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Original Article

Role of Laryngopharyngeal Reflux in the Pathogenesis of Otitis Media with Effusion

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OBJECTIVE: To determine whether there is an association between otitis media with effusion and laryngopharyngeal reflux in children.

MATERIALS and METHODS: This study included 31 children with otitis media with effusion. The pepsinogen level in the middle ear fluid of all patients was measured by sandwich enzyme-linked immunosorbent assay. Each patient's middle ear fluid was investigated for *Helicobacter pylori (H. pylori)* using the Campylobacter-like organism (CLO) test. The middle ear pepsinogen levels were compared with those in the serum. The correlation between pepsinogen levels and *H. pylori* positivity in the middle ear fluid was investigated.

RESULTS: The mean middle ear pepsinogen level (211.69 ng/mL) was significantly higher than that in the serum (24.18 ng/mL) in patients with otitis media with effusion. The middle ear aspirates of six patients (19%) were positive for *H. pylori*, and the correlation between *H. pylori* positivity and increased pepsinogen levels in the middle ear fluid was statistically significant in patients with otitis media with effusion.

CONCLUSION: We detected higher pepsinogen levels and *H. pylori* positivity rates in the middle ear fluid than in the serum of patients with otitis media with effusion. These results support the role of laryngopharyngeal reflux in the pathogenesis of otitis media with effusion.

KEYWORDS: Otitis media with effusion, laryngopharyngeal reflux, pepsinogen, *Helicobacter pylori*

INTRODUCTION

Otitis media with effusion (OME) is the presence of non-purulent effusion within the middle ear and is a common disease during childhood. This condition must be given special attention because it may cause hearing loss in children and can irreversibly damage the middle ear mucosa. The pathogenesis of OME can be caused by adenoid diseases, allergic rhinitis, immunological diseases, or laryngopharyngeal reflux (LPR) [1]. One potential cause of OME is the reflux of gastric contents into the region of the nasopharyngeal mucosa, which initiates an inflammatory process [2]. This pathophysiological mechanism has been frequently questioned in recent studies [3, 4, 5]. Animal studies have shown that reflux leads to eustachian tube dysfunction. The eustachian tube is immature, and its angle is wider in children. Therefore, gastric contents can more easily reach the middle ear inducing an inflammatory process [6].

Helicobacter pylori (*H. pylori*), a gram-negative bacterium that causes chronic infection, is present in more than half of the global population. Developed countries have a 20%-50% prevalence of the infection, whereas that in developing countries is as high as 90% [7, 8]. By the age of 10 years, approximately 75% of children are infected with *H. pylori*. Many body regions other than the gastrointestinal tract have been investigated for the presence of this microorganism. In recent years, *H. pylori* has been found in adenoid tissue, nasal polyp tissue, and middle ear fluid [9].

Few studies have investigated the relationships among OME, pepsinogen levels, and *H. pylori* presence, separately [10, 11]. To our knowledge, this is the first study to evaluate the relationship of pepsinogen levels, both in the serum and middle ear fluid, with the presence of *H. Pylori* by detecting it with the Campylobacter-like organism (CLO) test from the middle ear fluid in the pathogenesis of serous otitis media in pediatric patients. Our aim in this study was to investigate the association between OME and LPR by evaluating the levels of pepsinogen and presence of *H. pylori* in the middle ear fluid.

MATERIALS and METHODS

Thirty-one pediatric patients with OME were included in this study that was from May 2012 to January 2013. The exclusion criteria were Down's syndrome, cleft palate, eustachian tube dysfunction, neurological disease, and immune deficiency. Nineteen children (38 ears) who had a history of recurrent otitis media (more than three attacks in 6 months or more than four attacks in 1 year) and who were treated with standard medical therapy and documented as clinically improved and type A tympanogram were included in the control group. All patients in the OME group and control group had no reflux symptoms clinically and no medication history of reflux disease.

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The diagnostic criteria for OME were as follows:

1) One or more of the following three otoscopic findings: an undertoned appearance of the tympanic membrane secondary to lack of aeration, the presence of an air-fluid level behind the transparent membrane, and tympanic membrane atrophy or retraction pockets
2) Type B or C tympanogram; absence of acoustic reflex
3) Evidence of air-bone gap on audiogram

Patients with clinical manifestations of chronic tonsillitis, recurrent tonsillitis, or tonsil or adenoid hypertrophy causing obstruction also underwent adenoidectomy or adenotonsillectomy.

