Monitoring of oral and nasal exhaled nitric oxide in eosinophilic chronic rhinosinusitis: A prospective study

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ABSTRACT

Background: *We aimed to examine the effect of different therapeutic modalities on levels of fractional concentrations of exhaled nitric oxide (FeNO) in patients with eosinophilic chronic rhinosinusitis (ECRS).*

Methods: *Thirty-six ECRS patients with nasal polyps were treated either medically or surgically. Oral and nasal FeNO levels were measured using an electrochemical NO analyzer initially and at 1 and 6 months. The mRNA expression and localization of nitric oxide synthase (NOS) isoforms in sinus mucosa and nasal polyps were analyzed by real-time polymerase chain reaction (PCR) and immunohistochemistry.*

Results: *The mean oral FeNO levels in the surgical group had decreased significantly from 50.9 to 36.8 ppb 6 months after endoscopic sinus surgery. All patients in this group showed significantly higher nasal FeNO levels after treatment. The mean nasal FeNO levels were 62.3 ppb at 1 month and 93.6 ppb at 6 months. Mean oral and nasal FeNO levels in the medical group after treatment remained unchanged when compared with the baseline levels. Positive immunoreactivity of inducible NOS (iNOS) was observed in both epithelial cells and submucosal inflammatory cells. Real-time PCR analysis showed significant up-regulation of iNOS and IL-5 mRNA expression.*

Conclusion: *A combination of oral and nasal FeNO measurements is useful to monitor the extent of inflammation in CRS patients. The increase in nasal FeNO in the surgical group indicates prompt recovery of NO release from healed sinus mucosa through the opened sinus ostia. Reduction of oral FeNO levels may reflect a cessation of the underlying lower airway inflammation that is characteristic of ECRS.*

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The human paranasal sinuses are the major source of nitric oxide (NO) production and make a large contribution to the NO levels detected in the nasal cavity.[1] In this sense, the monitoring of fractional concentrations of exhaled NO (FeNO) has the potential to be a reliable and noninvasive marker of inflammatory conditions in human nasal airways.[2–5] Nasal NO levels are reported to be decreased in most patients with chronic rhinosinusitis (CRS), but some contradictions remain in the findings, and the results are often heterogeneous.[6–10]

In the present study, we prospectively examined changes in oral and nasal FeNO levels in patients with eosinophilic CRS (ECRS) after different therapeutic modalities. ECRS was summarized as an entity of intractable chronic sinus inflammation accompanied by the infiltration of numerous activated eosinophils in the paranasal sinus mucosa and nasal polyps.[11,12] The phenotype of ECRS is applicable for CRS with nasal polyps in Europe and the United States.[13] There is so far limited information available for changes in FeNO levels after medical or surgical treatment in ECRS patients. We also carried out a molecular analysis of paranasal sinus mucosa. The mRNA expression of three NO synthase (NOS) isoforms and interleukin-5 (IL-5) was quantitatively analyzed by real-time reverse transcription-polymerase chain reaction (RT-PCR), and the immunohistological localization of inducible NOS (iNOS) and endothelial NOS (eNOS) was examined. We found that a combination of the oral and nasal FeNO measurement described here was useful to monitor the clinical course of CRS patients.

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METHODS

Patients

Thirty-six ECRS patients were recruited into a prospective study of medical (n = 12) versus surgical (n = 24) therapy. Thirty-two age-matched normal volunteers served as controls. The diagnosis of ECRS was based on the clinical symptoms, endoscopic examination, and CT scanning, in accordance with criteria proposed by the Japanese Rhinological Society.[11,12] All patients had shown multiple nasal polyps and characteristic mucus secretion with high viscosity. None of the patients had received topical or systemic steroids for at least 4 weeks before initial visits. Patients who had undergone previous sinus surgery were excluded. The CT images were subjected to radiological grading using the Lund-Mackay system.[14] The total sinus scores were calculated bilaterally (range from 0 to 24). In the medical group, all patients received a 6-month course of oral antileukotrienes and intranasal corticosteroid preparations. Endoscopic sinus surgery (ESS) was performed on patients in the surgical group under general anesthesia. After the surgery, all patients were prescribed antibiotics for 5–7 days. After discharge, they received the same treatment as the medical group on an outpatient basis. Evaluation of postoperative endoscopic appearance was performed on patients in the surgical group at 6 months.[11] They were classified based on the condition of ethmoid sinus mucosa, *i.e.*, well healed or containing edematous regions. The study protocol was approved by the Institutional Review Board at the Hiroshima University School of Medicine.

