

Reproducibility of nasal lavage in the context of the inhalation challenge investigation of occupational rhinitis

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ABSTRACT

Background: The nasal lavage (NAL) method is increasingly used to assess changes in upper airways inflammation in the investigation of occupational rhinitis (OR). A good reproducibility of the method is fundamental to accurately assess changes in markers of inflammation in nasal secretions before and after inhalation challenges. The main objective of this study was to assess the short-term reproducibility of cells and cellular markers of inflammation in NAL in the setting of specific inhalation challenge (SIC) investigating OR. An ancillary objective was to assess the reproducibility of NAL in the context of two different SIC methodologies.

Methods: Twenty-five subjects attended the laboratory for 2 separate days of NAL performed within the same week. On the first visit subjects underwent NAL before a SIC sham session and on the second visit before a SIC with the active agent. These prechallenge NAL measurements obtained on both days were used to analyze the reproducibility of the NAL method.

Results: The reproducibility for cell differential counts was satisfactory for neutrophils (intraclass correlation coefficient [ICC] = 0.68), for eosinophils (ICC = 0.95), for macrophages (ICC = 0.77), and for epithelial cells (ICC = 0.73). The reproducibility of total cell counting was poor (ICC = 0.12). The reproducibility of ECP concentrations was satisfactory (ICC = 0.67). Eosinophil counts were reproducible in the context of two different challenge methodologies.

Conclusion: The NAL method was shown to be sufficiently reproducible to be considered useful for the monitoring of upper airways inflammation during the investigation of OR by SIC.

(Am J Rhinol 22, 271–275, 2008; doi: 10.2500/ajr.2008.22.3165)

Key words: Asthma, diagnosis, inhalation challenge, nasal lavage, occupation, occupational asthma, occupational rhinitis, reproducibility, rhinitis

The assessment of the inflammatory status of the upper airways is of rising interest in respiratory research. Means such as biopsy and cytology have been used to characterize the cellular and humoral response of the nasal mucosa to environmental and occupational agents.^{1–3} These methods assess directly normal and inflammatory events but their invasive character restricts their systematic use in clinical practice and in research. Sampling nasal secretions offers an alternative objective means to assess upper airways inflammation.

Analogously to the utilization of induced sputum for the assessment of lower airway inflammation in the investigation of occupational asthma (OA), the nasal lavage (NAL) method has been used to investigate occupational rhinitis (OR) in epidemiologic and experimental studies.^{4–6} Because of inter-individual variability, studies in which subjects serve as their own control are more suitable for assessing the reproducibility of NAL.⁷ As such, specific inhalation challenges (SICs) used to diagnose OA and OR offer a good research setup. In the SIC investigation of OR, subjects are challenged during a control and active challenge days either by mimicking working exposure conditions within a small challenge room (the realistic method) or by means of a generator of particles and aerosols (the closed-circuit method). The latter methodology

delivers during the challenge low and stable concentrations of the suspected agent that, in theory, cause less irritation to the respiratory tract when compared with the “realistic method.”^{8,9} Physiological changes in the nose resulting from the controlled exposition to the agent are monitored before and after the challenge. The monitoring of upper airways inflammation by sampling and analyzing nasal secretions is a relevant parameter to be considered in OR investigation protocols. However, the selected method must be sufficiently reliable to monitor changes in airways inflammation to be included in such a protocol.

The main objective of this study was to assess the short-term reproducibility of NAL measurements of total cell count (TCC), cell differentials, and eosinophilic cationic protein (ECP) values in the context of SIC. We also wanted to evaluate the reproducibility of NAL measurements in the context of two different SIC methodologies (realistic and closed-circuit methods).

METHODS

Subjects and Protocol

We analyzed data from 25 subjects (17 men and 8 women), aged 26–58 years with a history suggestive of OA referred to the Hôpital du Sacré-Coeur de Montréal for SIC during the period from August 2005 to February 2007. A parallel assessment of nasal responses that included the performing of NAL was offered to these subjects. The evaluation of the nose was not offered if (1) subjects reported a history compatible with recent common cold, rhinosinusitis, or allergic rhinitis exacerbation; (2) subjects were on medications for nasal symp-

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toms; (3) subjects had antecedents of recent nasal surgery; and (4) subjects had significant structural abnormalities such as nasal septum perforations and nasal polyposis. Subjects with antecedents of seasonal allergic rhinitis were studied outside their allergy seasons.

