Reference ranges for induced sputum eosinophil counts in Korean adult population

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Background: Induced sputum analyses are widely utilized to evaluate airway inflammation in asthmatics. However, the values have not been examined in Korean adults.

Objective: The purpose of this study is to determine reference ranges for induced sputum eosinophils and their influencing factors in Korean adults.

Methods: A total of 208 healthy nonasthmatic adults were recruited. Sputum induction and processing followed the international standard protocols.

Results: Adequate sputum samples were successfully collected from 81 subjects (38.9%). The upper 90 percentile for sputum eosinophil was calculated as 3.5%. The median value of eosinophil count percentage was significantly higher in subjects with atopy than those without atopy (median, 1.6%; range, 0–11.0% vs. median, 0%; range 0–3.6%, p=0.030). However, no significant correlations were found with age, gender, body mass index, smoking status, blood eosinophil, or fractional exhaled nitric oxide levels.

Conclusion: Current study was the first attempt to determine the reference ranges of induced sputum eosinophils in Korean adults. The cutoff value for sputum eosinophilia was 3.5%, and was significantly associated with atopy.

Key words: Eosinophils; Sputum; Healthy volunteers; Reference values

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INTRODUCTION

Induced sputum analysis is one of representative tests to define airway inflammatory phenotype in asthma [1]. It has been standardized about a decade ago, and has been widely used in research and practice due to its reproducibility and less invasiveness [2, 3]. Induced sputum eosinophil is still one of the most useful biomarkers for asthma [1], as it has clinical utility in phenotyping asthma or predicting the treatment response [1, 4, 5]. However, currently available reference levels (defined as more than 3% of eosinophil count in total cell count) have been derived from Western population studies [6-10], but not have been examined in Korean adults yet. Considering the potential heterogeneity in fractional exhaled nitric oxide (FeNO) levels among ethnic groups [11], induced sputum profiles also need to be examined specifically for Korean populations. In this study, we aimed to determine reference ranges for induced sputum eosinophil in healthy Korean adults.

MATERIALS AND METHODS

Subjects

Healthy adult volunteers were recruited by public posting from visitors to Seoul National University Hospital, Seoul, Korea. The recruitment was carried out from November 2010 to September 2011. Inclusion criteria were (1) age over 19-year-old, (2) no history of respiratory tract disease in previous 1 month, (3) no evidence of systemic infection in previous 3 months, and (4) absence of asthma related symptom and airway hyperresponsiveness (defined as methacholine provocative concentration causing 20% fall in forced expiratory volume during 1 second [PC_{20}]≤16 mg/mL). Finally, 208 healthy volunteers were recruited, and all of them underwent induced sputum tests. All the participants were given written informed consents. The study protocol was approved by the Institutional Review Board of Seoul National University Hospital (IRB no. 1012-061-344).

Sputum induction and processing

First, we check baseline forced expiratory volume during 1 second (FEV_1). They were pretreated with salbutamol 2 puff as a bronchodilator. Sputum was induced with hypertonic saline 4.5% via ultrasonic nebulizer, and was collected into a petri dish which was placed on the ice. FEV_1 was checked every 5 minutes during 20 minutes. The sample was kept at 4°C, and sputum processing was performed within 4 hours of induction, with minor modifications of the European Respiratory Society standard protocol [12]. We removed saliva with the pipet and determined the volume of sputum. The equal volume of 0.01M dithioerythritol was added, and we gently mixed them. These were filtered with 100-µm cell strainer or mesh on 15-mL tube and centrifuged with 1,000 or 2,000 rpm for 10 minutes at 4°C. We aspirated supernatant 1 to 1.5 mL into a cryotube and froze them at −20°C or −80°C. The cell pellet was resuspended with 1-mL phosphate buffered saline (PBS). The volume of 10-µL suspension was added with 90-µL trypan blue. We determined cell counts and then dilute cell suspension with PBS to obtain 1×10^5 cells/mL. The cytospin was done at 500 rpm for 5 minutes at 4°C, and the Diff Quick stain was done. Finally we counted cells as %, except the epithelial cells.

Skin prick tests

The inhalant allergen skin prick test was performed to determine atopic status. Major inhalant allergens were evaluated, including Dermatophagoides pteronyssinus, Dermatophagoides farinae, cat, dog, tree mix, grass mix, ragweed, mugwort, cockroach, Alternaria spp., and Aspergillus fumigatus. Normal saline and histamine were used to negative and positive control. Atopy was defined if there are positive results (median wheal size ≥3 mm or larger than size of histamine, and median flare size ≥10 mm) to one or more allergen.