Measurements of NO

Measurements of the FeNO level were performed using a handheld electrochemical analyzer (NObreath; Bedfont Scientific, Ltd., Rochester, U.K.) according to the American Thoracic Society/European Respiratory Society guidelines.[15,16] For oral FeNO measurements, subjects first inhaled ambient air with a nose clip and then exhaled for 16 seconds at a constant flow rate of 50 mL/s through a disposable mouthpiece. For nasal FeNO measurements, subjects were advised to exhale transnasally with their mouth closed into the device with a nose adaptor as described elsewhere.[17] Each measurement was performed in triplicate, and the mean value was used for analysis. The

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Table 1 **Background and baseline characteristics of the study population**

	Normal Control	ECRS Patients	
		Medical Group	Surgical Group
No.	32	12	24
Age	50 (30–78)	57.9 (44–77)	56.2 (32–75)
Asthma	—	9	14
CT score	—	14.2 (7–21)	16.9 (10–24)
Oral FeNO (ppb)	14.8 (2–40)	40.1 (8–116)*	50.9 (7–103)*
Nasal FeNO (ppb)	45.4 (15–98)	59.3 (30–123)	50.6 (12–130)

Data are shown as mean with ranges in parenthesis.
**$p < 0.01$ indicates significant difference compared with the control group. Oral and nasal FeNO values in ECRS patients were measured before treatment.*
ECRS = eosinophilic chronic rhinosinusitis; FeNO = fractional exhaled nitric oxide.

patients in both groups were examined at pretreatment and 1 and 6 months after treatment.

Immunohistochemistry

Mucosal specimens from ethmoid sinuses and nasal polyps were obtained from 12 patients in the surgical group at the time of surgery. In each case, the specimens were divided and either fixed in 4% paraformaldehyde for immunohistochemistry or, alternatively, immersed in RNA later solution (Ambion, Austin, TX) for real-time RT-PCR. Punch biopsies from the inferior turbinate were also performed for PCR analysis. Anti-human iNOS (NOS2) mouse monoclonal antibody (clone 2D2-B2) was from R & D Systems (Minneapolis, MN), and anti-human eNOS (NOS3) rabbit polyclonal antibody was from Thermo Scientific (Fremont, CA).

Immunostaining was performed on 5-μm-thick cryostat sections. For antigen retrieval, sections were immersed in Histo VT One (Nacalai Tesque, Kyoto, Japan) at 70°C for 40 minutes. The sections were then incubated overnight at 4°C in the presence of the primary antibodies. The color development was performed using the streptavidin-biotin amplification technique (ChemMate EnVision kit; Dako, Glostrup, Denmark). Peroxidase activity was visualized by the diaminobenzidine solution. Sections were counterstained with Mayer's hematoxylin. Negative controls were performed omitting the primary antibody or using an isotype control antibody from the same species.