It was not the objective of this study to assess the reproducibility of a nasal challenge test monitored by NAL but to assess the reproducibility of two baseline NAL measurements in the context of our SIC protocol investigating OR. Thus, study subjects underwent NAL on 2 separate days of SIC within the same week. NAL was performed before the start of each SIC session. The NAL was performed at the same time of the day (8:00 A.M.) after a 20-minute resting period. In each SIC session subjects were challenged with a control agent on the first visit and with the suspected occupational agent on the next visit. The NAL was performed before each challenge to avoid the effect of the exposure on the measurements and therefore to achieve physiological conditions as stable as possible for the assessment of the reproducibility of the measurements on the two visits. With this approach the measurement in the first visit was not influenced by any exposure and the measurement in the second visit might have some influence only from the exposure on the control day. Usually, we observe no clinically significant reaction after the exposure to the control agent; however, if such an influence was likely, it may be evidenced in the reproducibility of the two measurements.

Study subjects were challenged during the control day by means of the closed-circuit method ($n = 15$) or the realistic method ($n = 10$). The outcome measurements included the total and differential cell count and concentrations of ECP. Approval for this study was obtained from the Hospital Medical Ethics Committee and informed consent was obtained from all subjects. Participation was voluntary.

NAL Technique and Processing

The NAL technique used in this study was adapted from the procedure described by Naclerio *et al.*¹⁰ With the subject in a sitting position with the head flexed 45° backward, 5 mL of prewarmed at 37°C isotonic saline (0.9%) is instilled into one nostril. Previously, the subject was instructed to avoid breathing and swallowing and say “k-k” repeatedly for 10 seconds to prevent the fluid from being swallowed during the procedure by closing the velopharynx. After 10 seconds the subject expels the fluid into a plastic container previously stored in a refrigerator. This is followed by an NAL performed in the other nostril. The sample is collected and pooled in the same container and placed immediately on ice. Within 2 hours the NAL sample was processed. Sample volume was measured and the TCC was calculated in a hemocytometer. The supernatant was obtained by centrifugation of the sample volume at 3300 rpm for 8 minutes at 4°C and then kept frozen at -80°C until analysis. The pellet was resuspended in 0.5 mL of PBS containing 0.1% (w/v) bovine serum albumin fraction. Cytocentrifuge preparations were made by using 100 μ L of the remaining resuspended cell suspension. The preparation was centrifuged at 450 rpm and slides were stained with Wright-Giemsa to perform differential cell counts. Slides were examined blindly. Leukocyte counts were expressed as a percentage of 300 cells counted and determined by means of light microscopy. Nasal fluid samples were concentrated by centrifugation on Centricon centrifugal filter device YM-3 (Milli-

pore; FisherScientific, Montreal, PQ, Canada) with a 3000-molecular weight cutoff as previously described.¹¹ ECP levels in NAL concentrated samples were measured using a commercially available Elisa Kit (MBL Co., Ltd., Nagoya, Japan) following the manufacturer's instructions. Optical densities were determined using the ELISA reader Elx 808iu (Bio-Tek Instruments, Inc., Richmond, VA), and calculations were performed using the KC4 software (Bio-Tek Instruments, Inc.). Data were adjusted for the factor of concentration and expressed as nanograms per milliliter.¹¹

Statistical Analysis

Reproducibility of NAL measurements was determined for sample volume, total cell counting (TCC), neutrophils, eosinophils, macrophages, epithelial cells, and ECP; lymphocytes were not analyzed because the samples showing these cells were few and the numbers of cells were too low. Reproducibility was expressed by computing intraclass correlation coefficient (ICC) and was graphically represented by the Bland-Altman plots¹² for two visits (MedCalc Statistical Software, Mariakerke, Belgium). Significance was accepted at a 95% level. Differences in the percentage of cells between visits were compared by the Wilcoxon rank sign test and a 5% level of significance was applied. Data were analyzed using the statistical package SPSS 14.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Overall, the technique used to perform the NAL was well accepted by subjects. Four subjects discontinued the evaluation after the first NAL because they were not able to follow the instructions or they considered the procedure uncomfortable.

