ORIGINAL

A nocturnal decline of salivary pH associated with airway hyperresponsiveness in asthma

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Abstract : Salivary pH is associated with esophageal acid reflux and neutralization of esophageal acid. In this study, we assessed the association between nocturnal decline of salivary pH and airway hyperresponsiveness. Salivary pH was serially assessed in 9 patients with mild asthma (7 men and 2 women ; mean age 33.3 years ; mean %predicted $FEV_{1.0}$ 89.4%) and 10 healthy volunteers (6 men and 4 women ; mean age 31.2 years) using a pH indicator tape. The buffering capacity of saliva was defined as the median effective dose (ED_{50}) for acidification of saliva with 0.01 N HCl, and airway responsiveness was defined as the dose of methacholine producing a 35% fall in Grs (PD_{35}-Grs). There was a significant correlation between the values obtained from the pH indicator tape and those obtained from the electrometric pH meter. Using the indicator tape for sequential monitoring, we observed a nocturnal fall (△pH) in salivary pH in all subjects. A significant correlation was found between airway hyperresponsiveness (PD_{35}-Grs) and either △pH or ED_{50} in mildly asthmatic patients. Vagal reflux dysfunction might contribute to nocturnal salivary pH as well as to airway hyperresponsiveness in mild asthmatics. J. Med. Invest. 57 : 260-269, August, 2010

Keywords **:** *airway responsiveness, asthma, circadian rhythm, gastroesophageal reflux, salivary pH*

INTRODUCTION

Gastroesophageal reflux (GER) contributes to various oropharyngeal symptoms including oral erosion and chronic cough (1, 2). Additionally, a high prevalence of GER in patients with asthma has been widely reported (3-5). Ruling in or out a diagnosis of GER is important in the clinical management of oral and respiratory symptoms. As esophagitis is now found in fewer than 50% of GER patients, its manifestations as determined by endoscopy are underdiagnosed as GER. The most sensitive and specific test for GER is 24-h esophageal pH monitoring (6), but patients find this uncomfortable and inconvenient. Therefore, another objective method is required for the assessment of symptom severity and to improve patient response to treatment.

Salivary pH is associated with esophageal acid reflux and neutralization of esophageal acid. Dental erosion, which is caused by acid exposure in GER, shows an association with acidity in saliva (7). The initial salivary pH is correlated with pH in the lower esophagus and is higher in GER subjects than in control subjects (8). In addition, Katz *et al.* (9)

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detected nocturnal gastric acid breakthrough (NAB), defined as nighttime periods exceeding 1 h with a gastric pH below 4.0, in 67% of normal subjects ; and it was previously reported that there is a nocturnal decline of salivary pH in healthy subjects (10). Saliva is an important determinant of esophageal acid neutralization in patients with NAB as well as in GER patients (8).

Based on the reported number of reflux episodes occurring during 24-h esophageal pH monitoring, airway hyperresponsiveness to methacholine challenge tends to increase as GER worsens (11). The mechanisms of symptoms with GER are suggested to be tissue injury by microaspiration, vagal reflux, and neuroinflammatory reflux through the release of tachykinins, including substance P and neurokinin A (12, 13). It remains unclear if the mechanisms of airway hyperresponsiveness are influenced by GER.

In the present study, we used indicator tape to conduct sequential measurement of nocturnal salivary pH and examined salivary buffering capacity, which was defined as the median effective dose (ED_{50}), a concept modified to apply to HCl neutralization. After establishing this method to measure salivary pH at home, we assessed the relationship between the percentage of decline of saliva pH during the nighttime and airway hyperresponsiveness in mildly asthmatic patients.

MATERIALS AND METHODS

Subjects

Nine patients with mild asthma (7 men and 2 women ; 33.3±2.2 years, ranging from 25 to 45 years) and 10 age-matched healthy volunteers (6 men and 4 women ; 31.2±1.8 years, ranging from 27 to 42 years) were enrolled in the present study. All patients with asthma satisfied the criteria for asthma published by the National Institutes of Health (14). The patients were treated with fluticasone propionate 200 μg bid (n=2) and short-acting β_2 agonist inhalation alone as required (n=7). None of the patients used sustained-release theophylline or oral corticosteroids, none smoked, and all refrained from taking medication for 24 h before the study. All healthy volunteers were nonsmokers and had no history of esophageal symptoms or symptoms suggesting any other disease. All subjects were required to avoid caffeine-containing drinks for 12 h before testing. Alcoholic beverages were also to be avoided from the day before measurement until completion of all saliva sampling. In addition, none of the subjects tested took any anti-acids, H_2-blockers, or proton pump inhibitors for at least 14 days prior to the study. Informed written consent was obtained from all subjects. This study was approved by the Ethics Committee of Tottori University.

