Original Article / Çalışma - Araştırma

Pepsinogen identification in the middle ear fluid of children with otitis media with effusion

Efüzyonlu otitis medialı çocukların orta kulak sıvılarında pepsinojenin saptanması

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ABSTRACT

Objectives: This study aims to investigate the presence and concentration of pepsin/pepsinogen in middle ear fluid and to discuss the potential mechanisms involved in the pathogenesis of this condition.

Patients and Methods: A total of 33 children (21 boys, 12 girls; mean age 5.7±2.4 years; range 3 to 13 years) diagnosed with otitis media with effusion and scheduled for operation were enrolled into the study. Fluids aspirated from the middle ear were assessed for the presence of pepsinogen and albumin and blood samples were drawn simultaneously for comparison.

Results: Mean pepsinogen concentration was statistically significantly higher in middle ear fluids compared with serum samples (262.4 ng/mL [range: 211.7 ng/mL - 301.1 ng/mL] versus 102.6 ng/mL [range: 80.7 ng/mL - 134.5 ng/mL], respectively) ($p<0.001$). On the other hand, mean albumin concentration was significantly lower (1.1 g/dL [range: 0.01 g/dL - 9.5 g/dL] versus 5.8 g/dL [range: 0.9 - 9.5 g/dL], respectively) ($p<0.001$). The highest pepsinogen concentration was detected in patients with purulent effusion (275.3 ng/mL).

Conclusion: Our findings support the theory of gastro-esophageal reflux related pepsinogen transition to the middle ear and indicate that pepsinogen may a reliable biochemical marker for the assessment of gastro-esophageal reflux.

Keywords: Gastro-esophageal reflux; otitis media with effusion; pepsin; pepsinogen.

ÖZ

Amaç: Bu çalışmada orta kulak sıvısında pepsin/pepsinojen varlığı ve konsantrasyonu araştırıldı ve bu durumun patogenezindeki olası mekanizmalar tartışıldı.

Hastalar ve Yöntemler: Efüzyonlu otitis media saptanan ve ameliyat planlanan toplam 33 çocuk (21 erkek, 12 kız; ort. yaş 5.7±2.4 yıl; dağılım 3-13 yıl) çalışmaya alındı. Pepsinojen ve albumin varlığına ilişkin orta kulaktan aspire edilen sıvılar değerlendirildi ve karşılaştırma için eş zamanlı olarak kan örnekleri alındı.

Bulgular: Orta kulak sıvılarında ortalama pepsinojen konsantrasyonu serum örnekleriyle karşılaştırıldığında istatistiksel olarak anlamlı şekilde yüksekti (sırasıyla 262.4 ng/mL [dağılım: 211.7 ng/mL - 301.1 ng/mL]'ye kıyasla 102.6 ng/mL [dağılım: 80.7 ng/mL - 134.5 ng/mL]) ($p<0.001$). Diğer yandan, ortalama albumin konsantrasyonu anlamlı olarak düşüktü (sırasıyla 1.1 g/dL [0.01 g/dL - 9.5 g/dL)'ye kıyasla 5.8 g/dL [0.9 - 9.5 g/dL]) ($p<0.001$). En yüksek pepsinojen konsantrasyonu pürülan efüzyonlu hastalarda saptandı (275.3 ng/mL).

Sonuç: Bulgularımız orta kulağa gastro-özofageal reflü ilişkili pepsinojen geçiş teorisini desteklemekte ve pepsinojenin gastro-özofageal reflünün değerlendirilmesi için güvenilir bir biyokimyasal belirteç olabileceğini göstermektedir.

Anahtar Sözcükler: Gastro-özofageal reflü; efüzyonlu otitis media; pepsin; pepsinojen.

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Otitis media with effusion (OME) is an inflammatory condition characterized by fluid collection in the middle ear space in the absence of local or systemic infection. Its prevalence is between 2.2 - 4.8% worldwide.[1] Chronic OME is associated with childhood hearing loss. Studies showed that 35 to 70% of children experience at least one OME episode during the preschool years.[2] While approximately 80% of these episodes heal spontaneously within two months, the remainder becomes chronic.[3]

Gastro-esophageal reflux (GER) has been increasingly implicated in the pathophysiology of OME. Physiologic GER observed in the newborn and in infants usually resolves by 12-15 months of age.[4] Persistent GER, however, may impair Eustachian tube function and cause inflammation. Additionally, the immature angulation of the Eustachian tube may give way to leakage into the middle ear space and thereby hamper mucociliary function, which helps keep the middle ear free of effusion.[5] Together these changes may lead to the formation of a culture media for bacteria within the middle ear.

