

Short communication

Ultrasonic nebulization of hypertonic solution: a new method for obtaining specimens from nasal mucosa for morphologic and biochemical analysis in allergic rhinitis

Melillo G, Balzano G, Stefanelli F, Iorio C, De Angelis E, Melillo E.
Ultrasonic nebulization of hypertonic solution: a new method for obtaining specimens from nasal mucosa for morphologic and biochemical analysis in allergic rhinitis.
Allergy 1998; 53: 794–797. © Munksgaard 1998.

Various techniques are used to collect specimens from the nasal mucosa for morphologic and biochemical analysis. The purpose of this study was to devise a method that overcomes some of the disadvantages (e.g., invasive procedure, samples not suitable for cytologic and biochemical analysis, lack of standardization, and poor reproducibility) of these techniques. The new method requires subjects, with neck extended, to inhale an ultrasonic nebulization of a hypertonic (3% NaCl) solution (UNHS) for 5 min. They then blow their nose into a Petri dish, one nostril at a time with the other one blocked. The secretions are dispersed with 0.1% dithiothreitol in phosphate buffer solution for 20 min. Total cell count (TCC) is evaluated, and the cellular suspension is divided into two aliquots: one is centrifuged and the supernatants are collected for eosinophil cationic protein (ECP) measurements; the other is cytocentrifuged and the slides, stained with Diff-Quik, are used for differential cell count. The results obtained with the UNHS and nasal lavage (NL) methods were compared. Eleven nonatopic healthy subjects and 19 allergic rhinitic patients were studied. Total cell count ($\times 10^5$) was significantly higher with UNHS than with NL (13.0 ± 12.3 vs 1.9 ± 1.6 ; $P < 0.01$). The differential cell count was similar with the two procedures. ECP levels ($\mu\text{g/l}$) were higher with UNHS than with NL (39.1 ± 38.2 vs 16.7 ± 41.2 ; $P < 0.01$). For evaluation of reproducibility, four healthy and six rhinitic subjects underwent UNHS on two occasions within 5 days, and the results of two samples (sample 1 vs sample 2) were analyzed. Reproducibility was good as to TCC, differential cell count, and ECP.

**G. Melillo, G. Balzano, F. Stefanelli,
C. Iorio, E. De Angelis, E. Melillo**

Fondazione "S. Maugeri", Divisione di
Pneumologia e Centro per l'Asma Bronchiale,
Centro Medico, Telesse Terme (BN), Italy

Key words: eosinophil cationic protein;
hypertonic saline solution; nasal lavage;
ultrasonic nebulization.

G. Melillo, MD
Consultant Chest Physician
Centro Medico di Telesse Terme
Loc. S. Stefano in Lanterria
82037-Telesse Terme (BN)
Italy

Accepted for publication 24 February 1998

One of the main difficulties in conducting studies of nasal disorders is the collection of nasal secretions. Various techniques are used to obtain specimens from nasal mucosa for the analysis of cells and soluble components (1). Several of these techniques are invasive, e.g., nasal biopsy (2), or partially invasive, e.g., nasal brush (2) and nasal scraping (1). The simplest noninvasive technique is nasal lavage, whereby 5–10 ml of isotonic saline

(37°C) is instilled in each nostril while the patient's head is stretched backward with the soft palate closed (3–5). From 10 to 15 s later, the lavage fluid is expelled into the collecting tube. In a modified version of this method (6), the lavage is performed with a size 12 F Foley catheter bearing an inflating balloon which is placed in the nasal vestibule. In yet another version, a nasal pool-device consisting of a compressible plastic container is used to

introduce 12 ml of fluid into the nasal cavity (7). Two methods that do not require nasal lavage are also used to collect nasal mucosa specimens. One is the filter paper method, modified by Mygind (8), in which two pieces of folded paper are placed in each nasal cavity and left for 15 min. The other is the mucosal imprints method, in which two imprints are made from the septal side of the nasal mucosa with a plastic strip to which cells become attached.

