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Pyroptosis and Oxidative Stress in Chronic Otitis Media

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Abstract

Objective: In this study, our aim was to evaluate the relationship between COM, pyroptosis, and oxidative stress.

Materials and Methods: This prospective study included 60 patients who underwent surgery for chronic otitis media (COM) and 28 patients as a control group. Pathological tissue was taken from the middle ear in the study group, whereas healthy tissue was taken from the middle ear of patients who underwent cochlear implant surgery and had no signs of infection in the middle ear in the control group. Preoperative serum samples were obtained from all patients. Pyroptosis parameters, Nod-Like Receptor Protein 3 (NLRP3) and Interleukin-1 beta (IL-1β) were investigated. The oxidative stress parameters, including the total antioxidant status (TAS), Total Oxidant Status (TOS), and Oxidative Stress Index (OSI), were also examined.

Results: Of the 88 patients included in the study, 52 were men and 36 were women. The patients' ages ranged from 18 to 59 years, with a mean of 31.07±11.45years. In our study, there was a significant difference in NLRP3, IL-1β, TAS, TOS and OSI values in all case groups compared with the control group in both tissue and serum samples ($p<0.001$).

Conclusion: The significant variation in NLRP3, IL-1β, TAS, TOS and OSI values in tissue and serum compared with the control group indicates that pyroptosis and oxidative stress may play an important role in the presence of COM and cholesteatoma. Understanding the role of pyroptosis in the developmental stages of COM is very important, and elucidation of this relationship may lead to the development of a new treatment method.

Keywords Chronic otitis media · pyroptosis · NLRP3 · IL-1β · oxidative stress

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INTRODUCTION

Chronic otitis media (COM) is an inflammatory process affecting the tympanic and mastoid cavities. It is also associated with perforation of the tympanic membrane and purulent discharge from the external auditory canal (1). Despite mortality rates having decreased compared to the past, it remains a disease with a high morbidity rate. Therefore, it is an important social problem (2). Factors including heredity, infection, autoimmunity, cytokines, Eustachian tube dysfunction, osteoclast function, endotoxins and oxidative stress-triggered lipid peroxidation metabolites have been implicated in the chronic inflammation of otitis media, despite the pathophysiology not being fully elucidated (3, 4). Pyroptosis is one of the non-apoptotic programmed inflammatory cell death pathways that results in cell swelling and rupture, and releasing many pro-inflammatories (5). Activated pyroptosis releases IL-1 and IL-18, which may cause infection and chronicity by various mechanisms (6). Earlier studies suggested that the NLRP3 inflammasome, involved in the pyroptosis pathway, could be important in middle ear infection (7). Pyroptosis is strongly associated with several human diseases, including tumours. Researchers have been trying to target pyroptosis for cancer treatment by controlling tumour cell proliferation, migration and invasion (8). Oxidative stress is an imbalance resulting from an increase in the release of reactive oxygen species (ROS) and a decrease in the antioxidant defence (9). Studies have reported that oxidative stress is possibly related to the aetiology of COM (10). Given the frequent occurrence of COM, along with its lengthy treatment and follow-up, elucidating the etiopathogenesis and developing treatment modalities are crucial for alleviating the financial and time burdens on patients, physicians, and the community (11). Identifying factors associated with COM could be critical for preventing the disease. In this study, we demonstrated the presence of pyroptosis in COM as a component of the multifactorial inflammatory disease and evaluate its relationship with oxidative stress.

MATERIALS AND METHODS

Between June 2021 and January 2022, a controlled prospective study was conducted at the Department of Ear, Nose and Throat Diseases of the Harran University Research and Practice Hospital Hospital, involving 60 patients who required surgery for COM and 28 patients who were operated on for a cochlear implant. Ethical approval was obtained from the Harran University Faculty of Medicine Clinical Research Ethics Committee before the start of the study (Date: 25.04.2021, No: 39). Detailed information about the study was provided to all the participating patients. Informed consent was obtained through voluntary subject briefing, and consent forms were signed by all participating patients.

Comprehensive anamnesis was obtained and an Ear, Nose and Throat was examined. Audiometric and radiologic evaluations were performed on all patients. The gender and age of the patients, the operated ear, the histopathological status of the middle ear mucosa, the condition of the ossicles, and the type of surgery performed were recorded. Patients with comorbidities, chronic medication use, a history of previous surgery on the same ear, or congenital cholesteatoma were excluded from the study. The case groups were classified into three categories according to the pathology of the tympanic cavity.

