

The Degree of Gene Profile Expression for Tumor Necrosis Factor-alpha Gene Level in the Pediatric Tympanosclerotic Ear

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Abstract

Background: Tympanosclerosis, which can sometimes follow middle-ear inflammation, is the calcification of middle-ear connective tissue, especially the tympanic membrane. Numerous disorders, including chronic otitis media (OM) with effusion, are characterized by tumor necrosis factor (TNF) propensity to induce and maintain inflammatory reactions. **Aim:** This study aims to measure the expression of the TNF gene in the plasma of those who have tympanosclerosis as a result of ventilation tube insertion. **Patients and Methods:** In this study, 160 patients were included. The patients were split into two groups, Group A, which consisted of 120 patients and was subjected to inclusion and exclusion criteria, and Group B, which consisted of 40 volunteers. The patients underwent a full clinical examination, a history taker, and a laboratory investigation of TNF gene expression (quantitative real-time polymerase chain reaction). **Results:** According to TNF gene expression, there was a statistical difference between the groups; the mean TNF in the cases group (3.87) was statistically higher than the control group (1), $P=0.001$. TNF levels statistically differed according to the quantity of tubes inserted ($P=0.005$) and the length of the secretory OM ($P=0.042$). TNF levels were not statistically different according to medication use or acute OM bouts. **Conclusion:** In comparison to the control group, the level of TNF gene profile expression in a few middle-ear structures with tympanosclerosis was statistically significant.

Keywords: Otitis media, polymerase chain reaction, Tumor necrosis factor- α

INTRODUCTION

The condition known as tympanosclerosis, which can occasionally follow middle-ear inflammation, is the calcification of connective tissue in the middle ear, including the tympanic membrane. Tympanosclerosis begins as a mass of sclerotic material that resembles cheese but eventually hardens and turns into bone-like material.^[1]

Hyaline content gradually builds up across the tympanic membrane in tympanosclerosis, which is also characterized by inflammatory hypertrophic submucosa in the middle-ear cavity. Mobilization is hampered by calcification along tympanic membranes and ossicles.^[2]

According to the pathogenesis theory for tympanosclerosis, the connective tissue portion of these structures is activated by infection, inflammation, or trauma involving any level of local immunological hypersensitivity. When a middle-ear

infection is severe, the inner surface of the drum exhibits more permeable mucosal changes, and the lamina propria is affected by edematous ground substance that is absorbing water and middle-ear disease components. It can participate in the process since immunoglobulin components are found in irregular middle-ear secretions. Adsorption and repair assist the body in responding immunologically to the damaged connective tissue if it is defective, sensitizing this area.^[3]

Every time there is a persistent middle-ear effusion, the submucosa reacts by depositing collagen. As a result, the distinctive lamellated plaque structure can be visualized as a series of onion layers, with each "onion sheet" representing a middle-ear effusion event and the associated fibrogenesis that describes the lamellated structure of tympanosclerotic plaques.^[4]

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A number of disease-related inflammatory responses, including chronic otitis media (OM) with effusion, appear to be greatly influenced by tumor necrosis factor (TNF), which may also play a critical role in their maintenance (COME). To control the pro-inflammatory effects of these cytokines, cytokine inhibitors have recently been discovered. This prompted us to postulate that a discrepancy in the ratio of pro-inflammatory cytokines and inhibitors favoring pro-inflammatory cytokines was the cause of the COME's prolonged inflammation.^[5]

TNF is created by macrophages in response to viruses and bacterial endotoxins as triggers. Another significant source of this cytokine is mast cells. It has also been demonstrated that TNF promotes the production of histamine by human mast cells and basophils. Fast extracellular release of preformed (TNF), histamine, proteoglycans, and increased mRNA (TNF) are all caused by IgE-dependent mast cell activation.^[6]

It has been a routine practice to estimate cytokine concentrations using enzyme-linked immunosorbent assay (ELISA). Middle ear effusions (MME) cytokines were precisely identified by responsive ELISA sandwich. Results revealed a significant rise in (TNF) the allergy-positive group. TNF-alpha (TNF- α) in MEEs may be a factor in OME's continued allergy symptoms.^[7]

The purpose of this study was to examine the gene expression of TNF in individuals with tympanosclerosis brought on by ventilation tube insertion.

PATIENTS AND METHODS

The 120 patients and 40 volunteers who participated in this prospective, randomized, controlled research. All patients were chosen from the Benha University Hospital ORL clinic. The Benha University Faculty of Medicine's ethics committee gave the study the thumbs up. Each individual voluntarily signed an informed consent form. Two groups of patients were created according to inclusion and exclusion criteria, Group A consists of 120 patients, whereas Group B consists of 40 volunteers who have no history of ear conditions or other systemic diseases and appear to be healthy controls.

