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Title: Effect of Spirometry on Exhaled Nitric Oxide Values in Asthmatic Children.

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To the Editor

Measurement of fractional exhaled nitric oxide (FeNO) is important for asthma management. Regarding interpretation of FeNO, the American Thoracic Society (ATS) / European Respiratory Society (ERS) guidelines recommended that FeNO should be done first because hyperventilation maneuvers like spirometry have been shown to transiently reduce the exhaled NO level (1,2,3,4). However, in daily clinical practice, there are some situations where we want to evaluate FeNO after hyperventilation. Because it is unclear how hyperventilation affects assessment of FeNO, this study was conducted to clarify the influence of hyperventilation (spirometry) on FeNO, and the reliability of the FeNO data obtained in such situation.

Thirty-six asthmatic patients aged 6 to 16 were enrolled in this study between November 2016 and October 2017. They were inpatients or outpatients who visited regularly to our hospital. None of them had acute exacerbation of asthma needing systemic corticosteroids.

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The all subjects performed two consecutive measurements of FeNO (Pre-NO-1 and Pre-NO-2). Secondly, they were randomized to two groups that underwent measurement of forced vital capacity by spirometry three or five times in succession (FVC-3 group and FVC-5 group, respectively), and one measurement (Post-NO) was done after spirometry. The FeNO value was obtained after approximately one minute and the interval between the Pre-NO-1 and Pre-NO-2 tests was kept as short as possible. We investigated how much the FeNO value changed after spirometry and whether the number of times spirometry was performed affected FeNO.

Measurement of FeNO was performed according to the National Institute for Health and Care Excellence guideline (5) using an electrochemical analyzer (NIOX VERO[®]; Aerocrine, Solna, Sweden). According to the product information for NIOX VERO[®], the accuracy range was ± 5 ppb for measured values ≤ 50 ppb or $< 10\%$ for measured values ≥ 50 ppb (6). We defined patients with value outside this range as an outlier, while patients within the range were defined insider.

Measurement of FeNO and FVC was done by an experienced pediatric pulmonologist. This study was approved by the ethical review board of our hospital (approval no. F28-8).

Firstly, we compared patient characteristics between the FVC-3 and FVC-5 groups by Student's t-test or Fisher's exact test. Total IgE and the measured FeNO values were log-transformed before analysis. Subsequently, the paired t-test was employed to detect differences among Pre-NO-1, Pre-NO-2, and Post-NO for all subjects and for each frequency of FVC measurement. Adjusted mean values of Pre-NO-2 and Post-NO were compared between the FVC-3 and FVC-5 groups. They were obtained by analysis of covariance, including each preceding measurement of FeNO and the different frequencies of FVC measurement (3 times vs. 5 times); the adjusted mean value of Pre-NO-2 was calculated with adjustment for Pre-NO-1, while the adjusted mean value of Post-NO was calculated with adjustment for Pre-NO-2. Finally, logistic regression analysis was performed to examine possible risk factors for having an outlier defined above. Number of times regarding asthma exacerbation per year were assigned based on International study of asthma and allergies in childhood (ISAAC). Intensity of eosinophils in nasal discharge were categorized into 3 levels. A

P-value <0.05 was considered to indicate statistical significance. All statistical analysis was done by using STATA version 14.0 (Stata Corp., Texas, USA).

The 33 of 36 subjects completed the study protocol (19 were assigned to the FVC-3 group). There were no significant differences in background factors between FVC-3 and FVC-5 groups (Table 1).

The result of FeNO in all subjects and in each spirometry group were described in Table 2. When all subjects were combined, mean FeNO values were 24.4 (95% confidential interval: 7 - 71) ppb for Pre-NO-1, 24.9 (9 - 68) ppb for Pre-NO-2, 22.7 (7 - 69) ppb for Post-NO, respectively. Pre-NO-2 was 0.5 ppb higher than Pre-NO-1. Although there was no significant difference between Pre-NO-1 and Pre-NO-2 values ($P = 0.383$), the Post-NO values measured after spirometry were lower than Pre-NO-2 ($P < 0.01$).

Table 2 also shows the result of FeNO in crude value and adjusted mean values by frequency of spirometry maneuvers. In order to assess the influence of the number of spirometry maneuvers, we compared each FeNO values between FVC-3

and FVC-5 group. Pre-NO-1 values were 22.3 (15.4 - 32.3) ppb in the FVC-3 group and 27.5 (19.6 - 38.7) ppb in the FVC-5 group, respectively (p for difference = 0.398). The adjusted mean Pre-NO-2 values were 24.9 (23.4 - 26.5) ppb in the FVC-3 group and 25.0 (23.2 - 26.9) ppb in the FVC-5 group, respectively (p = 0.936), while the corresponding adjusted mean Post-NO values were 22.3 (20.4 - 24.3) ppb and 23.4 (21.1 - 26.0) ppb, respectively (p = 0.459).

When we consider the relationship of Pre-NO-2 values to the change between Pre-NO-2 and Post-NO, only five of 33 subjects were outliers. We performed regression analysis to compare the characteristics between those five subjects and the others. (Table 3). Patients who had frequently exacerbation per year (p = 0.026), higher eosinophils in blood and nasal smear (p = 0.031 and 0.019, respectively), and Pre-NO-2 over 30 ppb (P =0.038) were likely to become an outlier.