Questionnaire for GER diagnosis

GER was diagnosed by a questionnaire based on a self-assessment reflux questionnaire (ReQuest), which asked for respiratory and digestive condition, as well as symptoms of GER (heartburn, regurgitation of acid into the mouth, retrosternal pain, dysphasia) (15).

Measurement of salivary pH

In order to assess accuracy in measuring salivary pH, saliva was collected in a 50-ml centrifuge tube at least 1 h after eating, drinking, or tooth-brushing at our office. The collected saliva was filtered through gauze to remove particulate matters. A small quantity of filtered saliva was applied drop-wise to pH indicator tape (Advantec, Chiba, Japan) with a narrow pH range (pH 4.5 to 7.5 or pH 4.5 to 8.5). Acidity of the same saliva was also measured with a pH meter (MP220 ; Mettler Toledo, Schweizenbach, Switzerland) and values were compared with those obtained from the indicator tape.

Monitoring of pH

To evaluate the circadian rhythms of salivary pH, all subjects provided saliva samples collected at home. Since smokers have lower salivary pH and buffering capacity than nonsmokers (16), only nonsmokers were recruited for the present study. Additionally, since some foods and drinks affect salivary pH (17), we requested that all subjects allowed their mouths to fill with saliva and then swallow it, so that saliva sampled subsequently would be fresh. During the daytime, subjects were required to measure salivary pH several times, and to avoid sampling for a 1-h period after eating or drinking. For saliva sampling during the nighttime, subjects were asked to collect samples from a supine position three times per night : soon after going to bed around 0 : 00 AM, upon awakening at midnight, and upon waking around 7 : 00 AM. A small volume of saliva was placed on a strip from the indicator tape (Advantec), and the color of the strip was then compared with colors representing values on a chart ; pH determinations were recorded.

Titration curves

In order to assess the median effective dosage (ED_{50}) for acidification of saliva with 0.01N HCl, saliva was collected during the daytime in our office and these samples were used to establish a titration curve for saliva, using a pH meter in 1 ml of saliva with successive additions of 0.01 N HCl (pH 2.2, equivalent to gastric juice pH).

Pulmonary function tests

Forced expiratory volume at one second ($FEV_{1.0}$) was measured three consecutive times by a dry spirometer (Minato, Tokyo, Japan) and the highest value was recorded. Airway responsiveness was then obtained by methacholine inhalation test (Astograph TCK-6000CV ; Chest, M.I. Inc. ; Tokyo, Japan) (18). Airway hyperresponsiveness was first measured by dose response curves of respiratory resistance (Rrs) and respiratory conductance (Grs ; Grs=1/Rrs) during cumulative inhalation of methacholine and was then assessed as the dose of methacholine producing a 35% fall in Grs (PD_{35}-Grs).

Data analysis

The reproducibility of salivary pH measured by pH indicator tape was examined both within samples and between examiners. Within-sample reproducibility was calculated by comparing five different parts of the same sample ; between-examiner reproducibility was calculated by comparing measurements of the same sample reported by the subject and those reported by an independent observer, blind to each others' results. Agreement between pH indicator tape values and pH meter values was assessed by linear regression analysis as well as by a Bland-Altman plot (19). Changes in pH (ΔpH) were calculated as follows :

$$\left[\frac{\text{Mean for nighttime salivary pH} - \text{Mean for daytime salivary pH}}{\text{Mean for daytime salivary pH}}\right] \times 100.$$

The relationship between the buffering capacity of saliva and the initial salivary pH obtained during the daytime or ΔpH was analyzed using linear regression. Comparison between groups was made by Mann-Whitney non-parametric test. The accepted statistical significance was $p < 0.05$.

