics or bronchitics from healthy subjects, but they are not interchangeable[28].

The technical differences between those two methods are only at the very beginning of the processing where 100 to 1000 mg of sputum free of salivary contamination is selected and mixed with 10% Sputalysin solution equivalent 4 times of selected weight with the first method.

Comparison of different sputum induction protocols

Study authors	Saline centrations	lation tion	FEV1 ısurements	›ulizer ›ut	Expectoration interval
Ref. 15	3%, 4% and 5%, increased at 10 min. intervals.	5 min. periods up to 30 min.	Baseline and every 5 minutes.	ml/min	ry 5 min.
Ref. 8	3%, 4% and 5%.	n. each	Baseline and every 7 minutes.	ml/min	ry 7 min.
Ref. 4	4.5%	5 min. periods up to 20 min.	Baseline and every 5 min.	l/min and 2.5 ml/min	ry 5 min
Ref. 2	or 4.5%	5 min. periods up) min.	Baseline and every 5 min.	l/min	Every 5 min. or they want to do so.
Ref. 6	4.5%	1, 2, 4, 8, 16 min.	Baseline and after each inhalation.	Not recorded	After each inhalation

Conclusions

The proposed sputum induction protocol proved to be safe, simple and effective method to obtain sputum sample but was contaminated by squamous cells and did not produce cells from the lower airways. Sputum Induction Protocol based on F.E. Hargreave work was far superior producing an adequate sputum samples with cells from lower airways and minimal squamous cells contamination. Study data demonstrated that further research is needed with larger samples in order to draw conclusions about correlation between impulse oscillometry and spirometry measurements. Initial methylene blue staining of sputum smear is a useful tool in identifying grossly contaminated sample by squamous cells.

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Appendices

Appendix A: Population Characteristics

Subject	**Gender**	**Age**
21	Male	33
22	Female	51
23	Female	51
24	Male	27
25	Male	19
26	Male	19
27	Male	34
28	Male	20
29	Male	22
30	Female	41
31	Male	20
32	Female	23
33	Male	46
34	Female	24
35	Male	46
36	Female	34
37	Female	23
38	Female	23
39	Female	22
40	Female	25
41	Male	34
42	Male	41
43	Male	46
44	Male	31
45	Male	20

Appendix B: Expectorate Volume Measurements (in Milliliters)

	Group 1	Group 2	Group 3	Ungrouped
Subject	After 4 minutes	After 8 minutes	After 15 minutes	After 4 to 15 minutes
21	.	6.00	.	6.00
22	7.50	.	.	7.50
23	.	6.30	.	6.30
24	10.00	.	.	10.00
25	7.00	.	.	7.00
26	5.00	.	.	5.00
27	5.00	.	.	5.00
28	4.80	.	.	4.80
29	4.90	.	.	4.90
30	9.00	.	.	9.00
31	5.00	.	.	5.00
32	4.90	.	.	4.90
33	30.00	.	.	30.00
34	4.80	.	.	4.80
35	4.90	.	.	4.90
36	.	.	5.00	5.00
37	.	.	7.50	7.50
38	.	.	5.00	5.00
39	.	.	5.80	5.80
40	.	.	7.70	7.70

Appendix C: Total Cell Count

Subject	Total Cell Count per Milliliter	Percentage of Squamous Cells (%)
21	1,100,000	33
22	1,320,000	33
23	310,000	81
24	360,000	42
25	720,000	40
26	980,000	54
27	450,000	67
28	1,090,000	58
29	690,000	42
30	610,000	21
31	800,000	38
32	3,690,000	3
33	380,000	26
34	440,000	23
35	770,000	35
36	530,000	32
37	360,000	57
38	1,050,000	31
39	432,000	25
40	1,100,000	26

Appendix D: Spirometric Measurements (FEV1 in Litres)

Subject	Predicted	Baseline	1st Induction	2nd Induction	3rd Induction
21	4.51	5.04	4.87	5.06	.
22	2.67	3.23	2.89	.	.
23	2.75	3.73	3.79	3.84	.
24	4.69	3.62	3.82	.	.
25	4.58	4.36	4.34	.	.
26	4.58	4.22	4.62	.	.
27	4.32	4.00	3.43	.	.
28	3.85	3.68	3.31	.	.
29	4.38	3.37	2.75	.	.
30	3.26	2.73	3.00	.	.
31	3.99	4.10	3.86	.	.
32	3.96	3.00	2.98	.	.
33	3.97	4.74	4.73	.	.
34	2.90	2.93	2.87	.	.
35	3.97	3.93	4.18	.	.
36	3.19	3.11	3.05	3.01	3.05
37	3.39	3.39	3.07	3.07	3.04
38	3.23	3.58	3.44	3.51	3.39
39	3.33	3.07	2.90	2.92	2.90
40	3.67	2.95	2.90	2.73	2.81

Appendix E: Impulse Oscillometry Measurements (R5)

Subject	Predicted	Baseline	1st Induction	2nd Induction	3rd Induction
21	2.75	3.36	3.84	4.14	.
22	3.87	4.32	4.30	.	.
23	3.87	5.28	4.44	4.48	.
24	2.69	2.71	3.47	.	.
25	2.60	3.62	3.64	.	.
26	2.60	4.00	3.52	.	.
27	2.76	2.46	2.15	.	.
28	2.62	2.79	3.01	.	.
29	2.63	2.85	2.82	.	.
30	3.70	8.67	9.16	.	.
31	2.61	3.46	4.37	.	.
32	2.96	4.47	3.69	.	.
33	2.90	3.66	3.94	.	.
34	3.41	5.34	4.35	.	.
35	2.90	4.07	3.73	.	.
36	3.57	5.17	4.67	4.79	5.26
37	3.40	3.02	3.10	3.19	2.90
38	3.40	2.73	3.16	2.82	3.16
39	3.38	4.73	4.97	4.86	4.97
40	3.43	4.56	4.93	5.28	4.84

Appendix: F Medical Questionnaire

Subject number:

Induced Sputum Study Medical Questionnaire

Date ______________________________________

1) **Gender:** Male Female

2) **How old are you?** ____________________________

3) **What is your current occupation?**

____

4) **Do you currently have, or have you ever had any of the following conditions listed below?**

YES NO CHEST PAIN, PALPITATIONS, IRREGULAR HEART BEAT, OR HEART DISEASE?

YES NO HIGH BLOOD PRESSURE?

YES NO ASTHMA, BRONCHITIS, EMPHYSEMA, OR OTHER LUNG OR BREATHING DISORDERS?

YES NO DIFFICULT OR HEAVY BREATHING

Appendix: F (Continued)

YES NO A LARGE AMOUNT OF PHLEGM PRODUCTION

YES NO PREGNANCY?

IF YOU HAVE CIRCLED YES TO ANY OF THE ABOVE QUESTIONS, PLEASE EXPLAIN:

____

____

5) **Do you have any health problems or past medical history of health problems that you have seen a physician for?**

a.____

b. ____

c. ____

d. ____

e.____

6) **Are you taking any medications?**

a. ____

b. ____

c. ____

d. ____

e. ____

Appendix F: (continue)

7) **On what date were you last ill? ___________________________________**

8) **What did you have? illness ______________________________________**

9) **Do you or anybody in your family have any allergies**?