The cholesteatoma group included those patients in whom cholesteatoma tissue or lamellae were detected during surgery, and the pathology report confirmed the diagnosis of cholesteatoma. The granulation group comprised patients in whom hypertrophic granulation tissue was identified during surgery, and the pathology report confirmed the diagnosis of inflammatory granulation tissue. The de-epithelialization group (chronic nonsuppurative otitis media) included patients with isolated tympanic membrane perforation without ear discharge for at least 3 months and no signs of preoperative local inflammation. Tissue samples were collected from the pathological tissues in the tympanic cavity for the patient groups. The control group comprised patients who had no history of ear surgery or COM and who had undergone a cochlear implant operation. On otomicroscopic examination, the tympanic membrane was intact. During cochlear implant surgery, intact mucosa and/or bone fragments taken from the mastoid bone antrum of the middle ear were collected as healthy tissue. Preoperative blood samples were collected from patients who underwent COM surgery and from the control group who underwent cochlear implantation.

A 5 mL blood sample was collected from each participant using gel biochemistry tubes. The samples were then centrifuged at 4000 rpm for 12 min to separate the blood serum. The serum was then placed in two separate Eppendorf tubes for each patient and frozen at -20°C. Hemolyzed products were not used. Tissue samples collected from the middle ear cavity during surgery for investigation, including cholesteatoma, granulation tissue, de-epithelialization tissue from the tympanic membrane, and middle ear taken for control purposes, were frozen by liquid nitrogen at -80°C. A phosphate-buffered saline (PBS) solution of ten times the volume of the samples was prepared in Falcon tubes. The samples were immersed and stored at 2-8°C in these tubes.

After homogenisation (Ultra Turrax T25 IKA WERKE-USA) and centrifugation at 3000 rpm for 20 min at room temperature, they were stored at -20°C until biochemical analysis. The NLRP3, IL-1β kits were sourced from the Bioassay Technology Laboratory (Shanghai Korain), while the TAS, TOS, and OSI kits were obtained from the Rel Assay Diagnostics kit (Turkey). The OSI score was expressed as TOS/(TAS*10).

Statistical analysis

SPSS 24.0 (IBM SPSS Corp., Armonk, NY, USA) was used for the statistical analysis. Since the Shapiro-Wilk normality test analysis showed a non-normal distribution of data, non-parametric tests were used. The Mann–Whitney U test was used for the analysis of the two independent groups. For the analysis of more than two independent groups, the Kruskal-Wallis test was applied, and pairwise comparisons between these groups were interpreted using the Bonferroni correction for p-values. The relationship between two continuous variables was tested using Spearman's rank correlation analysis, and categorical variables were evaluated using the chi-square test. A two-tailed p value of <0.05 was set as statistically significant.

RESULTS

The study analysed 88 cases, of which 60 were in the treatment group and 28 in the control group. Gender distribution was similar between the groups with 52 male and 36 female patients (p=0.197). The mean age of the patients was 31.07±11.45(range 18-59). Age distribution was not statistically different between groups (p=0.938). The table presents information on the gender, age, affected ear, ossicular involvement status, and type of surgery for the cases (Table1).

Significant differences were observed among the groups based on the TOS, TAS, OSI, NLRP3, and IL-1β values measured in the tissues (Table 2). The TOS values were higher in the cholesteatoma, granulation, and de-epithelialization groups than in the control group (p<0.001, p=0.003, p<0.001, respectively). TAS values were lower in all groups compared with the control group (p<0.001). OSI, NLRP3, and IL-1β values were higher in all groups compared with the control group (p<0.001). Additionally, the NLRP3 value was higher in the cholesteatoma group than in the de-epithelialization group (p=0.006).

Significant differences were observed among the groups based on the TOS, TAS, OSI, NLRP3, and IL-1β values measured in the serum (Table 3). According to this analysis, higher TOS values were observed in the granulation and de-epithelialization groups than in the control group (p=0.001, p<0.001 respectively). The cholesteatoma group exhibited a lower TOS value compared with the de-epithelialization group (p=0.004). Lower TAS value and higher OSI, NLRP3, and IL-1β values were determined in all groups than in the control group (p<0.001). Furthermore, a higher NLRP3 level was found in the cholesteatoma group than in the de-epithelialization group (p=0.002).