RESULTS

Subjects

The characteristics of the patients with asthma are presented in Table 1. All subjects with asthma had

Table 1. **Patient characteristics**

Patient	Sex	Age	$FEV_{1.0}$	PD_{35}-Grs	Atopy	GER*
1	M	34	88	3.13	No	Yes
2	M	33	92	3.13	No	No
3	F	25	102	6.25	No	No
4	M	29	94	3.13	Yes	No
5	F	28	81	6.25	No	No
6	M	45	83	0.2	Yes	Yes
7	M	40	85	1.56	No	Yes
8	M	28	98	3.13	Yes	Yes
9	M	38	82	0.78	No	Yes

$FEV_{1.0}$, forced expiratory volume in one second expressed as a percentage of predicted; PD_{35}-Grs, the dose of methacholine producing a 35% fall in respiratory conductance; GER, gastrooesophageal reflux.
*GER is diagnosed by the questionnaire based on a self-assessment reflux questionnaire (ReQuest).

mild symptoms and mild airflow limitations (% predicted $FEV_{1.0}$; 89.4±7.5, mean±standard deviation). Concomitant GER was suspected in 5 of the 9 asthmatic patients (56%) based on responses to the ReQuest.

Accuracy of salivary pH measured by pH indicator tape

When measuring salivary pH by indicator tape, *within-sample* repeatability calculated as the mean intra-sample coefficients of variation (CVs) for salivary pH was 1.6%, with a range of 0.4 to 3.0%. In addition, the correlation coefficient of the *between-examiner* results was 0.93. Fifty saliva samples were collected from 10 healthy controls during the daytime to confirm the accuracy of the measurement of salivary pH by pH indicator tape. A highly significant linear relationship was confirmed between the values obtained with the pH indicator tape and those obtained with an electrometric pH meter (R^2= 0.94, Fig. 1a). Figure 1b shows the Bland-Altman plot of the difference between salivary pH measured by pH indicator tape and pH measured with a meter vs. their mean values. The tape was found to show slight overestimation of salivary pH under highly acidic conditions and slight underestimation under highly alkaline conditions.

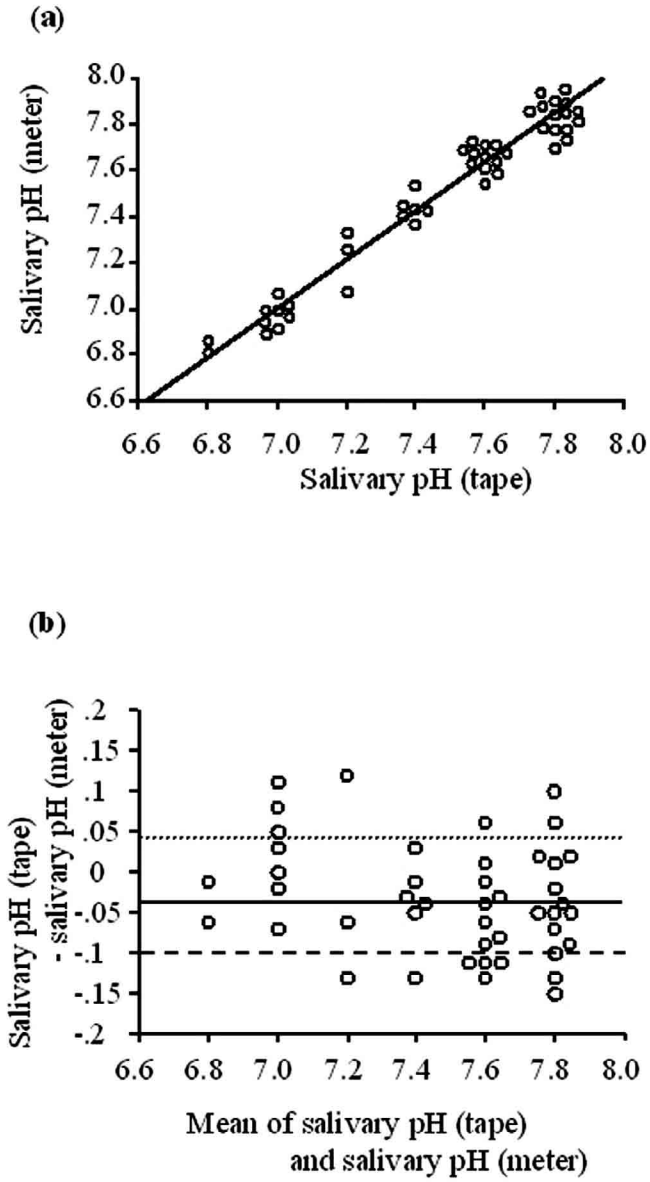

Figure 1. Accuracy of salivary pH measured by pH indicator tape. A significant linear relationship is evident between values obtained with pH indicator tape and with those obtained using a pH meter (a). A Bland-Altman plot (b) shows the relationship between differences in salivary pH from the mean values measured with pH indicator tape and differences in salivary pH from the mean values based on measurements with the meter. Mean bias (——), +2SD (……), and -2SD (- - -) lines are shown.

