

Reproducibility of measurements of exhaled NO, and cell count and cytokine concentrations in induced sputum

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Reproducibility of measurements of exhaled NO, and cell count and cytokine concentrations in induced sputum. M. Purokivi, J. Randell, M-R. Hirvonen, H. Tukiainen. ©ERS Journals Ltd 2000.

ABSTRACT: Sputum induction is a noninvasive, well-tolerated method for studying airway inflammation. When induction with hypertonic saline is repeated at short time-intervals (<24 h), the cell profile of sputum has not been reproducible. To determine the proper interval between sampling cell profiles and cytokine contents of sputum samples that had been induced 48 h apart, were compared. In addition, the inducible nitric oxide synthase (iNOS) expression of sputum cells was compared to the levels of exhaled nitric oxide (NO).

Sputum induction and measurement of exhaled NO was performed in 31 healthy nonatopic volunteers. Cell differentials were counted. Concentrations of interleukin (IL)-4, IL-6, tumour necrosis factor (TNF) α , eosinophil cationic protein (ECP) were measured in sputum supernatant, and iNOS was determined.

Reproducibility of cell counts was high ($r=0.836$ total cells, $r=0.762$ neutrophils, $r=0.966$ eosinophils, $r=0.742$ macrophages). IL-4 ($r=0.398$), IL-6 ($r=0.566$), TNF α ($r=0.658$) and ECP ($r=0.501$) were also less reproducible in healthy volunteers. Consistent with the low levels of NO in the exhaled air (18.5 ± 2.6 ppb and 19.3 ± 2.8 parts per billion (ppb) on the two study days, $r=0.976$, $p=0.0000$), expression of iNOS was not detected.

In conclusion, in healthy subjects, induced sputum cell counts are reproducible. Even though the success rate in nonatopic populations is relatively low, sputum induction appears to be a valid method for detecting inflammatory changes within the airways, when being performed 48 h apart.

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There has been wide interest in developing noninvasive methods to determine lower airway inflammation in different respiratory disorders. Measurement of inflammatory mediators in induced sputum provides a direct method to investigate airway inflammation in different respiratory conditions including asthma. Sputum induction by nebulized hypertonic saline has been found to be safe and well tolerated [1–3]. However, the saline inhalation affects the production of inflammatory markers, since neutrophilia and loss of macrophages has been reported in samplings repeated within 24 h [4]. This suggests that a longer time interval is required between two samplings of induced sputum, when it is used for the evaluation of allergen challenge. Thus, the time interval in which possible effects of saline inhalation disappear, needs to be defined.

Defining the cytokine profile in induced sputum could widen the view to inflammatory state of lower respiratory tract. Previously the attempts to measure cytokines by using bronchoalveolar lavage samples have failed because of dilution caused by the large volume of saline needed for sampling [5]. In sputum induction this problem is avoided, at least when low volume of nebulized saline is used [6].

Since not all patients are able to produce sputum, despite induction with hypertonic saline, other noninvasive methods to detect airway inflammation are needed. One of the most promising is the measurement of exhaled nitric oxide (NO) by a chemiluminescence analyser [7]. NO plays an important role as an immune defence molecule. The excessive production of NO by inducible nitric oxide synthase (iNOS) promotes the classical signs of inflammation and causes tissue injury. Changes in exhaled NO correlate with the number of eosinophils and increased eosinophilic cationic protein (ECP) values in induced sputum [8, 9]. The role of proinflammatory cytokines in the upper respiratory tract has previously been discussed in relation to indoor air problems as well as in relation to viral infection. Therefore, a more comprehensive spectrum of inflammatory markers, than those referring only to allergy, should be assessed from induced sputum, if the symptoms of the patient refer to inflammation but are not caused by asthma or immunoglobulin (Ig)E-mediated allergic reaction.

The aim of the present study was to evaluate the effect of saline inhalation on cell counts and cytokine concentrations in induced sputum repeated 48 h apart. The reproducibility of total cell count, cell differentials and ECP as well as concentrations of interleukin 4 (IL)-4, IL-6 and tumour necrosis factor (TNF) α in the supernatant of induced sputum were analysed in healthy volunteers. Moreover, the reproducibility of NO measurements by chemiluminescence analyser were studied, and the expression of iNOS in the cells of induced sputum was compared to levels of exhaled NO.