Each of the methods described above has some disadvantage: unsuitability of some samples for cytologic and biochemical analysis, poor reproducibility of the results, and lack of standardization. Moreover, most of these methods are at least partially invasive or require the collaboration of the patient. Here we describe a new method that seems to overcome some of the disadvantages of the methods currently used to assess nasal inflammation.

Material and methods

Subjects

Eleven nonatopic healthy subjects (age $\pm$ SD, 38 $\pm$ 15 years, five men) and 19 allergic rhinitic patients (age 27 $\pm$ 10 years, 15 men) were studied. Ten of the latter had seasonal symptoms and nine had perennial symptoms. Only two rhinitic patients were on regular inhaled steroids, and this therapy was continued throughout the study period. For the purpose of this study, allergic rhinitis was defined as skin test positivity to one or more allergens, a history of seasonal or perennial symptoms (sneezing, itch, nasal discharge, and blocking), and positive rhinoscopy findings (nasal congestion consequent to swollen mucous membrane). The most common inhalant allergens were used for the skin prick tests: house-dust mite (*Dermatophagoides pteronyssinus* and *D. farinae*), dog and cat dander, *Alternaria alternata*, *Cynodon dactylon*, *Poa pratensis*, *Holcus lanatus*, *Parietaria judaica*, *P. officinalis*, *Olea Europaea*, and *Artemisia vulgaris* (Lofarma Allergeni, Milan, Italy). The healthy subjects had no history of allergy or of recurrent or chronic nasal disease; they were skin prick test negative to the above-mentioned allergens and had negative rhinoscopy.

Study design

Each individual enrolled in the study underwent a standard nasal lavage (NL) and an ultrasonic nebulization of hypertonic (3% NaCl) solution (UNHS) on two different days within 1 week in random order. The results of the two methods (total and differential cell count and eosinophil cationic

protein [ECP] measurement) were compared. Ten subjects underwent UNHS on two different days within 5 days, and the reproducibility of total and differential cell count and ECP measurement was evaluated.

Nasal lavage

Nasal lavage was performed according to Naclerio et al. (9). Briefly, 5 ml of normal saline (0.9% NaCl) at 37°C was instilled into each nostril with the patient stretching the head bent backward and with the soft palate closed. Ten seconds later, the subjects flexed their neck and expelled the solution into a plastic basin. The sample was transferred to a 10-ml polypropylene tube, and the volume was recorded. The total cell count (TCC $\times$ 10⁵) was evaluated with a standard hemocytometer. The lavage fluid was then centrifuged at 1500 rpm for 10 min and the supernatant was collected and stored at -85°C until required for analysis. The cell pellet was resuspended in phosphate buffer solution to obtain a final cellular concentration of 2 $\times$ 10⁵/ml; 100 μ l was cytocentrifuged (Cytospin 3, Shandon, Pittsburgh, PA, USA) at 400 rpm for 5 min. Slides were stained with Diff-Quik (Dade AG, Duding, Switzerland). The Diff-Quik solutions allow rapid staining of smears and yield results comparable to those obtained with the May-Grunwald-Giemsa technique. Briefly, the air-dried smears are fixed for 5 s in 0.002 g/l fast green and methanol, stained for 5 s in 1.22 g/l eosin G in phosphate buffer at pH 6.6, and for 5 s in 1.1 g/l thiazin in phosphate buffer at pH 6.6, and rinsed in running water for 5 s. After staining, the differential cell count was performed by two separate investigators on at least 200 cells.

Ultrasonic nebulization of hypertonic solution (UNHS)

Induction of secretions. Ultrasonic nebulization of hypertonic solution was performed by inhalation of hypertonic saline solution (3% NaCl), generated by an ultrasonic nebulizer (Orion 2 Nova, Heyer, Germany) at maximum output power (4.4 ml/min), for 5 min while the patients had their necks extended to facilitate the penetration of the inhaled solution on the whole surface of the nasal mucosa, including the posterior nasal cavity. The particles inhaled through the nose had a mean size of 2.5–5.0 μ m. At the end of inhalation, the patients were asked to blow their nose forcefully into a Petri dish, one nostril at a time while blocking the other one.

