Association Between Pepsin in the Saliva and the Subjective Symptoms in Patients With Laryngopharyngeal Reflux

*Ah Ra Jung, *Oh Eun Kwon, †Jung Min Park, *Sung Hwa Dong, †Su Young Jung, ††Young Chan Lee, and *†Young-Gyu Eun, ††Seoul, South Korea

Summary: Objectives. Our study was designed to further evaluate the relationships between the saliva pepsin level and the symptoms and quality of life of patients with laryngopharyngeal reflux (LPR).

Study design. A prospective cohort study without controls.

Setting. Tertiary teaching hospital.

Subjects and methods. We analyzed 50 patients diagnosed with LPR by 24-hour multichannel intraluminal impedance pH monitoring. All subjects were instructed to collect saliva samples upon waking in the morning. The saliva pepsin levels were analyzed using enzyme-linked immunosorbent assay. The Reflux Symptom Index, Reflux Finding Score, Laryngopharyngeal Reflux-Health-Related Quality of Life, and Short Form 36 survey were administered.

Results. The pepsin was detected in the saliva of 41 patients with LPR (17.15 ± 20.42 ng/mL). Nine patients did not have pepsin in the saliva. There were no significant associations between the pepsin level in the saliva and Reflux Symptom Index, Laryngopharyngeal Reflux-Health-Related Quality of Life, or Short Form 36 of patients with LPR.

Conclusion. The saliva pepsin level is not significantly correlated with LPR symptoms or quality of life in LPR patients. It may be true that there is no association between pepsin levels and LPR symptoms, but this lack of association does not prove the lack of pathophysiological effect.

Key Words: Laryngopharyngeal reflux—Saliva—Pepsin—Symptom—24-Hour multichannel intraluminal impedance monitoring.

INTRODUCTION

Laryngopharyngeal reflux (LPR) involves the backflow of gastric acid into the laryngopharynx. Patients with LPR develop laryngitis with or without granulation or granuloma formation. Therefore, symptoms such as hoarseness, throat clearing, chronic cough, sore throat, and globus sensation arise. These symptoms certainly have an adverse effect on patient quality of life (QoL). Therefore, LPR treatment primarily focuses on symptom control, with the ultimate goal of improving QoL. The Reflux Symptom Index (RSI), Laryngopharyngeal Reflux-Health Related Quality of Life (LPR-HRQoL), and Short Form 36 survey were used to measure patients’ subjective symptoms and QoL.

Pepsin plays a significant role in the development of many reflux-related disorders. Samuels et al. demonstrated that pepsin levels in the saliva are significantly higher in patients with LPR than those in controls. Pepsin damages the mucosal membrane of the laryngopharynx more so than that of the esophagus. The laryngeal epithelium is far more sensitive to pepsin damage in the presence of acid than is the esophageal epithelium. This finding may help to explain why LPR and gastroesophageal reflux disease differ so greatly with regard to patterns and mechanisms of reflux, as well as clinical manifestations. One recent study assessed throat sputum samples using a pepsin immunoassay and found that this method is as sensitive and specific for LPR as is 24-hour double-probe (esophageal and pharyngeal) pH monitoring.

To our knowledge, there are no clear conclusions regarding the most effective diagnostic methods for identifying subjective symptoms. In addition, it is unknown how pepsin correlates to the subjective symptoms and QoL in LPR patients. Therefore, the aim of this study was to evaluate the associations between the pepsin level in the saliva and the symptoms and QoL of LPR.