Quantitative Real-Time RT-PCR Analysis

Cellular RNA was isolated using RNeasy mini kits (Qiagen, Valencia, CA). For cDNA synthesis, total RNA was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according to the instructions supplied by the manufacturer. Gene expression was measured on a 7300 real-time PCR system (Applied Biosystems) using TaqMan Gene Expression Assays. PCR primers for neuronal NOS (NOS1; Hs00167223_m1), iNOS (NOS2; Hs01075529_m1), eNOS (NOS3; Hs01574659_m1), and IL-5 (Hs00174200_m1) were used. PCR primers for GAPDH (Hs99999905_m1) were used as the reference gene. The results were analyzed using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta}$Ct). PCR cycles were run in triplicate for each sample. The Ct values for NOS1, NOS2, NOS3, and IL-5 were normalized to the value of GAPDH by calculating the change in Ct ($^{\Delta}$Ct). The mRNA expression levels were then quantified by calculating $2^{-\Delta\Delta}$Ct to account for the exponential amplification of the PCR. For quantification of mRNA levels, the average values obtained from the inferior turbinate were designated as one arbitrary unit.

Data Analysis

For multiple comparisons, screening of data for differences was first performed using ANOVA. If the analysis gave a significant result, further comparison was performed by the Mann-Whitney *U* test for between-group analysis. The comparison between each visit was assessed with the Wilcoxon rank sum test. A value of $p < 0.05$ was considered significant.

RESULTS

Change in FeNO Levels

The baseline characteristics of the study population are summarized in Table 1. No significant difference between the medical and the surgical groups was found in the baseline data of age distribution, proportion of asthma, or total CT scores. The ECRS patients in both groups showed significantly higher oral FeNO levels at pretreatment as compared with the normal group. On the other hand, there was no significant difference in nasal FeNO levels among the three groups.

Oral and nasal FeNO levels were assessed preoperatively and at 1 and 6 months after treatment (Figs. 1 and 2). The mean oral FeNO levels in the medical group after treatment were 44.3 ppb for 1 month and 36.3 ppb for 6 months and did not show a statistically significant difference when compared with the baseline levels. The mean oral FeNO levels in the surgical group were 46.4 ppb at 1 month and 36.8 ppb at 6 months. Twenty of 24 patients in this group showed a reduction in the oral FeNO level at 6 months, and the differences at these postoperative visits were both statistically significant. The mean nasal FeNO levels in the medical group were 59 ppb at 1 month and 62.8 ppb at 6 months and did not show a significant difference when compared with the baseline levels. On the other hand, nasal FeNO levels gradually increased after the ESS procedure in the surgical group. The mean nasal FeNO levels in this group were 62.3 ppb at 1 month and 93.6 ppb at 6 months, and the differences at these visits were both statistically significant. All patients in this group showed higher nasal FeNO levels at the 6-month follow-up when compared with the baseline levels.

In the surgical group, the endoscopic examination revealed edematous mucosa in most patients 1 month after surgery. The findings showed gradual improvement at the 6-month checkup. We compared the findings for the endoscopic appearance and the degree of postoperative FeNO changes in this group (Fig. 3). The examination of the ethmoid sinus mucosa at 6 months revealed 15 patients who were well healed and 9 patients with edematous regions. Well-healed patients tended to show a pronounced reduction in the oral FeNO levels and an increase in the nasal FeNO levels. However, the difference was not significant ($p = 0.11$ for oral FeNO; $p = 0.18$ for nasal FeNO).

Immunohistological Findings

Figures 4 and 5 show representative immunohistological images of the distribution of iNOS$^+$ and eNOS$^+$ cells in the ethmoid sinus mucosa and nasal polyps. In general, positive iNOS immunoreactivity was distinctly observed in ciliated epithelial cells, mainly in their

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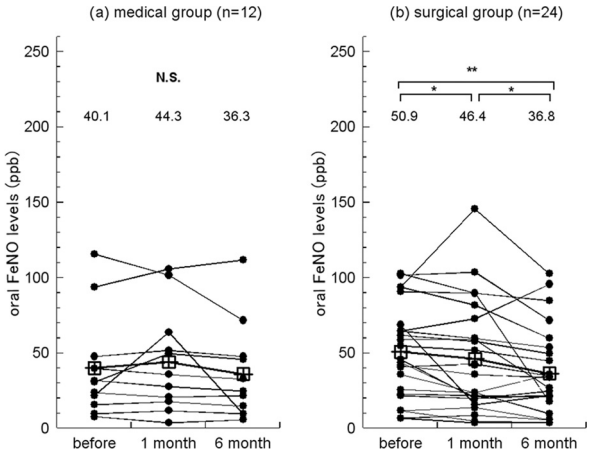

