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The effect of N-acetylcysteine on growth and biofilm formation in *Staphylococcus epidermidis* strains

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Aim: To examine the possible preventive effect of N-acetylcysteine (NAC) on biofilm formation in *Staphylococcus epidermidis* strains, which cause very important problems such as implant-associated infections in hospitalized and immunocompromised patients worldwide.

Materials and methods: The effects of varying concentrations of NAC on the formation of biofilm by 28 strains of *Staphylococcus epidermidis* was investigated spectrophotometrically by micro-ELISA assay.

Results: Significant differences in biofilm formation in the presence of various NAC concentrations were found in 15 methicillin-resistant *Staphylococcus epidermidis* and 13 methicillin-sensitive *Staphylococcus epidermidis* strains ($P < 0.05$).

Conclusion: It was found that slime formation was decreased due to the increased concentrations of the NAC treatments. Based on these findings, it is possible to use NAC as an antibiofilm agent on medical devices or in catheter lock solutions to prevent colonization and implant-associated infections.

Key words: *Staphylococcus epidermidis*, biofilm formation, indwelling device-associated infections, N-acetylcysteine

N-asetilsisteinin *Staphylococcus epidermidis* suşlarında üreme ve biyofilm oluşturma üzerine etkisi

Amaç: Bu çalışmanın amacı N-asetilsisteinin (NAC) hastaneye yatmış ve bağışıklık sistemi baskılanmış hastalarda, tüm dünyada implant ile ilişkili infeksiyonlar gibi çok önemli sorunlara neden olan *Staphylococcus epidermidis* suşlarında biyofilm oluşumundaki olası önleyici etkilerini incelemekti.

Yöntem ve gereç: NAC'nin çeşitli konsantrasyonlarının 28 *Staphylococcus epidermidis* suşu tarafından oluşturulan biyofilm üzerine etkisi mikroelisa yöntemiyle spektrofotometrik olarak test edildi.

Bulgular: NAC'nin çeşitli konsantrasyonlarının varlığında 15 metisilin dirençli *Staphylococcus epidermidis* ve 13 metisilin duyarlı *Staphylococcus epidermidis* suşlarında biyofilm oluşturma üzerinde anlamlı farklılıklar bulundu ($P < 0,05$).

Sonuç: NAC'nin artan konsantrasyonlarından dolayı yapışkan tabaka oluşumunda azalma görüldü. Bu bulgulara göre, kolonizasyonu ve yabancı cisim infeksiyonlarını önlemek için NAC' in medikal aletlerde ya da kateter solüsyonlarında antibiyofilm ajan olarak kullanımı mümkündür.

Anahtar sözcükler: *Staphylococcus epidermidis*, biyofilm oluşumu, yabancı cisim infeksiyonları, N-asetilsistein

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Introduction

The placement of indwelling medical devices into the human body for supporting and restoring function has become common practice in medicine (1). Indwelling device-associated infections demonstrate serious complications of high significance with related high morbidity and mortality rates, and also with high associated healthcare costs (2).

S. epidermidis is one of the most frequent causes of nosocomial infections (3) and the most common source of infections of indwelling medical devices such as intravascular catheters, cerebrospinal fluid shunts, prosthetic heart valves, peritoneal dialysis catheters, and prosthetic joints. *S. epidermidis*, which is a colonizer of human skin, can cause contamination of medical devices during insertion (4). The biofilm is broadly protective against host defenses (1). Infections of medical prosthetic devices in trauma and orthopedic surgery, which are difficult to treat with conventional therapies like antibiotics and surgery (5), may lead to removal of the implant to destroy biofilm-associated infections (6) and may lead to removal of the implant with function loss of the affected limb (7,8). The prevalence of methicillin-resistant *S. epidermidis* (MRSE) strains (9-11) and the emergence of vancomycin-resistant *S. epidermidis* (VRSE) further complicate the treatment of medical prosthetic device infections (12,13).