Circadian rhythms of saliva pH measured with pH indicator tape

When the circadian rhythms of salivary pH were monitored with pH indicator tape at home, all subjects showed a fall in salivary pH during the nighttime (midnight to 8 : 00 AM) compared to pH during the daytime (8 : 00 AM to midnight) (Fig. 2). Salivary pH values were found to be 6.42±0.31 (range, 6.2 to 7.2) during the nighttime and 7.14±0.39 (range, 6.5 to 7.8) upon waking in the healthy control group, while in asthmatic patients, salivary pH was 6.23±0.28 (range, 6.0 to 6.8) during the nighttime and 7.20±0.34 (range, 6.6 to 7.7) upon waking. A statistically significant difference was seen in both groups between nighttime and daytime values including the sample at the time of waking ($p<0.05$) (Fig. 3). The percentage of decline in salivary pH levels during the nighttime (ΔpH) was -14.2±4.5% in asthmatic patients and -9.1±3.3% in the healthy control group, and there was a significant difference in ΔpH between the healthy control group and asthmatic patients ($p<0.05$) (Fig. 4).

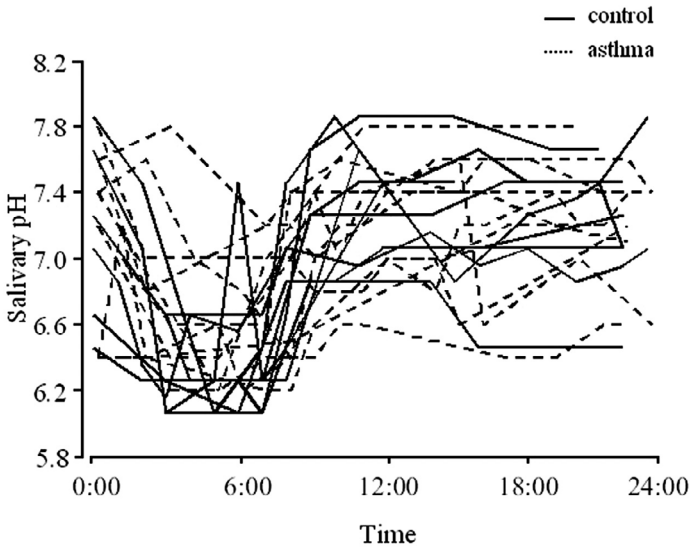

Figure 2. Circadian rhythms of salivary pH as measured with pH indicator tape in healthy controls (solid line) and mild asthmatics (dotted line). A separate line is shown for each subject. When the circadian rhythms of salivary pH were monitored with pH indicator tape, both healthy controls and asthmatic patients showed a decrease in salivary pH during the night (midnight to 8 : 00 AM) compared with daytime values (8 : 00 AM to midnight).