*Figure 1. Time-course changes in the level of oral fractional concentration of exhaled nitric oxide (FeNO) in eosinophilic chronic rhinosinusitis (ECRS) patients in the (a) medical group and (b) in the surgical group before and at 1 and 6 months after treatment. Time-dependent changes in each patient are indicated by connecting the individual points. Bars indicate mean values. *$p < 0.5$; **$p < 0.01$.*

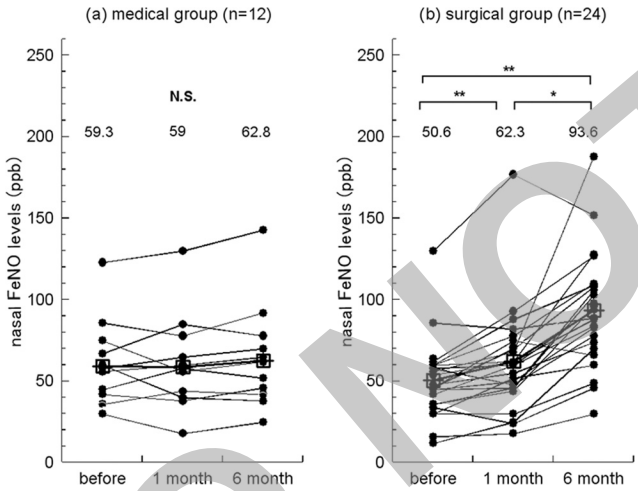

*Figure 2. Time-course changes in the level of nasal fractional concentration of exhaled nitric oxide (FeNO) in eosinophilic chronic rhinosinusitis (ECRS) patients (a) in the medical group and (b) in the surgical group before and at 1 and 6 months after treatment. Time-dependent changes in each patient are indicated by connecting the individual points. Bars indicate mean values. *$p < 0.5$; **$p < 0.01$.*

cytoplasm. A number of inflammatory cells, more or less widespread in the submucosal area, with eosinophils being predominant in the ethmoid sinus mucosa, also revealed positive staining. On the other hand, eNOS expression was constantly found to be positive in ciliated epithelial cells and endothelial cells around the capillary vessels. No significant eNOS staining was observed in submucosal inflammatory cells.

Real-Time RT-PCR Analysis

Quantitative real-time PCR was conducted to determine three NOS isoforms and IL-5 mRNA levels (Fig. 6). The ECRS patients showed significant up-regulation of iNOS and IL-5 mRNA expression in both ethmoid sinus mucosa and nasal polyps when compared with the inferior turbinate. On the other hand, mRNA levels for neuronal NOS (nNOS) and eNOS showed similar profiles to those of the inferior turbinate. In addition, a statistically significant difference was seen in

