Original Article

The role of *Helicobacter pylori* and gastroesophageal reflux disease in otitis media with effusion and evaluation of *MUC4, MUC5B* mucin genes and inflammatory cytokines in guinea pig middle ears

Emin Eren[1], Mustafa Deniz Yılmaz[2], Yeşim Çekin[3], Arsenal Sezgin Alikanoğlu[4], Hülya Eyigör[5], Gamze Öztürk Yılmaz[5], Ömer Tarık Selçuk[5]

[1]*Department of Otorhinolaryngology, Private Academy Hospital, Konya, Turkey*
[2]*ENT Specialist, Mustafa Deniz Yılmaz Clinic, Antalya, Turkey*
[3]*Department of Microbiology, Antalya Training and Research Hospital, Antalya, Turkey*
[4]*Department of Pathology, Antalya Training and Research Hospital, Antalya, Turkey*
[5]*Department of Otorhinolaryngology, Antalya Training and Research Hospital, Antalya, Turkey*

ABSTRACT

Objectives: This study aims to investigate the role of *Helicobacter pylori (H. pylori)* and gastroesophageal reflux disease (GERD) in otitis media with effusion (OME) and to examine the *MUC4*, *MUC5B* mucin genes in the guinea pig middle ears to analyze the inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1beta (IL-1β), and IL-8.

Patients and Methods: This study was conducted using eight guinea pigs. In the experimental group, *H. pylori* ATCC strain culture was injected transtympanically with microscopic guidance. The animals in the control group were injected with phosphate buffered saline (PBS) solution transtympanically. At 12, 24, 48, and 168 h after the injection, transtympanic PBS solution injection and aspiration of the middle ear were applied to all animals. After decapitation, the samples were taken.

Results: In the histopathological examination, grading according to the degree of inflammation showed no statistically significant difference between the two groups (p>0.05). No statistically significant difference was determined in the analysis results of *MUC4* and *MUC5B* genes in the middle ears of the two groups (p>0.05), the right and left ear values of IL-8, IL-1β, and TNF-α (p=0.999, p=0.610, and p=0.691, respectively).

Conclusion: Based on our study results, there was no relationship between *H. pylori* injection in the middle ear and the histopathological changes of the middle ear mucosa. The results of the analysis of *MUC4*, *MUC5B* genes, and the inflammatory cytokines did not support the cause-and-effect relationship between *H. pylori* and OME.

Keywords: Guinea pig, interleukin-1beta, *MUC4*, *MUC5B*, otitis media with effusion, tumor necrosis factor-alpha.

Otitis media with effusion (OME) is the most common cause of hearing loss in the pediatric age group in developed countries. It characterized by an accumulation of fluid over three months or more behind a healthy ear membrane without any signs or symptoms of local or systemic infection.

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Correspondence: Gamze Öztürk Yılmaz, MD. Antalya Eğitim ve Araştırma Hastanesi Kulak Burun Boğaz Kliniği, 07100 Muratpaşa, Antalya, Türkiye.
e-mail: gambu_9199@hotmail.com

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In the United States, 2.2 million cases of OME attack are diagnosed annually. Otitis media with effusion is a primary chronic inflammatory status and this inflammation is multi-factorial. Bacterial infections, a disorder in the mucociliary cleaning system, or tubal dysfunction can trigger this inflammation.[1]

Gastroesophageal reflux disease (GERD) is one of the most significant gastrointestinal system-related problems which is often seen in childhood and has been often mentioned in recent years as responsible for several unresolved medical problems. In animal models, it has been shown that the temporary inflammation of acidic gastric contents in GERD may be a reason for eustachian tube dysfunction and entry of the gastric contents to the middle ear cavity.[2] In several studies to date, pepsin/pepsinogen have been shown in the middle ear cavity of children with OME.[3] These studies have shown that pepsin/pepsinogen causes specific symptoms of GERD, is not produced from the middle ear mucosa and is not transferred from serum by diffusion. This has suggested that GERD is related to the pathophysiology of OME.[3,4] In a study conducted on rat middle ears, *Helicobacter pylori (H. pylori)* antigen was administered transtympanically to the rats and inflammatory cytokines were investigated to reveal the role of gastroesophageal reflux.[5] As a result of this study, it was concluded that *H. pylori* in the middle ear of patients with otitis media could affect the local immune response and that *H. pylori* could be considered to be strongly involved in the pathogenesis of otitis media.

