The Association of IL-1 Alpha Level and TNF Alpha Expressions on Bone Destruction in Chronic Suppurative Otitis Media and Cholesteatoma

Running Head: IL-1 Alpha, TNF Alpha, and Bone Destruction

Artono[1], Bakti Surarto[1], Nyilo Purnami[1], Fransiska Hutahaen[1], M. Reza Mahardhika[1]

[1]Department of Otorhinolaryngology - Head and Neck Surgery, Faculty of Medicine, Universitas Airlangga, dr. Soetomo General Hospital, Surabaya 60285, Indonesia

Corresponding Author: Artono

Department of Otorhinolaryngology - Head and Neck Surgery, Faculty of Medicine, Universitas Airlangga, dr. Soetomo General Hospital, Jalan Mayjen Prof. Dr. Moestopo no. 6-8, Surabaya 60285, Indonesia

Email: Artono@fkg.unair.ac.id

Phone: (+6231) 5501078 / (+62) 87853195395

ABSTRACT

Background: Bone destruction in patients with chronic suppurative otitis media (CSOM) and cholesteatoma is considered to be quite high. Bone destruction is caused by various inflammatory cytokines and osteoclasts including IL-1α and TNF-α. The imbalance between the resorption process by osteoclasts and the process of bone formation is also a causative factor for bone destruction. On top of that, the large number of patients is not supported by an equal amount of medical facilities and personnel to conduct operative procedures.

Objective: To analyze the associated of IL-1α level and TNF-α expression on the severity of bone destruction in CSOM and cholesteatoma patients.

Method: The total number of the subjects was 46 patients which group I (TNF-α) consisted of 26 individuals and group II (IL-1α) contained 26 individuals as well. The analysis was conducted in 2 different places (Solo, Indonesia and Surabaya, Indonesia). IL-1α expression was assessed by using ELISA kit at the absorbance rate of 450 nm whereas the rabbit anti-TNF-α polyclonal antibody was applied to examine TNF-α. The assessment of bone destruction was carried out during the operative procedure in Dr. Soetomo General Hospital Surabaya, Indonesia.

Result: Group I assessment resulted in severe bone destruction of 65.39% whilst group II showed severe bone destruction of 65.00%. This study revealed that TNF-α was categorized as strong positive (34.62%), moderate positive (42.30%), weak positive (19.23%), and negative (3.85%) with the value of $r=0.775$; $p\leq0.001$. On the other hand, the rate of IL-1α was attained as follows: 14.93±4.36 pg/ml, 22.75±12.18 pg/ml, and 31.98±14.16 pg/ml with the value of $r=0.625$; $p=0.003$.

Conclusion: There is a significant association between expression of TNF-α and IL-1α level on the severity of bone destruction in CSOM and cholesteatoma patients. Hence, it has been proven

that it is necessary to develop an additional therapeutic interventions to reduce TNF-α and IL-1α in CSOM and cholesteatoma patients.

Keywords: CSOM, Cholesteatoma, IL-1α, TNF-α, Bone destruction

INTRODUCTION

Chronic suppurative otitis media (CSOM) and cholesteatoma is one of the main health problems that can cause morbidity and mortality in the world (1). Complications of CSOM with cholesteatoma are mainly due to the process of temporal bone destruction. Temporal bone destruction can cause hearing loss, balance disorders, facial paralysis, periosteal abscess, and intracranial complications (2). The imbalance between the resorption process and bone formation occurs in CSOM patients with cholesteatoma. Osteoclasts, which is originated from monocyte/macrophage hematopoietic cells, plays an important role in bone resorption. Differentiation and function of osteoclasts are essentialy regulated by the receptor activator of nuclear factor κβ ligand (RANKL) or receptor activator of nuclear factor κβ (RANK). The RANKL/RANK bond will activate the osteoclastogenesis cascade (3, 4).