Pepsin often exists in the form of pepsinogen, which is secreted mainly by stomach mucosa cells and is completely deactivated in alkaline environments such as the middle ear cavity. The process of pepsinogen activation requires cleavage of 44 amino acids from the primary structure of pepsinogen to transform it into active pepsin form in acidic conditions. Pepsin is not detected in the middle ear under normal conditions, but pepsinogen (inactive) may be activated as pepsin (active) under stimulation of hydrochloric acid in gastric juice during reflux.[6]

Studies conducted so far have shown that pepsin/pepsinogen was present in up to 83% of middle ear effusions (MEEs). Tasker et al.[7] using enzyme linked immunosorbent assay (ELISA) found that pepsinogen concentration in the middle ear was 1000 fold higher than serum concentration. Albumin and fibrinogen levels in the middle ear space, however, were close to serum concentrations. These findings have suggested that the presence of pepsin in the middle ear might be of gastric origin.

In this study we aimed to identify the presence and assess the concentration of pepsin/pepsinogen in the middle ear fluid and to discuss potential mechanisms involved in the pathogenesis of this condition.

PATIENTS AND METHODS

Children who presented to the ear nose and throat department of Antalya Education and Research Hospital diagnosed with OME and scheduled for operation were considered for the study. The Antalya Education and Research Hospital ethics committee approved the protocol and written informed consent was obtained from the parents of the children. The study was conducted in accordance with the principles of the Declaration of Helsinki. Children with a diagnosis of Down syndrome, cleft palate and neurologic conditions known to increase the prevalence of GER disease were excluded. A total of 33 children (21 boys, 12 girls; mean age 5.7±2.4 years; range 3 to 13 years) were finally enrolled into the study. Middle ear aspirates were obtained from the middle ear space during myringotomy and were assessed for the presence of pepsinogen and albumin. Blood samples were drawn simultaneously to assess serum pepsinogen and albumin concentrations.

Otoscopic-audiologic evaluation

Tympanometric and pure tone audiometric testing (cooperative children underwent audiometric testing) were performed by the same audiologist using the same audiometer (AC-40 Clinical Audiometer, Interacoustics, Assens, Denmark) and tympanometer (Madsen Capella, GN Otometrics, Taastrup, Denmark) for all children. All children underwent tympanometric measurement and were classified according to the Jerger classification system.[8] The audiologist had no information about the otoscopic examination results. Otitis media with effusion was defined as effusion within the middle ear space persisting for at least three months and identified by either otoscopic or microscopic examination or B or C type tympanogram. Binocular otomicroscopy (Möller Hi-R, Germany) was performed on the anesthetized children by a specialist otolaryngologist, with magnification. The appearance of the tympanic membrane was described as either: dull (poor aeration), vascularized (presence of fluid) or retracted (structural alteration). Surgical interventions performed during the study were myringotomy, ventilation tube insertion, adenoidectomy and tonsillectomy. Adenoid tissue was assessed during nasal endoscopic examination and

adenoid hypertrophy was graded using the three level classification described by Wormald and Prescott.[9] Tonsillar size was graded using the Brodsky classification.[10]

Sampling and conservation of OM effusion

Samples of MEE were collected after myringotomy with a Juhn Tym-Tap (Medtronic Xomed, Jacksonville, FL, USA), which is a device for MEE suction, collection and stored at –20 °C. Each sample was classified as mucoid, serous or purulent MEE based on the gross appearance upon inspection with the naked eye. A mucoid MEE was a thick, viscid, and mucus like effusion that did not flow on inversion, while a serous MEE was a thin and watery effusion that flowed on inversion.[11] Blood samples were drawn prior to surgery to determine pepsinogen levels. Serum was separated by centrifugation at 3000 rpm for 10 minutes.