Antibiofilm activities of antibiotics or biocides combined with biofilm-specific agents such as N-acetylcysteine (NAC) (14), protamine sulfate (15), and dispersin B (16), which primarily target the extracellular matrix of biofilms, have been examined. Many drugs and compounds have been tested as biofilm inhibitors and some of these (e.g., silver, minocycline, rifampin, platinum, nitrofurantoin, chlorhexidine, and sulfadiazine) are used to coat catheters (17-20).

NAC, which is a nonantibiotic drug, has antibacterial properties. It is a mucolytic agent that disrupts disulfide bonds in mucus and, for this reason, it reduces the viscosity of secretions (21). In recent years, the actions of NAC in clinical applications have also broadened. NAC is widely used as a mucolytic agent and in the treatment of HIV, and its efficacy in chronic obstructive pulmonary disease and nephropathy has been reported (22). NAC, which is a glutathione precursor, provides neuroprotection by preventing oxidative damage (22,23). In addition, NAC is also used in the treatment of chronic bronchitis, cancer, and paracetamol intoxication (24,25). Acetylcysteine has been used to treat aspergilloma by local installation (26). NAC has been found to reduce biofilm formation by a variety of bacteria (21,27,28) and promote the disruption of mature biofilm (27,28).

Materials and methods

Bacteria

Fifteen MRSE and 13 methicillin-sensitive *S. epidermidis* (MSSE) isolates from samples from patients who visited the microbiology laboratory of Abant İzzet Baysal University Hospital (Faculty of Medicine, Abant İzzet Baysal University, Bolu, Turkey) were used as test microorganisms.

All of the isolates were identified as *S. epidermidis* according to colonial and microscopic morphology, positive catalase, and negative coagulase in the laboratory. All of the isolates were tested for methicillin resistance. The disk diffusion method was used with a 1-µg oxacillin disk (Oxoid, Basingstoke, UK). Isolates with zone sizes of ≤10 mm were considered methicillin-resistant.

Treatment with NAC

NAC was added to tryptic soy broth (TSB) (Merck, Whitehouse Station, NJ, USA) at concentrations of 0.03, 0.12, 0.5, and 2.0 mg/mL. Each concentration of NAC was analyzed separately. Isolates were inoculated into NAC-treated TSB and nontreated TSB as a control, which were then incubated at 37 °C for 24 h in the incubator. The process was repeated in triplicate.

Qualitative determination of the slime

Congo red agar method

For screening biofilm formation by *S. aureus*, bacteria were grown on Congo red agar (Merck) as described by Freeman et al. (1989) (29). Colony morphology was examined after holding at 37 °C for 24 h. A positive result was indicated by black colonies.

Tube method

The case study was verified by an assay, in which the biofilm formation by bacteria was additionally detected by another method described by Christensen et al. (1985). *S. epidermidis* isolates were inoculated in a polystyrene test tube that contained TSB and were then incubated at 37 °C for 24 h in the incubator (30). The biofilms that formed on the walls of the polystyrene test tube were washed twice with phosphate buffered saline (PBS) to remove the planktonic cells. The cells were then stained with safranin for 1 h. After discarding the safranin, the polystyrene test tube was washed twice with PBS and the process of air-drying the polystyrene test tube was conducted. If a visible film had lined the walls of the tube, slime production could be confirmed (30). The adherent bacterial films were measured spectrophotometrically at 540 nm in a microplate reader (Thermo Fisher, Waltham, MA, USA). This process was repeated with NAC-treated TSB at concentrations of 0.03, 0.12, 0.5, and 2.0 mg/mL to determine the effects of the NAC on the slime production of the isolates. The procedures were repeated in triplicate.

Quantitative determination of the slime

Nontreated TSB was used for the control. TSB supplemented with the different concentrations of NAC was also used. The optical density (OD) value of the inoculum was approximately 0.600 nm, as calculated with a spectrophotometer (Hitachi, Tokyo, Japan), and 200 µL of bacterial suspension that contained TSB was inoculated into a 96-well flat-bottomed sterile polystyrene microplate (LP Italiana, Milan, Italy). Some wells were left free of NAC as controls and the wells were incubated for 24 h at 37 °C.