Studies in Poland asserted that levels of tumor necrosis factor alpha (TNF-α), Interleukin 1 αlpha (IL-1α), and Interleukin 6 (IL-6) in cholesteatoma were higher than in granulation tissue (5). IL-1α increases RANKL expression in osteoblasts and macrophages. Interleukin-1α also affects fibroblasts and osteoclasts to produce prostaglandin E2 (PGE2) and collagenase which functions to degrade the matrix of bone (6). The expression of osteoprotegerin (OPG), RANKL, and TNF-α in cholesteatoma increases compared to the normal skin of the external auditory meatus (EAM) (7).

Release of TNF-α will induce RANKL, matrix metalloproteinase (MMP), nitric oxide (NO), and prostaglandin E2 (PG E2) as the factors in bone destruction. MMP protein will induce osteoclastogenesis, while RANKL stimulates osteoclastogenesis by activating nuclear factor κβ (NF-κβ). Thus, the number of osteoclasts will consequently increase (8). TNF-α cytokines are also able to work directly on the bone matrix, in this case, exposing the bone matrix to the

activity of osteoclasts. The increased NO and PG E2 cause OPG to decrease; hence, the number of osteoblasts reduces as well. The imbalance between the bone absorption process by osteoclasts and the process of bone formation by osteoblasts results in destruction of the bone (7).

The incidence of CSOM in the world ranges approximately from 65-330 million people, in which 60% (39-200 million) of them found to suffer from severe hearing loss. More than 90% of patients come from Southeast Asia, the Western Pacific, and Africa. The incidence of CSOM in Indonesia is estimated to be around 8.36 million people and the prevalence of CSOM in general is around 3.8% (9). The incidence of CSOM with cholesteatoma in developed countries is deemed to be low (0.6% to 1.1%), whilst the rate is higher (2.1%) in developing countries (1, 10). A research conducted in Dr. Soetomo General Hospital in 2007-2008 attained 61 cases of CSOM with cholesteatoma and mastoidectomy surgery was performed (11).

It has been reported that the number of CSOM patients in 2016-2018 treated at dr. Soetomo General Hospital Surabaya Indonesia was 66 patients with cholesteatoma. Around 90-100% of CSOM patients experience complications of cholesteatoma and require operative procedure. The capacity of operating room for Otolaryngology - Head and Neck Surgery cases is for ±200 patients for 1 year. It is estimated that 50 CSOM patients queue every month to be performed surgery at Dr. Soetomo General Hospital Surabaya, Indonesia. As a result, a long queue causes 6-month to 1-year of waiting time for the patients to receive further treatment. This condition requires a solution; therefore, CSOM patients with cholesteatoma are able to obtain alternative therapy to inhibit mastoid bone destruction. Based on the above description, it is deemed to be necessary to conduct a research in regards to the correlation of IL-1α level and

TNF-α expressions with the severity of bone destruction in CSOM patients with cholesteatoma in Dr. Soetomo General Hospital Surabaya Indonesia.

METHOD

The subjects of this study were CSOM patients with cholesteatoma who underwent mastoidectomy surgery at Dr. Soetomo General Hospital Surabaya Indonesia. The subjects were required to fulfill the inclusion criteria including CSOM patients with cholesteatoma and having sufficient pathological tissue for IL-1α and TNF-α examination. On the other hand, the exclusion criteria included a change in diagnosis of CSOM with cholesteatoma to congenital cholesteatoma or tuberculous otitis media before performing surgery and damage to cholesteatoma tissue which caused immunohistochemical examination could not be performed. The subjects were divided into two groups which were Group I for TNF-α examination and Group II performing IL-1α examination. Prior to the study being conducted, the subjects initially filled the informed consent.

This study applied analytic observational design conducted within the period of October 2016 to July 2017. This study was carried out in two different cities, namely Surabaya, Indonesia and Solo, Indonesia. The subject identification process took place in the inpatient room at Dr. Soetomo General Hospital Surabaya, Indonesia. Furthermore, the pathological tissue of cholesteatoma, MAE skin, and the severity of bone destruction were collected and assessed in the Operating Room of Dr. Soetomo General Hospital Surabaya, Indonesia. The IL-1α assessment was conducted at the Clinical Pathology Laboratory of Dr. Soetomo General Hospital, Surabaya, Indonesia whereas the immunohistochemical assessment (TNF-α) performed at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sebelas Maret, Solo, Indonesia. By using a cooperative sampling method, the number of samples in the IL-1α group was obtained in 20 subjects whereas there were 26 subjects in TNF-α group (Figure 1).