Sample processing. The secretions collected were immediately weighed and their weight was

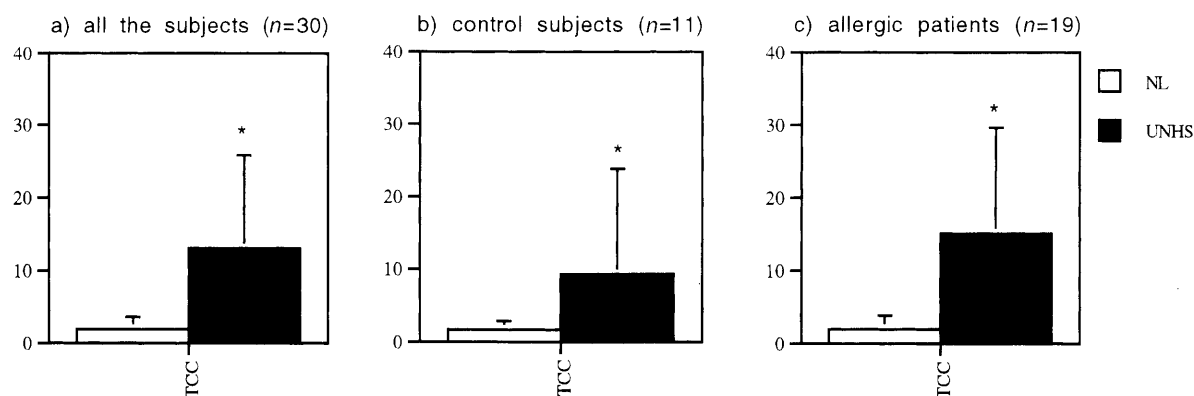


Fig. 1. Total cell count (TCC) obtained with nasal lavage (NL) and ultrasonic nebulization of hypertonic solution (UNHS) in: a) all subjects, b) control subjects, c) allergic patients. * $P < 0.01$.

recorded (mg). A volume (μl) of freshly prepared 0.1% dithiothreitol in phosphate buffer solution (Sigma, Milan, Italy) four times the weight in milligrams of the secretions was added to the secretions and the final volume was measured (ml). The sample was left at 37°C for 20 min and vortexed every 5 min to obtain cell dispersion. The sample was then filtered on sterile gauze, and a small volume ($20\ \mu\text{l}$) was used to evaluate TCC ($\times 10^5$) with a standard hemocytometer. The sample was then divided into two aliquots: one was diluted to obtain a final cellular concentration of $2 \times 10^5/\text{ml}$; $100\ \mu\text{l}$ of this sample was cytocentrifuged at 400 rpm for 5 min; the slides were stained with Diff-Quik, and a differential cell count was made on 200 cells by two separate investigators. The other aliquot was centrifuged at 4500 rpm for 10 min, and the supernatant was aspirated and stored at -85°C until required for analysis.

ECP measurement

ECP levels were measured in 20 nasal lavage supernatants and in 26 UNHS supernatants by the fluorimmunoenzymatic method with a commercially available kit (FEIA, CAP System, Pharmacia, Uppsala, Sweden), and the results were expressed in $\mu\text{g/l}$.

Statistical analysis

Correlations and comparisons between nasal lavage and UNHS were done with a linear regression test and the Mann–Whitney U -test for nonparametric data, respectively. The reproducibility was assessed with a linear regression test (sample 1 vs sample 2).