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Methods

Subjects

Thirty-one healthy, non-atopic volunteers, 25–58 (mean 43) yrs of age, selected from the staff at the Dept of Respiratory Medicine and Dept of Clinical Chemistry, were studied. They completed a questionnaire concerning their current health, previous inflammatory diseases, possible respiratory symptoms, occupational and housing conditions and smoking habits. None of them were using inhaled medication. The study was conducted according to the principles of the Declaration of Helsinki and it was approved by the Ethical Committee of the Kuopio University Hospital.

Study design

The subjects attended the laboratory twice, 48 h apart at the same time of the day. On the first visit they completed the health questionnaire, and peak expiratory flow rate (PEFR) (Wright Peak Flow meter; Airmed Ltd, Harlow, UK) [10] and exhaled NO were measured. After inhalation of salbutamol, sputum was induced. On the second visit the same measurements were repeated.

Sputum induction and processing

Peak expiratory flow rate (PEFR) was performed before and after the sputum induction, for safety reasons. The subjects inhaled two puffs of salbutamol (Buventol Easyhaler®) 100 µg·dos⁻¹, followed by inhalation of 5 mL nebulized, hypertonic (4%) saline for 15–20 min [11]. Saline solution was nebulized by an ultrasonic nebulizer, particle size 7.5 µm (Omron UI; Omron Healthcare GmbH, Hamburg, Germany). The collected sputum samples were examined within 2 h to avoid cell destruction [12]. Briefly, sputum plugs originating from the lower respiratory tract were separated and weighed. Freshly prepared dithireitol (DTT), (Sputolysin, Calbiochem corp., San Diego, CA, USA) was diluted in distilled water (1:10), and this solution was added to the sputum in a volume equal to two times the weight of the sputum portion [12]. Sputum was then shaken in a water bath at 37°C for 15 min. To ensure homogenization the sputum-DTT mixture was mixed using a vortex for 15 s every 5 min. This suspension was further diluted with phosphate buffered saline (Dulbecco's phosphate buffered saline (D-PBS); Life Technologies Ltd, Paisley, UK) in a volume equal to the sputum plus DTT. Then the suspension was filtered through a 41 µm nylon gauze (Millipore corporation, Bedford, MA, USA) to remove mucus and was centrifuged 790×g for 10 min. Total cell count (TCC) and cell viability (trypan blue exclusion method) were determined by using a haemocytometer. The supernatant was aspirated and frozen at -70°C.

Biochemical analyses

ECP (µg·L⁻¹) was analysed by radioimmunoassay (RIA, Pharmacia & Upjohn, Uppsala, Sweden). Cytokines were analysed using human IL-6, IL-4 and TNFα Duoset

enzyme-linked immunosorbent assay (ELISA)-kits (Genzyme, Cambridge, MA, USA). The samples were analysed by ELISA reader (iEMS Reader MF, Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Cytokine concentrations of samples were calculated by interpolating absorbances of samples to the standard curve. The detection limits for the ELISA were 2–5 pg·L iNOS was measured by Western blot analyses with antibody against iNOS (130 kDa) [13].

Cytospin

The cell pellet was resuspended in D-PBS to reach concentration of 1×10⁶ cells·mL⁻¹. The cell suspension was centrifuged (Shandon, Life Sciences International Ltd, Cheshire, UK) at 450 revolutions per min (rpm) for 6 min. The slides were fixed by ethanol and stained by May-Grünwald Giemsa for cell differential count from 500 cells. Only samples with cell viability >50% and squamous cell contamination <20% were considered adequate [11].