Data on cell differentials were available for 25 pairs. Data on ECP measurements were available for 10 pairs. Reproducibility of NAL cellular and humoral markers of inflammation is shown in Table 1. The analysis did not show differences statistically significant in the percentage of cells between visits. The reproducibility for all type of cells was satisfactory. The reproducibility of TCC was poor (ICC = 0.12). The reproducibility of ECP concentrations was satisfactory (ICC = 0.67). Figure 1 summarizes the reproducibility of cells percentage on the two visits.

Table 2 shows the reproducibility of cell components according to the type of SIC methodology used on the first visit in which subjects were challenged with a control agent. There were no differences in the percentages of cells between visits with both methods. Eosinophil counts were reproducible in the context of both SIC methods. The reproducibility of neutrophils (ICC = 0.75) and epithelial cells (ICC = 0.83) was satisfactory with the closed-circuit method and higher when compared with the “realistic method.” Macrophage counts showed good reproducibility with the realistic method and poor reproducibility with the closed-circuit method.

DISCUSSION

Methods to sample nasal secretions for the assessment of upper airways inflammation include absorption techniques (*e.g.*, filter papers and foams), suction techniques, and dilution techniques (*e.g.*, NAL, nasal pool, and Foley catheter).¹³ The selection of the technique should be based on its validity and

Table 1 Reproducibility of NAL measurements during SIC ($n = 25$ pairs)

Variable	Visit 1	Visit 2	ICC
Volume (cc)	5.4 ± 1.7	5.9 ± 1.1	0.53
TCC (10^6 / mL)	0.06 ± 0.09	0.04 ± 0.08	0.12
Neutrophils (%)	41.2 ± 37.5	45.8 ± 35.2	0.68
Eosinophils (%)	0.7 ± 1.6	0.7 ± 1.9	0.95
Macrophages (%)	6.7 ± 6.9	8.1 ± 8.6	0.77
Epithelial cells (%)	51.4 ± 35.6	43.9 ± 35.1	0.73
ECP (ng/mL*)	1.3 ± 1.8	0.9 ± 0.8	0.67

Numbers for visits represent mean and SD.

