Tumor Necrosis Factor Alpha Gene Level in Patients with Tympanosclerosis

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ABSTRACT

Background: Tympanosclerosis is the calcification of middle ear connective tissue, including tympanic membrane, which sometimes occurs after middle ear inflammation. Tumor necrosis factor (TNF)-α tends to induce and maintain inflammatory responses in various diseases, including chronic otitis media with effusion (COME).

Aim: To assay plasma level of TNF-α gene expression in patients with tympanosclerosis due to ventilation tube insertion.

Methods: Sixty patients were included in this study. Patients were divided into two groups: Group A: 60 patients, according to inclusion and exclusion criteria, and Group B: 20 volunteers. Patients were subjected to history taking, full clinical examination, and laboratory investigation of TNF-α gene expression [quantitative real-time PCR (qRT-PCR)].

Results: There was a statistical difference between groups according to TNF-α gene expression, as the mean TNF-α in the case group (3.87) was statistically higher than in the control group I, $p < 0.001$. There was a statistical difference in TNF-α level regarding the number of tube insertion ($p = 0.005$) and duration of secretory otitis media (OM) ($p = 0.042$). However, there was no statistical difference in TNF-α level regarding medication use or episodes of acute OM.

Conclusion: The degree of gene profile expression for TNF-α in selected middle ear structures with tympanosclerosis was statistically important, higher than the control group.

Keywords: Chronic otitis media with effusion, COME, OM, Otitis media, PCR, Polymerase chain reaction, QRT-PCR, Quantitative real time PCR, TNF-α, Tumor necrosis factor alpha.

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INTRODUCTION

Tympanosclerosis is the calcification of middle ear connective tissue, including tympanic membrane, which sometimes occurs after middle ear inflammation. Tympanosclerosis is a cheese-like mass of sclerotic material at initial stages that becomes tougher and then becomes a bone-like material.[1]

Tympanosclerosis is characterized by a gradual accumulation of hyaline content across the tympanic membrane and inflammatory hypertrophic submucosa in the middle ear cavity. Calcification along tympanic membranes and ossicles restricts mobilization.[2]

The pathogenesis hypothesis for tympanosclerosis entity is that infection, inflammation, or trauma involving any degree of local immunological hypersensitivity activates these structures' connective tissue portion. With an extreme middle ear infection, the drum's inner surface reveals more permeable mucosal modifications and the lamina propria presented by edematous ground material soaking up water along with components from middle ear disease. Immunoglobulin components present in irregular middle ear secretions allow it to engage in the process. If the connective tissue is impaired, the adsorption and repair helps the body to respond immunologically to the broken tissue, sensitizing this region.[3]

Each episode of chronic middle ear effusion results in a submucosal reaction manifested by collagen deposition. Thus, the characteristic lamellated plaque structure may be represented as several onion layers, each "onion sheet" reflecting an episode of middle ear effusion with its related fibrogenesis describing the lamellated structure of tympanosclerotic plaques.[4]

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Tumor necrosis factor (TNF)-α appears to play a crucial role in causing and maintaining several disease inflammatory reactions, like chronic otitis media with effusion (COME). Recently, cytokine inhibitors were isolated to regulate these cytokines' proinflammatory impact. This led us to hypothesize that the persistent inflammation of COME stemmed from a disparity in the ratio of proinflammatory cytokines and inhibitors favoring proinflammatory cytokines.[5]

TNF-α is produced by macrophages for triggers like bacterial endotoxin and viruses. Mast cells are also a very important source

of this cytokine. Also, it was shown that TNF-α stimulates human basophils and mast cells to generate histamine. IgE-dependent mast cell activation induces fast extracellular release of preformed TNF-α along with histamine and proteoglycans and enhanced mRNA TNF-α.[6]

ELISA has been commonly used to calculate cytokine concentrations. Responsive ELISA sandwich precisely detected MEE cytokines. Results showed a substantial increase of TNF-α in positive allergy community. TNF-α in MEEs can contribute to COME's persistence with allergy.[7]

This research aimed to test TNF-α gene expression in patients with tympanosclerosis due to ventilation tube insertion.

