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High TNF-α Levels in Active Phase Chronic Suppurative Otitis Media Caused by Gram-positive Bacteria

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Background: Chronic suppurative otitis media (CSOM) is a persistent inflammatory disease of the middle ear and mastoid cavity caused by pathogenic infection. CSOM has a fairly high incidence in developing countries and is the main cause of acquired hearing loss in children. Tumor necrosis factor alpha (TNF-α) is a significant inflammatory mediator in CSOM. This study aimed to analyze TNF-α levels in ear discharge and blood serum in active phase CSOM caused by Gram-positive and Gram-negative bacteria.

Materials and Methods: This research was an analytical observational study with a cross sectional design. Blood serum and ear discharge from CSOM patients were used in this study. Blood serum and ear discharge TNF-α levels were measured using enzyme-linked immunosorbent assay.

Results: From 26 CSOM subjects, 13 subjects were infected with Gram-positive bacteria and the 13 others were infected with Gram-negative bacteria. The majority of the subjects were male (53.8%) with an age range from 36-45 years (42.3%). The most common species of bacteria was *Pseudomonas aeruginosa*. Blood serum and ear discharge TNF-α levels were higher in samples that contained Gram-positive bacteria.

Conclusion: TNF-α levels in active phase CSOM caused by Gram-positive bacteria are higher than those which are caused by Gram-negative bacteria.

Keywords: *chronic suppurative otitis media, TNF-α, gram-positive, gram-negative*

Introduction

Chronic suppurative otitis media (CSOM) is a chronic inflammation in the middle ear and mastoid cavity with perforated tympanic membrane and history of prolonged or intermittent ear discharge (otorrhea) for more than 2 months caused by pathogenic infection.[1,2] otitis media causes the prescription of antibiotics in children which then causes antibiotic resistance. CSOM is a health problem with a fairly high incidence in developing countries and is the main cause of acquired hearing loss in children. This is due to the low level of the community's economy, nutritional status, and education.[3,4]

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The prevalence of CSOM worldwide ranges from 65-330 million cases. In Southeast Asia, the prevalence of CSOM is 5.2% of the entire population, while in Indonesia it is 3.0% of the entire population. In Indonesia, CSOM is found to be significantly higher in rural areas than in urban areas, and almost 40% of the entire population suffers from ear disease (13.9% external ear disease, 6.7% middle ear disorder, 16.8% hearing loss). The World Health Organization (WHO) estimates that in 2018 around 486 million cases of hearing loss occurred around the world with 3.6% and 0.27% of the cases being caused by active and inactive phase CSOM, respectively. Hearing loss due to CSOM is a major form of disability in Southeast Asia that causes a significant social and educational burden. Hearing loss is a serious concern in both children and adults. In children, hearing loss has a long-term effect on the development of communication, language, educational processes, and achievement. Meanwhile adult CSOM patients are a burden to individuals, families, and society.[3–6]

CSOM is a multifactorial disease due to interaction of bacteria, environment and host. Species of bacteria that are often identified are *Pseudomonas aeruginosa* which is a Gram-negative aerobic bacteria and *Staphylococcus aureus* which is a Gram-positive aerobic bacteria. CSOM can cause intratemporal and intracranial complications.[1] The development and use of appropriate antibiotics have reduced complications due to CSOM. However, as pathogens are resistant to various antibiotics, the incidence of complications has increased again.[7,8]

Infiltration of pathogens into the human body causes inflammatory reactions. Cytokines production is one of the mechanisms that cause inflammation.[9] Although cytokines are produced to modulate immune response to fight pathogenic infection, overproduction of cytokines is responsible for severe damage observed in infections caused by microorganisms.[9] Tumor necrosis factor alpha (TNF-α) is one of the cytokines that is crucial for immunity. TNF-α is a significant inflammatory mediator in otitis media. Bacterial infection induces the production of inflammatory mediators. TNF-α level is higher in Gram-positive bacterial infection than Gram-negative bacterial infection. Bacterial infection in otitis media, which in turn increases the TNF-α level, is considered to increase the probability of relapse, longer disease course and chronicity.[10]

Previous studies have been conducted to examine the proinflammatory mediators produced by the human body in response to pathogenic infections. However, the differences in TNF-α levels in active phase CSOM caused by different types of bacteria have not been elucidated yet. This study analyzed TNF-α levels in ear discharge and blood serum in active phase CSOM caused by Gram-positive and Gram-negative bacteria using enzyme-linked immunosorbent assay (ELISA).