Inclusion criteria

- The patients' ages ranged from 3 to 15
- Consistent MEE for 3 months
- The history of tympanostomy tube placements.

Exclusion criteria

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

Patients underwent a thorough physical examination, a history review, and a laboratory test to measure the expression of the TNF gene.

Sampling

Each patient had 2 ml of venous blood drawn, which was then placed in a plain, sterile vacutainer container. After allowing the blood to coagulate, it was centrifuged at 3000 rpm to

separate the serum, which was then placed in sterile Eppendorf tubes and stored at 80°C until RNA extraction.

Quantitation of TNF- α gene expression

The following procedures were used to evaluate TNF gene expression using quantitative real-time polymerase chain reaction (qRT-PCR):

- Total RNA Purification Kit (Jena Bioscience, Germany) total mRNA extraction from 100 μ l serum in accordance with manufacturer's instructions
- RNA UV spectrophotometric measurement was carried out using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA)
- At 260/280 nm, pure RNA preparations have an optical density ratio of 1.9–2.3.^[8]
- TNF qRT-PCR in two steps:
 - Reverse transcription (RT) of RNA into cDNA, the first step, it was carried out utilizing a Veriti™ Thermal Cycler and the Hisenscript RH (-) cDNA Synthesis Kit from iNtRON Biotechnology, Korea (Applied Biosystems, USA). Each sample's RT mix included nuclease-free water, enzyme solution, and 2 μ l of RNA template in addition to 10 μ l of 2X RT reaction solution (7 μ l). RTase inactivation was carried out after the RT mix had been incubated at 45°C for an hour.

Use of Step1 RT-PCR in the second step (quantitation of TNF mRNA using particular primers) (Applied Biosystem, Singapore). There were single-plex responses.

Master mix for Hera plus Sybr Green qPCR (Life Technologies Corporation, UK).

Human GAPDH served as the housekeeping gene. The following were the primers: TNF- α ; FP: 5'-CCCAGGCAGTCAGATCATCTTC-3' AGCTGCCCTCAGCTTGA-3', GAPDH; FP: TGAACGGGAAGCTCACTGG and RP: TCCACCACCCTGTTGCTGTA.^[9]

About 10 μ l of master mix, 1 μ l of FP, and 1 μ l of RP made up the qRT-PCR master mix.

6 μ l of nuclease-free water and 2 μ l of cDNA. Thermal cycling was performed under the following conditions: initial denaturation at 95°C for 2 min; cycling (45 cycles); denaturation at 95°C for 30 s; and annealing/extension at 60°C for 30 min. Each run included melting curve analysis to ensure the reaction's specificity.

Statistical analysis

Clinical outcomes were provided in a report type. The SPSS (Statistical Kit for Social Science) version 20 (IBM Corp., Armonk, NY, USA) was used to tabulate and analyze these results to derive the following descriptive statistics: mean, standard deviation, median, interquartile range for quantitative data, and frequency and delivery of high-quality data. Statistical analysis in a statistical comparison of multiple categories, the Student's *t*-test was used to determine the variance value – used to compare the means of two groups of quantitative data. Compare the means

Table 1: Comparison of tumor necrosis factor messenger RNA (relative quantitation) levels in instances of otitis media based on their medical histories

	<i>n</i> (60)	TNF- α mRNA (RQ), mean \pm SD	Statistical test	<i>P</i>
Medication use (antibiotics)				
Yes	59	4.53 \pm 1.72	St <i>t</i> =1.33	0.19
No	61	3.52 \pm 2.27		
Number of tube insertion				
1	67	4.78 \pm 1.95	St <i>t</i> =2.91	0.005**
>1	53	3.31 \pm 1.92		
Episodes of acute OM				
Once	36	4.31 \pm 2.35	<i>F</i> =2.43	0.097
Twice	7	5.06 \pm 2.1		
None	67	3.44 \pm 1.82		
Duration of secretory OM (months)				
3	67	4.46 \pm 1.72	St <i>t</i> =1.99	0.042*
>3	53	3.51 \pm 2.31		

*Significant, **Highly significant. St *t*: Student *t*-test, *F*: One way ANOVA, OM: Otitis media, TNF- α : Tumor necrotic factor-alpha, mRNA: Messenger RNA, RQ: Relative quantitation, SD: Standard deviation

Table 2: Relationship between the examined otitis media case's medical histories and tumor necrosis factor messenger RNA (relative quantitation)