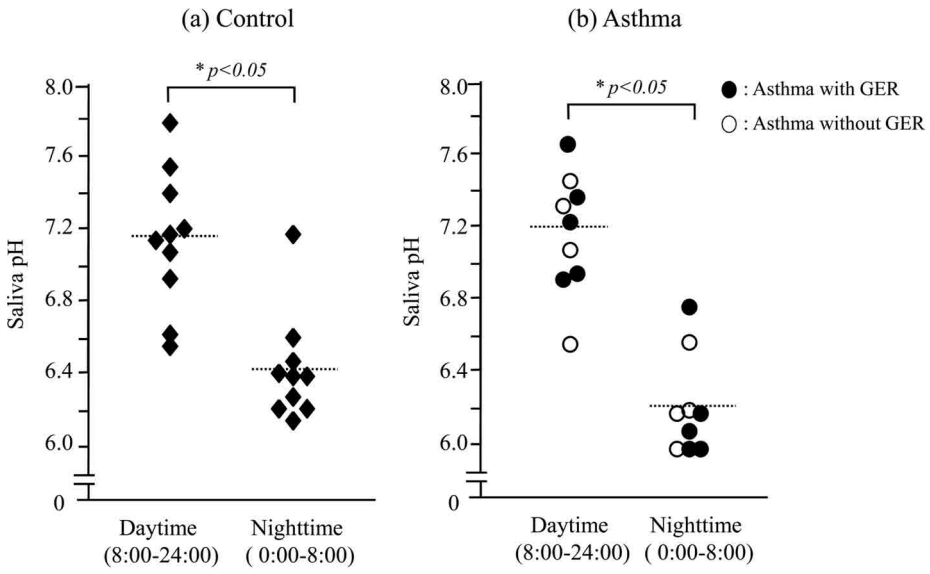

Figure 3. Comparison of daytime (8 : 00 AM to midnight) and nighttime (midnight to 8 : 00 AM,) salivary pH values between healthy controls (a) and mild asthmatics (b). There were significant differences in healthy controls and asthmatic patients between nighttime and daytime values including the sample at the time of waking ($p<0.05$)

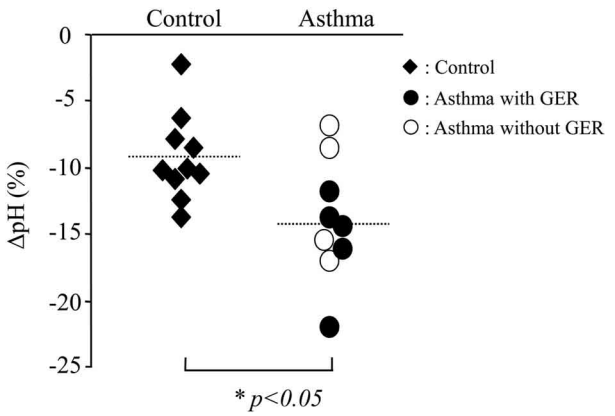

Figure 4. Comparison of the percentage of decline in salivary pH during the nighttime (ΔpH) between healthy controls and mild asthmatics. ΔpH was calculated as follows :

$$\left[\frac{\text{Mean for nighttime salivary pH - Mean for daytime salivary pH}}{\text{Mean for daytime salivary pH}}\right] \times 100.$$

A statistically significant difference was seen in ΔpH between the healthy control group and asthmatic patients ($p < 0.05$).

Buffering capacity of saliva (ED_{50})

A typical sigmoid titration curve was obtained for both healthy controls and mild asthmatics (Fig. 5).

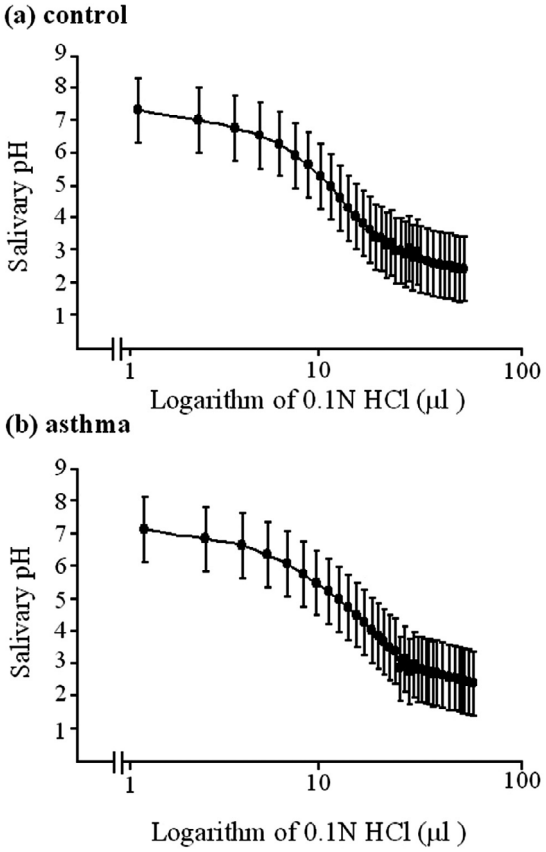

Figure 5. Titration curves for salivary pH in healthy controls (a) and mild asthmatics (b). To analyze the buffering capacity of saliva, 0.01 N HCl (pH 2.2, equivalent to the pH of gastric juice) was added to 1 ml of the subject's saliva. Salivary pH was monitored with a pH meter.