Materials and methods

Study Design and Subjects

A cross sectional study was conducted from January-May 2022 at the Ear, Nose, and Throat Polyclinic, Dr. Moewardi General Hospital, Universitas Sebelas Maret Hospital, and Sibela Health Centre, Surakarta. A total of 26 patients with active mucosal CSOM were enrolled in this study. Subjects were diagnosed with CSOM if they had repeated otorrhea within 2 months of recruitment. Subjects were excluded from the study if they had medical and/or surgical procedures or illnesses, such as infection and/or inflammation either in acute or chronic form. The study protocol was approved by Research Ethics Committee, Dr. Moewardi General Hospital (No. 397/III/HREC/2022) and all subjects signed the informed consent.

Ear Discharge Collection and Bacteria Culture

To prevent contamination by microorganisms from the external auditory canal, middle ear discharge was collected using sterile small cotton-tip swabs (Copan Diagnostics Inc, Jefferson, USA) under the direction of an otomicroscope. Swabs of purulent discharge were cultured on blood and MacConkey agar plates (Oxoid, Hampshire, England) overnight at 37°C. The microorganisms were identified using routine bacterial organism identification procedures.

Blood Serum Collection

Venous blood serum was collected from all subjects and placed in serum separator tube (SST) vacutainers. Fresh blood was allowed to clot for 30 minutes at 25°C before centrifugation at 3,500 rpm for 15 minutes. The serum was stored at -80°C for a day before it was used for further assay, then it was stored at -20°C.

ELISA

The serum and ear discharge TNF-α levels were determined using ELISA. The human TNF-α ELISA kit (Elabscience, Hubei, China) engages an antibody specific for human TNF-α. The standard curves were created

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using recombinant human TNF-α. Sample absorbance was measured at the wavelength of 450 nm.

Statistical Analysis

Data was analyzed using Statistical Package for the Social Sciences (SPSS) Statistic version 25 (IBM Corporation, Armonk, NY, USA). The normality of the data was tested with Shapiro–Wilk test. The Mann–Whitney test was used to determine whether there were significant differences in TNF-α levels in blood serum and ear discharge based on gender and types of bacteria. Statistical significance was defined as a $p<0.05$.

Results

The majority of the subjects in this study (11 subjects; 42.3%) were 36-45 years, with 14 male (53.8%) and 12 female (46.2%) subjects. The percentages of subjects who sustained conductive hearing loss (CHL) and moderate hearing loss (MHL) were 84% and 50%, respectively (Table 1). From a total of 26 subjects, there were 13 subjects that were infected with Gram-positive bacteria and 13 others were infected with Gram-negative bacteria. Furthermore, the most common bacteria causing CSOM was *P. aeruginosa* (30.8%) (Table 2).

TNF-α levels in blood serum and ear discharge were not normally distributed. Therefore, the data was expressed as median value (min-max) and analyzed with Mann-Whitney test. The blood serum TNF-α level was 21.06 (5.62-109.49) pg/mL, while the ear discharge TNF-α level was 28.53 (0.22-213.41) pg/mL. There was no significant difference between blood serum and ear discharge TNF-α levels in patients with active phase CSOM (p=0.367).

Table 1. Characteristics of research subjects.

Characteristics	Values
Age (years old±SD)	39.38±11.06
Gender, n (%)	
Male	14 (53.8)
Female	12 (46.2)
Degree of hearing loss, n (%)	
Mild	5 (19)
Moderate	13 (50)
Moderately severe	4 (15)
Severe	1 (4)
Profound	3 (11)
Types of hearing loss, n (%)	
CHL	22 (84)
MHL	3 (12)
Death ear	1 (4)

SD: standard deviation.

Table 2. Bacteria isolated from ear discharge samples.

Types of Bacteria	n (%)
Gram-positive	
Corynebacterium striatum	5 (19.2)
Staphylococcus aureus	2 (7.7)
Staphylococcus epidermidis	2 (7.7)
Enterococcus avium	1 (3.8)
Staphylococcus haemolyticus	1 (3.8)
Staphylococcus kloosii	1 (3.8)
Staphylococcus xylosus	1 (3.8)
Gram-negative	
Pseudomonas aeroginosa	8 (30.8)
Citrobacter koserii	2 (7.7)
Acinetobacter sp.	1 (3.8)
Klebsiella sp.	1 (3.8)
Providencia stuartii	1 (3.8)

The blood serum and ear discharge TNF-α levels in male subjects were lower compared with those in female subjects. There were no significant differences in serum and ear discharge TNF-α levels between male and female subjects with active phase CSOM (Table 3).