TNF- α mRNA (RQ)	Correlation coefficient	<i>P</i>
Antibiotics use	0.280	0.03*
Insertions of tubes: <i>n</i> instances of severe OM	-0.435	<0.001**
length of the secretory OM	0.27	0.03*
	0.253	0.052

*Significant, **Highly significant. OM: Otitis media, TNF- α : Tumor necrotic factor-alpha, mRNA: Messenger RNA, RQ: Relative quantitation

of more than two classes of quantitative data using the ANOVA measure (*F* value). Fisher's exact test and Chi-square metrics were used to conduct the intergroup analysis of categorical data. Discovering links between variables using the correlation coefficient. A *P* < 0.05 was statistically significant.

RESULTS

Patients in this study were split into two groups: 120 patients were assigned to Group A based on inclusion and exclusion criteria, and Group B consists of 40 volunteers who appear to be healthy controls and have no history of ear problems or other systemic disorders. Regarding age or gender, there was no statistical difference between cases and controls.

The mean TNF in the cases group (3.87) was statistically greater than the control group (1), *P* = 0.001, indicating a statistical difference between the groups based on TNF gene expression.

TNF levels statistically differed depending on the quantity of tubes inserted (*P* = 0.005) and how long secretory OM persisted (*P* = 0.042). Despite the fact that there was no statistically significant difference in the TNF level between medication use and acute OM episodes [Table 1].

TNF and (medication use and episodes of acute OM) showed a substantial positive correlation, whereas TNF and (number

of tube insertions) showed a significant negative correlation. TNF and secretory OM duration did not significantly correlate with one another [Table 2].

DISCUSSION

In comparison to the control group, plasma samples taken from individuals with tympanosclerosis had statistically significant increased levels of transcription of the (TNF) pro-inflammatory cytokine gene. These findings are consistent with those of Sari *et al.*^[6] who showed that total RNA was recovered from tissues using a modified version of the Chomczynski and Sacchi method. Using TaqMan probes and one-step multiplex PCR in real time, isolated RNA was used as a template. The ratio of the TNF gene's transcript level to that of the reference gene (β -actin) was used to estimate the quantity of mRNA. In comparison to middle-ear mucosa and tympanic membranes collected from corpses, the expression of the reference gene, TNF, was considerably higher in tympanic membranes taken from patients with tympanosclerosis (*P* = 0.00001). TNF gene expression had a median value of 1.41 in the control group and 24.78 in the study group.

According to Maxwell *et al.*,^[10] age was evaluated periodically and showed no statistically significant correlation with TNF (*P* = 0.46). When assessed nominally, variations remained significant (*P* = 0.02; 3.870.31 pg/mg of total protein for the initial tympanostomy vs. 2.660.41 pg/mg of total protein for previous tympanostomies) and TNF showed a significant tendency toward lower values as the number of tube placements increased (*P* = 0.008). The use of drugs was looked at nominally. When compared to children who did not receive perioperative antibiotics, TNF increased with antibiotic history but did not significantly differ. Although there was no statistically significant difference in TNF soluble receptor (TNFsolR) estimated depending on perioperative

antibiotic use, it appeared that TNF α was raised with antibiotic history.

It has been demonstrated that the toll-like receptor 4 gene variation genotype plays a role in the inflammatory process and it has been proven to reduce interleukin-6 (IL-6), IL-10, and TNF cytokine expressions, according to other research by Alpay *et al.*^[11] Reduced levels of these cytokines create a generalized inflammatory response that aids in the long-term conversion of macrophages into osteoclasts, which can result in tympanosclerotic plaque.

Yellon *et al.*^[12] found that children who had repeated tympanostomies had mean TNF levels that were roughly 14 times higher than those of children who had just had their first tympanostomy. The same correlation between IL-1 and IL-6 was discovered.

The number of prior tube insertions and the levels of TNF and IL-1 in MEEs did not, however, significantly correlate, according to Willett *et al.*^[13]

The interaction between TNF and IL-8 was found to be marginally statistically significant by Skotnicka and Hassmann^[14] ($r = 0.31$; $P = 0.056$). Regarding the levels of the other cytokines examined and IL-10, no correlation was discovered. Age, the length of the hearing loss, the number of acute OM episodes, and the observed cytokine concentrations did not correlate with one another. Tympanic membrane pathology and cytokine levels were not statistically correlated with otoscopic examinations.

CONCLUSION

In comparison to the control group, there was a statistically significant increase in the level of TNF gene expression in a few middle-ear structures.

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Conflicts of interest

There are no conflicts of interest.

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