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Article

The Nasal Nitric Oxide Response to External Acoustic Energy: A Pilot Study of Sampling Dynamics

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Abstract: Background: The paranasal sinuses serve as a reservoir of nitric oxide (NO), contributing to baseline nasal NO (nNO) levels. nNO has also been shown to increase transiently with humming, a response that may be blunted in severe rhinosinusitis. Blunting of the acoustically-induced nNO transient ("spike") has been proposed as a screening test for osteomeatal complex (OMC) obstruction in sinusitis. Preparatory to conducting a clinical evaluation study, to eliminate variation in patient effort during this maneuver, we evaluated the use of external acoustic energy—in place of humming—to elicit nNO transients, documenting the effects of varying stimulus amplitude and gas sampling rates. Methods: Non-smoking, non-asthmatic subjects with no history of chronic sinusitis or nasal polyposis underwent nNO measurements in triplicate under: (1) control (quiet) conditions, and (2) with 128 Hz external acoustic energy. In Experiment 1, twelve subjects were exposed to two different intensities of external acoustic energy at 3 L/min sampling rate. In Experiment 2, a subset of nine subjects was sampled with and without acoustic stimulation at three different gas sampling rates (1, 2, and 3 L/min). Results: Experiment 1: Subjects, as a group, showed intensity-related increases in nNO with increasing acoustic amplitude ($p < 0.01$). Experiment 2: independently, both applied acoustic energy and lower nasal gas sampling rates increased measured nNO levels ($p < 0.05$ to $p < 0.0001$). Longitudinally, baseline (quiet) nNO obtained on a repeated basis in the two experiments ($n = 9$) was highly reproducible ($R^2 = 0.84$; $p < 0.001$), and acoustically-stimulated nNO was moderately so ($R^2 = 0.50$; $p < 0.05$). Conclusions: Application of external acoustic energy is a practical alternative to humming for mobilizing NO from the paranasal sinuses, and could be more objectively applied in any future validation studies involving clinical sinusitis and/or OMC obstruction.

Keywords: paranasal sinuses; nasal cavity; nitric oxide; osteomeatal complex; acoustic stimulation

1. Introduction

Nitric oxide (NO), an endogenously produced gas in the human airway, has vasodilatory, bacteriostatic, and ciliastimulatory effects [1]. Fractional exhaled nitric oxide (FeNO) has proven clinically useful as an index of endobronchial inflammation in asthmatics [2]. In principle, nasal NO (nNO) could play a similar role as an inflammatory biomarker the upper airway, given the ability of bacterial infection (a feature of rhinosinusitis) to activate epithelial cells and stimulate NO flux in the sinus mucosa [3]. In point of fact, however, the empirical relationship between sinonasal inflammation and nNO is inconsistent [4–9]. Although the paranasal sinuses act as a major source for nNO, osteomeatal complex (OMC) obstruction can isolate sinus NO flux from the nasal cavity [10–12]. Thus, in chronic rhinosinusitis (with or without polyposis), baseline nNO has often been found to be paradoxically low despite widespread mucosal inflammation, and nNO levels can actually *rise* after appropriate medical or surgical treatment, suggesting restoration of OMC patency [13–20].

A variant of nNO sampling utilizes acoustic energy (normally from humming) to mobilize gases from the paranasal sinuses, with a resulting transient "spike" in nNO [21–27]. Since this response is often blunted in pan-sinusitis and/or polyposis, it has been proposed as a non-invasive index of OMC patency, and hence a screening test for severity of rhinosinusitis [28,29]. This maneuver has been well-documented, methodologically, with respect to the effects of sampling mode, humming frequency, and nNO depletion by repeated sampling ("washout") [23–27]. However, a limitation of this technique is the fact that humming during NO sampling requires a high degree of patient cooperation, resulting in potential variability in both acoustic frequency and amplitude, and the effects of altering acoustic amplitude are not well studied. Although external acoustic energy (potentially standardizable) has been used to elicit this phenomenon in a mechanical model, no published nNO data exist, to our knowledge, utilizing external acoustic energy *in vivo* [24,25]. We sought to further standardize this diagnostic maneuver (preparatory to conducting a clinical validation study) by pilot testing human subjects using an apparatus with which acoustic energy is applied by an *external* source (*i.e.*, audio oscillator, amplifier, and speaker). In doing so, we evaluated the effects of varying both applied energy level and gas sampling rate on the nNO response to acoustic stimulation. To our knowledge, we report the first *in vivo* human data demonstrating the acoustic response of nNO to externally-applied acoustic stimulation.