This present study was approved by the Ethical Committee of Dr. Soetomo General Hospital, Surabaya, Indonesia.

The assessment of bone destruction was performed during the mastoidectomy procedures based on the damage of bone tissue structure due to cholesteatoma. The severity of bone destruction was categorized into 3: mild including scutum erosion and ossicular erosion; moderate including tegmen destruction and entire ossicular destruction; and severe including the destruction of the entire osicle, labyrinthine bone, facial canal, or KAE posterior wall (5).

The overall IL-1α measurement procedure was conducted in Dr. Soetomo General Hospital Surabaya, Indonesia. Pathological tissue in the form of cholesteatoma tissue and MAE skin during surgery were put on plate and stored in a cooler box at 4°C for less than 2 hours. Afterwards, it was stored at -80°C in the Esco Lexicon II ULT freezer (Esco Technologies Inc., Hatboro, PA, USA). ELISA examination was executed by using human IL-1α ELISA kit for lysates (RayBiotech Inc, Georgia, USA). The absorbance value of the material at a wavelength of 450 nm was interpreted with a Single Humareader (HumaReader, Germany).

TNF-α expression was determined by immunohistochemical staining measured by Rabbit polyclonal TNF-α antibody (Bioss Antibodies, Woburn, MA, USA) and Olympus BX 51 microscope (Olympus Corporation) with 400x magnification. Each sample was assessed as many as 9 fields of view. On each field of view, the percentage of strong positive cell, moderate positive cell, and weak positive cell were calculated to determine the Intensity Distribution Score (IDS). The IDS formula is as follows: (3 x the percentage of strong positive cell) + (2 x the percentage of moderate positive cell) + (1 x the percentage of weak positive cell) + (0 x the percentage of negative cell) (12). Afterwards, the value of IDS was conversed into 4 kinds of TNF-α intensity level in which it is deemed as strong positive TNF-α if IDS is around 276.00-300.00; moderate

positive TNF-α if IDS is around 151.00-275.99; weak positive TNF-α if IDS is around 76.00-150.99; and negative TNF-α if IDS is around 0.00-75.99.

The collected data initially was assessed by using Shapiro-Wilk normality test with 95% confidence interval (CI). The rate of IL-1α in cholestetoma with MAE skin was compared by using Mann-Whitney U test and Independent t-test. The analysis of correlation of IL-1α and bone destruction was executed by using Spearman's rank correlation test. Similarly, the correlation of TNF-α and bone destruction was also analysed by using Spearman's rank correlation (p value < 0.05). The statistical analysis was carried out by applying IBM SPSS Statistics software version 23.0 (IBM Corp., Armonk, NY, USA).

RESULT

Subject Characteristics

The results of data collection found that most subjects were in the age range of 21-30 years (38.46%) and followed by the age range of 11-20 years (34.62%) in the TNF-α group. In the IL-1α group, the majority of subjects were between 21-30 years old (45.00%) and followed by the age of 11-20 years (30.00%). The majority of CSOM patients in the TNF-α group were male (57.69%) and Javanese (76.92%). Similarly, in the IL-1α group, the majority of the subjects were male (55.00%) and Javanese (70.00%). The detail of subject characteristics is presented in table 1.

Bone Destruction

In the TNF-α group, the majority of patients had severe bone destruction of approximately 65.39%. In the IL-1α group, the majority of patients had a severe bone destruction rate of 65.00%. Whereas, both TNF-α and IL-1α groups had 2 patients with moderate bone destruction in the moderate category (Table 2).

TNF-α in Cholesteatoma Tissue

Immunohistochemical examination of TNF-α in 26 subjects revealed several images which can be seen in figure 2. Most TNF-α expressions were in the moderate positive category of 42.30% and followed by a strong positive category of 34.62% (Table 3).