*Sample size for ECP was 10 pairs and measurements were taken within 48 hr

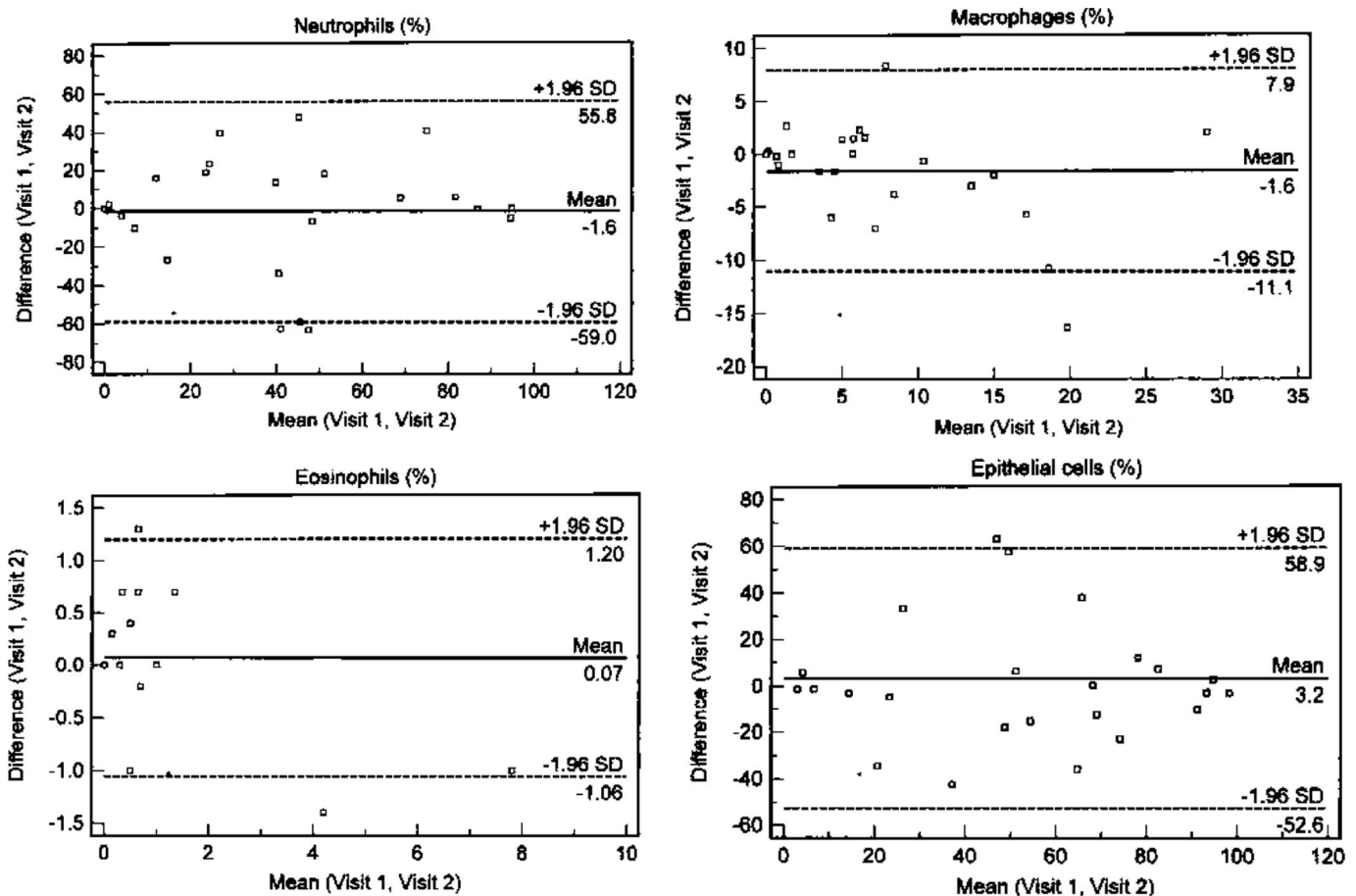


Figure 1. Bland-Altman summaries for the reproducibility of cell percentages in 25 pairs of NAL samples measured on two different visits. It is expected that 95% of the differences between measurements fall within 2 SD of the overall average difference. The plots indicate satisfactory reproducibility because most individual values are scattered around the mean difference and lie within the limits of agreement.

reproducibility and also on the characteristics and objectives of the study in which it is applied. In our study we adapted the NAL method described by Naclerio *et al.*¹⁰ because it is simple and rapid to perform and it has a good validity. These features were central in the context of our study that required repeated measurements of bronchial and nasal physiological parameters in the same patient during SIC. Also, we wanted to use a technique in which it was less likely to provoke a physiological response in the nose because nasal patency also

was simultaneously monitored by acoustic rhinometry during the SIC.

The Naclerio technique generally is reported as well tolerable but requires good collaboration from the patient. The technique causes some discomfort during the instillation of the saline solution into the nose and subjects have difficulties retaining the fluid and to prevent swallowing it. Most articles describing this technique indicate that the patient should be instructed to close the soft palate during the procedure to

Table 2 Reproducibility of cells in NAL based on type of challenge method

SIC Method	Neutrophils	Eosinophils	Macrophages	Epithelial Cells
Generator* (<i>n</i> = 15)				
Visit 1	44.8 ± 42.1	0.7 ± 1.9	4.0 ± 3.9	50.4 ± 41.2
Visit 2	48.5 ± 39.5	0.6 ± 2.1	4.2 ± 3.2	44.4 ± 40.8
ICC	0.75	0.96	0.28	0.83
Difference p-value	0.5	0.6	0.7	0.5
Realistic# (<i>n</i> = 10)				
Visit 1	35.7 ± 30.5	0.8 ± 1.1	10.6 ± 8.6	52.9 ± 27.3
Visit 2	41.8 ± 29.1	0.7 ± 1.5	14.0 ± 10.7	43.3 ± 26.2
ICC	0.48	0.91	0.78	0.38
Difference <i>p</i> value	0.6	0.7	0.1	0.3

The *p* value represents differences between the two visits in Wilcoxon test; numbers for visits represent mean and SD.