Results

As shown in Fig. 1, total cell count ($\times 10^5$) was higher in UNHS than in nasal lavage in the whole

study population (13.0 ± 12.8 vs 1.9 ± 1.6 ; $P < 0.01$), in the control subjects (9.3 ± 8.3 vs 1.7 ± 1.2 ; $P < 0.01$), and in patients with allergic rhinitis (15.1 ± 14.5 vs 2.0 ± 1.9 ; $P < 0.01$). There was no difference in differential cell count between UNHS and nasal lavage when subjects were considered as a whole or when patients were considered separately from healthy subjects. In our study, mononuclear cells were largely represented by monocyte-macrophages, and by rare lymphocytes or plasma cells. Epithelial cells, counted as a whole, were represented by goblet cells, ciliated cells, and basal epithelial cells in similar numbers.

As shown in Fig. 2, ECP levels ($\mu\text{g/l}$) were significantly higher in UNHS than in nasal lavage in the overall study group (39.1 ± 38.2 vs 16.7 ± 41.2 ; $P < 0.01$), in control subjects (19.8 ± 20.9 vs 1.7 ± 2.1 ; $P < 0.01$), and in patients with allergic rhinitis (46.3 ± 43.4 vs 16.9 ± 43.4 ; $P < 0.01$). Moreover, ECP was not detectable in 8/20 (40%) nasal lavage supernatants (three with rhinitis and five healthy subjects), and in only 2/26 (7.7%) UNHS supernatants (two healthy subjects).

There was a significant correlation between nasal lavage and UNHS for percentages of neutrophils ($r = 0.65$; $P = 0.0001$), eosinophils ($r = 0.7$; $P = 0.0001$), epithelial cells ($r = 0.81$; $P = 0.0001$), and mononuclear cells ($r = 0.5$; $P = 0.0046$), while there was no correlation between TCC and ECP levels (Table 1).

The reproducibility of UNHS was good for TCC, differential cell count, and ECP measurement (Table 2).

UNHS stimulates nasal secretory activity and increases nasal secretions by both the glandular and cellular component. Whereas the specimen collected by nasal lavage consisted of a mixture of a variable amount of instilled lavage fluid and secretions, the specimen collected by UNHS consisted exclusively of undiluted secretions. A significantly greater number of total cells was collected with the UNHS procedure in both healthy and

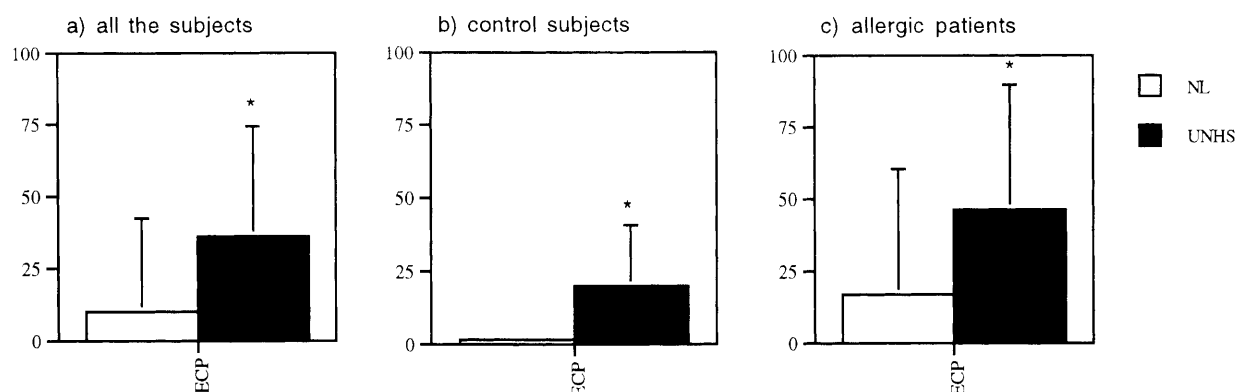


Fig. 2. Eosinophil cationic protein (ECP) obtained with nasal lavage (NL) and with ultrasonic nebulization of hypertonic solution (UNHS). * $P < 0.01$.