The blood serum TNF-α levels in samples that contained Gram-positive bacteria were significantly higher compared with those that contained Gram-negative bacteria. The ear discharge TNF-α levels in samples that contained Gram-positive bacteria were also higher compared with those that contained Gram-negative bacteria. However, there was no significant difference in ear discharge TNF-α levels in subjects with active phase CSOM caused by Gram-positive bacteria compared with Gram-negative bacteria (Table 4).

Discussion

CSOM often causes hearing loss. In this study, all patients had hearing loss with 22 patients (84%) suffering from CHL and 13 patients had MHL. The presence of perforation in the tympanic membrane as well as the presence of fluid that fills the ear canal and middle ear can interfere with sound transmission to inner ear and cause sound conduction disturbances, while hair cell damage due to bacterial infection and long-term use of topical antibiotics that causes absorption of

Table 3. TNF-α levels in active phase CSOM based on gender.

TNF-α Levels	Male (n=14)	Female (n=12)	*p* -value
Serum (pg/mL)	17.62 (8.46-97.38)	22.62 (5.62-109.49)	0.471
Ear discharge (pg/mL)	19.18 (0.22-120.93)	32.97 (11.68-213.41)	0.100

*$p<0.05$.

chemicals in round window membrane cause sensorineural sound disturbances.[11,12]

Bacterial infection is the dominant factor in most CSOM cases. The most common species of bacteria found in this study was *P. aeruginosa*. This was in accordance with previous research which stated that the main cause of CSOM in the tropics was *P. aeruginosa*, a Gram-negative aerobic bacterium. *P. aeruginosa* is recognized by immune cells but is able to defend against antimicrobial components through biofilm formation. This bacterium also has extracellular enzymes that cause tissue necrosis through epithelial damage, impaired blood circulation and loss of tissue protection.[13–17] In another study, it is stated that the most common cause of CSOM was *S. aureus*, followed by *P. aeruginosa*. This could be caused by differences in geographical variations.[18] Bacterial infection activates the innate and adaptive immune system. In the middle ear and the Eustachian tube, the innate immune system consists of structures with barrier functions (mucociliary clearance and mucous membrane), bacteria-sensing receptors, *i.e.* Toll-like receptors (TLRs) of the middle ear epithelium, as well as inflammatory and specific immune responses, such as effector cells (natural killer cells, macrophages, neutrophils, fibroblasts, eosinophils) recruitment, mast cells and mucosal dendritic cells (DCs), as well as peptides and proteins with antimicrobial activities (defensins and lysozyme). TNF-α, an early inflammatory cytokine that manifests in bacterial infections, is produced by the middle ear mucosa as a consequence of the accumulation of debris and bacterial metabolites in the middle ear.[19]

The biological effect of TNF-α on the systemic inflammatory response is most notable in the deposition of neutrophils and monocytes at the site of infection, induction of chemotaxis and leukocyte deposition, as well as molecular adhesion of vascular endothelial cells to leukocytes. TNF-α acts on cells that secrete it (autocrine), adjacent cells (paracrine), or cells located in different organs (endocrine). Previous studies stated that TNF-α as a biomarker for otitis media with persistent effusion and signs of chronicity. In the ear, TNF-α induces goblet cell hyperplasia, leading to excess production of thick mucus and enabling middle ear cell proliferation, which in turn promotes impaired mucociliary clearance. Low levels of TNF-α are found in the healthy body, and the level of this cytokine will increase in acute and chronic inflammatory conditions.[9,20–25]

There was no significant difference between blood serum and ear discharge TNF-α levels in patients with active phase CSOM. Therefore, the measurement of TNF-α levels in CSOM patients could be done both on blood serum samples and ear discharge. TNF-α levels in the ear discharge were higher compared with those in the blood serum. This phenomenon may be caused by bacterial infection that primarily took place in the middle ear where the ear discharge samples were obtained. However, there is no study that compares blood serum and ear discharge TNF-α levels in active phase CSOM patients.