2. Methods

Two experiments were conducted—the first examining the effect of varying applied acoustic energy level, and the second examining the effect of varying the nasal gas sampling rate—on the magnitude of the acoustically-induced transient in nNO.

2.1. Subject Recruitment

Subjects were recruited using flyers posted at a university research facility. Recruitment materials, including flyers and informed consent documents, were approved by the University of California, San Francisco Committee on Human Research. Inclusion criteria included ages 18–70, with or without rhinitis symptoms. Exclusion criteria included a self-reported history of asthma, chronic sinusitis, nasal polyposis, current tobacco use, serious cardiopulmonary disease, a past history of angioedema or anaphylaxis, or inability to comfortably breath-hold for 15 s. The two experiments occurred 18 months apart, with participants in Experiment 2 constituting a subset of participants from the initial study (*i.e.*, those who elected to participate in a follow-up study).

2.2. Study Procedures

2.2.1. Both Experiments

Nasal NO was sampled by aspirating from the right nostril at a rate in accordance with ATS (American Thoracic Society) and ERS (European Respiratory Society) recommendations [30]. For each trial, real-time NO was recorded for a 10 s period utilizing a Sievers 280i NO Analyzer (GE Analytical Instruments, Boulder, CO, USA) calibrated on each testing day, per factory instructions. The upper and lower airways were isolated during sampling by a combination of breath-holding and velo-palatine closure. Make-up air was directed to the left nostril through a T-piece communicating with a speaker enclosure (Figure 1). The flow rate for aspirated air (from the right nostril) was controlled using a vacuum rotameter (Model RMA-26-TMV, Dwyer Instruments, Michigan City, IN, USA). The targeted total flow rates were achieved including an adjustment for the contribution of the NO analyzer internal pump. Sampling occurred in triplicate under both unstimulated (quiet) and stimulated (128 Hz sinusoidal energy) conditions. A four minute recovery period was allotted between samples to minimize the "washout" effect of repeated acoustic stimulation.

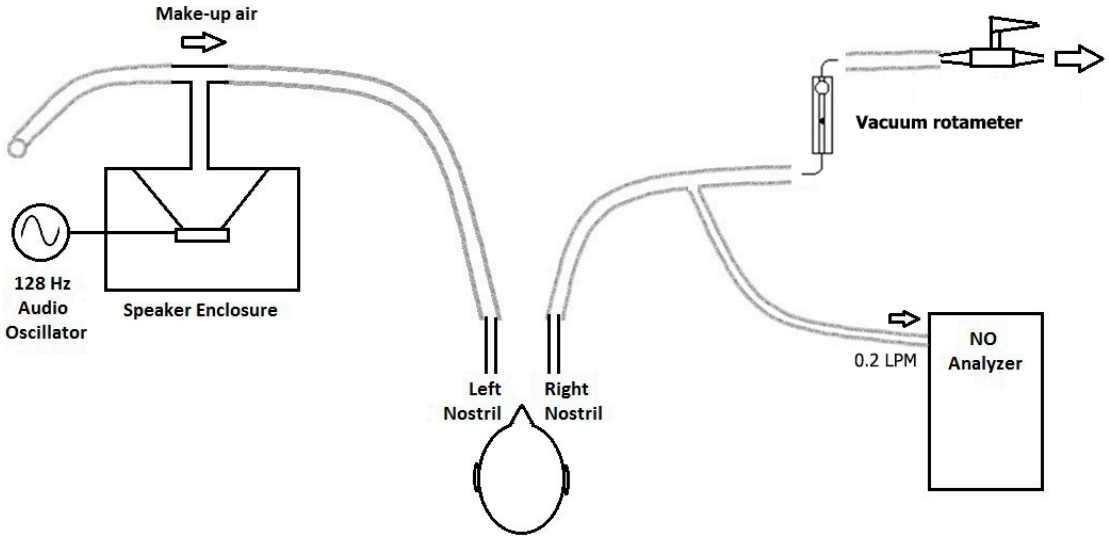


Figure 1. Schematic diagram of sampling apparatus.