Salivary pH was found to decline with progressive additions of 0.01 N HCl. The buffering capacity of saliva was defined as the median effective dosage (ED_{50}) for the acidification of saliva with 0.01 N HCl. The ED_{50} was 85.5± 22.0 μl (range, 45 to 120 μl) in the healthy control group and 88.0± 24.0 μl (range, 50 to 120 μl) in mildly asthmatic patients. There was no significant difference between the two groups (Fig. 6a).

Figure 6b shows the relationship of ED_{50} to initial salivary pH measured during the daytime and ΔpH in each individual. Despite the lack of a significant correlation between ED_{50} and initial salivary pH measured during the daytime (R^2=0.24, p=0.12), a significant correlation was found between ED_{50} and ΔpH in both the healthy control group (R^2=0.71, p< 0.01) and in mildly asthmatic patients (R^2=0.86, p< 0.01).

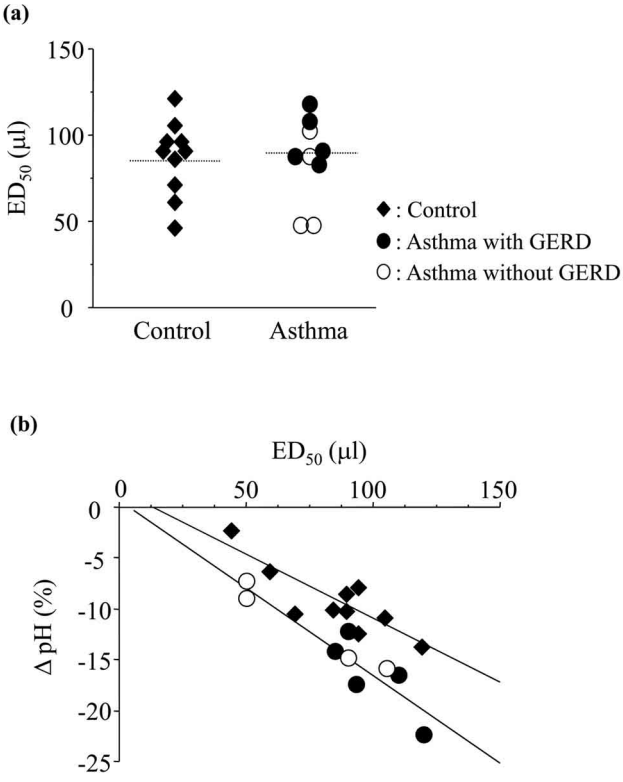

Figure 6. Comparison of the buffering capacity of saliva (ED_{50}) between healthy controls and mild asthmatics (a) and relationship between percentage of decline of salivary pH during the nighttime (ΔpH) and ED_{50} (b). Saliva was collected during the daytime to analyze salivary buffering capacity, which was defined as the median effective dosage (ED_{50}) for the acidification of saliva with 0.01N HCl (pH 2.2, equivalent to the pH of gastric juice). ΔpH was calculated as follows :

$$\left[\frac{\text{Mean for nighttime salivary pH - Mean for daytime salivary pH}}{\text{Mean for daytime salivary pH}}\right] \times 100.$$

Association between airway hyperresponsiveness and the buffering capacity of saliva (ED_{50}) or the percentage of decline of salivary pH during the nighttime (ΔpH)

When we assessed the dose of methacholine producing a 35% fall in respiratory conductance (PD_{35}-Grs) as an indicator of airway hyperresponsiveness, PD_{35}-Grs was calculated as 3.06± 2.12 (range, 0.2 to 6.25) (Table 1). PD_{35}-Grs was found to correlate significantly with ED_{50} (R^2=0.61, p< 0.05) as well as with ΔpH (R^2=0.46, p< 0.05) (Fig. 7). In asthmatic patients with stronger airway hyperresponsiveness, both ED_{50} and ΔpH were higher. There was no significant association between airway hyperresponsiveness and daytime salivary pH values.