Identification of pepsinogen

The presence of pepsinogen in OME samples was assessed by ELISA using the human pepsinogen I ELISA kit (Hu Pepsinogen I ELISA, DIAsource ImmunoAssays S.A, Belgium). Prior to ELISA analysis, samples were kept in room temperature for degradation. Then these samples were added to assay calibrators. Control samples, patient serum and middle ear samples were added into the streptavidin coated microwells. A mixture containing biotinylated anti-human pepsinogen I capture antibodies and horseradish peroxidase (HRP) conjugated anti-human pepsinogen I detecting antibodies was added. Following an initial incubation period of one hour, streptavidin biotinylated antibodies on the microplate walls break away and form the streptavidin-biotinylated antibody-pepsinogen I- HRP-antibody immune complex. Free proteins and non-bound HRP conjugated antibodies went through the next washing step. Thereafter, TMB-substrate solution was added into the microwell plate and incubated for 20 minutes. At the end of the incubation period the reaction was stopped. The absorbance was read at 450 nm in Victor 3 model 1420 Multilabel Microplate Reader (Perkin Elmer MA, USA).

Albumin in serum and middle ear fluid was measured by colorimetric BCG (Bromocresol Green) method using the Albumin liquicolor kit (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) in Perkin Elmer LAMBDA 25 UV/Vis Spectrophotometer.

Statistical analysis

Data analysis was performed using the SPSS version 11.5 for Windows software (SPSS Inc., Chicago, IL, USA). The distribution of continuous variables was assessed by Shapiro Wilk test. Continuous variables were presented as mean ± standard deviation or median (min.-max.) and categorical variables as n (%). Pepsinogenin serum samples and middle ear aspirates were compared either by Mann-Whitney U test or Kruskal Wallis test, depending on the number of groups compared. The correlation between adenoid and tonsil grade, albumin level, age and pepsinogen level was assessed by Spearman's correlation test. Albumin and pepsinogen levels in serum and middle ear aspirates were compared using the Wilcoxon signed rank test. Statistical significance was set at $p<0.05$.

RESULTS

While 15.2% (n=5) had a history of ear nose and throat surgery, 84.8% did not (Table 1). Mean pepsinogen concentration was significantly higher in middle ear aspirates compared with serum samples [262.4 ng/mL (range: 211.7 ng/mL - 301.1 ng/mL) vs. 102.6 ng/mL (range: 80.7 ng/mL - 134.5 ng/mL), respectively] ($p<0.001$). Mean albumin concentration was significantly lower [1.1 g/dL (range: 0.01 g/dL - 9.5 g/dL) vs. 5.8 g/dL (range: 0.9 g/dL - 9.5 g/dL), respectively] ($p<0.001$) (Figure 1) (Table 2).

Microscopic ear examination showed that the tympanic membrane was vascularized in 15.2% (n=5) of children, dull in 69.7% (n=23) and retracted in 15.2% (n=5). When children were classified according to the effusion characteristics, 57.6% (n=19) had mucoid, 15.2% (n=5) purulent and 27.3% (n=9) serous effusions. Middle ear fluid

Table 1. Demographic characteristics (n=33)

	n	%	Range	Mean±SD
Age (year)			3-13	5.7±2.4
Gender				
Male	21	63.6		
Female	12	36.4		
History of surgery	5	15.2		

SD: Standard deviation.

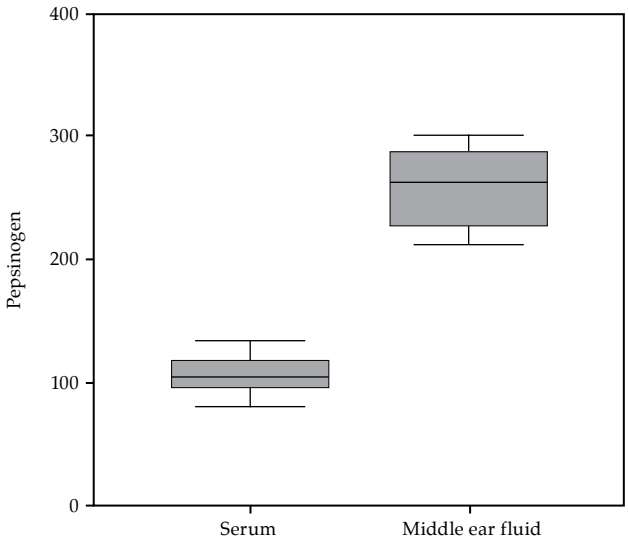

Figure 1. *Pepsinogen concentrations in serum and middle ear fluid samples.*

pepsinogen levels did not significantly differ among groups stratified according to effusion types. The highest pepsinogen concentration was found in patients with purulent effusion (275.3 ng/mL).