Time-weighted average nasal NO level for each sampling condition was determined from the area-under-curve (AUC) for 10 s of sampling. AUC, in turn, was measured using ImageJ software version 1.43u (National Institutes of Health, Bethesda, MD, USA). Data were entered and analyzed utilizing JMP version 12 software (SAS Institute, Cary, NC, USA) in a repeated measures ANOVA model. nNO values were averaged over three trials for each sampling condition. A "*p*" value of <0.05 was taken as significant.

2.2.2. Experiment 1

For three trials each, nasal gas was aspirated from the right nostril at 3.0 L/min (the upper limit of the ATS/ERS recommended range). Sampling took place under three conditions: unstimulated (quiet), and with make-up air supplied to the left nostril carrying acoustic stimulation at two different intensities (64 and 78 dBA @ 30 cm).

2.2.3. Experiment 2

For three trials each, nasal gas was aspirated from the right nostril at each of three different rates: 1.0, 2.0, and 3.0 L/min (all within the ATS/ERS recommended range). Each sampling rate was employed under both unstimulated (quiet) conditions and with acoustic stimulation of 78 dBA @ 30 cm (the higher of the two acoustic intensities employed in Experiment 1).

2.2.4. Longitudinal Analysis

For the nine subjects who participated in both experiments, it was possible to examine repeatability for both baseline (quiet) nNO and for acoustically-stimulated nNO at the single consistent amplitude/sampling rate combination of 78 dBA @ 3.0 L/min.

3. Results

Subject demographics were as follows: For Experiment 1, twelve subjects (including nine females), ranging in age from 24 to 64 years (mean, 44.1), were tested. For Experiment 2, nine subjects (including six females) ranging in age from 25 to 66 years (mean, 48.3), were tested. Although roughly half of subjects reported having been previously diagnosed with (allergic or nonallergic) rhinitis, allergy status was not further characterized, as the emphasis in this study was evaluating test characteristics (rather than correlating response with subject characteristics).

A representative tracing of real-time nNO levels taken under both baseline and acoustically stimulated conditions appears in Figure 2.

3.1. Experiment 1

Results appear graphically in Figure 3. Individual mean nasal NO levels sampled at 3 L/min ranged from 66–182 ppb under quiet conditions, 106–386 ppb with low-amplitude acoustic stimulation (128 Hz; 64 dBA @ 30 cm), and 122–750 ppb at higher amplitude (128 Hz; 78 dBA @ 30 cm). A paired analysis of nasal NO levels across subjects showed a significant upward trend in nNO with increasing applied acoustic energy ($p < 0.01$).

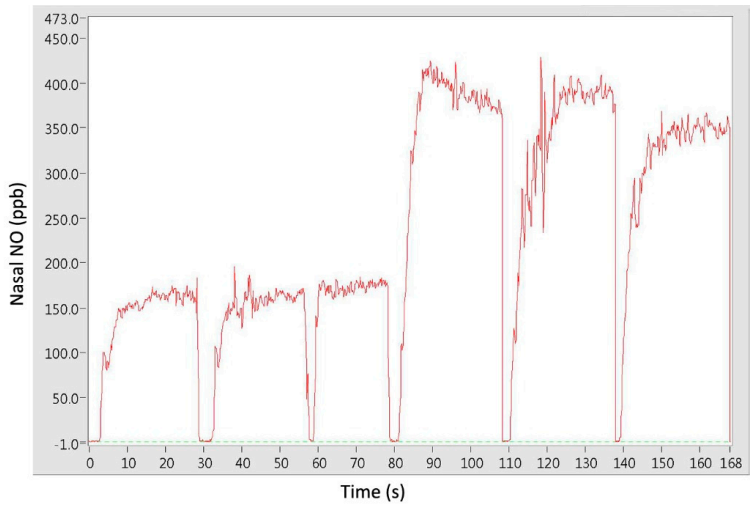


Figure 2. Representative nNO tracings under quiet (left three tracings) and acoustically stimulated (right three tracings) conditions (data from Experiment 2; sampling at 1.0 L/min).

