



Research paper

Novel nasal secretion collection method for the analysis of allergen specific antibodies and inflammatory biomarkers

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ABSTRACT

Background: The analysis of immunological markers in nasal secretions provides valuable information for studying nasal mucosa diseases and monitoring immunotherapy and immunity to vaccines administered locally. However, the concentration of biomarkers is highly variable in nasal secretions because of the diversity of collection methods. A nasal secretion collection device was developed to increase detectability of the assay, standardize the sampling technique, eliminate unknown dilution factor, and minimize analyte loss during the sample processing.

Objective: To develop and demonstrate the performance characteristics of a novel nasal secretion collector and its advantages over nasal lavage techniques.

Methods: Characteristics of absorption and recovery of the liquid or proteins by different types of polyurethane foam were evaluated. The concentration of immunoglobulins, inflammatory mediators and allergen specific antibodies was comparatively measured in nasal secretions collected by the novel nasal secretion collector and nasal lavages.

Results: The concentrations of cytokines, eosinophil cationic protein, and tryptase in nasal secretions obtained by the nasal secretion collector were at least 8-fold higher than those tested in nasal lavages. Furthermore, the levels of immunoglobulins and the grass/weed pollen allergen specific antibodies were 6- to 290-fold increased when the nasal secretion collector was used. The nasal secretion collector was easy to use, non-invasive, and caused minimal discomfort to subjects during sampling.

Conclusion: A novel nasal secretion collector shows a significantly higher detectability and reproducibility than nasal lavages for analyzing immunological markers in nasal secretions.

Clinical implication: The nasal secretion collector represented an approach for collecting nasal secretions and can be applied for routine evaluations of nasal immune responses induced by mucosal vaccinations or infections, inflammations and therapeutic interventions.

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Abbreviations: NS, nasal secretions; NSC, nasal secretion collector; ECP, eosinophil cationic protein; Ig, immunoglobulin; Ab, antibody; TG, timothy grass; SW, short ragweeds.

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1. Introduction

Appropriate collection and processing of nasal specimens is of paramount importance for evaluating immune responses induced by immunotherapy and vaccines administered locally. Although the collection, processing and storage of serum specimens do not pose specific problems, the collection of nasal secretions (NS) requires special attention and coordinated efforts. There are no known about optimal methods for collecting the most of external secretions, including NS. A standardized NS collector (NSC) may undoubtedly facilitate

uniformity of collection procedures and allow for an accurate evaluation of nasal immune responses induced by immunotherapies and vaccinations or inflammatory mediators.

Rhinitis and sinusitis are inflammatory diseases of the lining of the nasal and paranasal cavities. Upper respiratory tract diseases belong to the most frequent manifestations of mucosal disorders and are diagnosed based on history, clinical examination, rhinoscopy, allergy testing, imaging, functional testing, nasal cytology (cytobrush) (Watelet et al., 2004). However, the biochemical and immunological analyses of NS can provide additional information on mucosal innate and adaptive immunities.

To investigate the immune responses in nasal mucosa following a local immunization or inflammation, a trace amount of antibody, cytokines and inflammatory mediators has been detected in the NS (Noah et al., 1995; Bachert et al., 1998; Wilson et al., 1998). However, various methods to collect NS yield heterogeneous materials and results, which result in difficult data comparison. Currently used sampling NS techniques were classified as the collection of spontaneous secretions, washes and absorption techniques. Collection of spontaneous NS is practicable in patients with nasal hypersecretion as result of nasal mucosa diseases (Heikkinen et al., 1999; Niehaus et al., 2000). However, in healthy individuals the amount of spontaneously secreted fluid from nasal mucosa is often insufficient (Klimek et al., 1999). Blown secretions were the way to collect the NS, but the quantities of NS obtained were extremely variable. Microsuction, aspiration and nasal lavages are described to optimize the NS collection with the least discomfort for the patients and the best reproducibility for the examiner. The most commonly used technique in clinical practices is nasal lavages by instilling 2.5–5 ml of sterile saline with a sterile plastic pipette or plastic syringe, which results in an unpredictable dilution factor of NS (Millqvist et al., 2005). In addition, swallowing the saline buffer and choking often occur. Thus, a maximum cooperation of the patient is required, which is not always possible for children or comatose patients. The main concern for the analysis is the concentrations of analytes in nasal lavages that reveal a significant variability and frequently fall below the lower detection limits of the assays (Watelet et al., 2004). The slightly improved method is using ultrasonic nebulization of 4.5% saline water for 5 min and then blowing one of the nostril (Ventura et al., 2007). This method is likely suitable for differential cell counting in NS. An alternative technique for collection of NS is using a collector with absorptive properties, such as synthetic electrophoresis wick (Kristiansen, 1984), cotton, filter paper, nasal swab (Walsh and Falsey, 1999), and sinus packs (Watelet et al., 2004). Although the absorption method provides sufficient amounts of undiluted NS, it has been thought to irritate nasal mucosa and therefore to contaminate the concentration of analytes by blood. In addition, the filter paper method collects the NS that is derived from a limited area of the nasal mucosa so that the volume of NS is often insufficient. Thus, it is universally accepted that the methods of NS collection directly influence the results obtained (Watelet et al., 2004). Accurate information from NS collected with an improved method could impact on our understanding of the pathogenesis in nasal inflammation or infection and the mechanisms underlying immunotherapy, as well as open new perspectives for diagnostic tools.

The present study focused on developing a novel method to collect NS using the polyurethane foam. Various polyurethane foams were evaluated for their characteristic performances based on fluid and protein recovery. Further, the comparison of nasal secretions collected by the NSC and nasal lavages was evaluated by measuring allergen specific IgA, IgG, IgE Abs, inflammatory cytokines and total immunoglobulins of allergic rhinitis subjects. The NS collected using the NSC demonstrated a superior detectability, reproducibility and patient acceptability over nasal lavages for analyzing local immunological and inflammatory mediators.

2. Materials and methods

2.1. Subject

A total of 18 patients, 10 women and 8 men, with seasonal allergic rhinitis caused by ragweed or mixed grass extracts were studied during the allergic season in Northwest Carolina, from mid-January to mid-May. Their median age was 35 years; range 24–45 years. All had allergic rhinitis documented by the exacerbation of symptoms (the symptom scores were increased 2 or more times when compared to non allergic seasons) during the grass or ragweed pollen seasons and a positive skin prick test (wheal diameter ≥ 4 mm) to ragweed (1/10 wt/vol) or timothy (100,000 BUA/ml) extract. Monitoring of clinical manifestations was done by a classic grade scale from 0 to 3 (0, no symptoms; 1, mild; 2, moderate; and 3, severe), in which the symptoms included 1) itching nose (pruritus) 2) sneezing; 3) nasal congestion; and 4) watery rhinorrhea. The subjects were randomly divided into two groups based on sampling methods. Group I consisted of 8 subjects (4 women and 4 men). Nasal lavage samples were collected from Group I at a clinical visit prior to nasal provocation test. Group II consisted of 10 subjects (6 women and 4 men) from which the nasal secretion was self-collected using the novel NSC for 10 samplings on 10 different days. Pooled NS samples from 10 different days for each subject in group II were analyzed. Additionally, 36 patients from a separate clinical study, but with the same enrollment criteria were added for the total IgA in NS collected with the NSC, so were 18 patients for TG and SW specific IgA Abs. Subjects who had symptoms or physical signs suggestive of renal, hepatic, or cardiovascular disease; nasal polyps; a displaced septum; or perennial rhinitis were excluded from the study. Furthermore, patients who had a history of an upper respiratory infection or acute sinusitis within 30 days before the enrollment, had received immunotherapy in the past 2 years, or had used any decongestant nasal sprays or cromolyn sodium within 2 weeks were also excluded from the study. Pregnant or lactating women were not permitted to join the study. The Institutional Review Board of Greer Laboratories Inc. approved the study. Each participant signed informed consent before the enrollment of the trial.