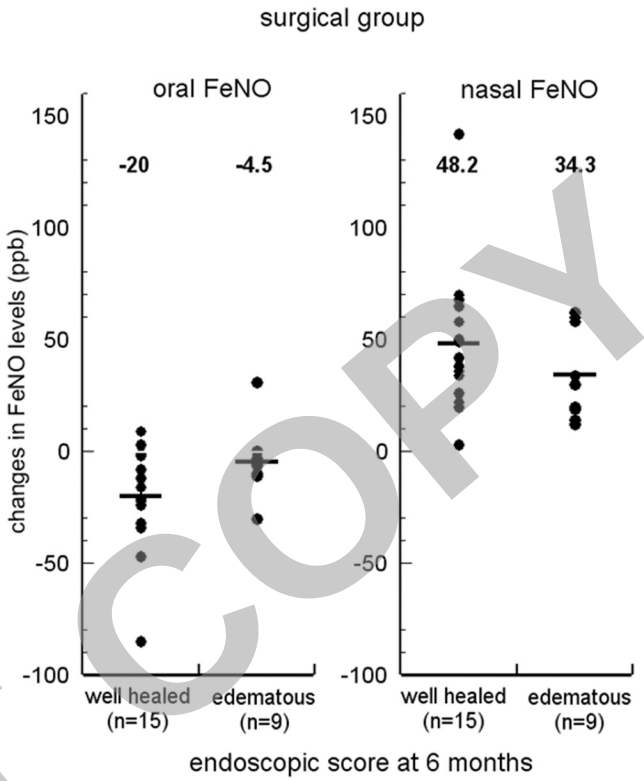

Figure 3. Comparison of the endoscopic appearance and the degree of post-operative fractional concentration of exhaled nitric oxide (FeNO) changes in the surgical group. Bars indicate mean values.

the level of iNOS mRNA expression from these patients, with ethmoid sinus mucosa showing higher levels of mRNA expression compared with nasal polyps.

DISCUSSION

In this study, both upper and lower airway FeNO levels were measured sequentially in a short period of time using a handheld analyzer. Several reports indicate that nasal exhaled NO tends to decrease in patients with acute and chronic sinusitis, mainly because of hampered ventilation of gaseous NO through occluded sinus ostia.[6,18,19] Weschta *et al.*[19] recently assessed oral and nasal FeNO levels in CRS patients using a handheld NO analyzer. They found that, in CRS patients, mean oral FeNO (23.9 ppb) was higher than in controls (15.6 ppb), whereas CRS patients with nasal polyps had lower nasal FeNO levels (19.7 ppb) than did healthy controls (40.3 ppb). The results of previous studies on FeNO levels are in line with those of the present study. We also found significantly higher oral FeNO levels at pretreatment in the ECRS patients when compared with those in normal subjects. On the other hand, there was no significant difference in the nasal FeNO levels among the three groups. We consider that the unchanged nasal FeNO levels in ECRS patients despite impaired sinus ostial patency before treatment might be related to augmented NO production in the inflamed paranasal sinus mucosa. This idea is supported by the present immunohistological and RT-PCR findings, which indicated the consistent expression of increased iNOS activity in the sampled specimens. Elevated levels of oxidized NO metabolites such as nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite ($ONOO^-$) have been detected in CRS patients.[20] Such an amount of NO metabolites could be autotoxic for the surrounding epithelium and lead to persistency of the disease.

The beneficial outcomes of ESS have been well described in ECRS patients.[21] In addition, to prevent disease recurrence, supplementary

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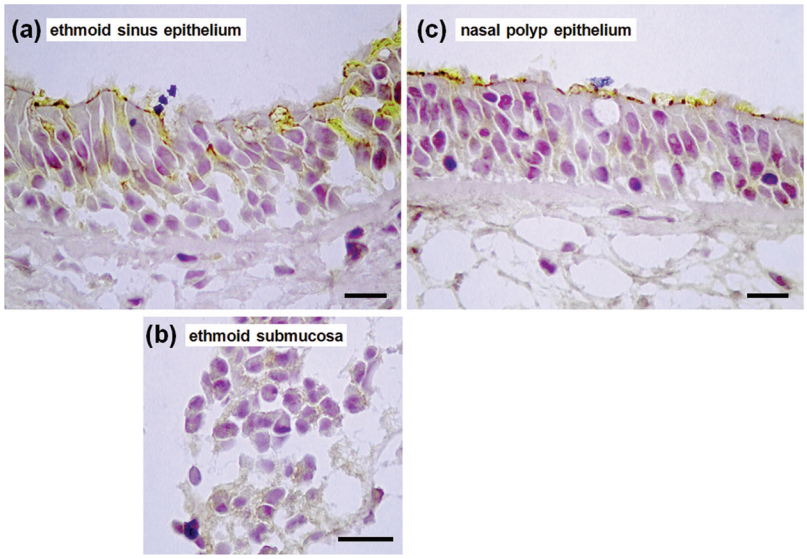

