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Otology

The effect of N-acetyl cysteine on biofilm layers in an experimental model of chronic otitis media

L'effetto della N-acetil cisteina sullo strato di biofilm in un modello sperimentale di otite media cronica

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SUMMARY

Objectıve. The aim of this study was to investigate the efficacy of N-acetylcysteine (NAC) on biofilm layers and on the course of disease in chronic otitis media.

Methods. Twenty-five rats that were induced with chronic otitis media (COM) were separated into three groups. In Group 1 (N = 18), 0.2% ciprofloxacin + 0.1% dexamethasone sodium phosphate + 0.5 mg/ml NAC solution was locally injected to the right ear of the rats; in Group 2, (N=18) 0.2% ciprofloxacin + 0.1% dexamethasone sodium phosphate was locally injected to the left ear of the rats. No treatment was applied to either ear of rats in Group 3 (N = 5). Histopathological and scanning electron microscope (SEM) evaluations were performed in all groups.

Results. SEM revealed biofilm formation in all COM induced groups. No significant difference was seen between groups 1 and 2 in terms of suppuration levels, fibrosis, inner ear involvement, infection staging and biofilm formation ($p > 0.05$).

Conclusıons. In this study, while histopathological and SEM evaluation revealed no effect of 0.5 mg/ml NAC on the biofilm layer in COM-induced rats, further studies with NAC at different concentrations are still needed on different types of experimental animals.

KEY WORDS: chronic otitis, biofilm, N-acetylcysteine, rat

RIASSUNTO

Obıettıvo. *Lo scopo di questo studio è quello di valutare l'efficacia della N-acetilcisteina (NAC) sullo strato di biofilm e sul decorso della malattia nell'otite media cronica.*

Metodo. *Venticinque ratti con otite media cronica (COM) sono stati divisi in tre gruppi. Nel gruppo 1 (N = 18) sono stati iniettati 0,2% di ciprofloxacina + 0,1% di desametasone sodio fosfato + 0,5 mg / ml di soluzione NAC all'orecchio destro dei ratti; nel gruppo 2 (N = 18) sono stati iniettati localmente 0,2% di ciprofloxacina + 0,1% di desametasone sodio fosfato all'orecchio sinistro dei ratti. Nessun trattamento è stato applicato alle orecchie inoculate dei ratti nel gruppo 3 (N = 5, entrambe le orecchie). Sono state eseguite valutazioni istopatologiche e valutazioni al microscopio elettronico (SEM) mediante scansione.*

Rısultatı. *SEM ha documentato la formazione di biofilm in tutti i gruppi indotti con COM. Nessuna differenza statisticamente significativa è stata osservata tra i gruppi 1 e 2 in termini di livelli di suppurazione, fibrosi, coinvolgimento dell'orecchio interno, stadiazione dell'infezione e formazione di biofilm ($p > 0,05$).*

Conclusıone. *Anche se in questo studio la valutazione istopatologica e SEM non ha rivelato alcun effetto del 0,5 mg/ml NAC sullo strato di biofilm nei ratti indotti dalla COM, per arrivare a una conclusione migliore è necessario eseguire ulteriori studi con di NAC a diverse concentrazioni su differenti tipi di animali da esperimento.*

PAROLE CHIAVE: *otite cronica, biofilm, N-acetilcisteina, ratto*

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Conflict of interest
The Authors declare no conflict of interest.

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Introduction

Biofilm is a community of micrororganisms residing in a gelatinous layer with polymeric structures and its production starts by attaching to a surface [1]. Biofilm formation may take place in lifeless surfaces *in vivo* or *in vitro*. The microorganisms in biofilms are more resistant to antimicrobial agents than planktonic cells, as they have barriers impeding contact with antimicrobial agents and decreasing sensitivity [2]. Biofilm has also the characteristic of protecting the organism from osmotic stress, phage debris, toxic compounds and antibiotics.

Various bacterial proteins (biofilm) are produced by bacteria in chronic otitis media (COM), which increase the adhesion and penetration of them into middle ear mucosa [3]. It is considered that in resistant COM, otorrhoea is possibly derived from biofilm layer [3]. In cases of COM in which otorrhoea and inflammation cannot be relieved in spite of medical treatment, surgical intervention is used as an alternative treatment which aim to destroy the damaged osteitic tissue.