2.2. Preparation of nasal secretion collector

The reticulated polyurethane foams tested were resopore, polyether-110 pores per inch (PPI); polyester-60 PPI, and polyether-45 PPI (kindly provided by Swiss Tex, Greenville, SC). The foams were received as sheets of varying thickness.

The foams were cut into cylinder shape (12 mm diameter; 24 mm in length) by using a home-made cutting device. For ease of breathing and handling the NSC during the sample collection, a channel of 3 mm in diameter was cut in the center of the foam roll and was used to insert a 45 mm length of hollow plastic tube (4 mm in diameter) (Fig. 1, right panel).

2.3. Collection of NS samples

The method of nasal lavages was performed according to the method described by Naclerio R.M. et al. (1983). To summarize, nasal lavage samples in Group I were collected by instilling sterile 3 ml of physiological saline water into each nostril, and the fluid was expelled 10 s later by the subject into a plastic collection device. About 3–5 ml of the nasal secretion can be collected by the nasal lavages. Nasal lavage samples were first vigorously shaken for homogenization of the mixture of sol and gel phases and were aliquoted. The aliquots of the nasal lavage samples were kept in frozen at -80°C until assayed. NS in Group II were collected using the NSC on days when the subjects experienced a rhinorrhea. The foam roll was inserted on the floor of the cavity about 1 in. between the septum and inferior turbinate for at least 5 min. The foam roll containing NS was pulled out from the nostril and inserted into the inner tube that was in the outer centrifuge tube (Sarstedt Inc) (see Fig. 1, left panel). The whole tube was stored in a -20°C or -80°C freezer within 15 min after the sample collection. The NSC can collect 100–400 μl of NS in each sampling. About 0.4–2.4 ml of the total NS was collected in 10 sampling from each individual. At the laboratory, the sample was thawed, centrifuged at 3000 g for 20 min to recover the fluid (Fig. 1). The extracted NS was aliquoted in 100 μl per vial, and stored at -80°C until assayed.

2.4. Measurement of BSA, ECP, tryptase, cytokines, total Igs, and grass/weed pollen specific Abs in NS

Bovine serum albumin (BSA) concentration was measured by Micro BCA protein assay kit (Pierce, IL). NS ECP and tryptase

were measured using UniCAP system (PharmaciaDiagnostics, Inc.). Cytokines: IL-1, EGF, MCP-1, IL-6, IL-7 and TNF- α were measured by using a Bio-Plex suspension array, the Luminex xMAP system (BioRad Laboratories). Total IgA, IgG, and grass/weed pollen specific Abs in NS were measured by ELISA as previously described (Lu, 2000) (Lü et al., 1999) (Lü et al., 1994). A standard pooled high immune sera (Greer Laboratories Inc. Lot #: ZE-P9) was used as the calibrator for quantitative Abs measurement. The concentration of grass- and weed specific Abs in the standard pooled high immune sera was determined using the Phadia UniCap calibrators. The timothy grass (*Phleum pratense*) (TG) and short ragweed (*Ambrosia artemisiifolia*) (SW) used for skin prick testing during patient selections and ELISA were manufactured by Greer Laboratories Inc.

2.5. Stability test

To examine the percentage of the recovery, evaporation and retention of NS after being collected with the NSC at different storage conditions. The NSCs in triplet were loaded with 100 μl of PBS then stored at room temperature, 4°C , -20°C and -80°C for 1, 2 and 4 weeks. The NSCs (the whole tube without an inner tube, the inner tube containing foam, and the whole collector) were weighted prior to and after the storage at different temperatures and for 1, 2, and 4 weeks. The secondary weighing was performed after centrifuging the loaded NSC at 3000 g for 20 min. The differences in weight of PBS for the whole collector without an inner tube, for the inner tube only, and for the whole collector, between being loaded and after being stored at different temperatures and durations, were calculated for the volume of the recovery, retention and evaporation respectively.

2.6. Statistics

The data were presented as mean + s.d. from duplicate measurements. The Mann Whitney test was used to determine a significant difference in the biomarker concentrations at 95% level between the nasal lavages and NSC. A linear regression test was used to compare the initial and the recovered protein concentrations during processing of NS using the NSC. A *p* value less than 0.05 was considered significant.

3. Results

3.1. The maximum absorbance and percentage of the recovery of phosphate buffer saline (PBS)

The absorption capacity and the recovery profiles for the foams with 110, 60 and 45 PPI are shown in Fig. 2. The 110 and 60 PPI foams demonstrated an equal capacity of PBS maximum absorption in an average of 1.8 ml (Fig. 2A). Both of them absorbed 0.2 ml more PBS than the 45 PPI foam. Further, the three types of foam in the same size were tested for the PBS recovery. The foams were loaded with serial volumes of PBS ranging from 50 μl to 500 μl and centrifuged at 3000 g for 20 min. The recovered PBS was weighed and the volume was calculated using a density parameter of 1 g/ml. The higher density foam, 110 PPI, exhibited a lower liquid recovery rate when the volume of PBS was less than 200 μl .

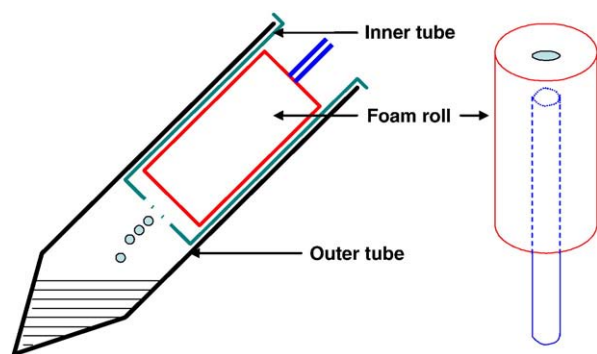


Fig. 1. A schema of the NS collector (left panel) and extraction of NS from the foam roll by centrifugation (right panel). Design of the NSC includes the cylinder shape of the polyurethane foam roll and plastic tube in its center. The foam roll was inserted in the centrifuge tube that consists of inner and outer parts. The non-diluted nasal secretions were obtained following centrifugation. NSC: nasal secretion collector.