Overall, 9.1% (n=3) of children had grade 0 tonsillar hypertrophy, 6.1% (n=2) grade 1, 45.5% (n=15) grade 2, 33.3% (n=11) grade 3 and 6.1% (n=2) grade 4 tonsillar hypertrophy. Children with a history of tonsillar surgery were classified as grade 0 tonsillar hypertrophy. Overall, 15.2% (n=5) were classified as grade 1, 66.7% (n=22) as grade 2 and 18.2% (n=6) as grade 3 adenoid hypertrophy.

DISCUSSION

Otitis media with effusion is primarily an inflammatory condition of multifactorial etiology. Major precipitating factors for OME are Eustachian tube dysfunction and middle ear mucosa inflammation. Several disorders such as viral and bacterial infections, cleft palate and adenoid vegetation may impair Eustachian tube function. Gastro esophageal reflux has been implicated in the pathogenesis of OME and has therefore been a popular topic of research recently. While otitis media and GER are common pediatric disorders, establishing a causal relationship between the two is challenging.[12] Several researchers have shown that a significant number of children with OME had concurrent GER disease and some others demonstrated that children with GER disease displayed a high prevalence for OEM. Toros et al.[13] demonstrated that pepsinogen levels in patients with chronic serous otitis media with GER symptoms were higher than in those without GER symptoms. Rozmanic et al.[14] found that 55.6% of children with OME had concurrent GER disease. Velepic et al.[3] found that 60% of children with chronic tubotympanic disease suffered concurrent GER disease.

Table 2. Comparison of serum and middle ear fluid pepsinogen concentrations under various conditions

Variables	Serum pepsinogen			Middle ear fluid pepsinogen		
	%	Range	*p*	%	Range	*p*
Gender						
Male	102.6	80.7-123.9	0.542	262.4	211.7-301.1	0.593
Female	102.4	85.1-134.5		268.9	215.1-297.1	
Tympanic membrane						
Vascularized	103.2	92.6-123.9	0.694	262.4	221.2-291.5	0.623
Dull	102.6	80.7-134.5		261.4	211.7-301.1	
Retracted	98.1	83.8-125.6		275.3	243.9-291.7	
Effusion type						
Mucoid	103.2	83.8-125.6	0.958	262.4	215.1-301.1	0.762
Purulent	98.5	80.7-127.6		275.3	211.7-287.3	
Serous	101.6	92.6-134.5		261.4	221.2-297.1	
History of surgery						
No	102.8	80.7-134.5	0.338	261.4	211.7-301.1	0.448
Yes	95.3	83.8-125.6		275.3	221.2-291.7	