N-acetyl cysteine (NAC) is a precursor of glutathione and produces neuroprotection by preventing oxidative damage [4,5]. In addition to its primary use, it is also utilised in chronic bronchitis, cancer, paracetamol intoxication and aspergilloma [6-8]. NAC exerts an eradicating effect on biofilm layer produced by various bacteria [9-12]. The effect of NAC, on COM associated biofilm layer produced by primary pathogen *P. aeruginosa*, has been demonstrated [12]. Although the effect of NAC on biofilm layer was demonstated in infections produced by *P. aeruginosa* in respiratory and renal systems, its effect in COM treatment is controversial. The aim of the present study was to investigate the effect of NAC on biofilm layer formation in COM produced in a rat model. We also investigated the role of NAC in preventing the involvement of the inner ear in COM.

Materials and methods

The present study was carried out with 30 healthy male Wistar albino rats at a weight of 180-220 gm with the approval of ethics committee of Ankara Training and Investigation Hospital (13 May 2014, No. 0018). The principles of Helsinki declaration for laboratory animals were applied. All animals were followed and cared in accordance with a protocol approved by institutional animal care group that was compliant with experimental ethical principles and animal protection laws in Turkey. Animals were isolated in standard cages throughout the experimental period and were given food and water ad libitum.

Surgical technique

The surgical procedure was carried out by the same surgeon in all rats under general anaesthesia using ketamine hydrochloride (90 mg/kg) and xylazine hydrocloride (10 mg/kg). After otoscopic examination and otoacoustic emission tests were performed, 30 rats with normal findings were included in the present study. As in previous studies with chronic otitis model [3,13,14], tympanic membranes of 25 rats (TM) were perforated with a proportion of 75% and 10^6 colony *P. aeruginosa* strains (strain - ATCC -27853) were inoculated into the middle ear via the perforation. The same inoculation procedure was repeated one week later. Subsequently, microscopic examination was carried out twice weekly for three weeks and rats were followed up without treatment. Three weeks after the last inoculation, rats were examined under sterile conditions and external ear cultures were obtained. Otomicroscopic examination of all rats revealed COM and purulent discharge in the outer ear canal. The cultures demonstrated the presence of *P. aeruginosa* infection in all rats. Meanwhile, 2 rats were lost due to malnutrition. Treatments were then started; to right ears of 18 rats, (Group 1) 0.2% ciprofloxacin + 0.1% dexamethasone sodium phosphate + 0.5 mg/ml NAC and to left ears 0.2% ciprofloxacin + 0.1% dexamethasone sodium phosphate was administered (Group 2). These medical treatments were administered for 4 weeks twice a day locally by injection in the external auditory canal. In Group 3, 10 ears of 5 rats were not administered any medical treatment after inoculation. In Group 4, 10 ears of 5 rats were investigated as a control group without undergoing any procedure. Animals were decapitated after deep anaesthesia with the same protocol.

Histomorphological evaluation

After temporal bones were removed, they were fixed in 10% formalin and after decalcification paraffin blocks were prepared. 4 μm sections from paraffin blocks were stained with haematoxylin-eosin, PAS and masson trichrome stains. In the sections examined with light microscope, lesions produced by infection in external ear, middle ear, inner ear and the presence of mast cells and suppuration to determine improvement levels and development of fibrosis. As the variation in quality and quantity of lesions made it difficult to obtain repeatable objective data, fibrosis and suppuration, which are more suitable for quantitative evaluations, were evaluated semiquantitatively (-, +, ++, +++). Inner ear involvement was shown as present or absent (+, -).

In addition, stage/level of infection was evaluated histomorphologically; lesions produced by varying combinations of different histomorphological findings were classifed into acute suppurative (suppuration/fibrosis: ++ or +++/- or +) (Fig. 1) or chronic suppurative (suppuration/fibrosis: ++ or

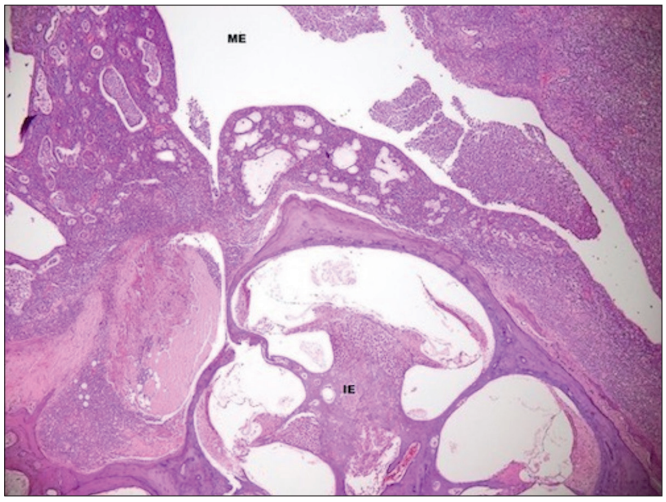

Figure 1. Group1 rats, acute suppurative inflammation in middle ear (ME); there is no involvement in inner ear (İE), routine histological characteristics (suppuration +++, fibrosis-).