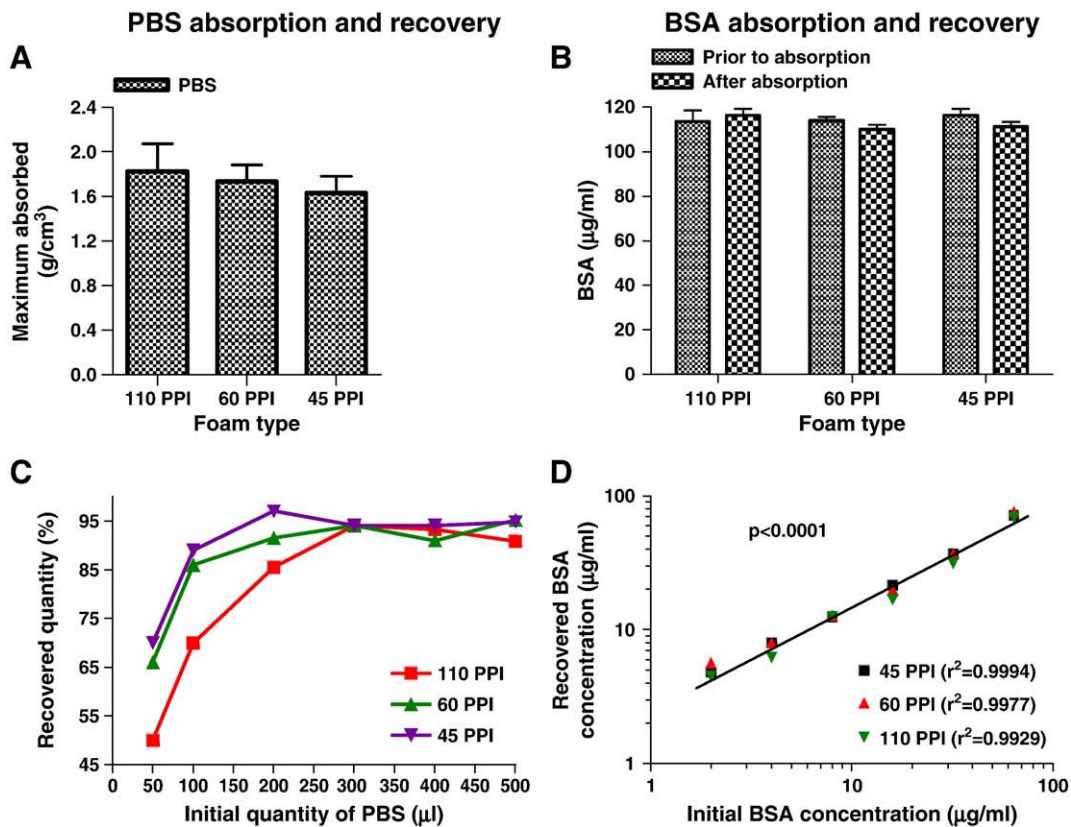


Fig. 2. The PBS and BSA absorption or recovery by different types of foam. A: The maximum PBS absorption by foam type 110, 60, and 45 PPI. The maximum absorption for PBS was tested using 1 cm³ of foams. Data presented were the mean value plus s.d. of triplicate foams (1 cm³) that were in different types. One cm cube of the foam can absorb 1.8 ml of PBS. The three types of foam showed approximately the same capacity for retaining the PBS. There were no statistically significant differences in maximum absorption of PBS between the 3 types of foam. B: The maximum BSA recovery by foam types 110, 60, and 45 PPI. The concentration of BSA was measured by BCA assay in the PBS-BSA solution prior to and after absorption by 3 types of foam. There were no statistically significant differences in BSA concentration between prior to and after absorption by 3 types of foam. C: The percentage of PBS recovery following centrifugation. The percentage was calculated as the weight of recovered PBS divided by the weight of initial loaded PBS on the foam. A slightly low recovery rate was found for the foam 110 PPI when the loaded PBS volume was below 200 µl. However, the recovery rate was approximately the same for all types of foam when the loaded PBS volume was more than 300 µl. D: The correlation in BSA concentration between the initial loaded BSA solutions (300 µl) and the recovered BSA solutions. A linear regression test was used with 95% confidence intervals. The r^2 value was shown for each type of foams.

However, when the loading volume was increased to equal or above 300 µl, all types of foam had approximately the same recovery rate (95%) (Fig. 2C). The 60 and 45 PPI foams had 80% of the recovery rate when the loading volume was 100 µl. Whereas, the 110 PPI foam demonstrated only 70% of the recovery rate (Fig. 2C).

3.2. The BSA absorption and recovery

A significant difference in BSA concentrations between prior to and after absorption for the three types of foam was not found (Fig. 2B). All types of foam used had less than 5% of BSA retention after the centrifugation at 3000 g for 20 min. In addition, when serial concentrations of BSA were tested, the concentrations of the recovered BSA were significantly correlated to that of the loading BSA concentrations ($p < 0.0001$) for all three types of the foam used (Fig. 2D). The correlation factor (r) was 0.9994 for the foam 45 PPI, 0.9977 for the foam 60 PPI, and 0.9929 for the foam 110 PPI (Fig. 2D).

3.3. IgA and IgG concentration in whole human saliva prior to and after absorption by polyurethane foams

To test the absorptive ability of the polyurethane foams, and its influence for immune mediators measurement by ELISA, the human saliva samples were used because it can be easily obtained in a large volume and the best mimic of the nasal secretions in biochemical compositions and viscosity. Human whole saliva was collected by Salivette (Sarstedt Inc.) from two individuals (Fig. 3). Five ml of saliva was absorbed with polyurethane foams and extracted by centrifugation at 3000 g for 20 min. The IgG and IgA concentration in the original and the recovered saliva were measured by a sandwich ELISA as previously described (Lu, 2000). The data demonstrated that there was no significant difference in salivary IgA and IgG concentration between prior to and after absorption with the 110 PPI, 60 PPI and 45 PPI foams. The data presented were the mean value of 3 dilutions of the saliva samples in duplicate wells (Fig. 3). The whole saliva samples obtained by this experiment involving the use of the NSC for the absorption and extraction demonstrated the

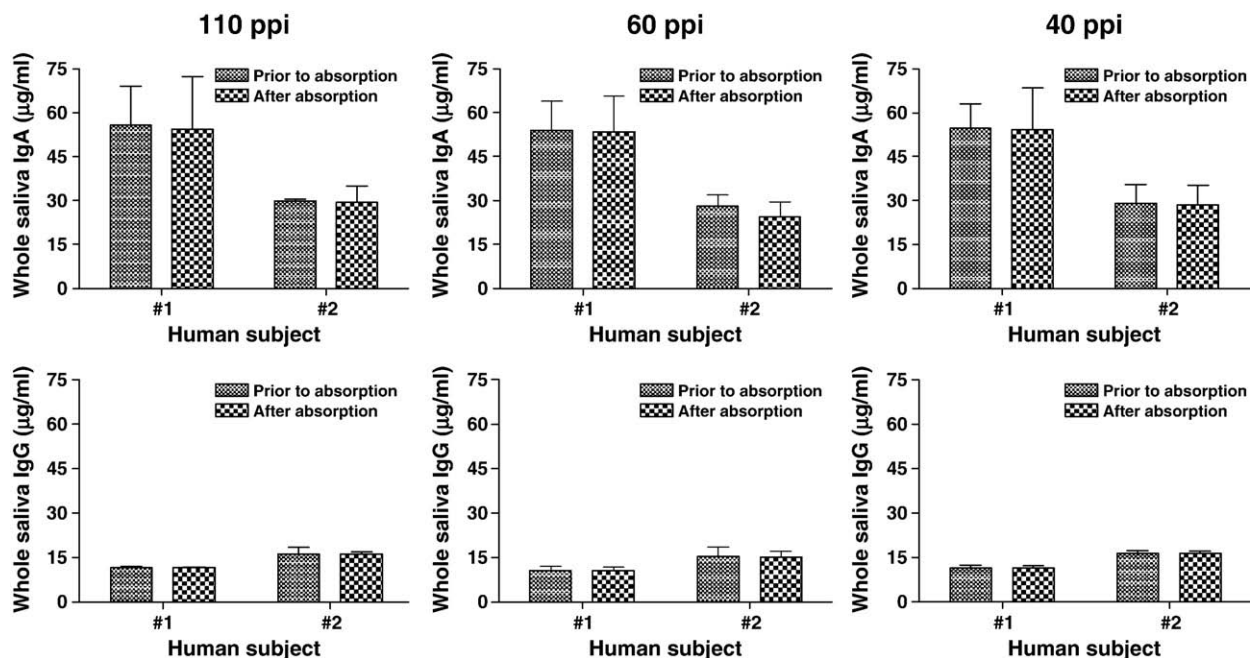


Fig. 3. The concentration of total IgA and IgG in whole human saliva prior to and after absorption by the NSC. Two normal human subjects were selected for the test. The Igs concentration was measured in saliva prior to and after absorption by the NSC. There were no significant differences in salivary Ig levels between prior to and after absorption by all types of foam tested. NSC: nasal secretion collector.

results in an agreement with our previous separate studies stating that the level of total IgA was higher than that of IgG in whole saliva (Lu, 2000).