The biofilm formation by bacteria was detected by the method described by Christensen et al., as follows (30). The biofilms that formed on the plates were washed twice with PBS to remove the planktonic cells. The cells were then stained with safranin (EMD Chemicals Inc., Gibbstown, NJ, USA) for 1 h. After removal of safranin from the microplate, the microplate was washed twice with PBS, followed by the air-drying of the wells. Adherent bacterial films were measured spectrophotometrically at 540 nm in a microplate reader (Thermo Fisher). This process was repeated with NAC-treated TSB at concentrations of 0.03, 0.12, 0.5, and 2.0 mg/mL to determine the effects of NAC on the slime production of the isolates.

Determination of the slime index

Following the 24-h incubation of the isolates, which were treated with different concentrations of NAC, the growth of *S. epidermidis* was confirmed with a micro-ELISA reader instrument (Thermo Fisher). The OD value of the biofilm corresponded with the OD value of the bacterial growth determined spectrophotometrically, before the aspiration of the culture, in order to compensate for the partial inhibition in growth caused by the NAC, and this was called the slime index (SI). The result was expressed as a percentage relative to the control without NAC. For this purpose, the following formula was applied: SI = 100 × (mean density of biofilm with treatment / mean growth with treatment) / (mean density of biofilm without treatment / mean growth without treatment) (21).

Statistical analysis

The Friedman test was used to determine the existence of differences in the different concentrations of NAC. The comparison between the different concentrations of NAC was carried out with the 2 related sample tests (Wilcoxon test) where significant differences were present. At the beginning of the study, $P < 0.05$ was set as statistically significant.

Results

In this study, 28 investigated strains of *S. epidermidis* were found to be biofilm-producing. The results determined by spectrophotometric assay for growth and biofilm formation in the presence of different concentrations of NAC are shown in Table 1.

It was found that there were significant differences in biofilm formation and growth among the different concentrations of NAC ($P < 0.05$). In addition, there were significant differences in the biofilm formation of MSSE and MRSE among the different concentrations of NAC. The decrease in the OD of the biofilms was probably in direct relation to the NAC concentration (Table 1). NAC applied at 4 different concentrations showed the same effect on the biofilm formation and growth of MSSE and MRSE ($P < 0.05$).

Table 1. Friedman test results showing the effects of different concentrations of NAC on the growth and biofilm formation of 28 isolates.

	NAC (mean ± SD)				df	N	P
	0.03 mg/mL	0.12 mg/mL	0.5 mg/mL	2.0 mg/mL			
SI	84.22 ± 26.04	76.90 ± 22.41	72.00 ± 2.42	76.96 ± 3.88	3	28	0.019*
Slime	79.72 ± 21.50	73.65 ± 26.14	66.00 ± 2.11	56.47 ± 2.07	3	28	0.000*
Growth	98.35 ± 26.83	95.57 ± 23.61	88.24 ± 1.75	77.02 ± 2.05	3	28	0.000*

*P < 0.05

At 4 different concentrations of NAC, 28 strains were found to decrease the biofilm formation, and NAC diminished the biofilm formation by 44% at a concentration of 2.0 mg/mL. The mean percentage of biofilm of all of the strains relative to the control at a concentration of 0.03, 0.12, 0.5, and 2.0 mg/mL of NAC was 79.72 ± 21.50%, 73.65 ± 26.14%, 66.00 ± 2.11%, and 56.47 ± 2.07% (P < 0.05), respectively (Table 1). NAC demonstrated a dose-dependent slime reducing activity at the 4 concentrations of NAC (Tables 1 and 2).

