

# Exhaled nitric oxide collected with two different mouthpieces: a study in asthmatic patients

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## Abstract

Techniques for collecting exhaled nitric oxide (ENO) recommend the use of antibacterial filters of 0.3  $\mu\text{m}$ . The aim of the present study was to compare the measurements of ENO obtained with two different filtering devices. Air samples from 17 asthmatic and 17 non-asthmatic subjects were collected by a recommended off-line technique using two different mouthpieces: 1) the Sievers disposable tool (A) under a breathing pressure of 18  $\text{cmH}_2\text{O}$ , and 2) a mouthpiece containing a HEPA filter (B) under a breathing pressure of 12  $\text{cmH}_2\text{O}$ . The nitric oxide samples were collected into an impermeable reservoir bag. Values for ENO were compared using two-way repeated measures ANOVA followed by the Tukey test. Agreement was assessed by Bland-Altman analysis. ENO values obtained with mouthpieces A and B were comparable for asthmatic (mean  $\pm$  SEM,  $42.9 \pm 6.9$  vs  $43.3 \pm 6.6$  ppb) and non-asthmatic ( $13.3 \pm 1.3$  vs  $13.7 \pm 1.1$  ppb) subjects. There was a significant difference in ENO between asthmatics and non-asthmatics using either mouthpiece A ( $P < 0.001$ ) or B ( $P < 0.001$ ). There was a positive correlation between mouthpiece A and mouthpiece B for both groups. The Bland-Altman limits of agreement were considered to be acceptable. Mouthpiece B was less expensive than A, and these data show that it can be used without compromising the result. Our data confirm reports of higher ENO values in the presence of airway inflammation.

## Key words

- Exhaled nitric oxide
- Asthma
- Nitric oxide collecting apparatus
- Filter

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## Introduction

Nitric oxide (NO), a highly reactive gas involved in several pathophysiologic processes, is synthesized by endothelia and neuronal tissue cells and by macrophages. NO may participate in arterial pressure regulation, neuronal communication and immune system defense. High NO production may be associated with diseases such as sepsis, stroke and cerebrovascular disease, neuronal de-

generation, diabetes, arthritis and several chronic inflammatory conditions (1,2).

Asthma is a worldwide disease. Symptoms include dyspnea, cough and difficult respiration associated or not with wheezing that have been related to the release of inflammatory airway mediators (3). The treatment and control of asthma have been recommended by guidelines such as the Expert Panel Report 2 (National Heart, Lung, and Blood Institute, NIH, 1997) (4). During the

last few years, emphasis has changed from bronchospasm control, surely necessary, to the control of airway inflammation.

NO participates in the physiologic regulation of bronchomotor tonus, in diseases such as asthma (5,6). Exhaled NO (ENO) is elevated in asthmatic persons compared to non-asthmatic individuals (7,8) and is derived mainly from peripheral airways (9,10). The higher ENO values for asthmatic persons have been related to airway inflammation mainly when eosinophils are present (11). ENO is also elevated during a late allergic response (12) although they are reduced during inhaled corticoid treatment (13).

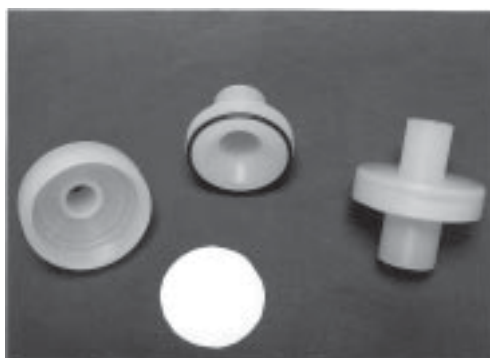
Recently the techniques used to collect and to measure ENO have been standardized (14). Included in the recommendations is the use of antibacterial filters with a filtering capacity of 0.3  $\mu\text{m}$ . A disposable filtering material is available from Sievers (Sievers Instruments Inc., Boulder, CO, USA). A second device has been constructed to use the high efficiency particulate air (HEPA) filters with similar filtering capacity to the Sievers filters.

The objective of the present investigation was to compare ENO measurements obtained from non-asthmatic healthy volunteers and asthmatic subjects using two filtering mouthpieces.

## Material and Methods

The protocol was approved by the Ethics Committee of the University Hospital and

Figure 1. A photograph of the mouthpiece containing the HEPA filter assembled and separated into three parts showing the screw portion where the two connect. The HEPA pad is located inside the device.



informed consent was obtained from the subjects.

Seventeen asthmatic and 17 healthy non-asthmatic volunteers were invited to participate in the study by providing exhaled air samples in order to compare ENO collected by the recommended off-line technique using two different devices (Figure 1): 1) the Sievers mouthpiece with a built-in 0.3- $\mu\text{m}$  bacterial filter (A), and 2) a mouthpiece containing a 0.3- $\mu\text{m}$  bacterial HEPA filter (Trox Technik, Curitiba, PR, Brazil) (B). Subjects were required to inhale orally up to their total lung capacity first through mouthpiece A and then through mouthpiece B, both attached to a Sievers sampling kit. This kit contains a pressure gauge and an inspiratory filter that reduces the NO level from the inhaled air to a very low concentration.