Fractional exhaled nitric oxide

FeNO was measured by using NIOX MINO (Aerocrine, Solna, Sweden), according to the guidelines [13]. Briefly, they were asked to avoid medication or food intake which could interfere with the results. They were also instructed to avoid smoking or exercise within one hour before on the day of testing [14]. Then, they inhaled over 2–3 seconds to total lung capacity through NIOX filter, and exhaled as an upper airway pressure of 5–20 cm H_2O. FeNO was measured during its plateau status at least for 3 seconds. As the lower detection limit was 5 parts per billion (ppb), the values <5 ppb were considered as 2.5 ppb. In this analysis, average levels of at least two acceptable measurements were utilized.

Statistical methods

Statistical analyses were performed with SPSS ver. 10.0 (SPSS Inc., Chicago, IL, USA). As sputum inflammatory cell counts were not normally distributed, their distributions were described as

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median and ranges. The associations between cell counts and clinical factors were analyzed by the Mann-Whitney tests and the Spearman correlation tests. Results were considered significant when two-sided \( p \) values <0.05.

RESULTS

Baseline characteristics

From 208 healthy volunteers who underwent induced sputum tests, 81 samples (38.9%) were eligible for analyses. The reasons for ineligibility were inadequate quality of sputum (n = 20), and failure to induce sputum (n = 107), presumably due to the lack of airway inflammation. Baseline characteristics of the study subjects were described in Table 1. Female were predominant (66.7%) and a mean age was 52.3 ± 13.1 years. Body mass index values were over 23.7 ± 3.4 kg/m\(^2\). Most of subjects (77.8%) were never-smoker and 20 subjects (31.3%) had atopy. There was no subject who had peripheral blood eosinophilia (≥500/μL). Most of subjects (72.8%) had low value of FeNO (<25 ppb) and the maximum value of FeNO was 49 ppb. Minimum value of sputum eosinophil was 0% and maximum value was 11%.

Distribution of inflammatory cells in induced sputum of healthy subjects

The distribution of induced sputum inflammatory cells in healthy subjects was described in Table 2. Macrophage and neutrophil were predominant cells in induced sputum. Macrophage had a median of 71.3% (range, 0–97.3%), and the upper 90 percentile of 89.2%. Neutrophil had a median of 24.7% (range, 0.7–100%), and the upper 90 percentile of 96.0%. Eosinophil had a median of 0% (range, 0–11%), and the upper 90 percentile of 3.5%. The distribution of induced sputum eosinophil was also presented in Fig. 1.

Associations of sputum inflammatory cells with demographic and clinical parameters

Associations of sputum eosinophil or neutrophil counts with demographic and clinical parameters were analyzed (Tables 3, 4). For eosinophils, subjects with atopy showed significantly higher counts than those without atopy. Distribution of induced sputum eosinophil (%) according to presence of atopy was presented in Fig. 2. However, no significant correlations were found with age, gender, smoking, body mass index, allergic rhinitis, peripheral

DISCUSSION

The present study reported, for the first time, the normal range of induced sputum eosinophils among Korean healthy adults. The range (3.5% as 90 percentile) was comparable to those from previous studies conducted in Western populations [6, 7].

Airway inflammation is an important pathogenesis underlying airway diseases such as asthma, chronic obstructive pulmonary disease, or chronic cough [15]. Therefore, to obtain airway sample

Table 1. Baseline characteristics of healthy adults who successfully underwent induced sputum analyses (n = 81)

| Characteristic                      | Value          |
|-------------------------------------|----------------|
| Male gender                         | 27 (33.3)      |
| Age (yr)                            | 52.3 ± 13.1    |
| Body mass index (kg/m\(^2\))        | 23.7 ± 3.4     |
| Smoking status                      |                |
| Never-smoker                        | 63 (77.8)      |
| Ex-smoker                           | 15 (18.5)      |
| Current-smoker                      | 3 (3.7)        |
| Atopy (n = 64)                      | 20 (31.3)      |
| History of allergic rhinitis        | 39 (48.1)      |
| Pulmonary function test             |                |
| FEV\(_1\) (% of predicted value)    | 111.9 ± 24.6   |
| FVC (% of predicted value)          | 104.9 ± 28.5   |
| FEV\(_1\)/FVC (%)                   | 85.4 ± 8.2     |
| PC\(_{20}\) > 16 mg/mL              | 81 (100)       |
| Peripheral blood eosinophilia       |                |
| (calculated eosinophil count ≥ 500/μL) | 0 (0)         |
| FeNO (ppb)                          | 19.3 ± 10.1    |
| Differential cell counts in induced sputum (%) |          |
| Macrophage                          | 71.3 (18.5–84.5)|
| Neutrophil                          | 24.7 (14.2–63.0)|
| Eosinophil                          | 0 (0–2.0)      |

Values are presented as number (%), mean ± standard deviation or median (interquartile range).