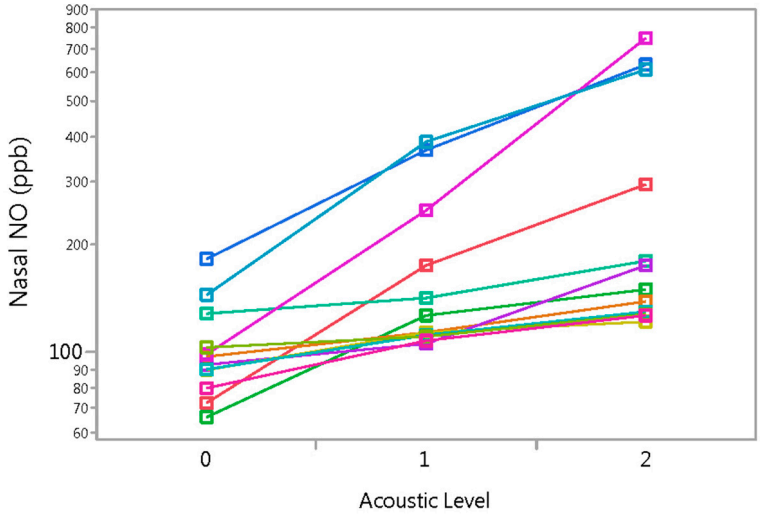
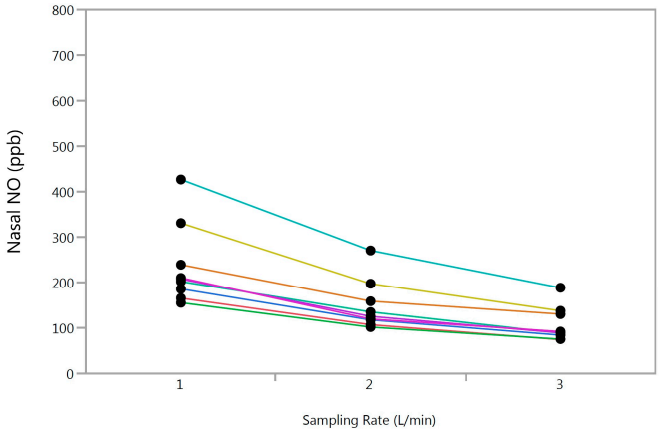


Figure 3. nNO levels from Experiment 1 (n = 12). Nasal sampling at 3.0 L/min under three acoustic conditions: Quiet ("0"); 64 dBA @ 30 cm ("1"); and 78 dBA @ 30 cm ("2"). A paired analysis across subjects showed a significant upward trend with increasing applied acoustic energy ($p < 0.01$).

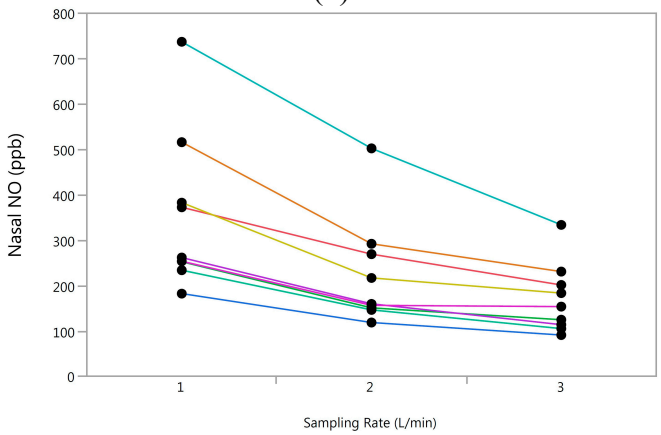
3.2. Experiment 2

Results appear graphically in Figures 4 and 5. Under baseline (quiet) conditions, individual mean nasal NO levels ranged from 74–188 ppb when sampled at 3.0 L/min, 102–270 ppb when sampled at 2.0 L/min, and 155–426 ppb when sampled at 1.0 L/min. Under acoustically stimulated conditions (128 Hz; 78 dBA @ 30 cm), corresponding NO levels ranged from 93–335 ppb, 120–503 ppb, and 184–737 ppb. A paired analysis of nasal NO levels across subjects demonstrated that baseline (quiet) nNO increased significantly with decreasing sampling rate ($p < 0.0001$; Figure 4a). A similar sampling rate effect was seen with acoustic simulation at 128 Hz ($p < 0.0001$; Figure 4b). Across sampling rates, application of acoustic energy consistently raised nNO levels ($p < 0.05$ at 1.0 and 2.0 L/min; $p < 0.01$ at

3.0 L/min; Figure 5a–c). On a multivariate basis, there was no significant interaction between these two factors (*i.e.*, sampling rate and acoustic stimulation acted independently; interaction factor $p > 0.05$).