Once the subject had inhaled to total lung capacity, a gentle exhalation maneuver into the Mylar bag (Sievers) was performed through the sampling kit without any breath-holding (mean oral NO). The volunteer using the A mouthpiece was instructed to achieve a breathing pressure of 18  $\text{cmH}_2\text{O}$  monitored by the pressure gauge, which gave a flow rate of 250 ml/s. During this expiratory time, he was instructed to push in a stainless steel valve of the sampling kit after 3 s of exhalation and to continue for about 2 s, producing a collected volume larger than 50 ml (duplicate samples). This procedure was repeated using the B mouthpiece with the volunteer reaching a breathing pressure of 12  $\text{cmH}_2\text{O}$ , which gave a flow rate of 200 ml/s. The breathing sample was collected into an NO-impermeable reservoir bag (Mylar bag) with a volume capacity of 1.5 liters. The bags were sealed and subsequently analyzed for ENO by chemiluminescence (Sievers, model NOA 280).

The resistance of the Sievers sampling kit with the A or B mouthpiece was determined using an Inter 5 ventilator (Intermed, São Paulo, SP, Brazil) with the application of a quadratic constant flow.

## Statistical analysis

Statistical analysis was performed using the SigmaStat 2.0 software (SigmaStat for Windows version 2.03, SPSS Inc.). All data are reported as means  $\pm$  SEM. ENO values were compared by two-way repeated measures analysis of variance for one factor of repetition followed by the Tukey test for multiple comparisons, with the level of significance set at a  $P < 0.05$ . The Pearson correlation coefficient was used in order to compare the devices. Agreement of the results obtained with the two mouthpieces was determined by the Bland-Altman method calculated as the mean difference and the standard deviation of the differences between A and B NO values. This mean added or subtracted to doubled standard deviation gives the "limits of agreement" (15).

## Results

The measurements of ENO using mouthpieces A and B for asthmatic patients ( $42.9 \pm 6.9$  vs  $43.3 \pm 6.6$  ppb) or for non-asthmatic subjects ( $13.3 \pm 1.3$  vs  $13.7 \pm 1.1$  ppb) were the same (Figure 2). ENO from asthmatics was higher than ENO from non-asthmatics using either device A ( $42.9 \pm 6.9$  vs  $13.3 \pm 1.3$  ppb,  $P < 0.001$ ) or device B ( $43.3 \pm 6.6$  vs  $13.7 \pm 1.1$  ppb,  $P < 0.001$ ).

There was a strong positive correlation between the results obtained with filters A and B for both groups (Figure 3).

The Bland-Altman diagrams plot the difference in the exhaled nitric oxide measured between techniques (Figure 4). The agreement between techniques is demonstrated by a plot of differences against mean values of exhaled nitric oxide. In this plot the solid bar represents the mean difference and broken lines indicate  $\pm 2$  SD from the mean. The figure shows the various measures to be reproduced because the individual points on the Bland-Altman plot are randomly scattered around the overall average difference

and most points fall within 2 SD of the overall difference. A random scatter with several points outside the 2 SD would indicate a weak agreement.

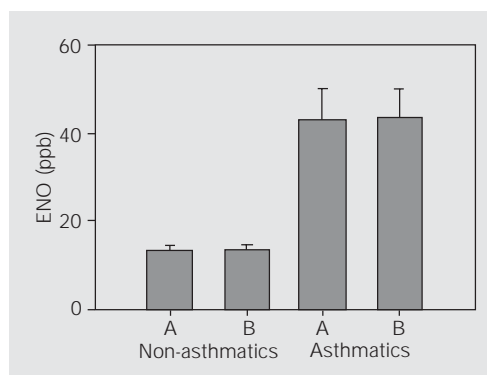


Figure 2. Exhaled nitric oxide (ENO) obtained with the different devices for asthmatic (N = 17) and non-asthmatic subjects (N = 17). There was no difference in ENO between mouthpieces A and B for asthmatic patients or non-asthmatic subjects. However, there was a difference in ENO when asthmatic patients were compared with non-asthmatics using either device A ( $P < 0.001$ ) or device B ( $P < 0.001$ ).

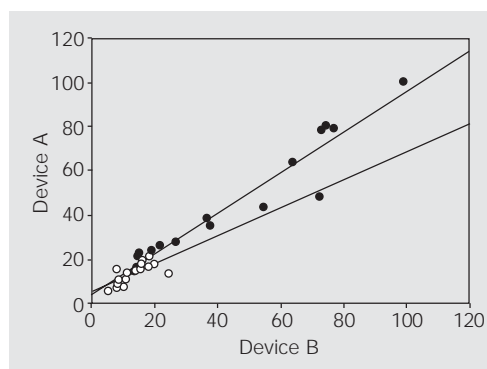


Figure 3. Correlation curve of exhaled nitric oxide (ppb) obtained from non-asthmatic (open circles) ( $r = 0.508$ ) and asthmatic subjects (filled circles) ( $r = 0.930$ ). There was a positive correlation between the different devices for both non-asthmatic and asthmatic subject data.