Figure 4. Immunohistological photographs of inducible nitric oxide synthase (iNOS; NOS2) expression in the ethmoid sinus mucosa and nasal polyps sampled from a patient in the surgical group. Ciliated epithelial cells and inflammatory cells are stained positively. (b) Note that iNOS^{+} inflammatory cells are scattered predominantly in the ethmoid sinus mucosa (scale bar = 20 μm).

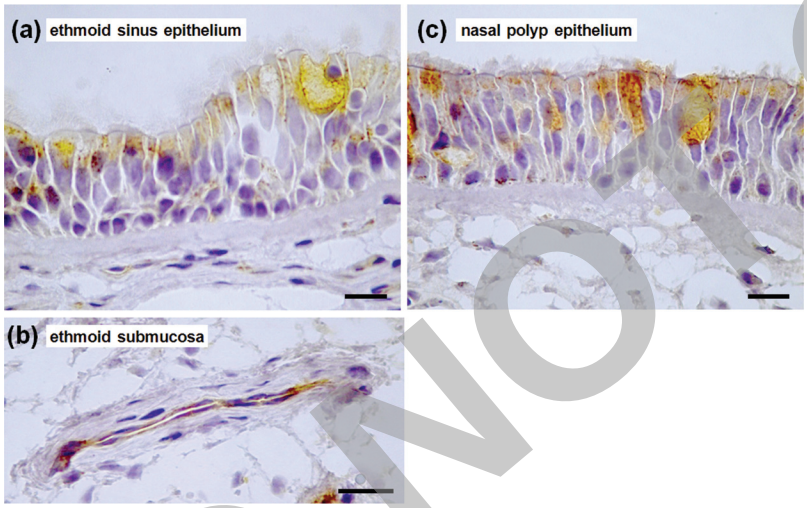

Figure 5. Immunohistological photographs of endothelial nitric oxide synthase (eNOS; NOS3) expression in the ethmoid sinus mucosa and nasal polyps sampled from a patient in the surgical group. (a and c) Ciliated epithelial cells and (b) vascular endothelial cells are stained positively (scale bar = 20 μm).

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medical treatment such as the use of topical steroids and antileukotrienes is often necessary. Therefore, the time course measurement of FeNO levels may provide an objective tool for evaluating the therapeutic response of ECRS. Ragab *et al.*[22] found an inverse correlation between nasal NO levels and the extent of sinus disease in untreated CRS patients. In their study, after both medical and surgical treatment, the percentage rise in nasal NO levels correlated with changes in symptom scores, endoscopic changes, and surgical scores. However, there was no significant correlation with age, sex, smoking status, or allergy. In the present study, the nasal FeNO levels in the ECRS patients also increased as a consequence of a combination of medical and surgical therapies, thus suggesting that treatment may result in recovery of a high output NO level from constitutive sources.[8] It could be anticipated that the ciliated epithelium of the paranasal sinuses regained its normal ability to produce NO that passed through the sinus ostia.

The higher prevalence of bronchial asthma in ECRS patients was associated with increased oral FeNO levels than those in normal controls (mean, 46.8 ppb versus 14.8 ppb). This represents a larger production of endogenous NO derived from lower airways in these patients. Interestingly, the mean oral FeNO levels in the surgical group showed a significant reduction of >20% at 6 months. The results are consistent with a recent study by Delclaux *et al.*[23] showing improvement in nasal polyposis after treatment not only to increase nasal but also to decrease bronchial NO concentrations. The interpretation of oral FeNO levels for clinical applications is a topic gaining a lot of attention. The recent American Thoracic Society guidelines suggest a reduction of at least 20% or >10 ppb in FeNO to indicate a significant response to anti-inflammatory therapy from one visit to the next.[24] However, further studies are required to objectively assess time-course changes in the lower airway function in these patients.