In the present study, we aimed to determine the role of *H. pylori* and GERD in OME, which has a multifactorial etiology, to analyze the *MUC4*, *MUC5B* genes and the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1beta (IL-1β), and IL-8, in the middle ear, and to histopathologically evaluate the middle ear mucosa.

PATIENTS AND METHODS

Sample preparation

The approval for the study was granted by the Animal Experiments Local Ethics Committee (No: B.30.2.AKD.0.05.07.00/86, Date: 02.09.2013) and was supported by the local Ethics Committee (No: 81266704/71, Date: 04.09.2013).

The study used a total of eight guinea pigs (3 males, 5 females), with an average age 12 months and average weight of 934,5 g. The animals were randomly divided into two groups as the study group (Group D) and the control group (Group C), and each animal was marked with its sex and group. General anesthesia was applied. Using a microscope, *H. pylori* American Type Culture Collection (ATCC) bacterial strains strain was injected through the transtympanic route in the study group and phosphate buffered saline (PBS) was administered in the same way to the control group. The guinea pigs were evaluated in paired groups of one from the study group and one from the control group at 12, 24, 48, and 168 h (D1, C1; D2, C2; D3, C3; D4, C4) and, at each time interval, the middle ear of both the study group and control group animal was

Table 1. Characteristics of the study and control groups

Group	Weight (gr)	Sex	Time of sacrifice post-procedure	Age (months)
D1	802	Male	12th hour	12
D2	865	Male	24th hour	12
D3	776	Female	48th hour	11
D4	1,134	Female	168th hour (7th day)	13
C1	1,117	Female	12th hour	13
C2	703	Female	24th hour	11
C3	1,296	Female	48th hour	13
C4	783	Male	168th hour (7th day)	11

D: Study group; C: Control Group.

Table 2. Grading according to the inflammation severity and histopathological findings of the middle ear specimens

Grade	Histopathological findings
0	No inflammation
1	Minimal, occasional single inflammatory cells
2	Mild, a few single or clustered inflammatory cells
3	Moderate, intense inflammatory cells that have infiltrated the epithelium
4	Severe, intense inflammatory cell infiltration to the extent of masking underlying structures

irrigated (Table 1). The samples were taken from the middle ear aspirates and middle ear mucosa. The tissue samples were examined under a light microscope by a single-blinded pathologist. In the histopathological examination, grading was made according to the severity of inflammation.

MUC4, MUC5B mucin gene analysis

The tissue samples taken for *MUC4* and *MUC5B* genes were centrifuged. Using the QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany), complementary deoxyribonucleic acid (cDNA) was obtained with reverse transcription. The primers used for reverse transcription-polymerase chain reaction (RT-PCR) are listed as follows: *MUC4* forward primer with 23 bases (TCC TCT TTG TGG GAC TCC TTC TA), *MUC4* reverse primer with 20 bases (TTG CCA GTC TGA CGG GAG AA), *MUC5B* forward primer with 22 bases (AGA CCT TTG CCA TGT ACT CAG C), and *MUC5B* reverse primer with 21 bases (TGT GCG TGT AGG TGT AAG GCA). The gamma actin gene was used as the reference gene.

Analysis of IL-1β, IL-8, and TNF-α

The middle ear aspirate samples taken from the study and control group were stored at -20°C, until analysis. The IL-1β, IL-8, and TNF-α measurements of the middle ear fluid aspirate samples were made using the guinea pig IL-1β, IL-8, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory, Shanghai, China).