Table 4 shows the correlation coefficients and significance values for Spearman's rho correlation analysis, which was performed to assess the relationship between the parameters measured in the tissue and serum. TOS–OSI and TAS–OSI correlation values were omitted from the table due to

Table 1. Comparison of the characteristics of the groups

	Cholesteatoma (n=20)	Granulation (n=20)	De-epithelialization (n=20)	Control (n=28)	Total (n=88)
Gender					
Male	14 (70%)	13 (65%)	9 (45%)	16 (84%)	52 (59%)
Female	6 (30%)	7 (35%)	11 (55%)	12 (16%)	36 (41%)
Age, yr (mean±SD)	33.05±11.39	29.7±11.6	32.95±10.48	28.6±12.44	31.07±11.45
Ear					
Right	10 (50%)	9 (45%)	5 (25%)	14 (50%)	38 (43%)
Left	10 (50%)	11 (55%)	15 (75%)	14 (50%)	50 (57%)
Ossicular involvement					
Malleus	7 (37%)	1 (5%)	1 (5%)	-	9 (16%)
Incus	11 (58%)	4 (21%)	4 (21%)		19 (33%)
Stapes	9 (47%)	6 (32%)	-		15 (26%)
Type of surgery					
CWD	14 (70%)	4 (20%)	-	-	18 (45%)
CWU	6 (30%)	16 (80%)	-	-	22 (55%)

CWD: Canal wall down, CWU: Canal wall up

Table 2. Values of Pyroptosis and Oxidative Stress Parameters in the Tissue of Study Groups

	N	Cholesteatoma	N	Granulation	N	De-epithelialization	N	Control	P
TOS (μmol H_2O_2 Equiv/L)	20	13.18±2.66	20	12.29±1.99	20	14.73±6.35	28	10.79±3.61	<0.001
TAS (mmol Trolox Equiv/L)	20	1.09±0.05	20	1.13±0.04	20	1.11±0.09	28	1.51±0.06	<0.001
OSI (Arbitrary Unit)	20	1.22±0.27	20	1.09±0.19	20	1.35±0.62	28	0.71±0.25	<0.001
NLRP3 (pg/mL)	20	35.96±5.54	20	30.53±5.66	20	28.76±2.31	28	13.71±1.89	<0.001
IL-1β (pg/L)	20	771.96±80.22	20	703.33±74.13	20	760.68±74.92	28	331.16±32.59	<0.001

TOS: Total oxidant status, TAS: Total antioxidant status, OSI: Oxidative Stress Index, NLRP3: Nod-Like receptor protein 3, IL-1β: Interleukin-1 beta

Table 3. Values of Pyroptosis and Oxidative Stress Parameters in the Serum of Study Groups

	N	Cholesteatoma	N	Granulation	N	de-epithelialization	N	Control	P
TOS (μmol H_2O_2 Equiv/L)	20	12.20±2.50	20	13.51±1.46	20	14.59±1.86	28	11.42±1.29	<0.001
TAS (mmol Trolox Equiv/L)	20	1.03±0.28	20	0.98±0.14	20	0.96±0.25	28	1.63±0.18	<0.001
OSI (Arbitrary Unit)	20	1.28±0.50	20	1.41±0.25	20	1.67±0.66	28	0.71±0.13	<0.001
NLRP3(pg/mL)	20	23.76±3.46	20	20.85±2.31	20	18.80±1.47	28	8.07±1.17	<0.001
IL-1β(pg/L)	20	994.19±103.72	20	917.62±117.50	20	986.70±121.91	28	441.01±65.27	<0.001

TOS: Total oxidant status, TAS: Total antioxidant status, OSI: Oxidative Stress Index, NLRP3: Nod-Like receptor protein 3, IL-1β: Interleukin-1 beta,

Table 4. Spearman's rho Correlation Analysis of Parameters Measured in Tissue and Serum