Measurement of FeNO was very reproducible; the intraclass correlation was 0.952 (98% CI: 0.907 - 0.976) and the Kendall's tau-b =0.898 (p <0.001) based on two sets of FeNO measurements, before and after FVC. On the other hand, the difference between the Pre-NO-2 in FVC-3 and FVC-5 group, between the Post-NO in FVC-3

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and FVC-5 group did not reach the statistical significance; severity of asthma was much varied in the present 33 study subjects and range of the FeNO values was too large to detect a difference. It may be required asthmatic children with the same degree of disease control should be examined in future studies.

Although the mechanism leading to a decrease in FeNO after hyperventilation is still unclear, there have been a number of reports that airway constriction and airway caliber change are caused by repeating spirometry and this might decrease FeNO (2,3,4,7). Sputum eosinophil counts are generally considered to be gold standard as assessing eosinophilic airway inflammation (8). However, it is not easy for children. FeNO and blood eosinophilia can be presumable eosinophilic airway inflammation (9), and nasal eosinophilic inflammation might contribute to peripheral airway responsiveness (10). In our study, patients who have the factors mentioned above were possibly to have highly eosinophilic inflammation and hyperresponsiveness. Therefore, their Post-NO changed in the present study was over accuracy range by spirometry in daily practice use. Sample size might be limitations in this study, further investigation with a larger number of subjects will be needed to make our conclusion more robust.

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In conclusion, FeNO values significantly decreased after spirometry in the present study performed in daily practice. It seems however very unlikely the minimal difference, 2.2 ppb (from 24.9 to 22.7) affect the evaluation of patient's inflammation and control. We also should know that patient who had some clinical factors mentioned above might become an outlier. Especially, it should be noted that the patients with FeNO ≥ 30 ppb are possibly to change beyond the accuracy range of the device in clinical setting.

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x. Tables:

Table 1 Characteristics of the subjects

	All subjects (n=33)	FVC-3 group (n=19)	FVC-5 group (n=14)	FVC-3 vs. FVC-5
Male	22	12	10	P = 0.720
Age (years)				
Mean (95% CI)	10.4 (9.5 – 11.4)	10.4 (9.3 – 11.6)	10.4 (8.6 – 12.2)	P = 0.970
Allergic rhinitis	22	11	11	P = 0.278
Total IgE (IU/mL)				
Mean (95% CI)	634.8 (361.9 – 1113.6)	828.1 (428.7 – 1599.5)	430.5 (146.0 – 1269.6)	P = 0.386
Treatment of ICS	(n=32) 27	(n=19) 15	(n=13) 12	P = 1.000

FVC: forced vital capacity, ICS: inhaled corticosteroid, CI: confidence interval,

FVC-3: FVC performed 3 times in spirometry, FVC-5: FVC performed 5 times in spirometry.

Table 2 FeNO in all subjects and in each group

Order		Pre-NO-1	Pre-NO-2	Post-NO
FeNO(ppb) Mean (95% CI)	All subjects	24.4 (7 - 71)	24.9 (9 - 68)	22.7** (7 - 69)
		Crude values	Adjusted mean values	
	FVC-3	22.3 (15.4 - 32.3)	24.9 (23.4 - 26.5)	22.3 (20.4 - 24.3)
	FVC-5	27.5 (19.6 - 38.7)	25.0 (23.2 - 26.9)	23.4 (21.1 - 26.0)
	FVC-3 vs. FVC-5	P = 0.398	P = 0.936	P = 0.459

FeNO: fractional exhaled of nitric oxide, Pre-NO-1: the value of first FeNO before spirometry, Pre-NO-2: the value of second FeNO before spirometry, Post-NO: the value of FeNO after spirometry, FVC-3: FVC performed 3 times in spirometry, FVC-5: FVC performed 5 times in spirometry, FVC: forced vital capacity, CI: confidential interval, ppb: parts per billion, **;There was significant difference between Pre-NO-2 and Post NO (P<0.01).

Table 3 Risk factor of deviation from accuracy range

Variables	Outlier n=5	Insider n=28	Odds Ratio	95% CI	P value
Male	3	19	0.71	0.100 - 5.030	0.732
Age (years)	11.1	10.3	1.008	0.980 - 1.038	0.567
Total IgE (IU/mL)	851.3	601.3	1.166	0.610 - 2.226	0.642
Treatment of ICS	3	24	0.25	0.031 - 1.999	0.191
Number of spirometry performed					
3 times	4	15	0.288	0.029 - 2.917	0.292
Number of times exacerbation per year †					
None	0	5			
1-3	1	15	12.174	1.343 - 110.313	0.026
4-12	3	5			
≥13	1	0			
N.D.	0	3	-	-	-
Blood eosinophil count ($10^2/\mu\text{L}$)	6.6	2.4	1.345	1.027 - 1.762	0.031
Eosinophils in nasal discharge † †					
None	0	17			
1+	0	6	10.511	1.465 - 75.418	0.019
2+	3	0			
3+	1	1			
N.D.	1	4	-	-	-
Pre-NO-2 ≥30 ppb	4	7	12	1.141 - 126.12	0.038

† It was based on ISAAC questionnaire. ISAAC; International study of asthma and allergies in childhood. † † It was the degree of eosinophil in nasal mucosa. A value of none, 1+ , 2+, 3+ were assigned when no eosinophils, eosinophils were scattered, eosinophils detected as small cluster, eosinophils detected as large cluster,

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respectively. Pre-NO-2: the value of second FeNO before spirometry, ICS: inhaled corticosteroid, CI: confidence interval, N.D.: No Data, ppb: parts per billion

xi. Figure legends;

None