+++/++ or +++) depending on the severity and proportion of suppuration and fibrosis.

Ears in which suppuration/fibrosis were (+/+), (+/-) or (-/+), were classified as healed/improved (Fig. 2), because none of the ears undergoing treatment procedures were completely healed (suppuration/fibrosis: -/-).

Scanning electron microscopic (SEM) evaluation

Fresh specimens obtained from 5 ears of each group were fixed in 2.5% glutaraldehyde for 24 hours, irrigated in phosphate buffer (pH 7.4), fixed with 1% ozmium tetroxide in phosphate buffer (pH 7.4) and dehydrated in increasing alcohol concentrations. After dehydration, specimens were dried and mounted on metal frames with double sided adhesive tape. Subsequently, Bio-Rad (Hercules, CA) gold thick layer 150-A ° k were sprayed to specimens. SEM images were obtained with JEOL SEM ASID-10 (Tokyo, Japan) electron miscrosope at 500X-3000X magnification range.

The presence of biofilm was determined using SEM morphological findings such as three dimensional structure, variability in the size of microorganisms embedded in polysaccaride matrix and residue of multiple layers of tissue and microorganisms. 3 x 3 mm specimens sampled from middle ear mucosa were examined. With this investigation, the presence of biofilm was evaluated and in biofilm positive specimens, similar to other studies in the literature [15], and the presence of biofilm in less than 25% of all visualised surface areas was classified as (+) between 25-50% as (++) and over 50% as (+++) SEM analysis.

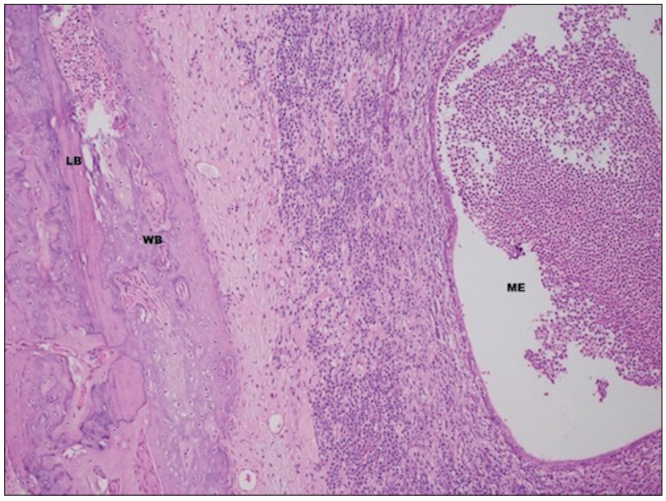

Figure 2. Group 1, residual necrotic lamellous bone (LB) fragments due to destruction in middle ear (ME) bone wall, caused by infection at the recovery process. Overlying new woven bone (WB) tissue and thin fibrous wall are seen on the surface of degenerated epithelium. Suppuration continues in the lümen (suppuration +, fibrosis +).

Statistical evaluation

Descriptive statistics were expressed with the number and percentage of cases both for nominal and ordinal variables. Nominal data were analysed with Fisher's exact test, while ordinal data were analysed with Mann Whitney U test. Data analysis was performed using IBM SPSS version 17.0 software (IBM Corporation, Armonk, NY, USA). A $p < 0.05$ was considered significant for all results.

Results

In all rats with experimental COM, cultures obtained three weeks after the last inoculation revealed the *P. aeruginosa* infection.

Histomorphological findings

Except for the control group, which did not undergo any procedure, in all ears and temporal bones varying degrees of supurative or chronic inflammation, granulation tissue, vascularisation, fibrous tissue development and partial appearance of cholesteatoma were detected. Mast cell involvement was observed in eustachian tubes and inner ears and for the most part ossicles and tympanic membrane were completely destroyed and were surrounded by suppuration and fibrosis, accompanied by inner ear involvement. Inner ear structures; cranial nerve VIII, spinal ganglions, cochlear and vestibulare bones were partially or completely destroyed and replaced b fibrous repair tissue filling the vestibulare and tympanic ducts.

It was observed that the inner ear was completely destroyed in a few ears in which suppuration disappeared and fibrosis

Table I. Histopathological findings of cases undergoing only treatment or NAC plus treatment.