GROUPS	Tissue		Serum	
	Correlation coefficient	P	Correlation coefficient	P
NLRP3–TAS	0.797	<0.001	−0.684	<0.001
IL-1β – TAS	0.699	<0.001	−0.736	<0.001
NLRP3 – IL-1β	0.623	<0.001	0.839	<0.001
OSI– IL-1β	0.602	<0.001	0.592	<0.001
NLRP3–OSI	0.568	<0.001	0.491	<0.001
IL-1β – TOS	0.530	<0.001	0.434	<0.001
TAS–TOS	0.507	<0.001	−0.418	<0.001
NLRP3–TOS	0.455	<0.001	0.335	0.001

TOS: Total Oxidant Status, TAS: Total Antioxidant Status, OSI: Oxidative Stress Index, NLRP3: Nod-Like Receptor Protein 3, IL-1β: Interleukin-1 beta

the mathematical relationship between them. A moderate positive correlation was found between TAS, NLRP3 and IL-1β levels in both the serum and tissue.

The cut-off values that could be used to distinguish the cholesteatoma, granulation, and de-epithelialization groups from the control group based on the ROC analysis performed to determine the diagnostic properties of the OSI, NLRP3, and IL-1β parameters measured in the serum are provided in Table 5.

DISCUSSION

COM is a prevalent disorder that often results in hearing loss. In children, hearing loss can impede speech and language development, whereas in older individuals, it can heighten the risk of developing dementia (12). Due to the diagnostic and therapeutic challenges, COM remains an important issue in Otolaryngology (13). Pyroptosis is a programmed cell death pathway that triggers inflammation. Pyroptosis involves cell swelling, rupture, and the release of pro-inflammatory factors (14). The discovery of inflammatory agents in the early 2000s was a major milestone in the understanding of the pathogenesis of inflammation, infections and the mechanisms of programmed cell death (15, 16). Pyroptosis is usually induced by intracellular pathogen infection and is part of the host defence system. The NLRP3 inflammasome collaborates with Toll-like receptors and nuclear factor kappa B to control the production of IL-1β and IL-18. Upon activation, the NLRP3 inflammasome leads to caspase-1 activation, which leads to IL-1β maturation (5). ROS are generated through endogenous biochemical reactions during oxygen metabolism (17). Oxidative stress is linked to a reduction in antioxidants or an increase in oxidant production (18). Overproduction of ROS can cause tissue injury by the chemical modification of proteins, carbohydrates, nucleotides and

Table 5. ROC Analysis Data for Diagnostic Values of OSI, NLRP3, and IL-1β Parameters

Disease	Parameters	Cut-off	AUC	Sensitivity	specificity	PPV	NPV	P
Cholesteatoma	OSI	≥0.84	0.909	0.90	0.86	0.82	0.92	<0.001
	NLRP3	≥14.5	1.000	1.00	1.00	1.00	1.00	<0.001
	IL-1β	≥717	1.000	1.00	1.00	1.00	1.00	<0.001
Granulation	OSI	≥1.04	0.996	1.00	0.96	0.95	1.00	<0.001
	NLRP3	≥13.6	1.000	1.00	1.00	1.00	1.00	<0.001
	IL-1β	≥687	1.000	1.00	1.00	1.00	1.00	<0.001
De-epithelialization	OSI	≥0.89	0.995	1.00	0.93	0.91	1.00	<0.001
	NLRP3	≥13.2	1.000	1.00	1.00	1.00	1.00	<0.001
	IL-1β	≥669	1.000	1.00	1.00	1.00	1.00	<0.001

N: Number, AUC= Area Under the Curve, PPV: Positive Predictive Value, NPV: Negative Predictive Value, p<0.01 is considered statistically significant

lipids, and may therefore contribute to the development of several pathologies (17). To the best of our knowledge, this is the first investigation of pyroptosis and oxidative stress in both tissue and serum together in COM.

Kariya et al. reported higher levels of NLRP3 in cholesteatoma and COM compared with those in normal controls (19). A study has shown that injecting lipopolysaccharide into the tympanic cavity of mice leads to a marked elevation of IL-1β and NLRP3 levels in the middle ear compared with control mice. These findings indicate that NLRP3 activation plays a role in the pathogenesis of otitis media (7).

The study revealed that both the tissue and serum levels of NLRP3 and IL1β were significantly higher in the cholesteatoma, granulation and de-epithelialization groups than in the control group. Moreover, NLRP3 levels were significantly higher in the cholesteatoma group than in the de-epithelialization group.