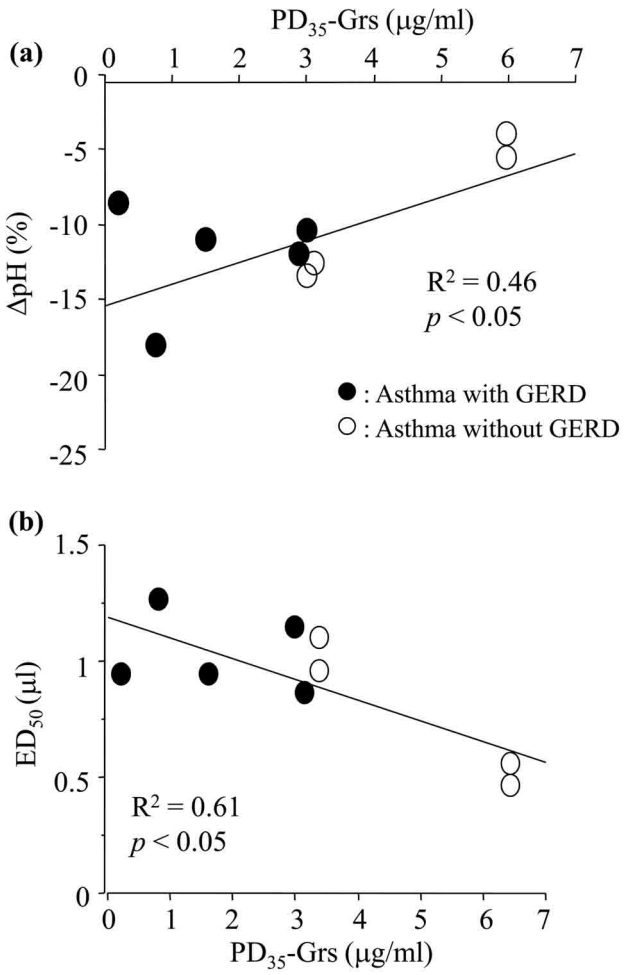

Figure 7. Relationship between airway hyperresponsiveness and either a decrease in salivary pH during the nighttime (ΔpH) or the buffering capacity of saliva (ED_{50}) in mildly asthmatic patients. PD35-Grs significantly correlates with both ED_{50} (R^2=0.61, p< 0.05) and ΔpH (R^2=0.46, p< 0.05).

DISCUSSION

In the present study we first established the validity of pH indicator tape for sequential measurement of salivary pH at home. We also found a relationship between the buffering capacity of saliva and a nocturnal decrease in salivary pH in both healthy control subjects and mildly asthmatic patients. Additionally, a significant correlation was found between a nocturnal decrease in salivary pH and airway hyperresponsiveness in mildly asthmatic patients.

Measuring salivary pH at frequent intervals with a pH meter is difficult ; it is therefore a matter of great interest to have an effective, accurate and easier method of measurement. In the present study, we found that the method using pH indicator tape is highly reproducible both within samples and between examiners for salivary pH measured by pH indicator tape. We also confirmed agreement between the results obtained with pH indicator tape and those obtained by pH meter by linear regression analysis. The overall accuracy of salivary pH measurements using the indicator tape was found to be high. Next, we examined our results with a Bland-Altman plot, again finding a significant correlation between the two methods. Nevertheless, scatter of the data-points from the mean value of the bias indicated that in many cases the indicator tape might significantly overestimate salivary pH under more acidic conditions and underestimate it under more alkaline conditions. We believe that the use of indicator tape is a useful and reliable method of measuring salivary pH.

The present study found that salivary pH during the nighttime is lower than that during the daytime in both healthy and mildly asthmatic subjects. Our results are consistent with those presented by Ionescu *et al.* (10), who demonstrated that there is a distinct diurnal behavior of salivary pH, with minimal values in the morning and maximal ones in the afternoon. Our study provides further evidence in favor of the idea that salivary pH declines throughout the night, not only in the morning. As lower esophageal pH values have been suggested to differ between daytime and nighttime (20), the nocturnal decrease in salivary pH may result from the reflux of esophageal acid. Additionally, given that NAB occurs commonly even in healthy subjects with no signs of esophageal exposure to acid evident on upper gastrointestinal endoscopy (21), diurnal fluctuations in salivary pH may be attributed to NAB.

We have demonstrated that salivary pH is more

reduced during the nighttime in mildly asthmatic patients than in normal subjects. GER in asthma patients is known to contribute to various oropharyngeal symptoms such as oral erosion and chronic cough (1, 2). Dental erosion, which is caused by acid exposure from GER, is also associated with salivary pH[7]. The decrease in salivary pH during the daytime in mildly asthmatic patients with concomitant GER was in high compared with that in asthmatic patients without GER. It considered that acid reflux had strengthened by the supine position during nighttime in asthmatic patients with GER. This result suggested that GER facilitated the decline salivary pH. The nighttime decrease in salivary pH may increase susceptibility to nocturnal symptoms including cough.