SUBJECTS AND METHODS

Subjects

This prospective clinical study included 50 patients. We determined the subject of the 24-hour multichannel intraluminal impedance (MII)-pH monitoring based on the patient’s symptoms and the inflammation of the larynx on endoscopy. Symptoms of suspected LPR included globus sensation, voice changes, chronic throat clearing, excessive throat mucus, chronic cough, andodynophagia. The inflammation of the larynx suspected of LPR included larynx swelling, redness of mucosa, subglottic swelling, contact granuloma, and thick mucus. Determination to perform 24-hour MII-pH monitoring was done by one expert (Eun YG). We also measured the Reflux Finding Score (RFS) through the larynx examination. The RFS ranged from 0 (normal) to 26, with a higher score indicative of a deteriorated laryngeal condition. Fifty patients underwent 24-hour MII-pH monitoring.
LPR was diagnosed in patients with one or more reflux events that reached the larynx and pharynx on 24-hour MII-pH monitoring.

We excluded patients who had previously been taking proton pump inhibitors, patients with a history of head and neck radiation or any esophageal or gastric surgery, patients who were pregnant, patients older than 75 years, and those with previously demonstrated salivary gland disease.

All included patients completed Reflux Symptom Index (RSI), LPR-HRQoL, and SF-36 questionnaires. Age, sex, medical history (such as hypertension and diabetes mellitus), and social history (such as smoking, alcohol, and coffee use) were determined from the patients’ medical records. Patients with signs and symptoms of LPR were determined for 24-hour MII-pH monitoring and participated in clinical studies if the patient agreed before 24-hour MII-pH monitoring. After that, questionnaires were administered, 24-hour MII-pH monitoring was performed, and saliva samples were collected.

Saliva collection and enzyme-linked immunosorbent assay
All subjects were instructed to collect saliva samples as soon as they woke up on the day of the 24-hour MII-pH monitoring. The levels of total pepsin in saliva collected upon waking were significantly higher than after meal or when patient complained symptoms. Subjects were asked to collect the early morning sample prior to eating, drinking, or brushing their teeth. We used 30-mL collection tubes containing 0.5 mL 0.01 M citric acid (pH 2.5), which is a pepsin-stabilizing agent. The samples were refrigerated at –80°C and analyzed within 2 months of collection. Saliva samples were centrifuged at 1000g for 15 min at 4°C. The supernatant was harvested.

We determined the pepsin levels in saliva samples using a pepsin enzyme-linked immunosorbent assay kit (Cloud-Clone, Houston, TX). Saliva was added to a precoated 96-well plate. Detection Reagent A was immediately added, and the plate was incubated for 1 hour at 37°C. The wells were washed and incubated with Detection Reagent B for 30 minutes at 37°C and then with TMB Substrate solution for 15 minutes at 37°C. The reaction was stopped using stop solution, after which the samples were immediately evaluated at 450 nm using a microplate reader. The assays were read by a single investigator who was blinded to both the patient clinical status and questionnaire results.

24-Hour MII-pH monitoring test
The dual-channel MII-pH catheter consists of a 2.3-mm polyurethane catheter that incorporates six impedance segments (each segment is 2 cm long) and 2 pH-measuring electrodes (ZepHr-Impedance/pH Reflux Monitoring System, Sandhill Scientific, Inc., Highlands Ranch, CO). After inserting the fiber optic scope into the nasal cavity to place the probe using direct visualization, a dual-channel MII-pH catheter was inserted transnasally to the opposite side and the blue visualization band was placed approximately 1 cm below the proximal pH sensor at the proximal edge of the upper esophageal sphincter.

The probe was attached to an external electronic data recorder for continuous 24-hour esophageal pH monitoring. The probe was removed the following day. The pH data were downloaded for analysis (ZepHr CompactFlash Card and Recorder, Sandhill Scientific, Inc.). Patients were instructed to record the time of each meal and to document any associated coughing, wheezing, heartburn, or regurgitation.

Each MII tracing was manually analyzed. A liquid reflux episode detected by impedance was defined as a retrograde drop in impedance >50% of the baseline in the distal 2 channels. A gas reflux episode was defined as an increase in impedance by 50% or greater in two consecutive channels with ohms >8000. A distal reflux episode was defined as an episode that reached the two impedance sensors closest to the lower esophageal sphincter. In contrast, a proximal reflux episode was defined as an episode that reached the two sensors closest to the oropharynx. These reflux episodes were classified as acidic if the pH dropped below 4 or nonacidic if the pH remained >4 during the episode. LPR was diagnosed if there were ≥1 proximal reflux episodes.