Measurement of exhaled nitric oxide

Exhaled NO was measured by a chemiluminescence analyser (Sievers Model 280 NOA; Sievers Instruments, Inc., Boulder, CO, USA) according to the European Respiratory Society (ERS) guidelines for the measurement of exhaled NO [14]. Subjects performed a slow vital capacity (VC) manoeuvre for 30 s against a fixed expiratory resistance, which eliminated contamination by nasal NO by closing the soft palate. The pressure level during exhalation was optimized by following the computer screen on-line to reach constant flow rate in exhale. Exhaled air was led through a nonbreathing valve into a Teflon tubing system connected to the analyser. Recordings were performed by the single-breath programme, and they were seen on a computer screen on-line. The subjects gave three exhaled samples. The relative standard deviation (SD) between collected samples was expected to be <10%. The detection limit for NO was 1 part per billion (ppb). Measurements were made in the same laboratory under constant conditions. The chemiluminescence analyser was calibrated daily by using zero air and a certified concentration of NO.

Statistics

Reliability coefficients and Bland-Altman plots [15] were used to describe reproducibility and intrapatient correlation of NO measurements, cell counts and cytokines. It was expected that 95% of the differences between measures <2SD. Exploratory data analyses revealed that the distribution of the TNFα values obtained in this study was not normal and therefore, logarithmic transformations were used to analyse the reliability coefficient. Differential cell counts are presented as geometric mean (range) except eosinophils which are presented as mean (range) due to their small number. Results of cytokine measurements are presented as mean±SEM. The statistical analyses were performed by using the SPSS/PC+ software package version 8.0 (SPSS Inc., Chicago, USA) and Bland-Altman plots were drawn by MedCalc software package version 4.2 (MedCalc Software, Mariakerke, USA).

Results

Sputum induction

Twenty of the thirty-one healthy volunteers (64%) succeeded in sputum production. One sample was rejected because of a low cell count (<500 cells per cytospin) and one because it contained a high number of squamous cells (>20%). Thus, statistical analyses of total cell count, cell differentials and biochemical markers were done with 18 sample pairs. No significant variability was noticed between PEFR values before and after sampling. The mean weight of sputum on the first measurement day was (mean \pm SEM) 356 \pm 78 mg and on the second 360 \pm 61 mg. The mean cell viability was 86% and 82% after 0 and 48 h respectively.

Cell differentials

With the exception of lymphocytes, all cell types were highly reproducible (fig. 1). The reliability coefficients of total cell count and cell differentials are shown in table 1.

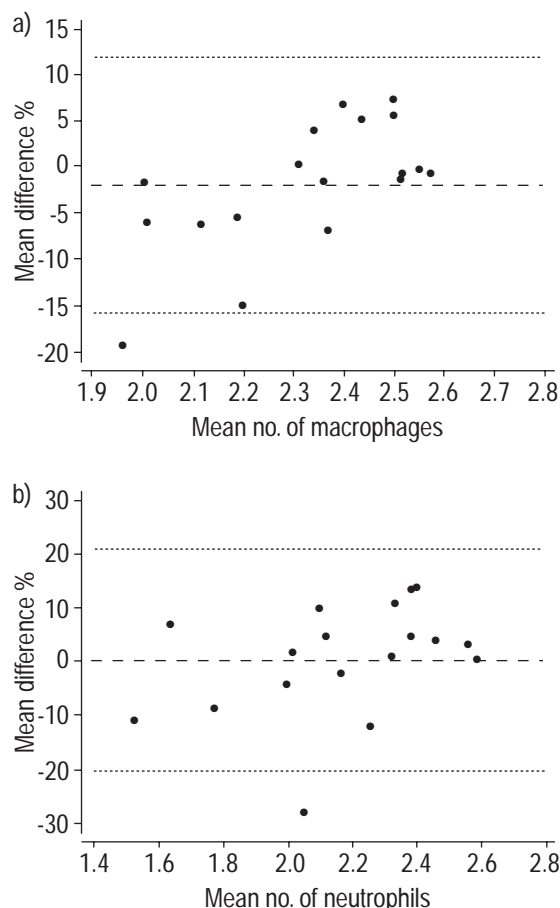


Table 1. – Reproducibility of differential cell count in induced sputum collected at 0 and 48 h

| | Cell number 10 ⁶ cells·L ⁻¹ | | r | p |
|--------------------------|---|------------|-------|-----|
| | 0 h | 48 h | | |
| Total cells | 561 (1699) | 440 (1944) | 0.836 | *** |
| Macrophages | 203 (334) | 221 (278) | 0.742 | *** |
| Neutrophils | 152 (373) | 148 (350) | 0.762 | *** |
| Eosinophils [#] | 2.0 (90) | 1.5 (55) | 0.966 | *** |
| Lymphocytes | 7.3 (27) | 9.0 (30) | 0.253 | |

Values of 18 subjects are expressed as geometric mean (range) except [#]: mean (range). ***: p<0.001.