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). In the histopathological evaluation of the differences between the groups, the Mann-Whitney U test was used for the variables which did not conform to normal distribution. The Fisher's exact test was applied to examine the relationship between the groups of

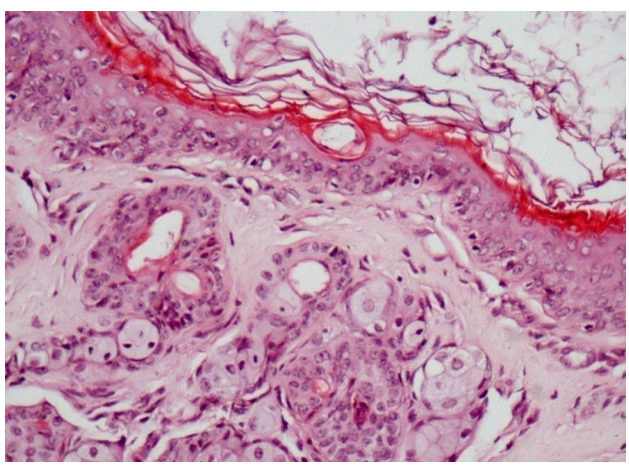

Figure 1. *No inflammatory cells, Grade 0 (D4), H-E, ×200.*

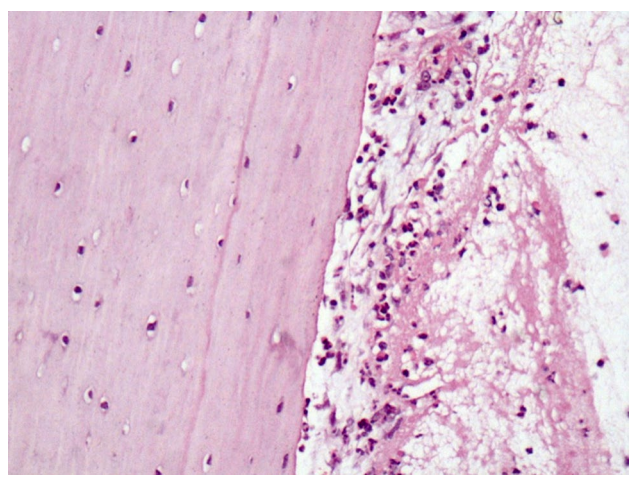

Figure 2. *Minimal, occasional single inflammatory cells, Grade 1 (D3), H-E, ×200.*

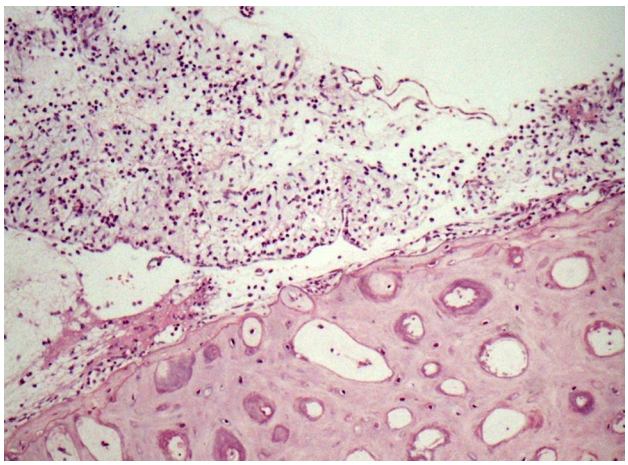

Figure 3. *Clusters of inflammatory cells, Grade 2(k1), H-E, ×100.*

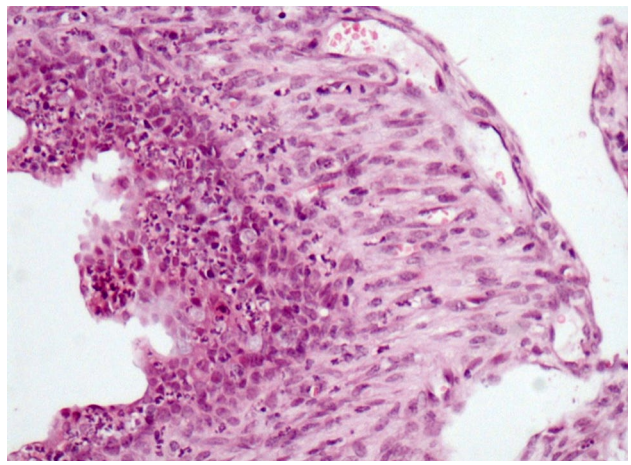

Figure 4. *Moderate, intense inflammatory cells infiltrating into epithelium, Grade 3 (K4), H-E, ×200.*

nominal variables of *MUC4* and *MUC5B* results. In the analysis of the difference between the values of two groups, conformity to normality was tested using the Shapiro-Wilk test. Where distribution was normal, the Student's t-test was used and, where the distribution was abnormal, the Mann-Whitney U test was used. A p value of <0.05 was considered statistically significant.