Measurement of Interleukin-1α Levels

The mean IL-1α cholesteatoma level was 26.79 ± 13.98 pg/ml with a minimum value of 10.68 pg/ml and a maximum value of 68.45 pg/ml. Meanwhile, the average IL-1α level on MAE skin is 4.14 ± 1.56 pg/ml with a minimum value of 2.20 pg/ml and a maximum of 5.81 pg/ml. Measurement of IL-1α level was performed in 4 CSOM patients. The comparison of mean IL-1α cholesteatoma levels with mean IL-1α MAE skin had a significant ratio of $p \leq 0.001$. The highest level of IL-1α cholesteatoma was between the ranges of 25.01 - 35.00 pg/ml as much as 35.00% (table 4).

Correlation of TNF-α Expression on Bone Destruction

There was a significant correlation between TNF-α expression and the severity of bone destruction in CSOM patients with cholesteatoma ($r = 0.775$; $p < 0.001$). There were 9 subjects who had TNF-α expression in the strong positive category and had a severe bone destruction (34.62%). TNF-α expression had an even distribution in each category of bone destruction, that is TNF-α expression in a positive category with 1 subject having mild bone destruction (3.85%), 2 subjects having moderate bone destruction (7.69%), and 8 subjects having severe destruction bone (30.80%). The correlation of TNF-α expression with the severity of bone destruction can be seen in table 5.

Correlation of IL-1α Levels on Bone Destruction

The mean IL-1α levels in bone destruction include mild of 13.87 pg/ml, moderate of 22.75 pg/ml, and severe of 30.85 pg/ml. The mean IL-1α levels in mild, moderate, and severe bone destruction can be seen in Table 5. Correlation of IL-1α levels with the severity of destruction is considered as significant ($r = 0.625$, $p = 0.003$).

DISCUSSION

TNF-α plays an important role in causing bone destruction although it uses different techniques in its examination, different sample characteristics, different ways of calculating the TNF-α immunoreactivity, and different antibodies. This study has proven that there is a significant correlation between TNF-α expression and the severity of bone destruction in CSOM patients with cholesteatoma (5, 13) . The release of TNF-α will induce RANKL, MMP, NO, and PG E2 as factors in bone destruction. MMP protein will induce osteoclastogenesis, while RANKL stimulates osteoclastogenesis by activating NF-κβ. Consequently, the number of osteoclasts will increase. Tumor necrosis factor-α is also able to work directly on the bone matrix, exposing the bone matrix to the activity of osteoclasts. Increasing NO and PG E2 causes OPG to decrease resulting the number of osteoblasts decreases. The imbalance between the bone absorption process by osteoclasts and the process of bone formation by osteoblasts results in destruction of the bone (7, 14, 15).

The comprehension of the bone destruction process that occurs in CSOM with cholesteatoma at the molecular level is expected to be a molecular basis for the development of future therapeutic strategies. TNF-α cytokines can be a new target in CSOM therapy. Research continues to be conducted to find ways to stop the inflammatory process by suppressing TNF-α; hence, the bone destruction can be inhibited. The use of anti TNF-α or TNF-α systemic inhibitors such as infliximab and adalimumab has been implemented abroad as therapy in Chron's disease (15, 16). Both of these medicines, etanercept and golimumab, are also used to treat ankylosing spondylitis (17).

Interleukin-1α is not the only cytokine that plays a role in bone resorption in CSOM with cholesteatoma. TNF-α cytokines also have an important role in the process. Studies in Japan

provide the fact that there is an increase in TNF-α and IL-1 which is detected in both acquired cholesteatoma and congenital cholesteatoma compared to MAE normal skin. The RT-PCR technique shows that messenger ribonucleic acid (mRNA) for IL-1α and TNF-α was detected at 5/5 acquired cholesteatoma, whereas in congenital cholesteatoma strong mRNA expression for TNF-α was found in 5/5 cases. However, it is deemed to be weak for IL-1α as it occurs only in 4/5 cases. The ELISA technique obtained higher IL-1α levels in acquired cholesteatoma than in congenital cholesteatoma, whereas TNF-α levels did not differ greatly in these two types of cholesteatoma (15, 18, 19).