3.4. ECP, tryptase, total IgA and IgG concentration in NS collected by nasal lavages and the NSC

Nasal lavage samples were collected from seven seasonal allergic patients by using 3 ml of PBS as described in the [Materials and methods](#) section. The NS from five seasonal allergic individuals were collected using the NSC (110 PPI foam, [Fig. 4A and B](#)). A significant difference was found in ECP and tryptase concentration between the nasal lavages and the NSC samples. The result demonstrated that the mean concentrations of nasal ECP and tryptase was increased 8-fold in NS samples collected using the NSC ($p < 0.01$). The mean values of ECP and tryptase in NS collected by the NSC were $40 \pm 15 \mu\text{g/ml}$ and $6 \pm 4 \mu\text{g/ml}$ respectively. Whereas, the mean values of ECP and tryptase in nasal lavages were $5.6 \pm 1.6 \mu\text{g/ml}$ and $1.1 \pm 0.2 \mu\text{g/ml}$ respectively ([Fig. 4B](#)). About 30% of nasal lavage samples had ECP level below the detection limit ($2 \mu\text{g/ml}$), and 85% of nasal lavage samples had tryptase level below the detection limit ($1 \mu\text{g/ml}$). However, 100% of NS collected by the NSC demonstrated ECP and tryptase levels above the detection limit. Thus, the detectability was 30–85% increased based on the biomarker analyzed.

The total IgA and IgG levels were measured in NS collected by both lavages and the NSC from 8 individuals ([Fig. 4C and D](#)). An additional data from 36 patients in a separate clinical study but with the same enrollment criteria was analyzed for total IgA measurement in NS collected by the NSC. The data of total IgA were pooled so that the total patient number was 44

for NS collected by the NSC. The median level of IgA was 34-fold higher in NS collected using the NSC than in nasal lavages ($p < 0.0001$). The mean of total IgA levels was $459.0 \pm 72.5 \mu\text{g/ml}$ (median: $287.6 \mu\text{g/ml}$; 25–75% percentile: 119.5 – 613.7) in NS collected by the NSC, and $11.9 \pm 2.60 \mu\text{g/ml}$ (median: $8.56 \mu\text{g/ml}$; 25–75% percentile: 7.45 – $18.78 \mu\text{g/ml}$) in nasal lavages. A similar result was found for IgG in NS, in which the median level of IgG in NS collected by the NSC was 22-fold higher than that of nasal lavages. The mean value of IgG in NS was $44.22 \pm 7.52 \mu\text{g/ml}$ (median: $38.21 \mu\text{g/ml}$; 25–75% percentile: 25.38 – $64.24 \mu\text{g/ml}$) when using the NSC, while a very low level of IgG with mean level of $3.55 \pm 1.64 \mu\text{g/ml}$ (median: $1.77 \mu\text{g/ml}$, 25–75% percentile: 1.10 – $4.12 \mu\text{g/ml}$) was detected in nasal lavages. Total IgA median concentration was 7.5 time higher than that of IgG in NS. Thus, a predominantly IgA was present in NS as detected by both lavages and NSC methods.

3.5. The levels of allergen specific Abs in NS collected by nasal lavages and NSC

The timothy grass (TG) and short ragweed (SW) specific Abs were quantitatively evaluated in NS collected by lavages ($n = 8$) and the NSC ($n = 10$) ([Fig. 5](#)). An additional data from 18 patients in a separate clinical study but with the same enrollment criteria was analyzed for TG and SW specific IgA measurement in NS collected by the NSC. The data of TG and SW specific IgA were pooled so that the total patient number was 28 for NS collected by the NSC. ([Fig. 5C and D](#)). The nasal lavages were serial diluted ranging from 1/2 to 1/16 for IgA, IgG and IgE Abs. The NS collected by the NSC were diluted ranging from 1/5 to 1/40 for IgA, IgG and IgE Abs. The data were the mean value from 2 to 4 dilutions and expressed as ng/ml for IgG/A Abs and

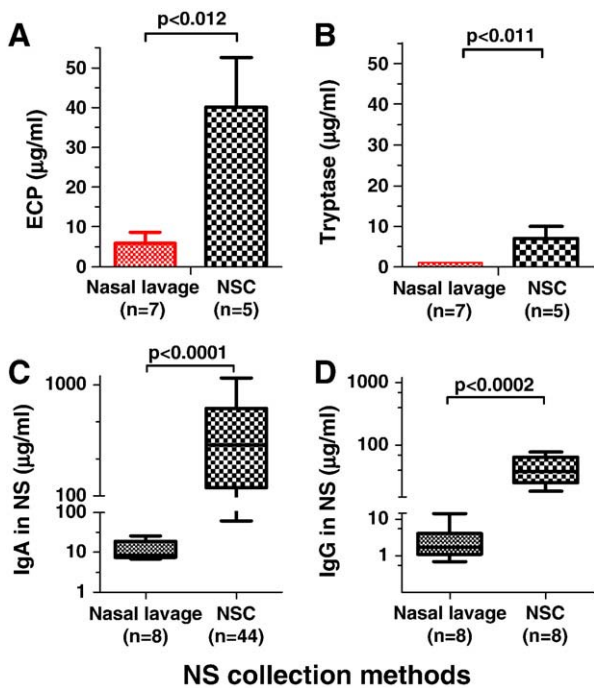


Fig. 4. Nasal ECP, tryptase, IgA and IgG levels in NS collected by lavages and the NSC. Seven nasal lavages and 5 NSC samples were tested for ECP and tryptase. A *p* value was noted on the top of the figure. The mean value and standard error bars were shown. A: ECP concentration in NS. The sensitivity of the detection was at least 8-fold increased when the NSC was used. B: Tryptase concentration in NS. C: Total IgA concentration in NS. Eight nasal lavages and 44 NSC samples were measured for IgA and 8 NSC samples for IgG. The median of total IgA levels in the NSC samples was at least 34-fold higher than that of nasal lavage samples. D: Total IgG concentration in NS collected by nasal lavages and the NSC. The median of total IgG levels in the NSC samples was at least 22-fold higher than that of nasal lavage samples. NS: nasal secretions; NSC: nasal secretion collector.

U/l for IgE Ab. In general, the level of TG specific IgA, IgG, and IgE Abs in NS was greater than the level of SW specific Abs. The Abs levels in NS collected by the NSC were significantly ($p < 0.01$) high when compared with nasal lavages for TG and SW specific IgE, IgA and IgG Abs (Fig. 5A–F). The median level of TG specific IgA was 8-fold higher than that of IgG in NS collected by the NSC (Fig. 5C and E). The median level of TG specific IgA and IgG Abs in NS collected by NSC was 287- and 180-fold respectively higher than that of TG specific IgA and IgG Abs in nasal lavages ($p < 0.0001$) (Fig. 5C and E). In NS collected by the NSC, the mean values of TG specific IgA and IgG Abs were 382.5 ± 70.05 ng/ml and 59 ± 21.11 ng/ml respectively (median: 287.5 ng/ml and 25–75% percentile: 113.0–585.5 ng/ml for TG specific IgA Ab; median: 36.92 ng/ml and 25–75% percentile: 8.3–106.2 ng/ml for TG specific IgG Ab). Whereas in nasal lavages, the mean values of TG specific IgA and IgG Abs were 3.46 ± 1.94 ng/ml and 0.37 ± 0.16 ng/ml respectively (median: 1.14 ng/ml and 25–75% percentile: 0.83–4.0 ng/ml for TG specific IgA Ab; median: 0.21 ng/ml and 25–75% percentile: 0.16–0.29 ng/ml for TG specific IgG Ab). The median of TG specific IgE Ab in NS collected by NSC was 11-fold higher than that of IgE Ab in nasal lavages ($p < 0.0001$) (Fig. 5A). In NS collected by the NSC, the mean value of TG specific IgE Ab was 186.5 ± 76.39 U/l (median: 84.65 U/l and 25–75% percentile: 53.71–236.7 U/l, whereas in nasal lavages, the mean value of TG