Production of cytokines

The measurements of IL-6 (r=0.567, p=0.006, fig. 2a) TNF α (r=0.658, p=0.014, fig. 2b), IL-4 (r=0.398, p=0.046, fig. 2d) and ECP (r=0.501, p=0.003, fig. 2c) were also reproducible (table 2).

Production of nitric oxide

Exhaled NO was analysed from all 29 volunteers twice, 48 h apart. The mean NO levels were 18.5 \pm 2.6 ppb and

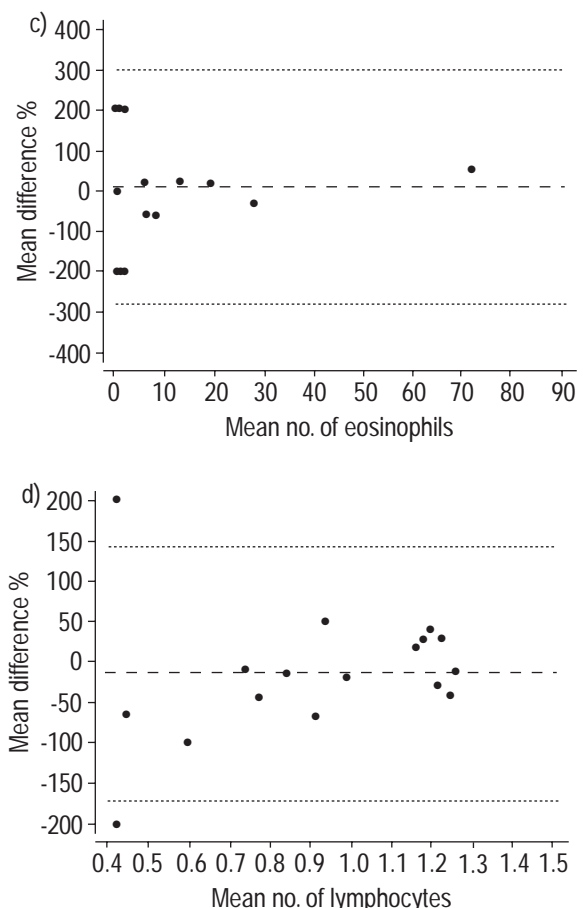


Fig. 1. – Reproducibility of differential cell counts: a) macrophages; b) neutrophils; c) eosinophils; d) lymphocytes in induced sputum (%) mean (---) \pm 1.96 SD (·····). Repeatability of measurements is expressed as proposed by BLAND and ALTMAN [15] and it is expected that 95% of the differences between measures are <2 SD. For each figure the differences of log values between 0 h and 48 h are plotted on vertical axis against the mean value of the two measurements. Due to small number of (or zero) eosinophils, they are expressed without log transformation.