	Group 2	Group 1	p-value
Suppuration			0.963†
-	4 (22.2%)	2 (11.1%)	
+	3 (16.7%)	8 (44.4%)	
++	4 (22.2%)	0 (0.0%)	
+++	7 (38.9%)	8 (44.4%)	
Fibrosis			0.963†
-	4 (22.2%)	2 (11.1%)	
+	5 (27.8%)	4 (22.2%)	
++	3 (16.7%)	11 (61.1%)	
+++	6 (33.3%)	1 (5.6%)	
Inner ear chacaracteristics			0.338‡
Normal	14 (77.8%)	17 (94.4%)	
Involved	4 (22.2%)	1 (5.6%)	
Infections stage			0.462†
Improved	5 (27.8%)	10 (55.6%)	
Acute suppurative	7 (38.9%)	1 (5.6%)	
Chronic suppurative	6 (33.3%)	7 (38.9%)	

† Mann Whitney U test; ‡ Fisher's Exact test.

was very mild. It was also found that inner ear involvement was more marked in groups that did not undergo treatment (Group 3) compared to the groups that underwent treatment (Group 1, Group 2) (Fig. 1). In the evaluation of osteoblastic and osteoclastic activities, no difference was found between Groups 1 and 2 (Fig. 2).

There was no significant difference between NAC+treatment group and only treatment group with respect to severity of suppuration and fibrosis, inner ear involvement or stage of infection ($p > 0.05$) (Tab. I). In group 3, no statistical comparison was performed with groups 1 and 2 since extensive suppuration, fibrosis, and inner ear involvement was detected in all ears.

In the control group, which did not undergo any procedure, suppuration, fibrosis and inner ear involvement were not observed.

SEM findings

In SEM imaging, biofilm formation was established in all groups. There was no significant difference between Groups 1 and 2 in terms of biofilm formation ($p > 0.05$) (Tab. II).

In the evaluation of SEM findings in Group 3, widespread biofilm areas were observed on the middle ear mucosa. As there was no treatment, together with polymorphonuclear leukocytes, many free bacteria, bacteria debris and ECM appearance were also observed (Fig. 3).

Table II. SEM findings in groups 1 and groups 2.

	Group 2	Group 1	p-value
SEM			0.690†
+	1 (20.0%)	2 (40.0%)	
++	4 (80.0%)	3 (60.0%)	
+++	0 (0.0%)	0 (0.0%)	

† Mann Whitney U test.

In SEM findings of Group 1 rats, widespread biofilm was observed along with polymorphonuclear leukocytes.

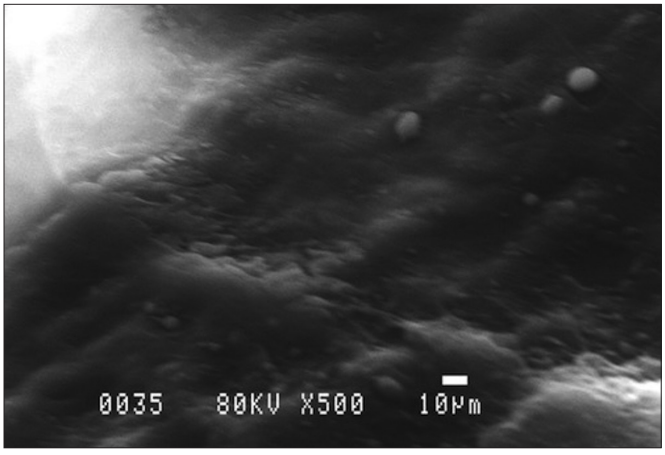

Figure 3. Group 1 rats, SEM appearance of biofilm layer.

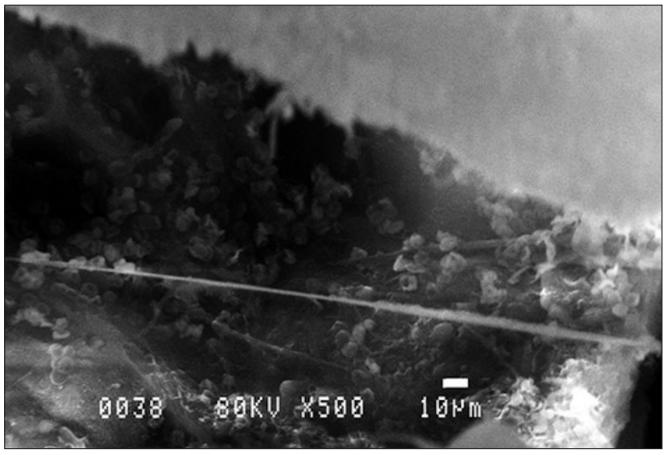

Figure 4. SEM appearance of biofilm layer in Group 2 rats.