*Closed-circuit method (particles and aerosols generator).

#Realistic method (recreating working conditions).

avoid swallowing the fluid but without a clear indication of the method to achieve this. Our technique in which subjects repeat continuously the letter “k” during the NAL to close the velopharynx was found satisfactory. It was inspired by the Proetz method¹⁴ used in otolaryngological practice to irrigate the paranasal sinus. With this technique the mean recovery of fluid in each visit was ~60% (Table 1). Most importantly, it was found comfortable by most subjects; a practical advantage is that it is easy to explain and understandable by the patient.

Despite its extensive use in research studies the reproducibility of NAL by the Naclerio method has seldom been examined. Using this technique Belda *et al.*¹⁵ assessed the reproducibility of cells and ECP concentrations in healthy and rhinitic subjects on two visits separated by 48 hours. Among subjects with rhinitis the reproducibility was good for neutrophils (ICC = 0.7), eosinophils (ICC = 0.9), macrophages (ICC = 0.6), and epithelial cells (ICC = 0.8). They observed a poor reproducibility for cell differential counts in normal subjects. The reproducibility also was poor for TCC and ECP concentrations in both healthy and rhinitic subjects. In agreement with Belda *et al.*¹⁵ we observed that cell differentials are reproducible in two consecutive visits within 1 week (average within 3 days; Table 1). We also observed that TCC has a poor reproducibility (ICC = 0.12), which may have an explanation in the range of variation of the sample volume between two visits; this NAL parameter is expressed per volume unit. According to this, TCC should not be considered as an outcome to monitor changes during SIC. In contrast to the study by Belda *et al.*¹⁵ we observed that ECP measurements taken within 48 hours were reproducible (ICC = 0.7) by the Naclerio method. Such a divergence is difficult to explain but some reasons may be found in methodological aspects regarding the study design and the processing of the NAL samples. Our study subjects were referred for SIC. The performing of SIC in our hospital is a standardized procedure in which the subject must attend two to four SIC sessions. NALs are performed at the same time of the day and subjects spend most of the day in the laboratory under well-controlled conditions. Thus, it is less likely that in the interval of time between visits the subject is exposed to environmental factors that may influence the

outcome measurements in the second visit. Although the processing of the samples was similar in many aspects in both studies, we did not use dithiothreitol (DTT) in the NAL protocol to disperse cells. The use of DTT in the processing of induced sputum samples has been questioned based on potential adverse effects on inflammatory mediators.¹⁶ The effect of DTT in NAL samples has never been examined. However, Belda *et al.*¹⁵ observed a better reproducibility in their ECP measurements (ICC = 0.6) using a different technique of NAL sampling in which a higher volume of fluid is instilled and remains for a longer time in contact with the nasal mucosa.

The analysis of NAL based on the methodology used to perform the SIC after the NAL on the first visit showed that except for macrophage counts, the reproducibility with the closed-circuit method was better than with the realistic method. The closed-circuit method was developed in our hospital in an attempt to challenge subjects with low, stable, and well-controlled levels of occupational agents.^{8,9} The exposure is better controlled than with the realistic approach where concentrations may at times be higher than the Threshold limit value, short-term exposure limit. Based on our findings these controlled exposure conditions seem to provoke a more controlled inflammatory response in the nose reflected in a good reproducibility of cells in NAL samples. Despite the fact that study subjects were exposed to a nonspecific agent during the control day and that the NALs were performed in most cases on consecutive days, there was no residual or carryover effect influencing the NAL measurement on the second visit. The interval of time between both NAL therefore was long enough to return to prechallenge baseline conditions.

A cutoff value that can be used to define a significant change in eosinophils counts in NAL after exposure to occupational agents has not been established. We found only one study¹⁷ that proposed a 5% increase in eosinophils as the criteria to define a nasal provocation test as positive in subjects exposed to high molecular weight agents. Based on the reproducibility in eosinophil counts observed in our study, it seems that a 4% increase in eosinophils after nasal provocation may be an adequate cutoff point to classify an NAL test

as positive if a cutoff value is set at 2 SD above the mean percentage change in eosinophils shown in Table 1.