RESULTS

Histopathological evaluation

The tissue samples were embedded in paraffin blocks. Slices of 4-μm in thickness were cut from the paraffin blocks, were stained with hematoxylin-eosin (H-E) and examined under a light microscope by a single-blinded pathologist. In the histopathological examination, grading was made according to the severity of inflammation. The grades according to this grading system are shown in Table 2. The histopathology grading results are listed. Control Group 1 (C1) is Grade 2, C2 is Grade 3, C3 is Grade 0, and C4 is Grade 4. Study Group (D1) is Grade 0, D2 is Grade 1, D3 is Grade 0, and D4 is Grade 0. Examples are shown below of the histopathological appearance for each grade of inflammation of the middle ear mucosa (Figures 1-4). The Mann-Whitney U test results regarding the differences between the groups in terms of grades are shown in Table 3. No statistically significant difference was found between the groups in terms of grades (p>0.05). Although not statistically significant, grades were higher in the control group.

Results of the analysis of *MUC4* and *MUC5B* mucin expression

The results of the analysis of *MUC4* and *MUC5B* expression are shown in Table 4.

The results of chi-square analysis investigating the relationships between the

Table 3. Results of the Mann-Whitney U test in terms of grade differences between the groups

	Grade				Mann-Whitney U test		
	n	Mean±SD	Median	Min-Max	Row mean	U	*p*
Control	4	2.0±1.4	2.50	0-3	5.88	2.500	0.114
Study	4	0.3±0.5	0.00	0-1	3.12		
Total	8	1.1±1.4	0.50	0-3			

SD: Standard deviation; Min: Minimum; Max: Maximum.

Table 4. Results of the analysis of *MUC4* and *MUC5B* expression

Row no	Sample name	*MUC4*	*MUC5B*
1	D1R	Absent	Present
2	D1L	Present	Present
3	D2R	Absent	Present
4	D2L	Absent	Present
5	D3R	Present	Present
6	D3L	Absent	Present
7	D4R	Absent	Absent
8	D4L	Absent	Present
9	K1R	Absent	Present
10	K1L	Absent	Present
11	K2R	Absent	Present
12	K2L	Present	Present
13	K3R	Present	Present
14	K3L	Absent	Present
15	K4R	Absent	Present
16	K4L	Absent	Present

groups and categories *MUC4* and *MUC5B* are shown in Table 5.

No statistically significant relationship was determined between the groups and the *MUC4* categories (p>0.05). Although not statistically significant, no *MUC4* was determined at the rate of 75% in both the study and the control groups.

No statistically significant relationship was found between the groups and the *MUC5B* categories (p>0.05). Although not statistically significant, *MUC5B* presence was observed at the rate of 87.5% in the study group and 100% in the control group.

Results of the analysis of inflammatory cytokines

The analysis results of the differences in the right ear, left ear, and general ear measurements are shown in Table 6.

No statistically significant difference was observed between the study and control groups in terms of the evaluation of the IL-8, IL-1β, and TNF-α values measured from the right and left ears.

The IL-8, IL-1β, and TNF-α analysis results are shown in Figures 5-7.

DISCUSSION

Helicobacter pylori is a Gram-negative, spiral-shape, micro-aerophyllic bacteria which has been shown to be related to several diseases, including various gastroduodenal diseases such as chronic gastritis, gastric ulcer, duodenal ulcer, and gastric carcinoma.[6] It may also cause extra-intestinal manifestations such as respiratory, vascular and autoimmune diseases.[7] *Helicobacter pylori* infection can be seen in middle ear diseases, as well;[8-10] however, its role in the upper respiratory tract infection and otitis media has not been clarified, yet.[11] In the current study, we found no statistically significant difference between the groups in terms of the inflammation grades of the histopathology results, the measured mucin

Table 5. Results of chi-square test in terms of the relationships between groups and *MUC4* and *MUC5B* categories