Questionnaires for subjective symptoms and quality of life
We evaluated patients’ subjective symptoms and QoL using three surveys. These were the RSI, LPR-HRQoL, and SF-36 version 2.0. The RSI not only evaluates the severity of LPR symptoms, but is also a highly validated survey, which includes nine questions that assess treatment response. The survey evaluates symptoms and their severity with a six-point Likert scale (0–5). RSI ranged between 0 and 45. A score of zero indicates no symptoms, whereas higher scores denote more severe symptoms.

The LPR-HRQoL is a reliable and valid QoL rating scale described by Carrau et al. This method can be used to evaluate the QoL of LPR patients through a simple survey, which includes 43 questions across five different categories of hoarseness, cough, throat clearing, swallow, and overall impact of acid reflux. The questionnaire uses a basic seven-point Likert scale question in four of the categories and a 10-point Likert scale for the overall impact of acid reflux. The LPR-HRQoL ranged from 5 to 314. Again, a score of zero indicates no symptoms, whereas higher scores denote more severe symptoms.

The SF-36 version 2.0 comprises 8 categories and 11 questions. It was designed and validated to assess quality of life in large population studies. The questionnaire includes eight items that measure physical and mental health outcomes of physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health. The percentage scores range from 0% (lowest or worst possible level of functioning) to 100% (highest or best possible level of functioning). Information from these items is used to construct the physical and mental component summary measures.

Statistical analysis
All statistical analyses were performed using SPSS software version 18.0 for Windows (SPSS for Windows, SPSS Inc., Chicago, IL). Continuous variables that were not normally distributed are expressed as median (interquartile range). Mann-Whitney U test was used for comparing two groups. The Spearman correlation was used to analyze the association between
questionnaires and average pepsin level in saliva. P values <0.05 were considered statistically significant.

RESULTS
A total of 50 patients were included in this study. The mean age was 51.7 (±13.0) years, with a 9:16 ratio of men to women (18 men and 32 women). Pepsin was detected in the saliva of 41 patients (pepsin-positive group) after waking (82%), with a mean level of 17.15 (±20.42) ng/mL. Nine patients did not have the pepsin in the saliva (pepsin-negative group) (Table 1). There were 16 patients with acidic reflux and 34 patients with nonacidic reflux. The mean pepsin levels were 16.82 ± 20.54 ng/mL and 16.42 ± 19.41 ng/mL, respectively, but not statistically significant. There were 26 patients with heartburn symptoms and 24 patients without heartburn. The mean pepsin levels were 16.16 ± 20.29 ng/mL and 18.23 ± 20.95 ng/mL, respectively, but not statistically significant.

Questionnaire results of 50 patients were as follows. Values were presented as median (interquartile range). The RSI was 14 (10–17), the RFS was 11.5 (10–13). The VOICE of LPR-HRQoL was 4 (1–23), the COUGH was 3.4 (1–13), the THROAT was 5 (3–13.8), the LIFT was 18.5 (13–30.3). The physical component summary of SF-36 was 45.7 (40.6–50.4), and the mental component summary was 48.6 (41.3–54.7). There were no significant differences in the RSI, RFS, LPR-HRQoL, or SF-36 scores between the pepsin-positive and pepsin-negative groups (Table 2). There were no significant correlations between RSI, RFS, LPR-HRQoL, SF-36 and average pepsin level in the saliva (Table 3).