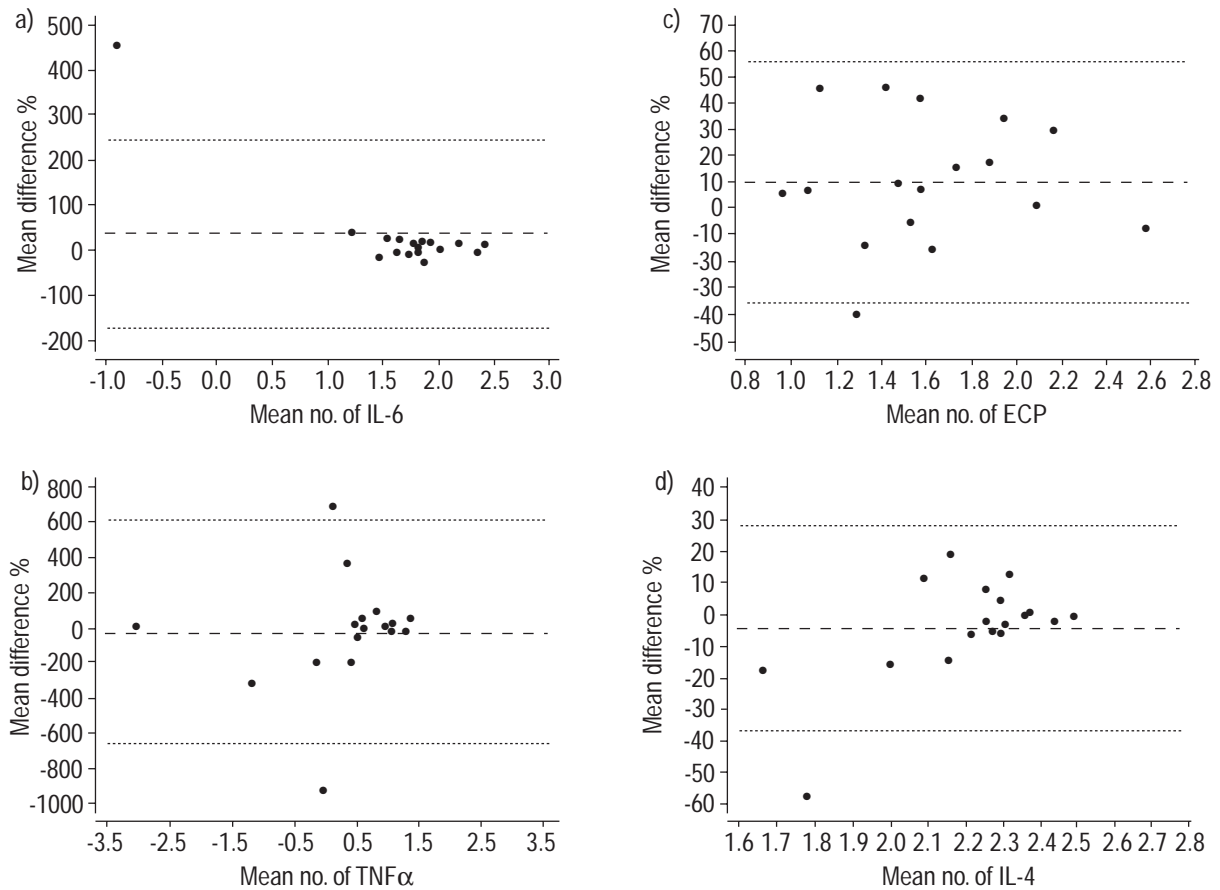


Fig. 2. – Reproducibility of cytokine measurements: a) interleukin (IL)-6 ($\text{pg}\cdot\text{mL}^{-1}$); b) tumour necrosis factor (TNF) α ($\text{pg}\cdot\text{mL}^{-1}$); c) IL-4 ($\text{pg}\cdot\text{mL}^{-1}$); and d) eosinophil cationic protein (ECP) ($\mu\text{g}\cdot\text{L}^{-1}$) in induced sputum (%), mean (---) \pm 1.96 SD (····). Reproducibility of measurements is expressed as proposed by BLAND and ALTMAN [15] and it is expected that 95% of the differences between measures are <2 SD.

19.3 \pm 2.8 ppb respectively on the two study days. ($r=0.976$, $p=0.0000$, fig. 3). Consistent with the low levels of NO in the exhaled air, expression of iNOS was not detected by Western blot analyses in the induced sputum samples (data not shown).

Discussion

The results demonstrate that systematic changes in cellular composition caused by saline inhalation [4, 16] can be avoided, when sputum induction is performed at least 48 h apart. In healthy volunteers the reproducibility of total cell count was high as well as that of eosinophils, neutrophils and macrophages. Consistent with previous studies the lymphocyte count was not reproducible [17]. The fairly small amount of saline used in the experimental

procedure to limit the inhalation time and to avoid the dilution of samples also enabled the measurements of cytokines in induced sputum. Despite a relatively short procedure of saline inhalation, 64% of healthy volunteers succeeded in sputum induction 48 h apart. The method was well tolerated with no changes in PEFV values measured before and after the saline inhalation.