specific IgE was 9.49 ± 2.56 U/l (median: 7.65 U/l and 25–75% percentile: 3.6–16.52 U/l). The TG specific Abs were undetectable in most of the nasal lavages at the dilution 1/2. The similar results were true for SW specific Abs in NS (Fig. 5B, D and F). The median of SW specific IgE and IgA Abs in NS collected by the NSC was about 6-fold higher than that of NS collected by nasal lavages. Whereas, the median of SW specific IgG in NS collected by the NSC was 15-fold higher than that of nasal lavages ($p < 0.0001$). In NS collected by the NSC, the mean values of SW specific IgA, IgE and IgG Abs were 40.83 ± 22.63 ng/ml, 256.2 ± 103 U/l and 35.5 ± 23.96 ng/ml respectively (median: 6.608 ng/ml, and 25–75% percentile: 1.92–37.18 ng/ml for SW specific IgA Ab; median: 23.6 U/l, and 25–75% percentile: 13.76–191.7 U/l for SW specific IgE Ab; and mean: 4.9 ng/ml, 25–75% percentile: 1.25–48.78 ng/ml for SW specific IgG Ab). Whereas, in nasal lavages, the mean values of SW specific IgA, IgE and IgG Abs were 1.82 ± 0.58 ng/ml; 5.83 ± 1.67 U/l; and 0.50 ± 0.20 ng/ml respectively (median: 1.06 ng/ml, 25–75% percentile: 0.75–2.62 ng/ml for SW specific IgA; median: 4.19 U/l, 25–75% percentile: 2.52–9.81 U/l for SW specific IgE; median: 0.26 ng/ml, 25–75% percentile: 0.04–1.17 ng/ml for SW specific IgG). These results indicated that the number of the NS samples above the detection limit was significantly increased for measuring allergen specific Abs in NS collected by the NSC when compared with NS collected by nasal lavages (Fig. 5). Thus, the trend of detectability was increased from 6- to 290-fold depending on the specificity of an isotype Ab measured in NS. The NSC remained to be a better choice to collect NS for analyzing NS mucosal immune responses.

3.6. Cytokine concentration in NS collected by nasal lavages and the NSC

The NS samples collected by both nasal lavages and the NSC were tested for the concentration of cytokines IL-1, EGF, MCP-1, IL-6, IL-7 and TNF- α (Fig. 6A and B). A relative high level of IL-1, EGF, and MCP-1 (mean value: 800 pg/ml) was present in NS collected by the NSC (Fig. 6A). A significant difference was found in cytokines (IL-1, EGF, MCP-1, IL-6, and IL-7) concentration between two NS collection methods ($p < 0.05$). The TNF- α was undetectable for most the NS samples collected by nasal lavages. Whereas, the TNF- α was within the detection limit of the assay using NS collected by the NSC for all the samples (Fig. 6B). The results again demonstrated that the detectability of NS cytokines was at least 8-fold increased when NS samples was collected using the NSC.

3.7. Stability tests

The stability test of the NSC in terms of its liquid recovery, evaporation and retention at various temperatures was performed. When 100 μ l of PBS was loaded to the NSCs, the recovered volumes were from 45 μ l to 60 μ l at room temperature, and from 60 μ l to 75 μ l at 4 $^{\circ}$ C, -20 $^{\circ}$ C, and -80 $^{\circ}$ C. There was no significant difference in the liquid recovery volume at storage conditions 4 $^{\circ}$ C, -20 $^{\circ}$ C, and -80 $^{\circ}$ C for the period of 1, 2 and 4 weeks. The recovered PBS volume was the lowest (45 μ l) when the NSC was stored at room temperature for 1 week (Fig. 7A).

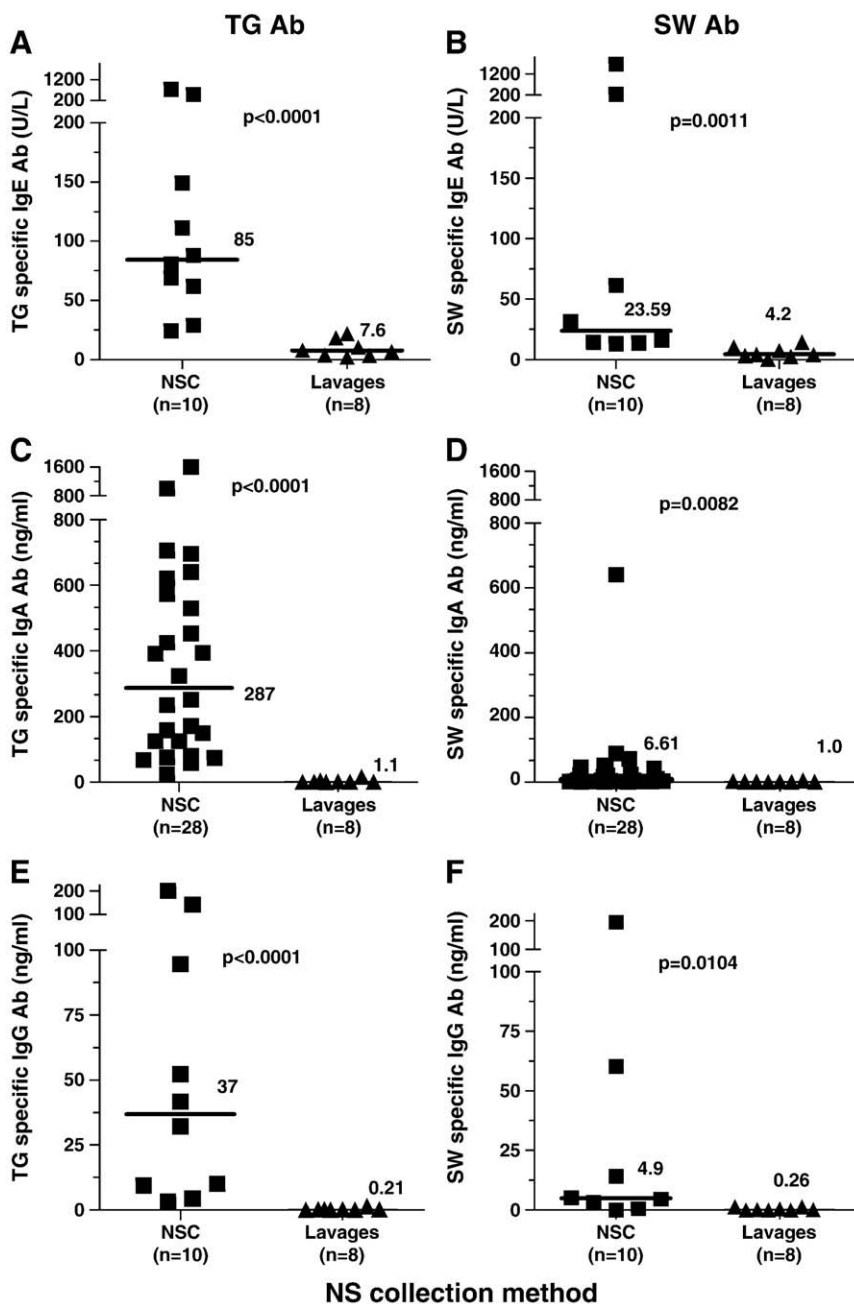


Fig. 5. Grass/weed specific antibodies in NS collected by nasal lavages and the NSC. Eight nasal lavages, 10 NSC samples for TG and SW specific IgE and IgG Abs and 28 NSC samples for TG and SW specific IgA Ab were tested. The median value was shown and represented by the horizontal bar. The *p* value was noted on the top of figures. A: TG specific IgE Ab levels. B: SW specific IgE Ab levels. C: TG specific IgA Ab levels. D: SW specific IgA Ab levels. E: TG specific IgG Ab levels. F: SW specific IgG Ab levels. The median levels of Ab specific to TG were from 11 to 290-fold higher in NSC samples than in nasal lavage samples using the quantitative ELISA, while the median levels of Ab specific to SW were 6–16 fold higher in NSC samples than in nasal lavage samples. The highest detectability was shown for TG specific IgA and IgG Abs (290- and 185-fold increased respectively) (panels C and E). TG: timothy grass; SW: short ragweed. NS: nasal secretions; NSC: nasal secretion collector.