Interleukin-1α is a cytokine that plays a role in inflammation and increases bone destruction directly or through increased osteoclastogenesis in CSOM with cholesteatoma. Various studies on IL-1α inhibition have been carried out on various diseases related to bone resorption, including rheumatoid arthritis, gout, ankylosing spondylitis, and erosive osteoarthritis. Several IL-1α inhibiting agents include anakinra, rilonacept, and MABp1. Anakinra has been used as a therapy for rheumatoid arthritis and ankylosing spondylitis (20, 21).

However, research on IL-1α antagonists as the target of therapy in CSOM with cholesteaoma has never been done. Providing recombinant IL-1RA can be a new target for adjuvant therapy in addition to surgery on CSOM with cholesteatoma. The aim is to reduce IL-1α activity so that it reduces the inflammatory process and inhibits the growth of cholesteatoma and bone destruction process.

CONCLUSION

In this study, there is a strong positive association between IL-1α levels on the severity of bone destruction in CSOM and cholesteatoma patients. Furthermore, it is attained that there is also a

strong positive correlation between TNF-α expression and the severity of bone destruction in CSOM and cholesteatoma patients.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Figure

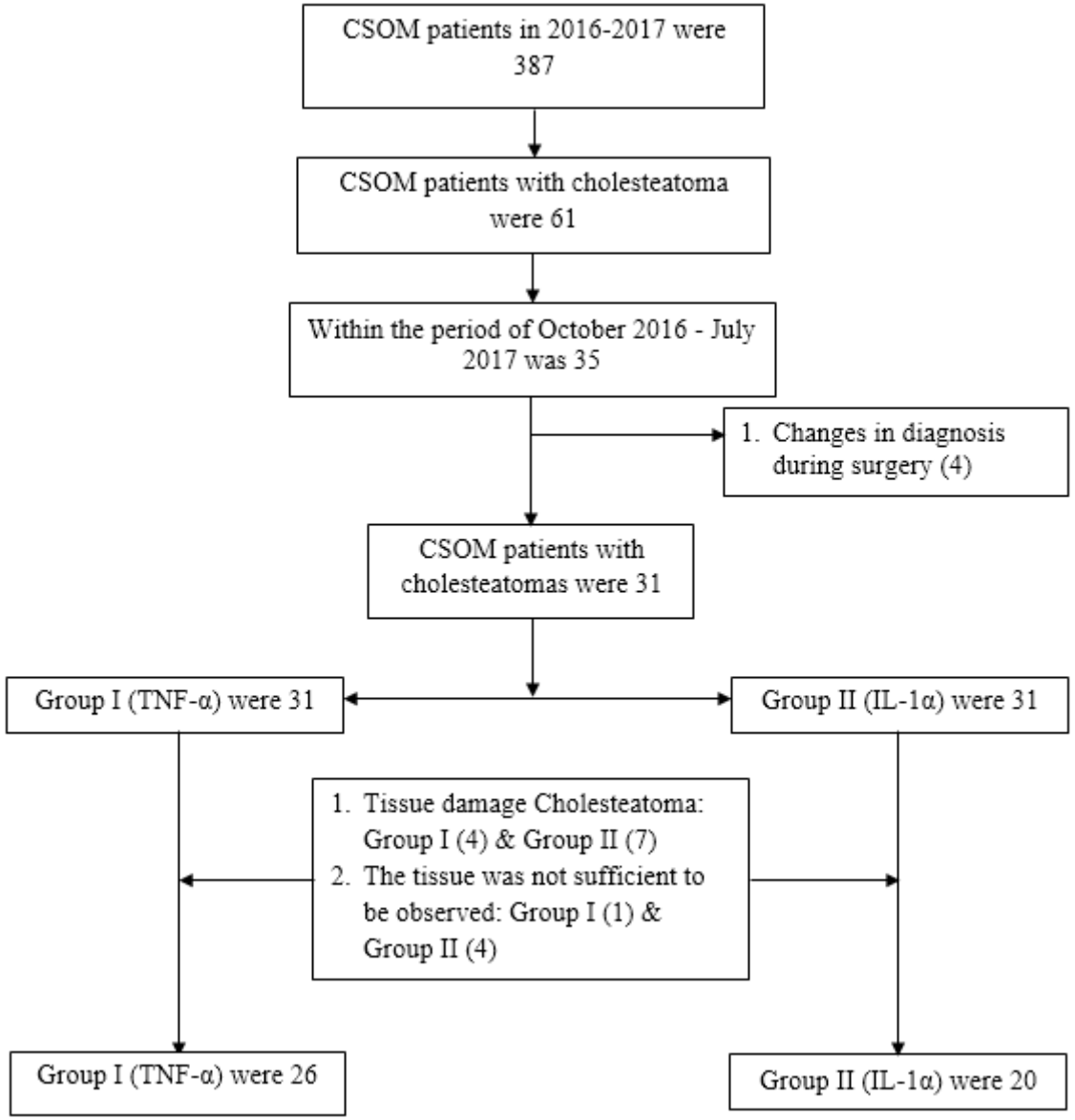

Figure 1. Flowchart of Subject Sampling

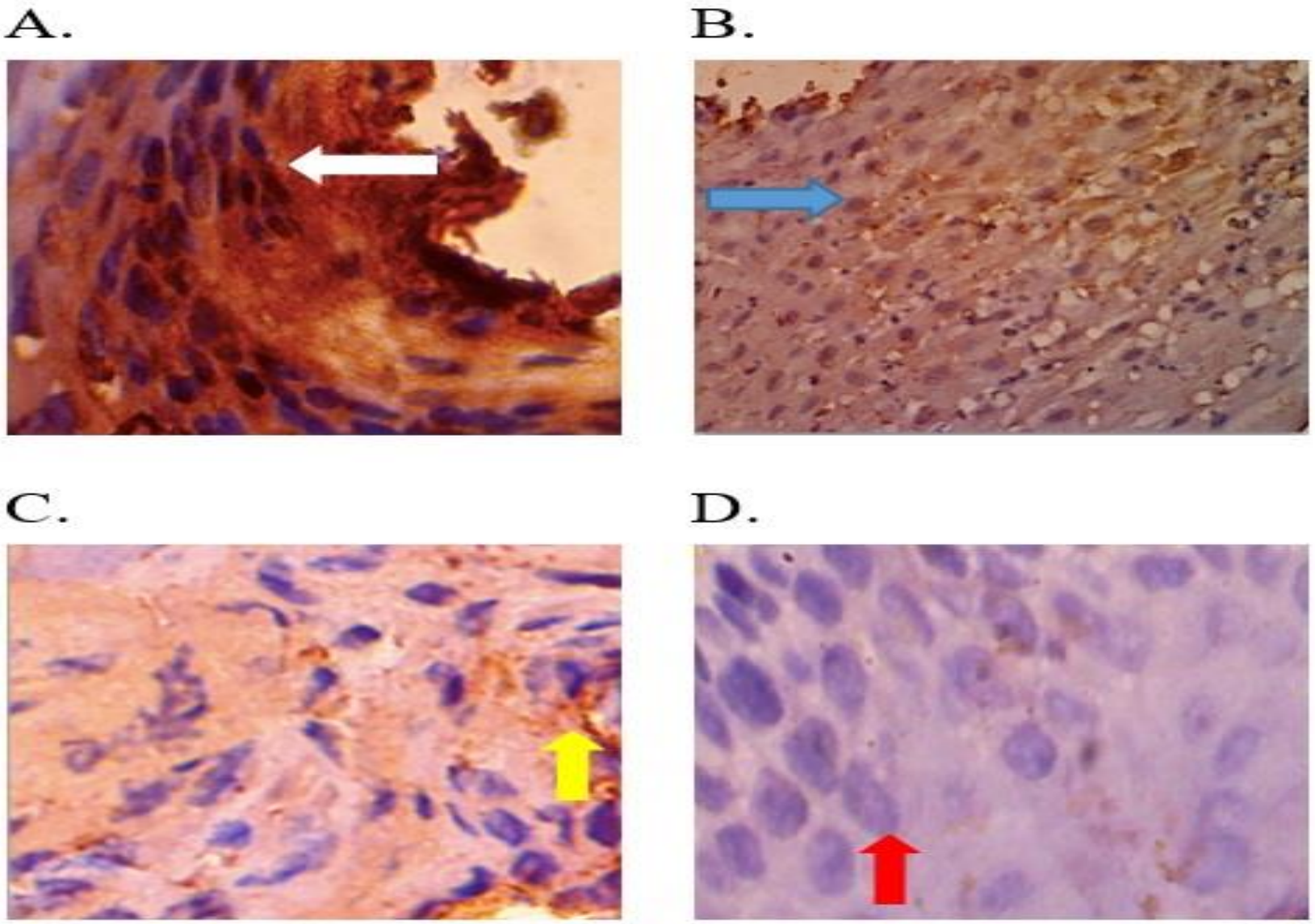

Figure 2. The intensity of TNF-α expression in cholesteatoma tissue. A. Strong positive is indicated by the dark brown cytoplasm (white arrow). B. Moderate positive (blue arrow). C. Weak positive (yellow arrow). D. Negative (red arrow).

TABLE AND LEGEND

Table 1. Subject Characteristics

Characteristics	IL-1α (n=20)		TNF-α (n=26)	
Age (years)	n	%	n	%
≤ 10	2	10.00	1	3.85
11 - 20	6	30.00	9	34.62
21 - 30	9	45.00	10	38.46
31 - 40	2	10.00	3	11.53
≥ 41	1	5.00	3	11.53
Sex				
Male	11	55.00	15	57.69
Female	9	45.00	11	42.31
Race				
Javanese	14	70.00	20	76.92
Maduranese	4	20.00	4	15.38
Banjarese	2	10.00	2	7.70

Table 2. Distribution of the Severity of Bone Destruction

Severity of Bone Destruction	IL-1α (n=20)		TNF-α (n=26)	
	n	%	n	%
Mild	5	25.00	7	26.92
Moderate	2	10.00	2	7.69
Severe	13	65.00	17	65.39

Table 3. Distribution of patients based on TNF-α expression

TNF-α expression	N=26	%
Negative	1	3.85
Weak Positive	5	19.23
Moderate Positive	11	42.30
Strong positive	9	34.62