Collection of Middle Ear Fluid Samples and Storage Conditions

Before starting the operations, blood sampling was performed to assess the serum pepsinogen level. The blood samples were centrifuged at 3000 rpm for 15 min and separated.

All patients in the OME group underwent myringotomy and tympanostomy tube placement under general anesthesia. Samples of the fluid in the middle ear were collected through a myringotomy incision using a chamber aspirator (Middle Ear Fluid Aspirator/Collector; Medtronic/Xomed Inc. Jacksonville, FL, USA).

In the control group patients who were without effusion, middle ear cavity lavage was performed through the myringotomy incision with the help of a dental syringe. Sterile saline (1 mL) was injected into the middle ear and aspirated. A total of 0.2 mL of the middle ear fluid sample was subjected to the CLO test. The remaining middle ear fluid and serum samples were placed on ice immediately after they were obtained and were then transferred to freezers at -20°C. The samples were stored at -20°C until the pepsinogen assay was performed. The fluid removed from the middle ear was classified as glue or serous.

Detection of *H. pylori* in the Middle Ear Fluid

The CLO test (Hpfast, Kimberly-Clark, USA) was used to detect *H. pylori* in the middle ear. The CLO tests (one per ear) were stored at 4°C before the operation and brought to room temperature immediately thereafter. Two drops of the middle ear fluid sample were added to the color chamber of the CLO test. The color change was evaluated after 24 h.

Detection of Pepsinogen Level in the Samples

A sandwich enzyme-linked immunosorbent assay (ELISA) (Human Pepsinogen A ELISA Kit; USCN Life Science Inc., Wuhan, China) was used to detect the pepsinogen in the samples stored at −20°C. Before ELISA was performed, the samples frozen at -20°C were allowed to dissolve to room temperature without the use of a heater. The specific experimental procedures were conducted according to the manufacturer's instructions. Known volumes of phosphate-buffered saline, pH 7.2, were added at room temperature. The dilution factor was calculated for each sample. The resulting samples of known volume were centrifuged at 3000 rpm for 1 h. The pepsinogen in each sample was assayed by ELISA. The absorbance values of the samples at 450 nm were determined using a microplate reader (ELx800 ELISA Plate Reader System and Analyzer; Bio-Tek Instruments Inc., Vermont, USA). The level of pepsinogen in each sample was determined by in silico analysis.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS version 18.0, IBM SPSS Statistics, IBM Corporation; Chicago, IL, USA)) was used for statistical evaluation. The Mann-Whitney U test, Wilcoxon signed-rank test, and Kruskal-Wallis analysis of variance were performed. The Mann-Whitney U-test with Bonferroni adjustment was used for pairwise comparisons. The Wilcoxon signed-rank test was used for comparisons between pepsinogen values in the middle ear fluid and those in the serum. A value of $p<0.05$ was considered to be statistically significant. The study protocol was approved by the local ethics committee. The patients and their parents were informed in detail, and their informed consents were obtained.

RESULTS

Sixty-one ears of the 31 patients with serous otitis media admitted to the ENT Clinic of Adana Numune Training and Research Hospital were included in this study as the OME group. Thirty-eight ears of the 19 patients with no effusion were included in this study as the control group. The mean age of the patients in the OME group was 7.03±3.07 years (range, 2-15 years). The mean age of the patients in the control group was 6.59±3.76 years (range, 2-14 years). Sixteen (51.6%) patients were male, and 15 (48.4%) were female in the OME group. Nine (47.3%) patients were male, and 10 (52.7%) were female in the control group. Between the two groups, no significant difference was observed in age and gender.

All patients in the OME group underwent placement of a ventilation tube in the ear; 14 underwent adenoidectomy and 5 underwent adenotonsillectomy during the same operation.