The SI indicates that there is a significant difference in biofilm formation between the concentrations of 0.03 and 0.5 mg/mL and between the concentrations of 0.03 and 2.0 mg/mL (P < 0.05) (Table 2). Therefore, the inhibitory effects of NAC on biofilm formation are the same at the concentrations of both 0.5 and 2.0 mg/mL (P < 0.05) (Table 2), and are higher than the inhibitory effect of the concentration of 0.03 mg/mL (Table 1).

At 4 different concentrations, NAC decreased the growth of 28 strains; this was found to be statistically significant (Table 1). The inhibitory effects of NAC on growth were the same at the concentrations of 0.03 and 0.12 mg/mL, and were less than the inhibitory effects of the concentrations of 0.5 and 2.0 mg/mL (Tables 1 and 2).

Discussion

The prevalence of MRSE strains (9-11) and the emergence of vancomycin-resistance in these species further complicate the treatment of biomaterial infections (12,13). Nosocomial staphylococcal foreign-body infections related to biofilm formation are a serious threat, demanding new and effective therapeutic and preventive strategies.

Several studies have been conducted to decrease the adherence of several bacteria, such as coagulase-negative *Staphylococcus*, on catheters by coating them with antiseptics and silver (18,20). The antibiofilm activities of antibiotics combined with NAC (14), protamine sulfate (15), and dispersin B (16) have been examined in recent studies. NAC was found to decrease biofilm formation by a variety of bacteria (21,27,28) and to promote the disruption of mature biofilm (27,28).

Table 2. Wilcoxon test results showing the effects of different concentrations of NAC on the growth and biofilm formation of 28 isolates.

		Concentrations of NAC (mg/mL-mg/mL)					
		0.12-0.03	0.5-0.03	2.0-0.03	0.5-0.12	2.0-0.12	2.0-0.5
	SI	0.076	0.011*	0.022*	0.200	0.203	0.615
P	**Slime**	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
	Growth	0.295	0.002*	0.000*	0.018*	0.000*	0.002*

*P < 0.05

In our study using 0.03, 0.12, 0.5, and 2.0 mg/mL of NAC, 28 strains lost 20%, 26%, 34%, and 44% of their capacity to produce biofilms. Juda et al. (19) studied the effect of EDTA on the formation of biofilm by *S. epidermidis* and showed that the adhesion and formation of the *S. epidermidis* biofilm on polychloride vinyl Nelaton and Thorax catheters was inhibited by EDTA at low concentrations (between 1 and 2 mmol/L) (19).

Our results show that NAC decreases growth-independent formation of biofilm, which is a major virulence factor of staphylococcal infections. For this reason, NAC may be an effective alternative for preventing indwelling prosthetic infections by *S. epidermidis*. This study has demonstrated that higher doses of NAC lead to lower levels of biofilm formation. In the presence of NAC at concentrations of 0.03 mg/mL or higher, the results become statistically significant. Four different concentrations of NAC showed the same effect on the biofilm formation and growth of MSSE and MRSE.

It would be appropriate to confirm these results by animal and clinical experiments. NAC could be administered through direct instillation, either intravenously or orally. However, it may be possible to obtain useful concentrations by local application to prevent the formation of biofilms and adherence of *S. epidermidis*. NAC may be incorporated into indwelling devices to prevent the adhesion of *S. epidermidis* on the devices. It is very difficult to remove an infected device, and the necessity of this may be prevented with the use of NAC. Indwelling device-associated infections, even by MRSE, may be prevented without using antibiotics or other chemicals, which may cause the development of resistance in bacteria.

In conclusion, our results suggest that NAC could prevent the formation of biofilms and adherence of *S. epidermidis*. NAC is a specific antibiofilm compound and might be used as an antibiofilm coating on medical devices or in catheter lock solution to prevent colonization and infection by biofilm pathogens. When incurable indwelling device-associated infections arise from *S. epidermidis*, NAC could be an alternative treatment to antibiotics. In this way, resistance to antibiotics in bacteria could be decreased.

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