All asthmatic patients inhaled corticosteroids and a part of corticosteroids was absorbed into gastrointestinal tract. Currently, there had been no report that inhalation of corticosteroid affected acid secretion of stomach and increased gastrointestinal disease. It was considered that use of inhalation corticosteroid did not affect reduce of salivary ph during nighttime. The amount of salivary secretion is a factor in determining salivary pH. In this study, we did not measure salivary secretion in each subject. The amount of salivary secretion might be a little in asthmatic patients compared with controls. In future study, measuring salivary secretion may be needed to confirm that salivary pH during nighttime in asthmatic patients is reduced easily more than healthy subjects.

In the present study, buffering capacity (ED_{50}) was also measured at various pH levels by acid titration. Saliva is an important determinant of esophageal acid neutralization in GER (8). We demonstrated that there is a significant correlation between ED_{50} and a nocturnal fall in salivary pH (ΔpH), but not initial salivary pH measured during the daytime, in both healthy and mildly asthmatic subjects. The buffering capacity of saliva involves three major systems : bicarbonate (HCO_3^-), phosphate and proteins show buffering capacity in saliva and are involved to different extents depending on the specific pH range in question. Since bicarbonate plays an important role in buffering capacity between pH 5 and pH 7 (22), it might be the dominant system within this pH range. Decreased salivary pH during the nighttime might involve attenuation of buffering capacity, which depends on salivation rates. Low secretion of saliva at night implies a lower bicarbonate concentration and more acidic pH, hence decreased buffering capacity. Given that the buffering capacity of saliva has been correlated with a high incidence of dental erosion attributed to GER (23), the low capacity of neutralization may induce oropharyngeal symptoms.

The present study provides further evidence that there is a significant correlation between a nocturnal decrease in salivary pH and airway hyperresponsiveness in mildly asthmatic patients. Bronchial obstruction by methacholine can trigger or aggravate GER through an increase in transient lower esophageal sphincter (LES) relaxations (11, 24). This association may indicate a dysfunction of the vagal nerve during the nighttime in asthmatic subjects. Asthmatics with GER show evidence of autonomic dysfunction, with many individuals having a hypervagal response (25). When esophageal acid events are followed by a fall in tracheal pH, peak expiratory flow (PEF) rates decrease (26, 27). Vagal pathways control LES pressure after muscle contraction. Since human esophageal smooth muscle expresses muscarinic receptor (28), we suggest that vagal reflex dysfunction might contribute to a large decrease in nocturnal salivary pH as well as to airway hyperresponsiveness in mild asthmatics.

The diagnosis and assessment of GER is quite complex. The majority of patients with GER have normal gross endoscopic findings (5), and those without esophagitis are sometimes said to have 'non-erosion reflux disease'. The most sensitive and specific test for GER is 24-h esophageal pH monitoring (6), but patients find this uncomfortable and inconvenient. Additionally, esophageal pH monitoring may be the only method of diagnosing GER in up to 32% of patients with reflux-induced cough (29). As symptoms of GER, such as heartburn, are frequently absent in patients with reflux-induced cough (21, 30), therapeutic trials may be less useful if no symptoms of GER are present. The ReQuest is a valid and reliable tool for measuring GER symptoms (15), but other methods are required for the objective assessment of symptom severity and patient response to treatment. The use of pH indicator tape is an easy and effective method that may permit the recruitment of large numbers of subjects with GER for investigation of that disorder ; future study is required to determine whether salivary pH levels can serve as a substitute for the assessment of symptom severity and patient response to treatment for GER despite ReQuest responses.

The pH indicator tape was found to be a valid method of measuring salivary pH at any time of day

or night. Salivary pH is affected by the reflux of gastric juice to the oral cavity as well as by the neutralization of esophageal acid. Asthmatic patients showed a greater nocturnal decrease in salivary pH, and a circadian pattern of salivary pH was found to be related to the acid neutralization capacity of saliva. Airway hyperresponsiveness is also correlated with a nocturnal decrease in salivary pH and the buffering capacity of saliva. Measurement of salivary pH using indicator tape could be applied to detect associations with oropharyngeal symptoms in GER.

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