In the present study, ECRS patients showed increased iNOS and IL-5 mRNA expression. On the other hand, mRNA levels for constitutive NOS isoforms appeared to be unchanged. NO in human paranasal sinuses is mainly produced by iNOS activities by the ciliary epithelium under normal conditions and by inflammatory cells in inflammation. The epithelial iNOS expression seems to be necessary for maintaining ciliary beat frequency at a level sufficient for optimal mucociliary clearing function.[1,20,25] Naraghi *et al.*[25] propose that in rhinosinusitis, iNOS expression in epithelial cells decreases, but NO production by iNOS in inflammatory cells increases significantly. In such a process, the local deposition of NO and its metabolites tends to increase Th2 cells that promote adherence and accumulation of eosinophils through the release of IL-4 and IL-5.[26,27] We hypothesize that pathological events that occur in ECRS may be partly related to this mechanism. As previously described, a high amount of NO may dissolve and metabolize in the acidic aqueous environment of the sinuses.[25]

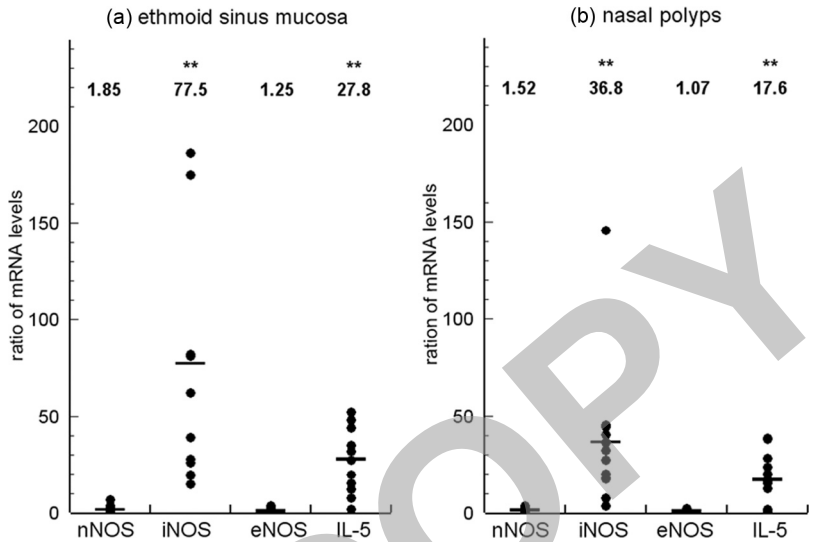

*Figure 6. Comparison of mRNA expression in ethmoid (a) sinus mucosa and (b) nasal polyps from eosinophilic chronic rhinosinusitis (ECRS) patients by real-time reverse transcription–polymerase chain reaction (RT-PCR). The mRNA levels of nNOS (NOS1), inducible nitric oxide synthase (iNOS; NOS2), l NOS (eNOS; NOS3), and interleukin (IL)-5 are normalized to GAPDH and expressed as ratios relative to controls (inferior turbinate mucosa; bars indicate mean values; **$p < 0.01$).*

The results of the present study imply a variety of roles of NO in the human nose and paranasal sinuses relevant to airway defense mechanisms, as well as being an inflammatory mediator. The establishment of standardized nasal NO measurements in CRS patients will be of great value to assess healing processes in relation to the underlying inflammatory status.

CONCLUSION

We prospectively examined the effect of different therapeutic modalities on FeNO levels in ECRS patients. The patients in the surgical group showed a significant increase in nasal FeNO levels from the baseline, indicating the prompt recovery of NO release from healed sinus mucosa. The reduction of oral FeNO levels in this group may also reflect a cessation of the underlying inflammation in the lower airway that is characteristic of ECRS.

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