Tympanometric tests were performed to diagnose OME. The tympanometry test results showed type B in 47 (77%) and type C in 14 (23%) of the 61 ears in the OME group. The tympanometry test results were type A for all patients in the control group

Pepsinogen Levels in Middle Ear Fluid and Serum

In the OME group, the serum pepsinogen levels ranged from 1.1 ng/mL to 89.2 ng/mL (mean, 24.18±12.90 ng/mL), and the middle ear fluid pepsinogen levels ranged from 1.5 ng/mL to 693.2 ng/mL (mean, 211.69±195.5 ng/mL) (Table 1). In the control group, the serum pepsinogen levels ranged from 1.2 ng/mL to 81.2 ng/mL (mean, 22.96±10.27 ng/mL), and the middle ear fluid pepsinogen levels ranged from 1.5 ng/mL to 34.8 ng/mL (mean, 13.06±10.37 ng/mL). In the OME group, the pepsinogen levels in the middle ear fluid were significantly higher than those in the serum ($p<0.001$).

Correlation among Pepsinogen Levels in Middle Ear Fluid and Tympanometric Results, CLO Test Results, and Type of Effusion

A comparison of the pepsinogen levels in the middle ear fluid according to the tympanometric evaluation results was performed. The pepsinogen levels in the type B group ranged from 1.7 ng/mL to

Table 1. Pepsinogen levels in the middle ear fluid and serum in the OME group

	Min-Max.	Mean SD
Pepsinogen levels in serum	1.1-89.2	24.18±12.9
Pepsinogen levels in middle ear	1.5-693.2	211.69±195.5

P<0.001
OME: Otitis media with effusion

693.2 ng/mL (mean, 221.91±203.87 ng/mL), and those in the type C group ranged from 1.5 ng/mL to 492.5 ng/mL (mean, 178.53±182.7 ng/mL). In the type A group, the pepsinogen levels in the middle ear was significantly lower than those the other two groups (p<0.001). There was no significant difference between the type B and type C groups (p=0.261) (Table 2).

A comparison of the pepsinogen levels in the middle ear fluid according to the CLO test results was also performed. The CLO tests were negative in all patients of the control group. In the OME group, the pepsinogen levels ranged from 36.3 ng/mL to 492.5 ng/mL (mean, 319.26±170.34 ng/mL) in the positive test group, and those in the negative test result group ranged from 1.5 ng/mL to 693.2 ng/mL (mean, 180.63±192.89 ng/mL) (Table 3). The pepsinogen levels in the middle ear fluid were significantly higher in the positive than in the negative CLO test result group (p=0.021).

The pepsinogen levels in the middle ear fluid were compared according to the type of effusion. The pepsinogen levels in the patients with glue effusion ranged from 36.3 ng/mL to 684.3 ng/mL (mean, 261.49±175.65 ng/ml), those in the patients with seromucinous effusion ranged from 32.8 ng/mL to 693.2 ng/mL (mean, 324.43±240.89 ng/mL), and those in the patients with dry ears (control group) ranged from 1.5 ng/mL to 34.8 ng/mL (mean, 13.06±10.37 ng/mL) (Table 4). The pepsinogen levels in the patients with dry ears were significantly lower than those in the patients in the other two groups (p<0.001).

Finally, the pepsinogen levels in the middle ear fluid of the patients with recurrent otitis media ranged from 5.9 ng/mL to 124.7 ng/mL (mean, 62.08±48.5 ng/mL), and those in the patients with non-recurrent otitis media ranged from 1.5 ng/mL to 693.2 ng/mL (mean, 228.01±198.72 ng/mL) (Table 5). The pepsinogen levels in the middle ear fluid of the patients without recurrence were significantly higher than in those with recurrence (p<0.021).

Table 2. Pepsinogen levels in the middle ear fluid according to the tympanometric evaluation

Tympanometry	Min-Max.	Mean SD
Type B	1.7-693.2	221.91±203.87
Type C	1.5-492.5	178.53±182.7
Type A (Control Group)	1.5-34.8	13.06±10.37

Table 3. Pepsinogen levels in the middle ear fluid according to the CLO test in the OME group

CLO test result	Min-Max.	Mean SD
Positive	36.3-492.5	319.26±170.34
Negative	1.5-693.2	180.63±192.89

p=0.021
CLO: campylobacter-like organism test

Table 4. The pepsinogen levels in the middle ear fluid according to the type of effusion