Studies have shown that oxidative stress may contribute to the development of COM with or without cholesteatoma (17, 20, 21). Additionally, it has been found that cases with cholesteatoma exhibit a greater imbalance between oxidative stress and antioxidant enzymes compared with cases without cholesteatoma (10). In our study, OSI values in both serum and tissue were significantly higher in COM with and without cholesteatoma. Additionally, this study showed decreased TAS and increased TOS levels in COM.

Our data on NLRP3, IL-1β, TAS, TOS values, and OSI scores are consistent with the literature. Unlike other studies, we strengthened our study by examining the tissue and serum samples together. In addition, we divided the COM group into cholesteatoma, granulation, and de-epithelialization subgroups. We found that there was no significant difference in pyroptosis and oxidative stress parameters within these groups.

Studies in which the neutrophil-to-lymphocyte ratio has been evaluated as an inflammatory marker (22), and studies that investigated a pro-inflammatory epithelial growth factor receptor ligand demonstrated the close relationship between the cholesteatoma group and inflammation (23). Similarly, this study revealed the impact of inflammatory pathways on COM with or without cholesteatoma. We found significantly higherNLRP3 and IL-1β levels in all three groups of COM.

Pyroptosis and parameters in the inflammatory pathway have also been the subject of etiopathogenetic studies regarding the inflammatory process in the upper respiratory tract (24-26).

Pyroptosis-related factors have a dual mechanism to both promote and inhibit tumour formation. The newly developed therapeutic strategies for tumour pyroptosis show great potential. Studies have revealed that chemotherapeutics and miRNAs may trigger tumour pyroptosis, thus inhibiting the malignant progression of tumours (27).

Although the exact mechanism of NLRP3 activation by ROS remains unknown, some studies have shown that ROS are involved in NLRP3 activation, thus increasing the inflammatory response (28-30). It has been demonstrated that the NLRP3-ASC pyroptosis pathway is initiated by ROS, and endothelial cell pyroptosis is prevented by N-acetylcysteine (NAC), which acts as an ROS scavenger (31). In their study, Nakahira et al. found that increased ROS production contributes to the inflammatory activation of NLRP3 (32). Heid et al. observed a decrease in caspase-1-mediated IL-1β secretion when certain ROS inhibitors or scavengers were administered (30). Hu et al. proposed that reducing IL-1β secretion could be achieved by inhibiting ROS activity and elevating antioxidant expression (33). Therefore, these findings imply that ROS may facilitate the onset of inflammation.

This study revealed a moderate correlation between TAS, NLRP3 and IL-1β levels in both the serum and tissue. In

addition, the positive correlation of IL-1β and NLRP3 with oxidative stress reveals the relationship between pyroptosis and oxidative stress.

This study revealed higher pyroptosis (NLRP3 and IL-1β) and oxidative stress (TAS, TOS, OSI) levels in COM patients than in the control group. This analysis was performed by evaluating each of the COM subgroups, including chronic suppurative otitis media (CSOM) with cholesteatoma, CSOM without cholesteatoma, and chronic nonsuppurative otitis media. The ROC analysis, which was conducted to measure the predictive values of serum OSI, NLRP3, and IL-1β, showed that these parameters exhibited excellent predictive power (AUC ≥ 0.9) (34). Given the excellent predictive power of these three parameters, it is considered that they may be used in conjunction with clinical findings and radiological evaluation for the early diagnosis of COM subgroups.

CONCLUSION

The pyroptosis parameters NLRP3 and IL-1β, as well as the oxidative stress parameters TOS and OSI, were detected to be markedly increased in the tissues and serum of the case groups compared with the control group, while the TAS values were markedly lower. Therefore, indicating that NLRP3, IL-1β and oxidative stress may play a role in the etiopathogenesis of COM with and without cholesteatoma. Additionally, considering the positive correlation between TAS values and NLRP3 and IL-1β, the relationship of pyroptosis with oxidative stress supports its role in inflammation. Currently, there is no approved targeted treatment that can successfully suppress the NLRP3 inflammasome. Understanding the importance of pyroptosis for the developmental stages of COM is crucial, and clarifying this relationship may lead to the development of a new treatment modality. Therefore, further studies on this topic are needed.

Data availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Harran University Faculty of Medicine Clinical Research Ethics Committee (Date: 25.04.2021, Number: 39).

Informed Consent: Informed consent was obtained through voluntary subject briefing, and consent forms were signed by all participating patients.

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