		Study group		Control group		Total		Chi-square analysis	
		n	%	n	%	n	%	Chi-square	*p*
MUC4	Absent	6	75.0	6	75.0	12	75.0	Fisher's exact test	1.000
	Present	2	25.0	2	25.0	4	25.0		
	Total	8	100.0	8	100.0	16	100.0		
MUC5B	Absent	1	12.5	0	0.0	1	6.3	Fisher's exact test	1.000
	Present	7	87.5	8	100.0	15	93.8		
	Total	8	100.0	8	100.0	16	100.0		

Table 6. Analysis of the differences in the right ear, left ear, and general ear measurements

Right ear measurement	Groups	n	Mean±SD	Median	Min-Max	*p*
IL-8 R	Control	4	6.5±4.5	4.39	3.96-13.28	0.564#
	Study	4	4.7±4.9	4.70	0.00-9.45	
IL-1β R	Control	4	0.3±0.7	0.00	0.00-1.32	0.850#
	Study	4	0.1±0.2	0.00	0.00-0.31	
TNF-α R	Control	4	11.9±1.5	12.27	9.89-13.30	0.386#
	Study	4	10.9±0.9	10.91	10.14-11.73	
Left ear measurement						
IL-8 L	Control	4	7.4±2.9	7.23	4.64-10.58	0.564#
	Study	4	14.7±12.4	14.59	0.67-28.93	
IL-1β L	Control	4	0.6±1.2	0.00	0.00-2.46	0.508#
	Study	4	1.3±1.8	0.67	0.00-3.81	
TNF-α L	Control	4	11.5±0.74	11.64	10.56-12.12	0.564#
	Study	4	12.0±1.3	12.07	10.75-13.25	
General ear measurement						
IL-8	Control	8	7.0±3.5	4.95	3.96-13.28	0.999#
	Study	8	9.7±10.2	8.77	0.00-28.93	
IL1-β	Control	8	0.5±0.9	.00	0.00-2.46	0.610#
	Study	8	0.7±1.4	.00	0.00-3.81	
TNF-α	Control	8	11.7±1.1	11.83	9.89-13.30	0.691+
	Study	8	11.5±1.2	11.36	10.14-13.25	

IL: Interleukin; IL-1β: Interleukin-1 beta; TNF-α: Tumor necrosis factor-alpha; # Mann-Whitney U test; + Student t test.

gene results, and the inflammatory cytokines. These findings do not support the hypothesis suggesting that *H. pylori* causes middle ear inflammation and OME.

In a study by Yilmaz et al.[12] of 38 pediatric patients with hypertrophic adenoid and/or chronic OME, the presence of *H. pylori* was investigated in adenoid tissues and middle ear

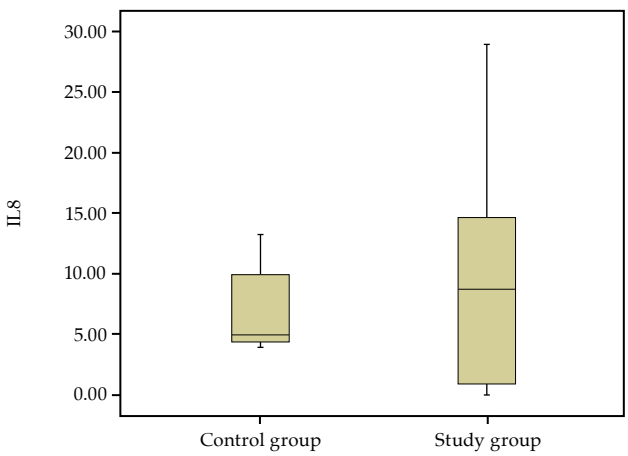

Figure 5. *IL-8 general analysis.*
IL: Interleukin.

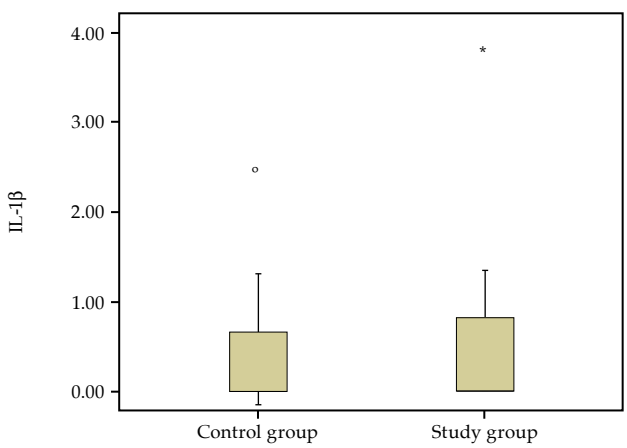

Figure 6. *IL-1β general analysis.*
IL-1β: Interleukin-1 beta.