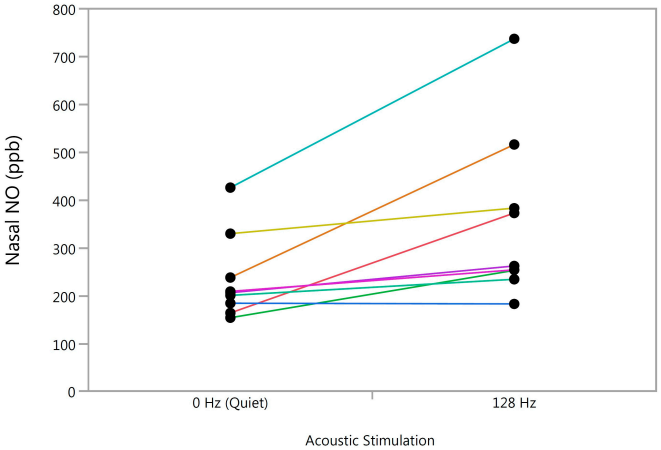


(a)



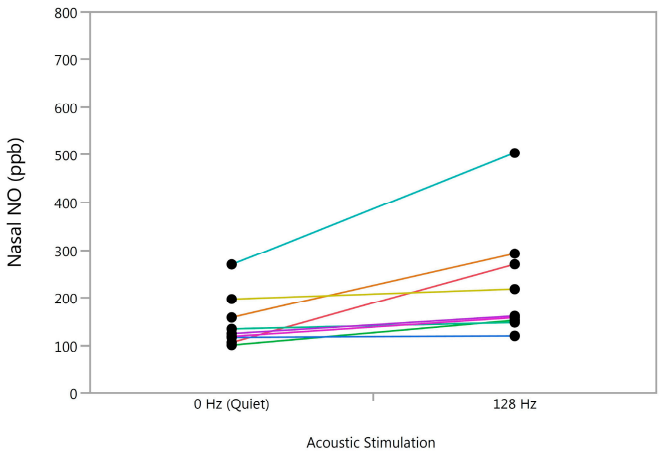
(b)

Figure 4. (**a**) Without acoustic stimulation; (**b**) with acoustic stimulation. nNO levels from Experiment 2 (n = 9), stratified by acoustic stimulation. Nasal sampling without (**4a**) and with (**4b**) acoustic stimulation (128 Hz; 78 dBA @ 30 cm). Decreasing sampling rate yielded higher nNO values under both quiet and acoustically-stimulated conditions ($p < 0.0001$).

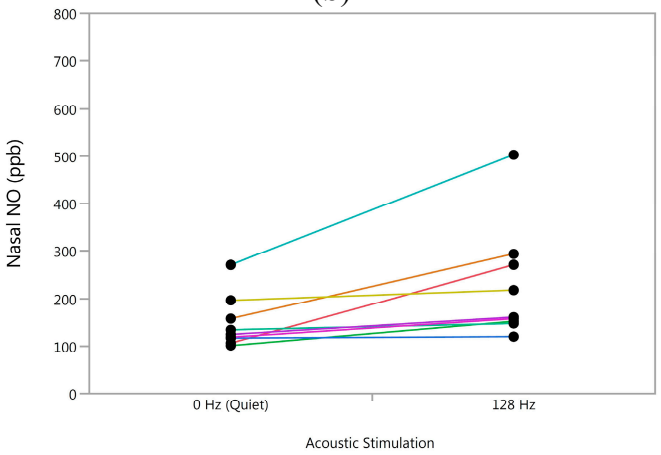


(a)

Figure 5. *Cont.*



(b)



(c)

Figure 5. (**a**) 1.0 L/min sampling; (**b**) 2.0 L/min sampling; (**c**) 3.0 L/min sampling. nNO levels from Experiment 2 (n = 9), stratified by sampling rate. Nasal sampling at 1.0 (**5a**), 2.0 (**5b**), and 3.0 L/min (**5c**) under two acoustic conditions: Quiet ("0 Hz") and 78 dBA @ 30 cm ("128 Hz"). Acoustic stimulation significantly increased nNO at all three sampling rates ($p < 0.05$ at 1.0 and 2.0 L/min; $p < 0.01$ at 3.0 L/min).