The percentage of the PBS evaporation volume was the highest (6%) at room temperature, while less than 1% of the liquid evaporation occurred when stored at refrigerator 4 °C, freezer –20 °C, and –80 °C for 1, 2 and 4 weeks (Fig. 7B). The percentage of the PBS retention volume in the inner tube with foam roll was 35–40% at room temperature, 4 °C, –20 °C, and

–80 °C. The highest (55%) percentage of the retention volume was observed when the NSC stored at room temperature for 1 week (Fig. 7B).

The NSC containing nasal secretions can be stored at 4 °C, –20 °C, and –80 °C for 1 month without having significant liquid evaporation (less than 1%) and retention when the

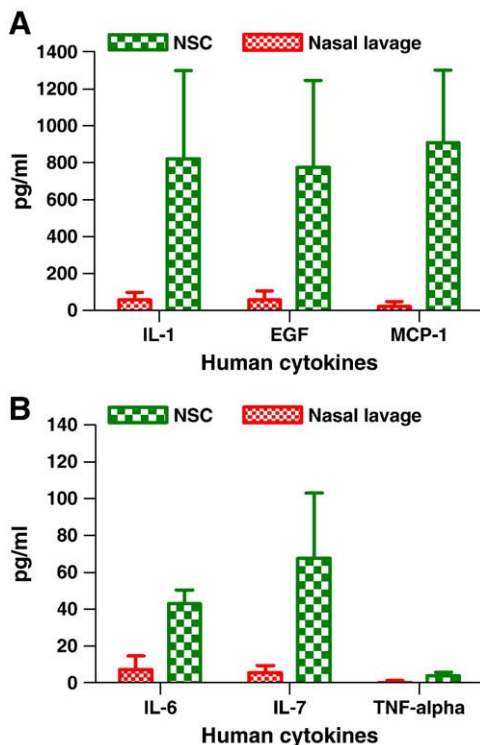


Fig. 6. Cytokine concentration in NS collected by nasal lavages and the NSC. Seven nasal lavage samples and 5 NSC samples were analyzed. The mean value and standard error bar were shown. A: IL-1, EGF, and MCP-1 concentration in NS samples. B: IL-6, IL-7, and TNF- α concentration in NS samples. The result again demonstrated that the detectability of NS cytokines was at least 8-fold increased when NS samples were collected using the NSC. NS: nasal secretions; NSC: nasal secretion collector.

loading PBS volume was 100 μ l. At the loading PBS volume as low as 100 μ l, the constant volume of the liquid retention was the main factor that caused the lower recovery rate.

4. Discussion

The variability of the results obtained in collecting NS is significantly influenced by the lack of a collection standard method. It appears that an efficient NS collector is needed for studying nasal mucosa immunity and evaluating immunotherapy and vaccine effects. The absorption technique described in this paper has not been challenged yet, and proves to be the most suitable in terms of reducing the variation, and increasing the detectability and the accuracy of the assays. An ideal NSC should possess the following attributes: 1) produce accurate results and a high detectability; 2) induce no nasal mucosa trauma and irritation; 3) maximize air flow passage through nasal cavity; 4) minimize protein retention on the absorptive material; 5) require minor patient's cooperation; 6) allow to collect sufficient non-diluted NS for multiple analyses; 7) offer easy handling and processing of the NS samples.

The present study shows that the novel NSC met the above criteria. The polyurethane foams at 110 and 60 PPI had acceptable capacity for the absorption and recovery of PBS or protein, and could be used for collecting nasal secretions on a daily practice. The number of subjects with detectable Abs

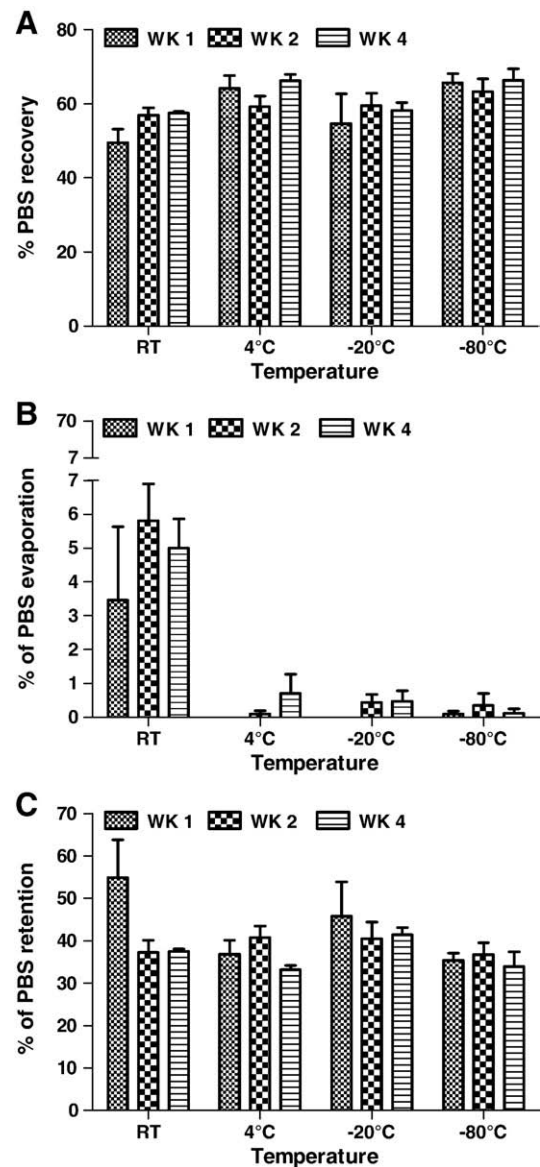


Fig. 7. A short term stability test, the liquid recovery, evaporation and retention of the NSC stored at different temperatures. The NSC samples were loaded with 100 μ l of PBS, the different parts of the collector (the whole collector without an inner tube, the inner tube only and the whole collector) were weighted for calculating the volume of recovery, evaporation and retention prior to and after storage conditions. The secondary weighing was performed after centrifuging the loaded NSC at 3000 g for 20 min. A: % of PBS recovery following the NSC stored at different temperatures for 1, 2 and 4 weeks. B: % of PBS evaporation following the NSC stored at different temperatures for 1, 2 and 4 weeks. C: % of PBS retention following the NSC stored at different temperatures for 1, 2 and 4 weeks. The NSC soaking with physiological water can be stored at 4 °C, -20 °C, and -80 °C for as long as 4 weeks without significantly reducing the sample volume. However, when the NSC was stored at room temperature for more than 4 weeks, the volume of samples was evaporated for at least 5%.

using the NSC was significantly increased when compared with the number of the subjects using the nasal lavage method. The NS collected by the NSC had at least 8-fold higher detectability than nasal lavages for measuring ECP, inflammatory cytokines and 6- to 290-fold higher detectability