In this study we used the ELISA method to assess pepsinogen concentration in middle ear fluid and serum samples. Pepsinogen tested positive in all the middle ear fluids. Pepsinogen concentration was significantly higher in the middle ear aspirates compared with serum samples, whereas, albumin concentration was lower. Average middle ear fluid and serum pepsinogen concentration were 262.4 ng/mL (range: 211.7 ng/mL - 301.1 ng/mL) and 102.6 ng/mL (range: 80.7 ng/mL - 134.5 ng/mL), respectively. Tasker et al.[7] in a study conducted on 65 patients identified pepsin/pepsinogen in the middle ear fluid of 59 patients (90.7%). Pepsin/pepsinogen concentration varied between 0.8 and 213.9 μg/mL and 19 (29.2%) displayed proteolytic activity. They used porcine stomach bound antibodies. The drawback of using such antibodies is that they may react with several blood proteins and affect the results. In a study conducted on 22 patients, Lieu et al.,[15] using ELISA, found that 77% of patients tested positive for pepsin/pepsinogenin the middle ear fluid. Pepsin/pepsinogen concentration in the middle ear fluid was measured between 1.33-275 μg/mL using the proteolytic activity and pepsinogen concentration between 2.68-196 μg/mL using ELISA. Abd El-Fattah et al.,[16] using ELISA, found that middle ear fluid pepsin concentration was between 0.085 and 5.020 μg/mL in 17 patients. In a study conducted by He et al.[4] 23 (10%) of 225 effusions tested positive for pepsin (concentration range: 12.5-687 ng/mL). Crapko et al.[17] identified pepsin in 18 (56%) of 32 effusions using the Western blot method (concentration range: 80-1000 ng/mL). O'Reilly et al.[18] determined that pepsinogen tested positive in 47% of purulent effusions. Among these the lowest rate was recorded in the mucoid group (20%). In our study 57.6% of effusions were mucoid, 15.2% purulent and 27.3% serous. A more recent study conducted by O'Reilly et al.[18] showed that 14% of a total of 893 effusions tested positive for pepsin (concentration range: 12.5-2303 ng/mL). Several studies have investigated the possibility of endogenous pepsin/pepsinogen production. Tasker et al.,[7] assessed the ear mucosa of three patients by immunohistochemistry but did not find any evidence of endogenous pepsin/pepsinogen production. In a comparative study, Lieu et al.,[15] failed to demonstrate pepsinogen-I messenger ribonucleic acid (mRNA) on infected mastoid mucosa samples as compared with gastric mucosa samples by using reverse transcriptase polymerase chain reaction (RT-PCR). This study had been criticized on the basis that it was not performed using infected middle ear mucosa samples. Although current evidence obtained from these studies refutes the possibility of endogenous pepsin/pepsinogen production, this issue merits further research because some studies provided evidence of pepsinogen isozymogen production in various tissues such as the lung and prostate and in several malignancies.

Animal studies have shown that the acidic gastric content may cause tubal dysfunction and pass into the middle ear space due to GER.[19] Wittenborg and Neuhauser[20] have radiologically demonstrated that the fluid within the nasopharynx could pass into the middle ear during physiological swallowing. Luo et al.[21] found that the expression levels of pepsinogen protein in adenoid samples in the OME group were significantly higher than those in the adenoid hypertrophy group; however, pepsinogen mRNA could not be detected in either group. These data suggest that the detected pepsinogen protein was not originally produced in adenoid samples, but likely originated from other processes, such as laryngopharyngeal reflux. Our study, in accordance with several previous studies, supports this theory.[7,15,19]

The fact that middle ear effusion contains several proteins such as albumin and fibrinogen suggests the possibility of diffusion of serum pepsinogen into the middle ear. Tasker et al.[7] compared fibrinogen and albumin concentration in the middle ear fluid and serum in eight and 10 patients, respectively. While average fibrinogen and albumin concentration in the middle ear fluid were 1.08 mg/mL (range: 0.30-2.30 mg/mL) and 23.82 mg/mL (range: 1.77-95.75 mg/mL), respectively, serum fibrinogen and albumin concentrations varied between 2.2-4.6 mg/mL and between 35-45 mg/mL, respectively. Several other studies reported similar results.[9,18] Our results are consistent with theirs. Albumin concentration was significantly higher in serum samples compared with the middle ear fluid samples [5.8 g/dL (range: 0.9-9.5 g/dL) vs. 1.1 g/dL (range: 0.01 g/dL - 9.5 g/dL)] ($p<0.001$). Pepsinogen concentration was significantly lower [102.6 ng/mL vs. 262.4 ng/mL, ($p<0.001$)]. While these findings support the view

that these proteins may actually diffuse into the middle ear space; the finding of Tasker et al.[7] that pepsin/pepsinogen concentration is almost 1,000 fold higher in the middle ear fluid compared with serum refutes this possibility.

Our study has several limitations. Performing 24h continuous esophageal PH monitoring and obtaining pepsinogen from the gastric juice could provide more sound evidence to establish causality between OME and GER, but both are invasive procedures and were not considered in the current study. Another limitation is that pepsinogen concentration in the middle ear fluid was compared with serum and not with gastric content because obtaining gastric content is also an invasive procedure. Finally, we could obtain middle ear fluid from both ears in only a small number of patients with bilateral OME and hence could not measure pepsinogen concentration separately.

In conclusion, we found that while pepsinogen concentration was significantly higher in middle-ear aspirates than in serum samples, albumin concentration was significantly lower. Our findings support the theory of GER related pepsinogen transition to the middle ear and suggest GER as a potential cause for OME. Further studies are warranted to better elucidate the pathophysiology of chronic serous otitis media.

Declaration of conflicting interests

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