PATIENTS AND METHODS

This prospective randomized controlled study was conducted on sixty patients and 20 volunteers. All patients were selected from ORL clinic of Benha University Hospital. This study was approved by the ethics committee of Faculty of Medicine, Benha University. Informed consents were signed by all subjects.

Patients were divided into two groups—Group A: sixty patients, according to inclusion and exclusion criteria, and Group B: 20 volunteers, with no history of any previous ear diseases and no any other systemic diseases as apparent healthy controls.

Inclusion Criteria

- Patients' ages were between 3 and 15 years old.
- Persistent MEE for 3 months.
- History of acute otitis media in the preceding 3 months.
- History of multiple placements of tympanostomy tubes.

Exclusion Criteria

- Systemic diseases.
- Acute upper respiratory tract infection.
- Craniofacial anomalies.

Patients were subjected to history taking, full clinical examination, and laboratory investigation of TNF-α gene expression.

Sampling

A venous blood sample (2 mL) was taken from each subject and put into a plain sterile vacutainer tube; blood will be left to clot and then centrifuged at 3000 rpm; serum was separated and put into sterile Eppendorf tubes and refrigerated at −80°C till the time of RNA extraction.

Quantitation of TNF-α gene expression: TNF-α gene expression was measured by quantitative real-time PCR (qRT-PCR) through the following steps:

- Total mRNA extraction from 100 μL serum by Total RNA Purification Kit (Jena Bioscience, Germany), as per the manufacturer's instructions. Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) performed RNA ultraviolet spectrophotometric quantification. Pure RNA preparations have 1.9-2.3 optical density ratio at 260/280 nm.[8]
- qRT-PCR for TNF on two steps:
  - The first step [reverse transcription (RT) of RNA into cDNA]: It was performed by HiSenScript™ RH(−) cDNA synthesis kit (Intron Biotechnology, Korea) using Veriti™ Thermal Cycler (Applied Biosystems, USA). The RT mix for each sample contained 2× RT reaction solution (10 μL), enzyme solution

(1 μL), RNA template (2 μL), and nuclease-free water (7 μL). Incubation of RT mix at 45°C for 1 hour was performed, followed by RTase inactivation at 85°C for 10 minutes.
  - Second step (quantitation of TNF mRNA using specific primers): StepOne real-time PCR (*Applied Biosystem, Singapore*) was used. Singleplex reactions occurred. Hera plus Sybr Green qPCR master mix (Life Technologies Corporation, UK) was used. Human GAPDH was the housekeeping gene.

The primers were as follows: TNF-α; FP: 5′-CCCAGGCAGT CAGATCATCTTC-3′, RP: 5′-AGCTGCCCCTCAGCTTGA-3′, GAPDH; FP: TGAACGGGAAGCTCACTGG and RP: TCCACCACCCTGTTGCTGTA.[9]

The master mix for qRT-PCR contained 10 μL master mix, 1 μL FP, 1 μL RP, 2 μL cDNA, and 6 μL nuclease-free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 minutes and cycling (45 cycles) that includes denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 30 minutes. Melting curve analysis was done in each run to confirm the specificity of the reaction.

Statistical Analysis

A report type reported clinical results. These results were tabulated and evaluated using the SPSS (Statistical Kit for Social Science) version 20 to obtain: for the data, descriptive statistics were measured in the form of mean and standard deviation, and median and interquartile range (IQR) for quantitative data. Frequency and qualitative data delivery. Analytical statistics: In statistical comparison of various categories, the value of variance was checked using one of the following tests: Student's t-test—used to equate the mean of two quantitative data groups; ANOVA measure (F value)—compare the mean of more than two quantitative data classes. The intergroup analysis of categorical data was conducted using Chi-square (χ^2-value) and Fisher's exact test (FET) measures. Correlation coefficient helps in finding the relationships among variables. Statistically important was a p-value < 0.05.