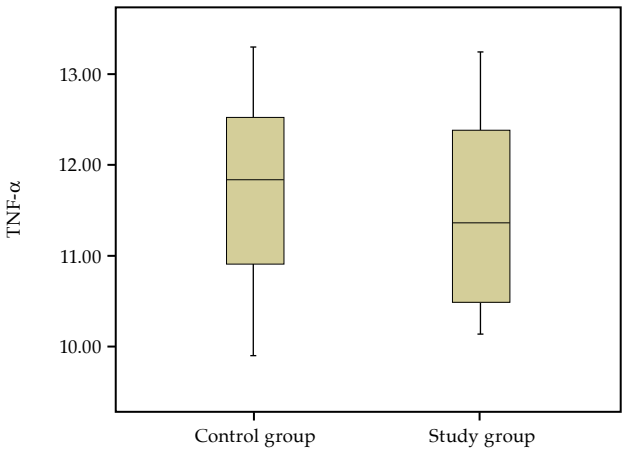

Figure 7. *TNF-α general analysis.*
TNF-α: Tumor necrosis factor-alpha.

mucoid aspirates of the children with chronic OME. The patients were separated into two groups: Group 1 (n=18) with hypertrophic adenoid + OME and Group 2 (n=20) with hypertrophic adenoid only. The *H. pylori* positivity was determined in 47% of the middle ear fluid samples of Group 1 and in the adenoid tissue of only one patient in Group 2.

It has been reported that GERD toward the larynx could be a potential inflammatory cofactor in upper respiratory tract diseases including OME in adults and children.[13] Gibson and Cochran[14] determined GERD in all the children with recurrent otitis media (ROM) in their study groups. In a study by Rozmanic et al.,[15] including 27 children with OME or ROM, GERD pathology was found in 56%. In the current study, the severity of the inflammatory response was examined following transtympanic middle ear injection of *H. pylori*. When the inflammation grades were examined, the highest grades in the control group were observed in C2 and C4 (Grade 3) and the highest grade in the study group in D2 (Grade 1). In the other study group subjects (D1, D3, D4), no inflammation was determined in the middle ear mucosa. There was no statistically significant difference between the groups in term of the grades (p>0.05). Although not statistically significant, the grades of the control group were higher.

Tasker et al.[13] reported the presence of gastric fluid in the middle ears of 65 children with chronic OME and confirmed with pepsin/pepsinogen levels that this fluid was of gastric origin in 91% of effusion samples. In the first study in the literature to determine *H. pylori* in the middle ear cavity, *H. pylori* was determined in 67% of middle ear aspirates of children with chronic OME. The studies by Tasker et al.[13] and Yilmaz et al.[12] confirmed that gastric fluid reached the nasopharynx, the eustachian tube, and the middle ear.

In the current study, no statistically significant relationship was determined between the groups and the *MUC4* categories (p>0.05). Although not statistically significant, 75% of both the study and control group comprised those with no *MUC4*.

There are conflicting reports in the literature related to *H. pylori* colonization in adenoid and tonsil tissues.[12] Unver et al.[16] determined the presence of *H. pylori* in the tonsil and adenoid tissue of 19 patients with the Campylobacter-like organism (CLO) test and *H. pylori* positivity was reported in 11 (58%) patients. Using the same method, Skinner et al.[17] determined no positivity in 50 tonsillectomy specimens. Similarly, Yilmaz et al.[18] did not obtain any positive result from the CLO test applied to 50 tonsillectomy specimens. Cirak et al.[19] used nested-PCR, which is a more sensitive method, and *H. pylori* positivity was found in seven (30%) of 23 tonsillectomy and adenoidectomy specimens.