Effusion type	Min-Max.	Mean SD
Glue	36.3-684.3	261.49±175.65
Seromucinous	32.8-693.2	324.43±240.81
Dry (Control Group)	1.5-34.8	13.06±10.37

p<0.001

Table 5. Pepsinogen levels in the middle ear fluid of patients with recurrent otitis media

Recurrence	Min-Max.	Mean SD
Yes	5.9-124.7	62.08±48.57
No	1.5-693.2	228.01±198.72

p<0.021

DISCUSSION

OME is one of the most common causes of hearing loss in developing countries. It is characterized by fluid collection in the middle ear without active infection. If the disease continues for more than three months, it is defined as chronic OME [2].

Evidence shows the presence of proliferation of mucous glands and goblet cells in the middle ear epithelium of patients with OME [12]. The level of mucous secretion is greater than that can be cleared by mucociliary activity, and mucin-rich fluid thus fills the middle ear. This fluid buildup results in hearing loss in the range of 15-50 dB.

OME is a chronic inflammatory disease with a multifactorial etiology. Many etiological factors have been proposed, including viral and bacterial infections of the upper respiratory system, deficiencies in mucociliary activity of the middle ear, dysfunction of the eustachian tube, and allergy [2]. Gastroesophageal reflux is one of the etiological factors for OME, especially over the last 10 years. Many studies performed over the last 10 years have attempted to elucidate the relationship between gastroesophageal reflux and OME.

Gastroesophageal reflux is defined as the reflux of gastric contents into the esophagus without retching or vomiting [9]. If the reflux reaches the upper esophageal sphincter level, it is called LPR [10]. Gastroesophageal reflux is physiologically seen in most newborns during the first year of life; thereafter, it is considered to be a pathological condition [12]. Pathological changes occur in the upper respiratory tract epithelium secondary to the reflux of gastric acidic contents. LPR is an accepted cause of chronic laryngitis, contact granulomas of the larynx, laryngeal stenosis, paroxysmal laryngeal spasms, and chronic cough [11, 13-16].

Studies performed over the last 10 years support the relationship between OME and LPR. The exact mechanism of LPR in the etiology of OME has not been clarified. There are four possible mechanisms of LPR in the etiology of OME: eustachian tube dysfunction due to LPR, stimulation of *Muc5b* gene expression in the middle ear epithelium by acidic content, proteolytic activity of refluxed pepsin in the middle ear, and stimulation of inflammation in the middle ear by refluxed *H. pylori* in the stomach.

Heavner et al. [4] investigated the effect of LPR on eustachian tube function in rats. The rats in the study group underwent injections of pepsin and hydrochloric acid, and those in the control group underwent injections of saline. In both groups, the passive opening and closing pressures of the eustachian tube before and after the injections were measured. The researchers found that eustachian tube

dysfunction developed in the study group. A similar animal study was performed by White et al. [5] They examined eustachian tube dysfunction by measuring the mucociliary clearance of the tube. These studies support the notion that LPR can cause OME by means of eustachian tube dysfunction.

The pH of the normal middle ear is alkaline. Aside from their pepsin activity, the gastric contents can have a degenerative effect on the middle ear epithelium because of their acidity. Block et al. [17] reported two main results in their animal study. First, they found that a particularly acidic pH shortens the lifespan of middle ear epithelium, which is physiologically alkaline. Second, they found that an acidic pH increases *Muc5b* gene expression in the middle ear epithelium. The *Muc5b* gene increases mucin production in middle ear. This result may explain the viscous mucin secretion in patients with OME.

The most frequently questioned parameter in the relationship between OME and LPR is the presence of pepsin/pepsinogen in the middle ear. We examined the pepsinogen levels and *H. pylori* colonization in the middle ear. Tasker et al. [2] were the first to investigate the presence and activity of pepsinogen in the middle ear. They compared the serum and middle ear fluid pepsinogen levels in 65 surgical patients with OME by ELISA; 56 patients (90.7%) were positive for pepsinogen. The middle ear pepsinogen level was 1000-fold higher than that in serum.