DISCUSSION
This study sought to determine the correlation between the pepsin level in saliva and the symptoms and QoL of LPR patients. Our study also did not identify any correlations between the pepsin level in saliva and the RSI, RFS, LPR-HRQoL, or SF-36. There were also no significant symptoms or variables of quality of life between the pepsin positive and negative groups. Although the pepsin in the saliva might be the indicator of the presence of LPR, this result means that the pepsin level in the saliva does not reflect the subjective symptoms and QoL of patients with LPR. Yadlapati et al.15 found that by using current normative thresholds, oropharyngeal pH testing and salivary pepsin analysis are not able to distinguish between healthy volunteers and subjects with a combination of laryngeal and reflux symptoms. As a result of the opposite, Spyridoulis et al.16 found a correlation between laryngeal findings of LPR patients and pepsin levels. They showed that there was weak correlation between the severity of laryngeal inflammation and the concentration of pepsin (r = 0.28).16 Pepsin was not detected in 9 of 50 patients. This result may indicate that pepsin alone does not cause LPR symptoms. As is known, many pathogens—gastric acid and bile—may be involved in the development of symptoms.

The LPR is associated with laryngopharyngeal sensory deficits from prolonged mucosal damage. These deficits may reduce the responsiveness of the laryngopharyngeal mechanoreceptors.17,18 The laryngeal mucous membrane in LPR is weak and has lower resistance against gastric acid and activated pepsin than does the membrane in healthy individuals. The larynx and pharynx do not have any mechanisms of acid elimination. Therefore, these structures are highly susceptible to chemical injury by acid.

### TABLE 1.
Baseline Clinical Variables for Subjects by Cohort

<table>
<thead>
<tr>
<th>Clinical Variables</th>
<th>No. of total patients</th>
<th>Age (mean ± SD)</th>
<th>Sex (male/female)</th>
<th>No. of pepsin detected after waking</th>
<th>Value of detected pepsin (median (IQR))</th>
<th>Note; Values were presented as median (interquartile range).</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of total patients</td>
<td>50</td>
<td></td>
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</tr>
<tr>
<td>Age</td>
<td>51.7 ± 13.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>18/32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of pepsin detected after waking</td>
<td>41 (82.0%)</td>
<td></td>
<td></td>
<td></td>
<td>6.96 (3.15–24.09)</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2.
Differences in the Pepsin-positive and Pepsin-negative Groups in Saliva After Waking

<table>
<thead>
<tr>
<th></th>
<th>Pepsin-positive Group (n = 41)</th>
<th>Pepsin-negative Group (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI score</td>
<td>14 (11–17)</td>
<td>12 (9–34)</td>
<td>0.710</td>
</tr>
<tr>
<td>RFS score</td>
<td>11 (10–13)</td>
<td>12 (11–13)</td>
<td>0.568</td>
</tr>
<tr>
<td>LPR-HRQoL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voice</td>
<td>4.5 (1–21.5)</td>
<td>2 (2–8)</td>
<td>0.882</td>
</tr>
<tr>
<td>Cough</td>
<td>4 (1–13)</td>
<td>3 (1–17)</td>
<td>0.823</td>
</tr>
<tr>
<td>Clear throat</td>
<td>5 (3–13)</td>
<td>4 (1–14)</td>
<td>0.551</td>
</tr>
<tr>
<td>Swallowing</td>
<td>7 (3–11)</td>
<td>5 (3–10)</td>
<td>0.901</td>
</tr>
<tr>
<td>Overall impact of acid reflux</td>
<td>18 (13–28)</td>
<td>25 (13–39)</td>
<td>0.486</td>
</tr>
<tr>
<td>SF-36</td>
<td>45.6 (38–50.5)</td>
<td>45.8 (43.8–47.5)</td>
<td>0.728</td>
</tr>
<tr>
<td>PCS</td>
<td>48.6 (41.2–54.8)</td>
<td>44.8 (42.3–52.2)</td>
<td>0.673</td>
</tr>
</tbody>
</table>

Note: Values were presented as median (interquartile range).