RESULTS

In this study, patients were divided into two groups—Group A: sixty patients, according to inclusion and exclusion criteria. Group B: 20 volunteers, with no history of any previous ear diseases and no any other systemic diseases as apparent healthy controls. There was no statistical difference between cases and controls regarding their age or gender.

According to TNF-α gene expression, there was a statistical difference between groups, as the mean TNF-α in the case group (3.87) was statistically higher than in the control group I, $p < 0.001$ (Fig. 1).

ROC curve was done to assess the validity of TNF-α mRNA (RQ) in the detection of cases; AUC was 0.979 (CI: 0.952–1.0), at a cutoff value of 1.33; sensitivity was 100, specificity was 80, PPV was 93.8, NPV was 100, and accuracy was 95% (Fig. 2).

There was a statistical difference in TNF-α level regarding the number of tube insertion ($p = 0.005$) and duration of secretory otitis media (OM) ($p = 0.042$). However, there was no statistical difference in TNF-α level regarding medication use or episodes of acute OM (Table 1).

There was a significant positive correlation between TNF-α, and medication use and episodes of acute OM, and there was a significant negative correlation between TNF-α and the number of tube insertion. However, there was no significant correlation between TNF-α and duration of secretory OM (Table 2).
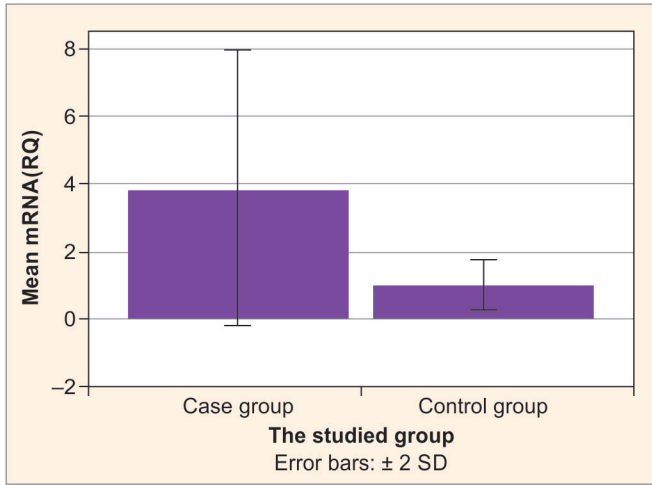
Fig. 1: Comparison between case and control groups according to TNF-α gene expression mRNA (RQ)
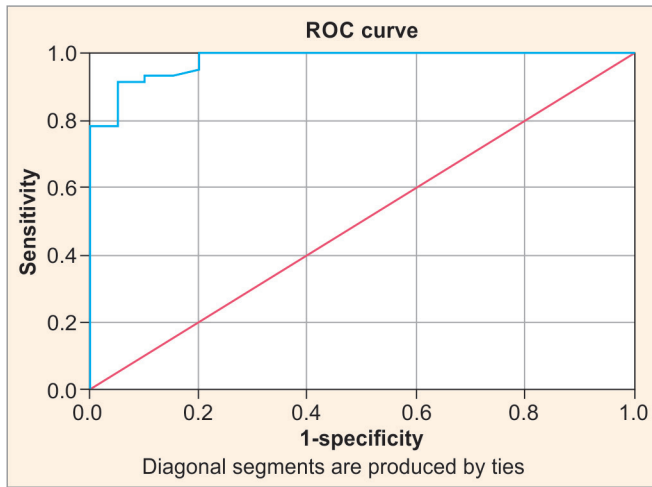
Table 1: Comparison between TNF-α mRNA (RQ) level in otitis media cases according to their medical history

		TNF-α mRNA (RQ)		Statistical	
	No (60)	Mean	±SD	test	p-value
Medication use (Antibiotics)					
Yes	29	4.51	1.73	St $t = 1.33$	0.19
No	31	3.51	2.28		
Number of tube insertion					
One	37	4.79	1.96	St $t = 2.91$	0.005[**]
More than one	23	3.30	1.91		
Episodes of acute OM					
Once	16	4.32	2.36	$F = 2.43$	0.097
Twice	7	5.05	2.0		
None	37	3.45	1.83		
Duration of secretory OM					
3 months	37	4.47	1.77	St $t = 1.99$	0.042[*]
>3 months	23	3.52	2.32		