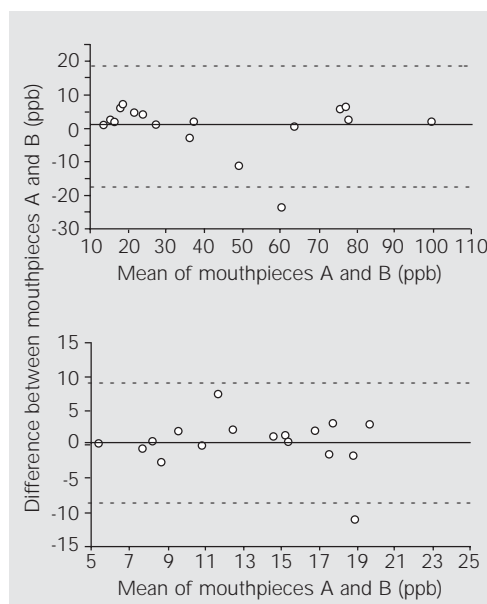


Figure 4. Bland-Altman diagram comparing exhaled nitric oxide (ENO) measured with the Sievers mouthpiece (A) and the mouthpiece containing a HEPA filter (B) in asthmatic patients (top) and non-asthmatic subjects (bottom). The mean difference was 0.49 ppb and the limits of agreement were 18.74 and -17.75 for ENO from asthmatic patients. These limits are acceptable and pertain to the 2 SD range of B-A analyses.

Mouthpiece resistance was checked by applying quadratic constant flow. Mouthpiece A under a constant flow of 16 l/min (260 ml/s) produced a sampling kit pressure of 18 cmH<sub>2</sub>O and in the Inter 5 ventilator a system pressure of 21 cmH<sub>2</sub>O. Mouthpiece B under a constant flow of 12 l/min (200 ml/s) produced an equal Inter 5 ventilator pressure of 21 cmH<sub>2</sub>O and a pressure of 12 cmH<sub>2</sub>O in the sampling kit.

### Discussion

The two mouthpieces, i.e., the Sievers device and the device containing a HEPA filter, were the same in their capacity to correctly collect ENO in higher or lower concentrations from asthmatic patients or healthy volunteers, respectively.

The main rationale behind our study was to compare the efficacy of the Sievers mouthpiece with a prototype manufactured in Brazil which costs approximately six times less than the imported mouthpiece. A significant part of the savings is that the mouthpiece of the HEPA filter apparatus is reusable.

The use of standardized ENO collecting methods has been recommended in order to avoid bias in the measurement (14). We have replaced the standardized piece that holds a built-in filter with a new one without changing the characteristics of the measurements. The pressure inside the sampling kit that provides the breathing flow to be redirected to the Mylar collection bag is different (12 cmH<sub>2</sub>O vs 18 cmH<sub>2</sub>O). However, this difference did not change the final amount of ENO measured. We tested its filtering performance in two situations: with asthmatic subjects, who might exhale higher levels of NO, and with healthy volunteers, from whom a lower ENO concentration is expected. Asthmatics may also not feel comfortable blowing into an apparatus with high airflow resistance, a situation probably well tolerated by non-asthmatics.

The pressure inside the mouth did not exceed 21 cmH<sub>2</sub>O for the two mouthpieces, as measured by tests performed in our laboratory using an Inter 5 ventilator. This limitation probably reflects the respiratory system charge imposed by these maneuvers. Although no other lower or higher pressure was tested, this pressure seems to be a good standard to reach 21 cmH<sub>2</sub>O in the mouth during the expiratory maneuver. None of the patients or volunteers complained about such effort.

The modified HEPA filter used instead of the original Sievers mouthpiece has a filtering capacity of less than 0.3 µm. This capacity was tested with heated dioctyl phthalate which creates a smoke averaging 0.3 µm. According to the Lydall Group technical support ([www.lydall.com](http://www.lydall.com)), this smoke is used to challenge the filter media at a flow rate of 5.33 cm per second with the penetration recorded. The HEPA filter media has a maximum efficiency of 99.97%, meaning that only 0.03% of the smoke within 0.3 µm penetrates the filter area. For this reason, our device can be considered to have the recommended antibacterial capacity (14).

The results showed a good Pearson correlation coefficient. However, even a good correlation may not reflect the agreement between the two results. In order to determine if the correlation coefficients would not be misleading with respect to the use of the new HEPA filter, the Bland-Altman method for clinical measurement comparison of any new technique with an established one was applied to the results obtained with these two mouthpieces (15). The agreement among the NO data obtained by devices A and B was confirmed by Bland-Altman analysis (15). This method estimates the average bias of one method relative to the other and also how the methods are likely to agree for an individual. Figure 4 shows that each of the limits of agreement is small enough to qualify both tools containing different filters as reliable to measure ENO in

both subject groups. For asthmatics the limits are wider than for non-asthmatics, probably because of the heterogeneity of the asthmatic group. However, it must be noted that there are large confidence intervals in the measurements with both filters.

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