Table 2. – Interleukin (IL)-4, IL-6, tumour necrosis factor (TNF) α and eosinophilic cationic protein (ECP) in induced sputum collected at 0 and 48 h

| Time point | IL-4 | IL-6 | TNF α | ECP [#] |
|------------|----------------|-----------------|-----------------|------------------|
| 0 h | 178 \pm 81.2 | 113 \pm 107 | 9.97 \pm 15.0 | 80.3 \pm 94.0 |
| 48 h | 189 \pm 65.7 | 65.1 \pm 56.1 | 5.24 \pm 5.39 | 59.4 \pm 100 |

Data presented as mean \pm SEM, the units are $\text{pg}\cdot\text{mL}^{-1}$ unless otherwise stated. #: units $\text{pg}\cdot\text{L}^{-1}$.

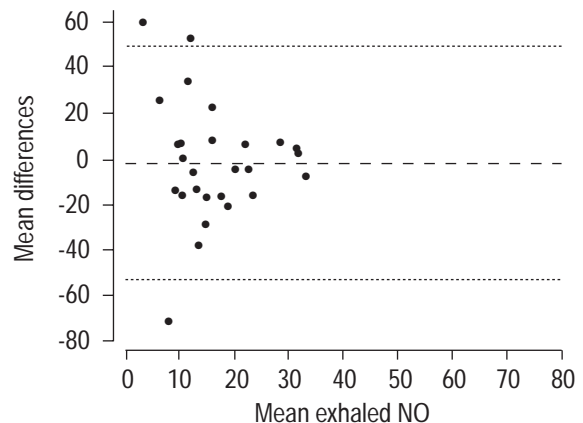


Fig. 3. – Reproducibility of measurements of exhaled NO. Reproducibility of measurements is expressed as proposed by BLAND and ALTMAN [15] and it is expected that 95% of the differences between measures are <2 SD.

According to the presented results the reproducibility of proinflammatory cytokines TNF α and IL-6 in induced sputum is high in a 48 h interval. Moreover, the present results indicate that low concentrations of TNF α and IL-6 can be detected in induced sputum from healthy subjects. These cytokines are of interest in searching for new diagnostic tools because of their central role in airway inflammation. They may be involved in allergic inflammation as well as in symptoms caused by occupational or indoor air exposures [18].

The levels of IL-4 were only low or moderate in the induced sputum of the healthy subjects in this study, however, they were still reproducible. In addition to low IL-4 concentrations, relatively small amounts of ECP in induced sputum suggest that the subjects did not suffer from IgE mediated allergy. The values at time point 0 were found to be slightly higher than those at 48 h, but the difference did not reach statistical significance.

In the present study, the measurement of NO from exhaled air of healthy subjects was highly reproducible. In search for the plausible source of exhaled NO, it was demonstrated that no expression of iNOS was detectable in induced sputum cells of healthy subjects. This agrees with the previous study of HAMID *et al.* [19], where iNOS was detectable in epithelial biopsies of asthmatic subjects but not in those of healthy volunteers. TNF α is known to induce iNOS expression in human lung epithelial cells [20]. The low level of TNF α detected is in concordance with the absence of iNOS in induced sputum samples studied here. This suggests that NO concentrations measured from the subjects by a chemiluminescence analyser, were induced by constitutive nitric oxide synthase (cNOS). Together with the finding that no active inflammation is recognized, and the concentrations of proinflammatory cytokines and number of macrophages are low, the origin of NO measured is likely to be bronchial epithelium [21]. NO produced by cNOS is involved in physiological regulation of airway function, whereas NO produced by iNOS is involved in inflammatory diseases of the airways and in host defences against infection.

In conclusion, the cell count in induced sputum and measurement of exhaled nitric oxide are reproducible and valid methods in detecting the inflammatory changes, when sputum induction is performed 48 h apart. Cytokines can also be reliably determined from induced sputum. Expression of inducible nitric oxide synthase was not detected from the samples of healthy subjects, its measurement may be a helpful tool in studying inflammatory processes and the origin of the nitric oxide.

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