Lieu et al. [18] examined fluid samples obtained from 36 middle ears of 22 children. The samples were subjected to ELISA, and proteolytic activity was evaluated. Pepsinogen positivity rates of 73% and 77% of the samples were identified by ELISA and proteolytic activity assay, respectively.

Nair et al. [19] investigated middle ear and serum pepsinogen levels by electrophoresis. They found that 61% of patients had middle ear pepsinogen positivity and that the pepsinogen level in the middle ear was 65-fold higher than that in serum.

Although pepsinogen in the middle ear may cause otitis media, it is not the sole cause because pepsinogen must be converted to its active form pepsin to have proteolytic activity. Conversion of pepsinogen to pepsin is possible only at an acidic pH. The physiological pH of the middle ear is alkaline. The presence of pepsin proteolytic activity in the middle ear indicates a strong relationship between OME and LPR.

Tasker et al. [2] found proteolytic activity in the middle ear samples 19 of 65 patients. He et al. [20] took samples from 225 middle ears of 152 children, and using a modified enzymatic method, they found pepsin activity in 23 of the 225 samples (10%). O'Reilly et al. [21] reported pepsin activity in 14% of 893 effusion samples.

Even in cases of high pepsinogen levels in the middle ear, a relatively low proteolytic activity may not be a precise indicator of pepsin activity. The effect of LPR is not static, but dynamic. After an episode of reflux, the pH of the middle ear decreases, and proteolytic activity begins. Compensatory mechanisms attempt to increase the pH. Irreversible pepsinogen inhibition is not possible at pH<8. Therefore, inactive pepsinogen can be activated by a subsequent reflux episode. Although the events at particular points in this process have been determined, they do not show the dynamic effect of LPR. It is possible that the middle ear proteolytic activity level is higher than that measured.

Pepsinogen in the middle ear can originate from three sources:

1. Diffusion of pepsinogen from blood: Tasker et al. [2] examined the pepsinogen, fibrinogen, and albumin levels in the serum and middle ear. They found no significant differences between the serum and middle ear fibrinogen and albumin levels, but the middle ear pepsinogen level was 1000-fold higher than that in the serum. Additionally, Nair et al. showed that the middle ear pepsinogen level was 65-fold higher than that in the serum [19]. The hypothesis of a vascular origin of middle ear pepsinogen was refuted by these studies.
2. Endogenous production of pepsinogen in the middle ear: Many studies have shown that enzyme activity can occur in cases of severe inflammation and chronic discharge. Production of pepsinogen in the middle ear is also possible. Pepsinogen isoenzymes are found in the lung, pancreas, prostate, and some malignant tissues. Liu et al. [18] found no pepsinogen 1 mRNA in infected mastoid mucosa by RT-PCR. Tasker et al. [2] found no evidence of pepsinogen production by immunohistochemical methods in middle ear biopsy specimens. Endogenous pepsinogen production is unlikely according to these studies.
3. Pepsinogen reaching the middle ear by LPR: LPR is the most likely source of pepsinogen in the middle ear.

We examined 61 samples of middle ear fluid from the 31 patients by ELISA in the OME group. Thirty of the 31 patients had significantly higher pepsinogen levels in the middle ear fluid than in the serum (211.69±195.5 vs 24.18±12.9 ng/mL), similar to the findings in the study by Tasker et al. [2].

The middle ear pepsinogen levels in the control group (13.06±10.37 ng/mL) were lower than those in ears with serous effusion (324.43±240.81 ng/mL) and glue effusion (261.49±175.65 ng/mL) in the OME group. He et al. [20] also reported lower pepsinogen levels in dry ears. This difference can be explained in two ways: first, lower pepsinogen levels cannot effectively cause effusion in the middle ear. Second, insufficient sampling. To obtain samples from dry ears, 1-mL saline was injected into the middle ear and was then aspirated. Such samples taken from the middle ear likely do not effectively and homogeneously reflect the actual biochemical parameters in the ear. For this reason, more effective sampling methods for evaluating dry ears should be developed.

The pepsinogen levels in our study are presented in ng/ml. Other studies presented pepsinogen levels in picograms and nanograms. These unit differences arise from methodological differences among studies. Thus, it is difficult to compare pepsinogen levels among studies. An effective comparison is possible only using gastric fluid as a positive control or serum as a negative control. We used serum as a negative control, as did other studies described earlier in the text.