3.3. Longitudinal Analysis

With regard to reproducibility, baseline (quiet) nNO values obtained on a repeated basis in the two experiments (n = 9) were highly correlated ($R^2 = 0.84$; $p < 0.001$). Acoustically-stimulated nNO values obtained under the single repeated stimulus conditions between the two experiments (*i.e.*, 128 Hz @ 78 dBA; 3.0 L/min sampling) were moderately correlated ($R^2 = 0.50$; $p < 0.05$).

4. Discussion and Conclusions

These results confirm the feasibility of applying external acoustic stimulation as an alternative to humming in assessing acoustic mixing of gases between the paranasal sinuses and nasal cavity. Although analogous results have been published employing a mechanical model of the paranasal sinuses, to our knowledge these are the first published human data of real-time nNO demonstrating the effectiveness of external acoustic stimulation [24,25]. These data also show a degree of stability in both baseline and

acoustically stimulated nNO measurements over time in a reference study population (*i.e.*, subjects without chronic rhinosinusitis or nasal polyposis).

This study had several limitations. For example, we did not compare clinical sub-groups, nor did we conduct a direct comparison of the test-retest reliability of nNO between external acoustic stimulation and humming. Nevertheless, significant long-term measurement stability was apparent in our sample. Of note, our previous attempt at clinical validation found that radiographically (CT-) documented OMC area predicted resting (quiet) nNO levels—but not the humming-induced "spike" in nNO [31]. Given the superior reproducibility of the acoustic stimulus utilizing external stimulation rather than humming, the results of a clinical validation study might conceivably differ using this variant method.

Another limitation concerned the choice of stimulus frequencies and amplitudes. A stimulus frequency of 128 Hz was chosen in light of published data suggesting that greater sinus NO mobilization occurred in the vicinity of 130 Hz, as compared to 150 or 450 Hz in a human model [24]. The selection of the two sound amplitudes was dictated by the observed dose-response curve, with 64 dBA eliciting a minimal nNO response, and 78 dBA nearing the threshold for an unpleasantly loud acoustic stimulus.

Methodologically, the American Thoracic Society (ATS) and European Respiratory Society (ERS) recommend nNO sampling in the range of 0.25–3.0 L/min [30]. In Study 1 we documented our technique using a rate at the high end of this range (3.0 L/min). In Study 2, we showed that lower sampling rates not only yield higher baseline nNO values (a previously-documented phenomenon), but also that acoustic stimulation and gas sampling rate *independently* affect sampled nNO levels. The higher baseline nNO levels obtained at lower sampling rates (2.0 and 1.0 L/min) link our findings with the experience of clinicians who are accustomed to using very low sampling rates (~0.2 L/min) to screen for such conditions as primary ciliary dyskinesia or cystic fibrosis. In the case of acoustic stimulation, each individual is sampled repeatedly and acts as his or her own control, so the choice of gas sampling rate can be made based upon factors other than population norms. Since nNO values tend to plateau more quickly at higher sampling rates (making them more amenable to automated measurement), higher sampling rates may be advantageous for this method.

Biologically, NO is an endogenous gas whose rate-limiting enzyme (NO synthase or "NOS") is differentially expressed in the nasal *vs.* paranasal sinus mucosae [32]. Further, NO flux between the paranasal sinuses and nasal cavity can be affected by multiple anatomical factors [33]. As a consequence, NO is difficult to model in the upper airway. Thus, despite NO's important bacteriostatic and ciliostimulatory effects *in vitro*, clinical interpretation of nNO levels, even in the presence of known inflammatory disease, is problematic.

In this context, although the nNO response to humming appears promising, much work remains to be done in terms of clinical validation. In addition to the above-cited studies linking radiographic and/or endoscopic OMC obstruction to a blunted nNO response to humming, this transient response has been shown to be normalize after effective medical therapy of nasal polyposis, consistent with restoration of OMC patency [34]. As noted above, however, in our earlier study of normal controls and allergic rhinitics without sinusitis, the magnitude of the humming-induced nNO transient did not correlate with radiographically-documented OMC cross-sectional area, leading one observer to characterize its blunting as a high-threshold, "all or none" response [31,35]. Given these uncertainties, along with the variability inherent in the humming maneuver, future clinical validation studies, in addition to including multiple clinical sub-groups to maximize external validity, might profitably examine whether the nNO

response to external acoustic stimulation yields more clinically-coherent results than does the response to humming.

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Author Contributions

Dennis Shusterman was responsible for the design and conduct of this study, as well as for the analysis of data and for the drafting of the manuscript.

Conflicts of Interest

The author declares no conflict of interest.

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