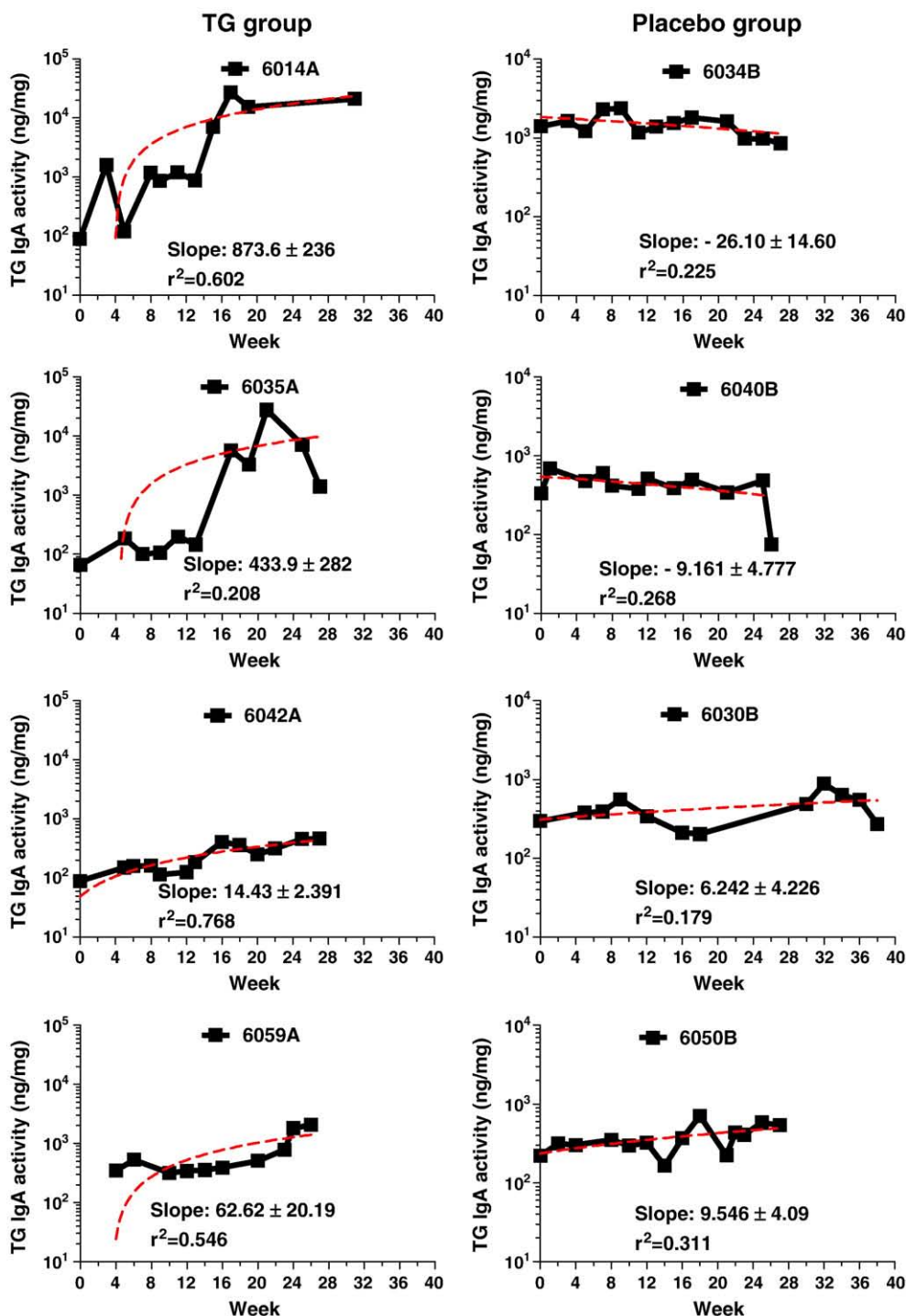


Fig. 8. Within-subject comparison for TG specific IgA activity in NS collected by the NSC in clinical immunotherapy trials. A within-subject comparison for the TG specific IgA activity in NS collected using the NSC from subjects enrolled for allergens sublingual therapy in treated and in placebo groups. Representative data were shown in the figure. The left panel was TG specific IgA activity in NS collected by the NSC from 4 individuals in the treatment group. The right panel was TG specific IgA activity in NS from the placebo group. Multiple NS samples were collected during a weekly visit for 40 weeks. The TG specific IgA activity was determined as the ratio of the ng/ml of TG specific IgA Ab in 1 mg/ml of total IgA ($\times 1000$). The subjects in TG treated group demonstrated a significantly increased IgA activity specific to TG in NS following daily escalation doses of TG delivered sublingually when compared with the placebo group. The dot line indicated the trend of IgA activity levels by a linear regression test. The slope and r^2 values were shown in each individual figure.

for grass/weed specific Abs. In addition, the NSC containing nasal secretions can be stored at 4 °C, –20 °C, and –80 °C for at least 1 month without having a significant liquid evaporation (less than 1%).

The NS is not a homogeneous fluid and it has considerable variations between individuals in terms of amounts, composition, physical properties, biological activities, and cellular contents (Baraniuk and Kaliner, 1991). Moreover, the NS has day-night differences in mucosal plasma proteins in common cold (Greiff et al., 1996). This study showed that there was large variability between-subjects regardless of the sampling method. The objective of the study focused on developing a NSC, and comparing NS collection methods on a similar population. The differences found were statistically analyzed based on the parameters of the mean, median and standard deviation. It was agreed that the measurement should be performed for within-subjects comparisons in which the individual serves as his/her own control. In fact, the NSC was the best suited for this purpose, in one of the randomized, double blind, placebo controlled, clinical trial study in phase IIa. The comparison has been made within-subject for the allergen (TG and SW) specific IgA activity in NS collected by using the NSC from subjects enrolled for allergens sublingual therapy in treated and placebo groups. Representative data were shown in Figs. 8 and 9. Some individuals demonstrated a significantly increased IgA activity specific to TG or SW in NS following daily escalation doses of TG or SW delivered sublingually when compared with placebo groups. However, because no NS were collected in this clinical trial with the nasal lavage method, the comparison between the two methods was not possible.

Several techniques such as nasal lavages and microsuction have been described for the collection of nasal secretions. Those techniques used to sampling NS were summarized in Table 1. Unlike other absorption methods with discs or cotton swabs, sufficient amounts of secretions can be collected by the NSC. The average volume of NS collected by the NSC was 200 µl (ranged from 20 to 1000 µl) depending on individual nasal mucosa inflammatory stages. An abundant NS can be collected from atopic subjects during seasonal allergy and common cold periods. In addition, the specimen processing procedure was extremely simple and rapid. The most important was the NS sample collected using the NSC yielded consistent and reproducible results for measuring a broad range of immunological mediators in NS.

NS derives from four major sources: goblet cells, sub-mucous glands, transepithelial ion, water transport and plasma transudation (Riechelmann et al., 2003). Lacrimal fluid and water condenses at the mucosal surface during expiration, and can make an additional contribution to NS (Riechelmann et al., 2003). Like other collection methods, secretions collected by the NSC may be mainly derived from goblet cells and submucous glands in the respiratory region, inferior turbinate of nasal mucosa as well as the secretions derived from mucosal lining of sinuses.

The polyurethane foam as the absorption material for collecting NS does not involve plasma transudation and tissue trauma (Riechelmann et al., 2003). The type of polyurethane foam (60–110 PPI) used in the present study had better fluid absorption capacity, and the shape of the foam is optimal for adapting human nasal cavities, allowing a maximum conduct

with nasal mucosa surfaces. Thus, the larger area of respiratory region secreting NS was covered. In addition, a relatively comfortable breathing during the sample collection can be reached. Further, the processing of NS sampling was simple and allowed the maximum recovery of nasal fluids. None of the subjects in a clinical trial phase IIa has experienced any adverse effects due to using the NSC, which was supporting the safety of the NSC for the applications in clinical trials.

The viscosity of NS is the important factor that allows pipetting an accurate volume of samples for the analysis. When the viscosity of NS is too high which is usually caused by the presence of a high level of sero-mucus contain, the pipetting of NS samples becomes difficult. The NS collected by the NSC contained less mucin due to the filtration effect of the foam during the centrifugation. Therefore, the NS sample was a clear liquid or had minimum viscosity that allowed for pipetting. In the case the mucin level was too high, a secondary centrifugation by transferring the NS to a new collector was needed.

Freely breathing through the nasal tracts during the sample collection is a main concern for patient's lung compliance and the quality of NS. The airflow tissue resistance of the upper airway in the nasal cavity may induce locally a blood flow rate increase and some degree of plasma transudation or immune cell infiltration. Most of the currently used nasal secretion samplers, such as sinus packs, filter paper strips or discs, could not allow the nasal air to flow adequately. Inserting a hollow tube in the center of the polyurethane foam roll could allow a relatively high level of air flowing through nasal cavity during the sample collection. In addition, the tube permits an easy handling of the foam roll by patients and investigators. Further, both polyurethane foam and plastic tube can be sterilized at a high temperature of 121 °C without changing the shape, absorptive ability and color (data not shown).