Another mechanism that may explain the role of LPR in the etiology of OME is the presence of *H. pylori*, which is a spiral-shaped, catalase-positive, oxidase-positive, protease-positive, and urease-positive forced microaerophilic mobile gram-negative microorganism.

It has no reservoir other than the human stomach. Transmission is oral-oral and fecal-oral. *H. pylori* is related acute and chronic gastritis, gastric and duodenal ulcers, gastric lymphoma, and gastric cancer and is an accepted carcinogenic agent [22].

There is no direct evidence of a relationship between *H. pylori* and gastroesophageal reflux. Our population showed 90% *H. pylori* positivity. Our aim in this study was to determine the source of *H. pylori* in the middle ear, which can originate from the stomach as a result of reflux. *H. pylori* can be used as a marker to show LPR, similar to pepsin and pepsinogen.

Yılmaz et al. [6] evaluated 22 children with OME (patient group) and 20 children who underwent surgery for other conditions (control group). They identified *H. pylori* in both the tonsil and adenoid samples by PCR and CLO methods. *Helicobacter* positivity was seen in 14 (64%) and 6 (30%) children in the patient and control groups, respectively; the difference was statistically significant.

Morinika et al. [23] evaluated smears from the middle ears of 15 patients who underwent surgery because of OME. The samples were examined by immunohistochemistry and CLO testing. Thirteen of the 15 samples (80%) were positive for *H. pylori*.

Chul-won et al. [24] examined the middle ear fluid of a group of pediatric patients. Sixty patients underwent insertion of a ventilation tube, and 32 of these 60 patients also underwent adenoidectomy. Thirty patients underwent adenoidectomy only. The presence of *H. pylori* was determined in both adenoid tissues and middle ear fluid by CLO testing and PCR. Only 6 of the 32 patients who underwent operations for treatment of OME were positive for *H. pylori* in their adenoid tissue according to CLO testing. Five of the 30 patients who underwent only adenoidectomy were positive on CLO testing. The difference was not statistically significant. However, 16 of the 60 patients (27%) were positive by CLO testing, and 18 of the 60 patients (30%) were positive by PCR.

In our study, 6 of the 31 patients (19.3%) showed CLO positivity in the OME group. All ears were CLO-negative in the control group. The sensitivity of the CLO test is concentration dependent. The ear fluid samples may not have contained a sufficiently high level to be detected by the CLO test, even when infected with *H. pylori*. The middle ear pepsinogen levels in CLO-positive patients were 319.26±170.34 ng/ml and were higher than those in the CLO-negative patients (180.63±192.89 ng/mL). This indicates that a higher concentration of *H. pylori* is needed to increase the sensitivity of the CLO test.

Many studies have reported a relationship between OME and LPR. The main question is whether the acidic pH and the presence of pepsin or the toxic effect of the presence of refluxed *H. pylori* is responsible for the pathological process. Yılmaz et al. [6] investigated this issue by prescribing antibiotic and acid suppression therapy for *H. pylori* eradication. Amoxicillin and clarithromycin are used to eradicate *H. pylori*. These antibiotics are also the most important medical treatment modalities for OME. The effect of antibiotic treatment for OME may be because of its eradication of *H. pylori* [7].

Serum and middle ear pepsinogen levels were evaluated in our study. The middle ear pepsinogen levels in our study were higher than those in the serum, supporting the notion that LPR plays a role in the pathogenesis of OME. The middle ear pepsinogen levels were 10-fold higher than those in the serum, which cannot be explained by vascular diffusion; instead, reflux may be the cause.

Additionally, in our study, 19.3% of the patients were CLO-positive in the OME group, whereas all the patients were CLO-negative in the control group. The pepsinogen levels in the middle ear fluid were significantly higher in the CLO-positive than in the CLO-negative patients. The exact relationship between OME and LPR and the role of antireflux therapy including the eradication of *H. pylori* from the middle ear in EOM treatment must be clarified in larger series.

Ethics Committee Approval: Ethics committee approval was received for this study from the local institutional review board (2012/72).

Informed Consent: Written informed consent was obtained from the parents of all participants who participated in this study.

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