FEV\(_1\), forced expiratory volume in 1 second; FVC, forced vital capacity; PC\(_{20}\), provocative concentration causing a 20% fall in FEV\(_1\); FeNO, fractional exhaled nitric oxide; ppb, parts per billion.
is a mandatory step for both of research and clinical purposes. This is why, induced sputum test has gained a considerable popularity as a safe noninvasive alternative to bronchoscopy which could be at risk of exacerbations [1]. As, asthma is now considered as a heterogeneous entity with potentially various inflammatory phenotypes [16, 17], the necessity of the assessment is to continue. In particular, sputum eosinophilia is a consistently important biomarker [18, 19]. Briefly, airway eosinophilia has been associated with airway obstruction, hyperresponsiveness, and corticosteroid response for asthma [20-22]. To determine this, the literature suggests the reference range as <2.4% [6, 7]. However, it should be noted that the reference values were obtained from relatively small numbers of studies conducted in Western populations [6, 7]. Gender effects on sputum eosinophils remain elusive, but our lack of the difference might have been related to a low number of male participants; previous studies found females had 0.274% higher eosinophils than males [6].

Our high failure rates in sputum induction (61.1%) could also have been a bias factor. In the present study, 51.4% of participants could not expectorate sputum despite efforts. The rate of inadequate sample was 9.6%, which was comparable to total failure rates of 11.4–19% in previous studies [6, 7]. We processed the samples within 4 hours of induction, which was known to be acceptable [24]. We used 100-µm mesh for improving slide quality, which was larger than 40-µm nylon net filter in the international standard protocol [12]; however, the step of mesh filtering does not influence the sputum eosinophils [25].

Clinical utility of sputum neutrophilia and its consistency is still controversial, and was not fully discussed in this article. The distribution of sputum neutrophils is wider than eosinophils, and
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Table 3. Associations of sputum eosinophil/neutrophil (%) with demographic and clinical parameters

| Parameter                | Eosinophils (%) | Neutrophils (%) |
|--------------------------|-----------------|-----------------|
| Gender                   |                 |                 |
| Male (n = 27)            | 0.7 (0–5.0)     | 22.2 (3.3–100)  |
| Female (n = 54)          | 0 (0–11)        | 20.7 (2.3–97.0) |
| p-value*                 | 0.275           | 0.920           |
| Smoking history          |                 |                 |
| Never-smoker (n = 63)    | 0.3 (0–11.0)    | 19.2 (2.3–99.0) |
| Ex-smoker (n = 15)       | 0 (0–3)         | 28.7 (10.3–100) |
| Current smoker (n = 3)   | 0.3 (0–1.0)     | 16.0 (3.3–62.0) |
| p-value*                 | 0.626           | 0.587           |
| Atopy                    |                 |                 |
| Atopy (n = 43)           | 1.6 (0–11.0)    | 20.0 (3.3–96.0) |
| Nonatopy (n = 20)        | 0 (0–3.6)       | 22.0 (2.3–100)  |
| p-value*                 | 0.030           | 0.284           |
| Allergic rhinitis        |                 |                 |
| History (+)              | 0 (0–11)        | 24.0 (3.3–100)  |
| History (-)              | 0.3 (0–9.3)     | 18.3 (2.3–97.0) |
| p-value*                 | 0.911           | 0.580           |

Values are presented as median (range).
* Mann-Whitney test.

Table 4. Bivariate correlations of sputum eosinophil/neutrophil (%) with demographic and clinical parameters

| Parameter                | Eosinophils (%) | Neutrophils (%) |
|--------------------------|-----------------|-----------------|
| Age (yr)                 |                 |                 |
| Correlation coefficient (rho) | 0.120           | 0.134           |
| p-value*                 | 0.286           | 0.234           |
| Body mass index (kg/m²)  |                 |                 |
| Correlation coefficient (rho) | 0.039           | 0.008           |
| p-value*                 | 0.730           | 0.945           |
| FeNO (ppb)               |                 |                 |
| Correlation coefficient (rho) | 0.094           | 0.057           |
| p-value*                 | 0.406           | 0.612           |
| Peripheral blood eosinophil counts (/μL) | 0.151 | 0.056 |
| p-value*                 | 0.287           | 0.694           |

FeNO, fractional exhaled nitric oxide.
* Spearman correlation test.

more likely to have normal distributions [6, 7]. Airway neutrophilia has been associated with infection, irritant exposure, obesity, or sometimes with corticosteroid-resistant asthma [26] Among normal subjects, neutrophilia also appeared to be associated with older age [8]. The major limitation to our study is a sample size. We originally
intended to analyze 150 or more normal subjects, but the success rate was lower than expected. Another limitation was the way of recruitment; participants were recruited from hospital visitors, which could not exclude the possibility of comorbidities other than asthma, respiratory diseases, or recent systemic infection. However, due to methodological considerations [24], sputum induction and processing should be performed with specialized instruments and very near to laboratories, making community-based sampling difficult.

In conclusions, the present study was the first attempt to determine the reference ranges of sputum eosinophils in Korean adults. Atopy was a clinical factor significantly associated with sputum eosinophilia.

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