Table 1. Correlations between total cell count (TCC), differential cell count, and eosinophil cationic protein (ECP) in nasal lavage and in ultrasonic nebulization of hypertonic solution (UNHS)

	r^*	P^*
TCC ($\times 10^5$)	0.10	0.5661
Neutrophils (%)	0.65	0.0001
Eosinophils (%)	0.70	0.0001
Mononuclear cells (%)	0.50	0.0046
Epithelial cells (%)	0.81	0.0001
ECP ($\mu\text{g/l}$)	0.28	0.2559

*Linear regression test.

Table 2. Reproducibility of UNHS for total and differential cell count and ECP levels

	r^*	P^*
TCC ($\times 10^5$)	0.81	0.001
Neutrophils (%)	0.72	0.002
Eosinophils (%)	0.90	0.0004
Mononuclear cells (%)	0.76	0.011
Epithelial cells (%)	0.75	0.0119
ECP ($\mu\text{g/l}$)	0.85	0.0037

*Linear regression test.

rhinitic patients, while there was no difference in the differential cell count. The latter finding suggests that UNHS does not stimulate the presence of a particular cell type, but rather increases the number of cells as a whole. Indeed, hypertonic solution at 3% exerts only a mucokinetic effect, and it does not irritate the airway mucosa (10).

This study shows that the samples obtained with the UNHS procedure are suitable for morphologic analysis and for the evaluation of soluble mediators. The reproducibility of the method is good for

all parameters considered. In conclusion, the UNHS method seems to be a sufficiently standardized and reproducible method, easily repeatable in the same patient, and is suitable for use also in children because minimal patient cooperation is required.

References

1. Pipkorn U, Karlsson G. Methods for obtaining specimens from the nasal mucosa for morphological and biochemical analysis. *Eur Respir J* 1988;1:856-62.
2. Godthelp T, Holm AF, Fokkens WJ, et al. Dynamic of nasal eosinophils in response to a non-natural allergen challenge in patients with allergic rhinitis and control subjects: a biopsy and brush study. *J Allergy Clin Immunol* 1996;3: 800-11.
3. Pipkorn U, Karlsson G, Enerbäck L. Nasal mucosa response to repeated challenge with pollen allergen. *Am Rev Respir Dis* 1989;140:729-36.
4. De Graaf-in't, Veld C, Garrelds IM, Koenders S, Gerth van Wijk R. Relationship between nasal hyperreactivity, mediators and eosinophils in patients with perennial allergic rhinitis and controls. *Clin Exp Allergy* 1996;26:903-8.
5. Nielsen LP, Bjerke T, Christensen MB, et al. Assessment of the allergic reaction in seasonal rhinitis: acoustic rhinometry is a sensitive and objective method. *Clin Exp Allergy* 1996;26:1268-75.
6. Wang JH, Devalia JL, Duvile JM, et al. Effect of six-hour exposure to nitrogen dioxide on early-phase nasal response to allergen challenge in patients with a history of seasonal allergic rhinitis. *J Allergy Clin Immunol* 1995;5:669-76.
7. Greiff L, Pipkorn U, Alkner U, Persson CGA. The "nasal pool" device applies controlled concentrations of solutes on human nasal airway mucosa and samples its surface exudations/secretions. *Clin Exp Allergy* 1990;20:253-9.
8. Mygind N. Immunology of nasal secretion. In: Mygind N, editor. *Nasal allergy*. Oxford: Blackwell Scientific, 1978: 198-216.
9. Naclerio R, Meier HL, Kagey-Sobotka A, et al. Mediator release after nasal airway challenge with allergen. *Am Rev Respir Dis* 1983;128:597-602.
10. Ziment I. Hypertonic solutions, urea and ascorbic acid. In: Braga PC, Allegra L, editors. *Drugs in bronchial mucology*. New York: Raven Press, 1989:137-43.