Abbreviations: RSI, Reflux Symptom Index; RFS, Reflux Finding Score; LPR-HRQoL, Laryngopharyngeal Reflux-Health-Related Quality of Life; SF-36, Short Form 36 Survey; PCS, physical component score; MCS, mental component score.
Previous studies have reported that LPR patients have higher levels of pepsin in the laryngeal lesions than do normal individuals; its presence may injure the larynx in LPR. 19

Recently, it was shown that pepsin remains stable at a pH of 7.0 and temperature of 37°C for more than 24 hours, retaining nearly 80% of its original activity on reacidification. With a mean pH of 6.8, the larynx may contain stable pepsin, which could potentially cause more damage with subsequent reflux episodes. 20 There is also evidence that pepsin is actively transported into and remains within laryngeal epithelial cells. 21 Intracellular structures such as Golgi bodies and lysosomes have a low pH (of 5.0 and 4.0, respectively). Therefore, pepsin could cause intracellular damage, even if the larynx itself is only exposed to inactive pepsin. 21

Several studies have suggested that pepsin measurement at the time of symptoms could be a sensitive diagnostic method for LPR. 22 Wang et al. 23 found that higher pepsin levels in the sputum were associated with higher RSI and RFS scores. The study also did not find any significant differences in the pepsin concentration based on the sputum collection methods (including oral and hypopharyngeal secretions). In our previous study, the level of pepsin in saliva collected upon waking were significantly higher than after meals or when the patients complained of the symptom. 24 In another review, Kim et al. 25 used Western blot analysis to measure pepsin in the saliva of patients with suspected extraesophageal reflux and in healthy controls. Pepsin was detected mainly in 45% of the samples of patients collected at the time of symptoms. However, it was not identified in any of the controls. Hayat et al. reported that patients with gastroesophageal reflux disease or hypersensitive esophagus had higher pepsin level in the postprandial period that in waking. 26 As there are many different reports, our saliva collection timing may not be the best timing. This will require additional research.

This study has several limitations. First, the study lacked a control group. However, we thought that a true control group of patients without LPR symptoms is not needed in this study. Because the study was designed as a comparison of patients with LPR who either have or do not have detectable salivary pepsin, normal controls are therefore not really necessary. Second, we used single morning sample. But single morning sample would decrease sensitivity of the method.

**CONCLUSION**

The pepsin level in saliva was not associated with subjective symptoms or QoL in patients with LPR. It may be true that there is no association between pepsin levels and LPR symptoms, but this lack of association does not prove the lack of pathophysiological effect.

**REFERENCES**


**TABLE 3. Correlations Between the Questionnaire and Average Pepsin Levels in the Saliva After Waking**

<table>
<thead>
<tr>
<th>Correlation Coefficient (r)</th>
<th>P Value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI score</td>
<td>−0.012</td>
<td>0.941</td>
</tr>
<tr>
<td>RFS score</td>
<td>0.069</td>
<td>0.670</td>
</tr>
<tr>
<td>LPR-HRQOL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voice</td>
<td>0.266</td>
<td>0.652</td>
</tr>
<tr>
<td>Cough</td>
<td>−0.063</td>
<td>0.694</td>
</tr>
<tr>
<td>Clear throat</td>
<td>−0.032</td>
<td>0.841</td>
</tr>
<tr>
<td>Swallowing</td>
<td>−0.103</td>
<td>0.521</td>
</tr>
<tr>
<td>Overall impact of acid reflux</td>
<td>0.053</td>
<td>0.743</td>
</tr>
<tr>
<td>SF-36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCS</td>
<td>−0.104</td>
<td>0.516</td>
</tr>
<tr>
<td>MCS</td>
<td>0.024</td>
<td>0.884</td>
</tr>
</tbody>
</table>

**Abbreviations:** RSI, Reflux Symptom Index; RFS, Reflux Finding Score; LPR-HRQOL, Laryngopharyngeal Reflux-Health Related Quality of Life; SF-36, Short Form 36 Survey; PCS, physical component score; MCS, mental component score.