As seen with other absorption methods (Table 1), the insertion of the NSC may be difficult (even with the help of a hollow tube fixed in the polyurethane foam) on patients presenting nasal polyposis and anterior septal deviation. In regard to these cases, the filter paper disc or nasal lavages method may apply.

These results demonstrated that the concentration of Igs/Abs in NS was not only varied between individuals, but also significantly influenced by the secretion collection methods. The NSC appears to be a better method for collecting NS to evaluate nasal mucosal immunity and to monitor the levels of biomarker changes within-subjects. The difference between the NS collected using the NSC and the NS collected using the nasal lavage method was that the NS from nasal lavages was highly diluted with an unknown dilution factor whereas the NS collected with the NSC did not involve a dilution factor. If the volume of NS samples collected with the NSC was not enough for multiple analysis, the NS could be diluted at a known dilution factor (3–5) in order to reach the lower and upper limit of the quantization range defined with the standard curve; thus, accurate results could be obtained (Fig. 4A and B).

It has been shown that nasal mucus secretion is a complex mixture of many substances and consists of about 95% water, 2% mucin, 1% salts, 1% of other proteins such as albumin, immunoglobulins (Igs), lysozyme and lactoferrin, kallikrein, and 1% lipids (Kaliner et al., 1984). Albumin is the most prominent protein in the nasal mucus, suggesting that

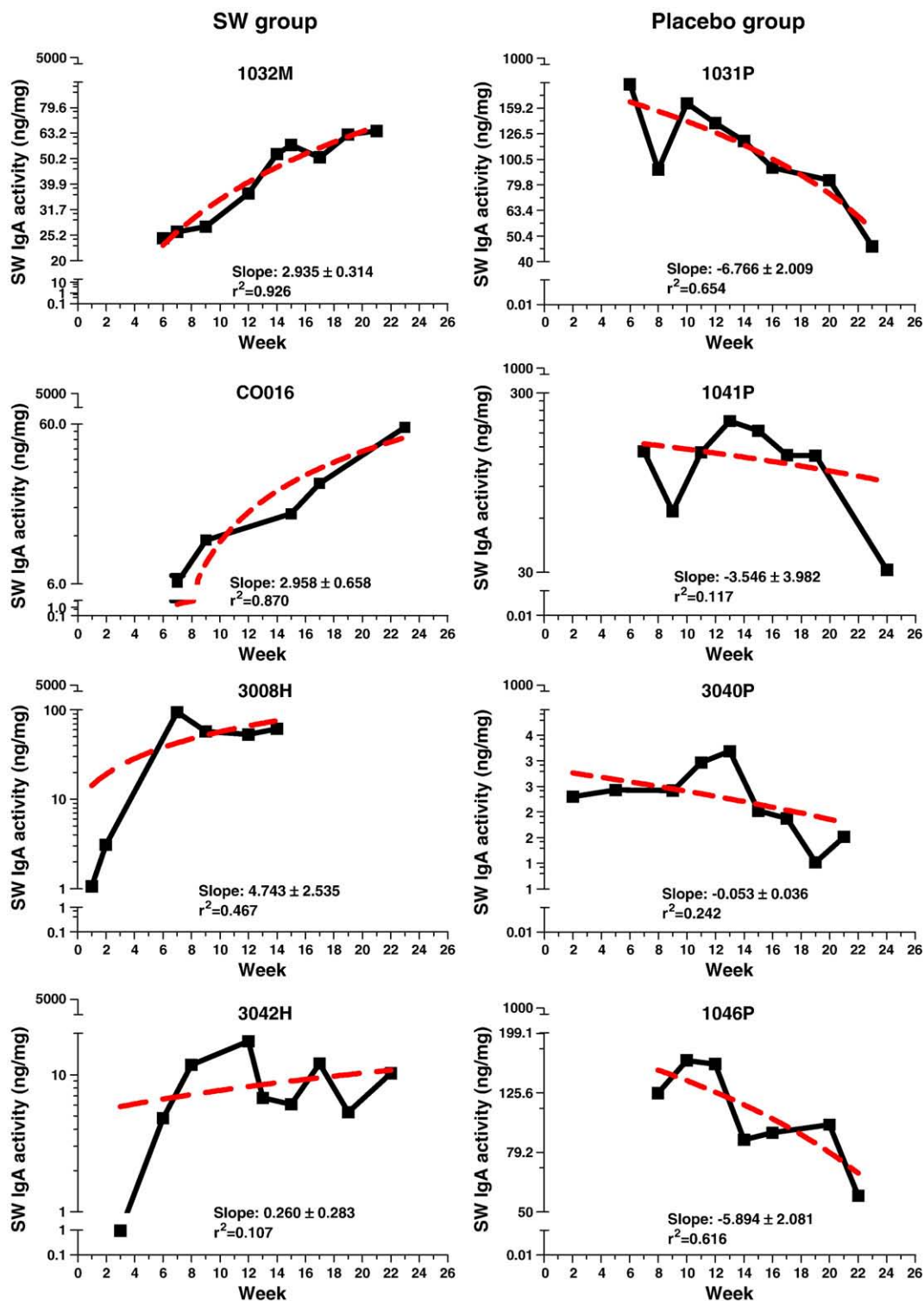


Fig. 9. Within-subject comparison for SW specific IgA activity in NS collected by the NSC in clinical immunotherapy trials. A within-subject comparison for the SW specific IgA activity in NS collected using the NSC from subjects enrolled for allergens sublingual therapy in treated and in placebo groups. Representative data were shown in the figure. The left panel was SW specific IgA activity in NS collected by the NSC from 4 individuals in the treatment group. The right panel was SW specific IgA activity in NS from the placebo group. Multiple NS samples were collected during a weekly visit for 40 weeks. The SW specific IgA activity was determined as the ratio of the ng/ml of TG specific IgA Ab in 1 mg/ml of total IgA ($\times 1000$). The subjects in SW treated group demonstrated a significantly increased IgA activity specific to SW in NS following daily escalation doses of SW delivered sublingually when compared with the placebo group. The dot line indicated the trend of IgA activity levels by a linear regression test. The slope and r^2 values were shown in each individual figure.

Table 1

Methods used to sampling nasal secretions.

NS collection method	Brief procedures and materials
Spontaneous secretions	- Nose blowing or collection of secretions dripping out of the nose - Suction and microsuction - Nose blowing or suction following stimulation (methacholine, histamine)
Washes	- Combined aspiration lavages - Spray blow - Nasal pool lavages - Standard lavage and sequential lavages - Ultrasonic nebulization of 4.5% saline for 5 min
Absorption	- Cotton, sterile pre-humidified gauze - Filter paper strips or discs, swab, synthetic electrophoresis wick - Cellular material (surgical cellulose sponges)

transudation occurs under normal conditions (Maremmanni et al., 1996). Furthermore, the IgA Ab produced by both the adenoid tissue and the nasal mucosa contributes significantly to the immune protection against inhaled bacteria and viruses (Bernstein et al., 1997). The NSC demonstrated the efficiency to sampling the mixture of the above substances to evaluate immunological responses, in particular for the allergen specific Abs, cytokines and local inflammation markers in NS of allergic patients. It may provide useful information for following the outcomes of an allergen sublingual immunotherapy (Mungan et al., 1999; Varga et al., 2003; Gidaro et al., 2005; Rienzo et al., 2005) as well as for the diagnostics of nasal chronic inflammation (Baroody et al., 2008) and upper respiratory infectious diseases (Hendley and Gwaltney, 2004, van Cauwenberge, 1996).

In conclusion, the NSC causes no trauma to nasal mucosa, it permits maximum liquids absorption, and it decreases nasal airflow tissue resistance. A high detectability and specificity of results in NS can be obtained with the connection to a reliable and reproducible analytical method based on a standardized NS samples collection tool.

This technique proved to rest on a feasible and quantifiable tool for sampling NS that could also be used for daily clinical practices, diagnostics, and other various research purposes.

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