St t, Student's t-test; F, one-way ANOVA; [*]Significant; [**]Highly significant; OM, otitis media; TNF-α, tumor necrotic factor-alpha; mRNA, messenger RNA; RQ, relative quantitation

Fig. 2: ROC curve of validity of TNF-α mRNA (RQ) in detection of cases

Table 2: Correlation between TNF-α mRNA (RQ) and medical history of the studied OM cases

TNF-α mRNA (RQ)	Correlation coefficient	p-value
Medication use (antibiotics)	0.281	0.03[*]
Number of tube insertion	−0.436	<0.001[**]
Episodes of acute OM	0.28	0.03[*]
Duration of secretory OM	0.252	0.052

[*]Significant; [**]Highly significant; OM, otitis media; TNF-α, tumor necrotic factor-alpha; mRNA, messenger RNA, RQ, relative quantitation

Discussion

The study showed statistically significant, higher transcription of the TNF-α proinflammatory cytokine gene in plasma samples taken from patients with tympanosclerosis, compared to the control group.

These results coincide with Mionskowski et al.,[6] who demonstrated that total RNA was isolated from tissues according to the Chomczyński and Sachi method with own modifications. Isolated RNA served as a template in the one-step multiplex PCR in real time using Taq Man probes. To determine the level of mRNA, the ratio of the transcript level for the TNF-α gene to the transcript level for the reference gene (β-actin) was determined. TNF-α gene expression in tympanic membranes obtained from patients with tympanosclerosis the reference gene was significantly higher than in the middle ear mucosa and tympanic membranes collected from the corpse ($p = 0.00001$). The median value of TNF-α gene expression was 1.41 in the control group and 24.78 in the study group.

Maxwell et al.,[10] said age was tested at intervals and had no statistically relevant association with TNF-α ($p = 0.46$). TNF-α showed a substantial tendency toward lower values as the recurrence of tube placements increased ($p = 0.008$) and variations remained significant when assessed nominally ($p = 0.02$; 3.87 ± 0.31 pg/mg of total protein for initial tympanostomy vs 2.66 ± 0.41 pg/mg of total protein for previous tympanostomies). Drug consumption was examined nominally. TNF-α increased with antibiotic history, but was not dramatically different from children without perioperative antibiotics. TNFsolR appeared to be increased with antibiotic history, but there was no statistical variation in TNFsolR calculated depending on perioperative antibiotic use.

Other research such as Alpay et al.[11] stated that TLR4 gene variant genotype has a role in the inflammatory process, and it has been shown to minimize IL-6, IL-10, and TNF-α cytokine expressions. The decreased expressions of these cytokines induce systemic inflammation that contributes to long-term transformation of macrophages into osteoclasts that can cause tympanosclerotic plaque.

According to Yellon et al.,[12] children under repeated tympanostomy had mean TNF-α levels around 14 times greater than children undergoing their first tympanostomy. The same relation was found for IL-1β and IL-6.

However, Willett et al.[13] found no significant correlation between the sum of previous tube insertions and TNF-α and IL-1β concentrations in MEEs.

Skotnicka and Hassmann,[14] observed a marginal statistical significance of the interaction between TNF-α and IL-8 ($r = 0.31$; $p = 0.056$). However, no relation was found between IL-10 concentrations and other cytokines tested. There was no correlation between age, duration of hearing loss, number of acute otitis media episodes, and cytokine concentrations measured. During otoscopic studies, no statistically significant correlation was observed between tympanic membrane pathology and cytokine levels.

CONCLUSION

The level of gene profile expression for TNF-α in selected structures of the middle ear with tympanosclerosis was statistically significant, higher in comparison with the control group.

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