In summary, in this study we observed that the reproducibility of cellular and soluble inflammatory markers of NAL samples on two separate occasions within 1 week was sufficiently reproducible to recommend this method for the monitoring of changes in upper airways inflammation in the investigation of OR by SIC. Changes in eosinophils >4% after SIC can be considered as significant. More studies should be conducted in a larger population on the reproducibility of cellular components in NAL with different sampling techniques and also addressing specifically the determination of a significant nasal reaction during challenge with NAL.

ACKNOWLEDGMENTS

The authors express their gratitude to the technician Carole Trudeau, who played a key role in the performing of the tests and processing of the samples. They also thank the technicians Sonia Boudreault, Lucero Castellanos, and Simone Chaboillez for their participation in the processing and analysis of the samples.

REFERENCES

1. Granstrand P, Nylander-French LA, Lacks G, et al. Absence of proinflammatory cytokine gene expression in nasal biopsies from wood surface-coating industry workers. *Acta Otolaryngol* 121:743–749, 2001.
2. Raulf-Heimsoth M, Wirtz C, Papenfuss F, and Baur X. Nasal lavage mediator profile and cellular composition of nasal brushing material during latex challenge tests. *Clin Exp Allergy* 30:110–121, 2000.
3. Varney VA, Jacobson MR, Sudderick RM, et al. Immunohistology of the nasal mucosa following allergen-induced rhinitis. Identification of activated T lymphocytes, eosinophils, and neutrophils. *Am Rev Respir Dis* 146:170–176, 1992.
4. Granstrand P, Nylander-French L, and Holmstrom M. Biomarkers of nasal inflammation in wood-surface coating industry workers. *Am J Ind Med* 33:392–399, 1998.
5. Howarth PH, Persson CG, Meltzer EO, et al. Objective monitoring of nasal airway inflammation in rhinitis. *J Allergy Clin Immunol* 115:S414–S441, 2005.
6. Larsson BM, Palmberg L, Malmberg PO, and Larsson K. Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid. *Thorax* 52:638–642, 1997.
7. Roponen M, Seuri M, Nevalainen A, et al. Nasal lavage method in the monitoring of upper airway inflammation: seasonal and individual variation. *Inhal Toxicol* 15:649–661, 2003.
8. Cloutier Y, and Malo JL. Update on an exposure system for particles in the diagnosis of occupational asthma. *Eur Respir J* 5:887–890, 1992.
9. Vandenplas O, Malo JL, Cartier A, et al. Closed-circuit methodology for inhalation challenge tests with isocyanates. *Am Rev Respir Dis* 145:582–587, 1992.
10. Naclerio RM, Meier HL, Kagey-Sobotka A, et al. Mediator release after nasal airway challenge with allergen. *Am Rev Respir Dis* 128:597–602, 1983.
11. Maghni K, Lemiere C, Ghezzi H, et al. Airway inflammation after cessation of exposure to agents causing occupational asthma. *Am J Respir Crit Care Med* 169:367–372, 2004.
12. Bland JM, and Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1:307–310, 1986.
13. Riechelmann H, Deutschle T, Friemel E, et al. Biological markers in nasal secretions. *Eur Respir J* 21:600–605, 2003.
14. Martin RA. The aspiration-irrigation maneuver. *Laryngoscope* 110:1271–1276, 2000.
15. Belda J, Parameswaran K, Keith PK, and Hargreave FE. Repeatability and validity of cell and fluid-phase measurements in nasal fluid: A comparison of two methods of nasal lavage. *Clin Exp Allergy* 31:1111–1115, 2001.
16. Woolhouse IS, Bayley DL, and Stockley RA. Effect of sputum processing with dithiothreitol on the detection of inflammatory mediators in chronic bronchitis and bronchiectasis. *Thorax* 57:667–671, 2002.
17. Gorski P, Krakowiak A, Pazdrak K, et al. Nasal challenge test in the diagnosis of allergic respiratory diseases in subjects occupationally exposed to a high molecular allergen (flour). *Occup Med (Lond)* 48:91–97, 1998. □

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