Table 4. IL-1α levels of cholesteatoma

IL-1α (pg/ml)	Mean ± SD	Min – Max	n (%)
5.00 – 15.00	12.71 ± 1.64	10.68 – 14.14	5 (25.00)
15.01 – 25.00	20.38 ± 2.79	16.85 – 23.77	5 (25.00)
25.01 – 35.00	30.76 ± 2.99	26.17 – 33.89	7 (35.00)
≥ 35.00	51.72 ± 14.99	39.53 – 68.45	3 (15.00)

Table 5. Correlation between IL-1α and TNF-α Levels with Severity of Bone Destruction

IL-1α (n=20)		r	p	TNF-α (n=26)				r	p
Mean±SD	Min – Man			-	+	++	+++		

Mild	14.93±4.36	10.68 – 20.39			1	5	1	0		
Moderate	22.75±12.18	14.14 – 31.37	0.625	0.003	0	0	2	0	0.775	0.000
Severe	31.98±14.16	13.69 – 68.45			0	0	8	9		

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Corresponding Author:	artono artono Universitas Airlangga Fakultas Kedokteran INDONESIA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universitas Airlangga Fakultas Kedokteran
Corresponding Author's Secondary Institution:	
First Author:	artono artono
First Author Secondary Information:	
Order of Authors:	artono artono
	Bakti Surarto
	Nyilo Purnami
	Fransiska Hutahaen
	M. Reza Mahardhika
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	interventions to reduce TNF-α and IL-1α in CSOM and cholesteatoma patients.
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