Higher levels of TNF-α in blood serum and ear discharge were found in female subjects. Blood serum and ear discharge TNF-α levels were not significantly different between male and female subjects. A study stated that higher levels of TNF-α were found in men. Male hormones (high testosterone and low estrogen) predispose to increased inflammatory responses. In females, hormonal changes play an important role in the alteration of cytokine levels. Several physiological, pathological, and therapeutic conditions can alter estrogen hormone levels, including menstrual cycle,

Table 4. TNF-α levels in active phase CSOM based on the types of bacteria.

TNF-α Levels	Gram-positive (n=13)	Gram-negative (n=13)	*p* -value
Serum (pg/mL)	33.87 (8.46-109.49)	13.62 (5.62-37.58)	0.005*
Ear discharge (pg/mL)	35.75 (0.22-213.41)	20.80 (0.46-55.92)	0.130

*$p<0.05$.

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pregnancy, menopause, and medications (corticosteroids, oral contraceptives, and hormone replacement therapy). It is also associated with diseases such as diabetes, atherosclerosis, and cardiovascular diseases. Increased levels of cytokine in women with estrogen deficiency are due to an increase in cytokine receptors and cofactors that facilitate the action of cytokines. The results of the present study were not in line with in this study. This may be caused by external hormonal factors in women, such as the use of contraceptives and the menstrual cycle that suppresses estrogen.[26,27]

In this study, serum TNF-α levels were significantly higher in samples that contained Gram-positive bacteria compared with those that contained Gram-negative bacteria. From the ear discharge samples, the TNF-α levels were higher in samples that contained Gram-positive bacteria compared with those that contained Gram-negative bacteria, although the TNF-α levels between both types of samples were not significantly different. Higher levels of TNF-α in Gram-positive bacteria were in accordance with the literature which stated that higher levels of TNF-α are found in infection caused by Gram-positive bacteria compared with infection with Gram-negative bacteria. Gram-positive bacteria have a thick peptidoglycan layer that surrounds a single layer of lipid membrane in their walls. The main components of the cell wall of Gram-positive and Gram-negative bacteria are lipoteichoic acid and lipopolysaccharide, respectively. Gram-positive bacteria have a thick peptidoglycan layer. This layer triggers the production of cytokines.[22,27–30] The Gram-positive component is recognized by TLR2 and TLR4 while the Gram-negative component is only recognized by TLR4. This causes a large influx of neutrophils and increases TNF-α levels higher in Gram-positive bacterial infections.[31] Gram-positive bacteria are strong inducers of TNF-α compared to Gram-negative bacteria while Gram-negative bacteria are strong inducers of interleukin-1 beta (IL-1β).[30]

CSOM is often preceded by an upper respiratory tract infection. Spread of infection to the middle ear triggers goblet cell hyperplasia. Inflammatory reactions in CSOM mediate the expression of mucin genes such as *MUC5B* and goblet cell hyperplasia through mechanisms that depend on inflammatory mediators. Cytokines are known to be involved in goblet cell hyperplasia. The common pathway is middle ear infection followed by inflammatory cell infiltration as well as goblet cell hyperplasia. The accumulation of mucoid effusion in the middle ear cavity is one of the manifestations of goblet cell hyperplasia. Mucus will accumulate in the middle ear cavity and this condition may be further aggravated by the ateration of Eustachian tube structure. The viscosity of mucus produced by the middle ear mucous cells, primarily mucin, is excessive.[19]

The relationship between TNF-α levels and types of bacteria in active phase CSOM demonstrates that TNF-α could be used as a prognostic marker for active phase CSOM. It is expected that the use of TNF-α as a prognostic marker could lead to a development of a strategy for antibiotics administration to prevent complications or worsening of CSOM.

Conclusion

TNF-α levels in active phase CSOM caused by Gram-positive bacteria are higher than those which are caused by Gram-negative bacteria. There is a significant difference in serum levels of TNF-α between active phase CSOM caused by Gram-positive and Gram-negative bacteria. However, TNF-α levels in ear discharge are not significantly different between active phase CSOM caused by Gram-positive and Gram-negative bacteria.

Authors Contribution

MRD, DP, and PWK were involved in concepting and planning the research. MRD performed the data acquisition/collection. MRD, DP, and PWK calculated the experimental data, performed the analysis, and interpreted the data. MRD drafted the manuscript and designed the figures. All authors took parts in giving critical revision of the manuscript.

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