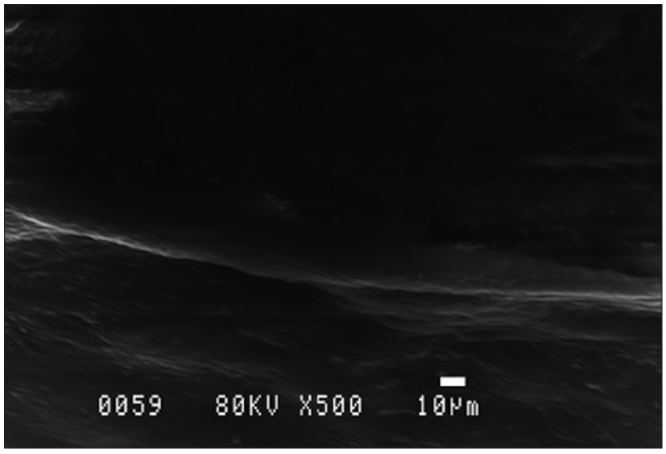

Figure 5. Control group SEM findings.

SEM findings were similar in Group 2 to those in Group 1. Widespread biofilm formation was observed along with blood components (Fig. 4).

In control group without chronic otitis (Group 4), the normal middle ear mucosa was observed without any biofilm formation (Fig. 5).

Discussion

To our knowledge, the present study is the first attempting to experimentally demonstrate the effect of NAC on biofilm formation in the middle ear and mastoid cells in COM, in terms of histopathological and SEM findings. In refractory COM, in spite of medical treatment to which primary pathogen, *P. aeruginosa*, is sensitive, otorrhoea and inflammation cannot be improved. NAC also exerts an eradicating effect on biofilm layers produced by various bacteria in addition to its other benefits [9-12,16,17]. The effect of NAC on primary pathogen of COM, i.e. *P. aeruginosa*, has been demonstrated in many in vivo and in vitro studies [12,16,17]. For this purpose, the efficacy of NAC has been investigated at different concentrations. In addition to other routes, NAC may also be administered locally. In the study by Özcan et al. [18], in which the effect of NAC in myringosclerosis in rats was investigated, 0.6 mg and 1.2 mg topical NAC was used, and it was demonstrated that it prevents the development of myringosclerosis. In the study by El-Feky et al. [16], aiming to determine the effect of the addition of NAC to ciprofloxacin treatment for biofilms on the surface of uretheral stents, 2-4 mg/ml NAC was used, and it was determined that the development of mature biofilms declined. Zhao et al. [12] added NAC at 0.5 mg /ml and 1 mg /ml to ciprofloxacin treatment and found that NAC decreased biofilm layer produced by *P. aeruginosa* and production of extrapolysaccharides. However, in that study, its effect on *P. aeruginosa* was investigated in patients with respiratory tract infections. Similar to this study, we used NAC at 0.5 mg/ml for eradication of biofilms. Herein, we found no significant difference between groups 1 and groups 2 with regard to severity of suppuration and fibrosis, involvement of inner ear, stage of infection or SEM findings ($p > 0.05$) (Figs. 1, 2). It may be thought that this is due to differences in experimental animals used, variations in dose, the infectious process in which NAC was used and different organs. In previous studies, NAC was used in vivo and in vitro in pseudomonal infections, and not in treatment of COM, which may also explain the discrepant results.

It has been demonstrated in various studies that NAC enhances osteoblastic activity and bone regeneration [19]. However, in the present study, in COM model, no difference was found between Group 1 and 2 in terms of osteoblastic and osteoclastic activity (Fig. 2).

There are few studies in the literature on the effect of NAC on COM treatment. In the only study carried out by Choe et al. [20], it was established that NAC with ciprofloxacin was effective in the treatment of refractory COM. Nevertheless, their study was performed with a small number of patients with no histopathological and SEM evaluation, and their conclusion was based upon solely clinical evaluation.

Severe and destructive lesions developing in all of the ears undergoing experimental treatment procedures made it difficult to evaluate the severity of histomorphological findings quantitatively, and hence to determine the grade of findings of infection and recovery. We hope that the present study will be a pioneer model that can guide future studies with biofilms and resistant infections. In future studies, lower microorganism concentrations can be used in conjunction with higher dosages of NAC. This method may better reveal the efficacy of NAC in the process of biofilm formation in COM.

Conclusions

Although there are many studies demonstrating the effect of NAC on biofilms, very few of them examined its efficacy in COM. In our study, we found no additional effect of topical NAC to the treatment of COM evaluated histopathologically and with SEM in the rat model. We suggest that further studies are required using different concentrations of NAC with different experimental animals in order to reach more definitive conclusions.

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