In the study by Yilmaz et al.,[12] despite a high rate of *H. pylori* in the middle ear effusion samples, *H. pylori* positivity was only determined in one of 38 adenoid tissue samples. Researchers who found a positive result for *H. pylori* reported that tonsil and adenoid tissue could have a reservoir role for *H. pylori*, particularly in the form of oral-oral transfer. This view is also supported by the results of previous studies showing bacteria in dental plaque, oral lesions, and saliva.[20-22] Yilmaz et al.[12] concluded that the probability of adenoid tissue as a reservoir for *H. pylori* infection was very low, as no *H. pylori* positivity was determined in the adenoid tissues of children with middle ear effusion samples positive for *H. pylori*. The presence of *H. pylori* was determined in the middle ear of children

with chronic OME and it was shown that *H. pylori* could have a role in the pathogenesis of OME.

In another study, Yilmaz et al.[23] investigated the possible a relationship between *H. pylori* and OME in children. When the culture and PCR results were analyzed combined, the middle ear of 10 (45%) of the study group patients and of two (10%) of the control group was positive for *H. pylori* and the difference was statistically significant (χ^2=6.453, p=0.011). The authors suggested that there was a higher probability of a relationship between *H. pylori* in the middle ear and stomach contents in the adenoid and tonsillar tissue with pharyngeal reflux. Acid reflux leads to mucosal inflammation in the nasopharynx and edema and impaired clearance of the eustachian tube, thereby, resulting in nasopharyngeal bacteria entering the middle ear. This is supported by high *H. pylori* colonization in adenoid tissue. It is thought that the adenoid tissue serves as a nest for the spread of *H. pylori* infection to the middle ear from the eustachian tube in OME patients. This is supported by significantly increased *H. pylori* colonization in the adenoid tissue of patients with OME, compared to patients with hypertrophic adenoid with no OME. The *H. pylori* colonization reaching the middle ear by means of GERD shows that the bacteria may have a role in the pathogenesis of OME. The recovery of OME with antibiotics supports this hypothesis. Therefore, it seems reasonable to evaluate OME cases resistant to medical treatment in terms of GERD and *H. pylori*.[23]

In a study by Eyigor et al.[24] including 55 operative specimens taken from 47 patients, although the rapid urease test was positive in three (5.5%) specimens, no *H. pylori* gene positivity was determined, suggesting that there was a low probability of adenotonsillary tissue as a reserve for *H. pylori* infections. These findings are also consistent with those of the current study and support our hypothesis.

In another study by Kariya et al[5] of 12 ears of six rats, the expression of inflammatory mediators was examined in otitis media induced with the *H. pylori* antigen. The middle ears were irrigated and the IL-1β concentrations were measured in the collected PBS solution. The IL-1β concentration at 48 h was significantly higher in the study group, compared to the control group (12 h, p=0.674; 24 h, p=0.868; 48 h, p=0.037; and 168 h, p=0.902). The mean TNF-α concentrations in the study group were also significantly higher than the control group at 24, 48, and 168 h (12 h, p=0.053; 24 h, p=0.007; 48 h, p=0.004; and 168 h, p=0.015). In the current study, by taking the IL-8, IL-1β and TNF-α values measured in the right and left ears of both groups, the differences were evaluated between the results of a general ear measurement. The mean IL-8 level was 4.95±3.55 (range, 3.96 to 13.28) pg/mL in the control group and 8.77±10.21 (range, 0.00 to 28.93) pg/mL in the study group, indicating no statistically significant difference between the groups (p=0.999). The mean IL-1β value was 0.00±0.93 (range, 0.00 to 2.46) pg/mL in the control group and 0.00±1.35 (range, 0.00 to 3.81) pg/mL in the study group, indicating no statistically significant difference between the groups (p=0.610). The mean TNF-α value was 11.71±1.14 (range, 9.89 to 13.30) pg/mL in the control group and 11.48±1.17 (range, 10.14 to 13.25) pg/mL in the study group, indicating no statistically significant difference between the groups (p=0.691).

As a limitation of this study, there was a small sample size within each group, so the results of this study should be interpreted in this context. Studies with larger sample sizes are needed.

In conclusion, our study results showed no significant relationship between the *H. pylori* injection and histopathological changes in the middle ear mucosa. In addition, the results of the analysis of *MUC4* and *MUC5B* genes and inflammatory cytokines did not support the relationship between *H. pylori* and OME. Nevertheless, further large-scale, well-designed studies are needed to draw a firm conclusion.

Declaration of conflicting interests

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