Staphylococcus epidermidis biofilms control by *N*-acetylcysteine and rifampicin

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Running title: *N*-acetylcysteine as therapeutic adjuvant

Abstract

Medical device-associated infections caused by *Staphylococcus epidermidis* usually involve biofilm formation and its eradication is particularly challenging. Although rifampicin has been proving to be one of the most effective antibiotics against *S. epidermidis* biofilms its use as a single agent can lead to the acquisition of resistance. Therefore, we assessed the combined effect of rifampicin with *N*-acetylcysteine (NAC) known by its mucolytic effect, in the control of *S. epidermidis* biofilms. Biofilms of two *S. epidermidis* strains (9142 and 1457) were treated with 1xMIC (4 mg/mL) and 10xMIC (40 mg/mL) of NAC and 10 mg/L (peak serum) of rifampicin alone and in combination. NAC at 40 mg/L alone or in combination with rifampicin (10 mg/L) significantly reduced (4 $\log_{10}$) the number of biofilm cells. Considering their different modes of action, the association of NAC with rifampicin constitutes a promising therapeutic strategy in the treatment of infections associated to *S. epidermidis* biofilms.

Key words: *Staphylococcus epidermidis*, biofilm, planktonic cells, *N*-acetylcysteine, rifampicin.

INTRODUCTION

Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) produce extracellular matrix, which is an important virulence factor. This polymeric matrix promotes bacterial adhesion and produces a biofilm that makes the eradication of microorganisms more difficult (1). *Staphylococcus epidermidis*, the most frequently isolated coagulase-negative staphylococcus, is the leading cause of infection related to implanted medical devices (IMDs). This is directly related to its capability to establish multilayered, highly structured biofilms on artificial surfaces. Bacterial biofilms are difficult to detect in routine diagnostics and are inherently tolerant to host defenses and conventional antibiotic therapies. Thus, device-related infections are notoriously difficult to treat and bacteria within biofilm communities on the surface of IMDs frequently outlive treatment, and removal of the medical device is often required for successful therapy (2). Additionally, the emergence of antibiotic-resistant bacteria and the slow progress in identifying new classes of antimicrobial agents have encouraged research into novel therapeutic strategies (3).

N-acetylcysteine (NAC) is a non-antibiotic drug that has antibacterial properties. It is a mucolytic agent that disrupts disulphide bonds in mucus and reduces the viscosity of secretions (4). NAC is commonly used in medical treatment of chronic bronchitis and cancer (5, 6) and is one of the smallest drug molecules in use (7). NAC affects several processes that are

important for bacterial biofilm formation on stainless steel surfaces, including a drastic reduction in extracellular polysaccharide production, and thus acts as an antibiofilm substance (8).

Previous results have demonstrated that rifampicin is the most effective of traditional antibiotics against *S. epidermidis* cells in biofilms (9-12).

Accordingly, the aim of this work was to determine the *in vitro* effect of NAC alone but specifically in combination with rifampicin on biofilms of two different *S. epidermidis* strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Two *Staphylococcus epidermidis* clinical isolate strains (9142 and 1457, good biofilm-producers) were used in this study. Both strains were provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA.

The culture media used tryptic soy broth (TSB) and tryptic soy agar (TSA), were prepared according to the manufacturer's instructions. All strains were inoculated into 15 mL of TSB from TSA plates not older than 2 days and grown for 24 (±2) h at 37°C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 5 min at 9500*g* and 4°C), and ressuspended in TSB adjusted to an optical density (640 nm) equivalent to 1 x 10^6 cells/mL and then used in the subsequent assays.

Solutions of NAC 4 mg/mL (1x MIC) and 40 mg/mL (10x MIC) (13) were prepared in TSB and stock solution of rifampicin (10 mg/L - peak serum concentration) (9, 14) was prepared in methanol.

Minimum inhibitory concentration (MIC)

MIC determination of the tested agent *N*-acetylcysteine and for each *S. epidermidis* strain (9142 and 1457) was carried with dilution range of 0.5-64 mg/mL.

The MIC was determined in 96 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 100 µL of a stock solution of NAC (64 mg/mL) and the dilution was realized with TSB, adding at the end 100 µL of a *S. epidermidis* cell suspension (1 x 10^6 cells/mL). Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 h. After incubation the minimum inhibitory concentration was determined with the lowest concentration able to inhibit bacterial growth.

The controls were not exposed to the antimicrobial agent tested. All experiments were carried out in triplicate and repeated three times.

Effect of NAC and rifampicin on biofilm cells

Biofilms were formed in 96 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 200 µL of a *S. epidermidis* cell suspension (1 x 10^6 cells/mL) in TSB supplemented with 0.25% of glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 h. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 200 µL of saline solution (0.9% NaCl; Merck). The biofilms were incubated for 24 h in fresh nutrient medium containing NAC (4 mg/mL and 40 mg/mL) or rifampicin (10 mg/L) alone or a combination of both NAC concentrations tested with rifampicin.

Crystal violet (CV) staining was used as indicator of total biofilm biomass. After exposure to the treatment agents, biofilms were washed with 200 µL of saline solution, then 250 µL of methanol was added and

allowed to act for 15 min. Afterwards methanol was removed and crystal violet was added (5 min). The wells were washed with water and finally, acetic acid 33% (v/v) was added. The absorbance was measured at 570 nm.

Another colorimetric method based on the reduction of XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phe-nylamino)carbonyl]-2H-tetrazolium hydroxide}; Sigma, St Louis, USA) was applied to determine cell activity (XTT is converted to a colored formazan salt in the presence of metabolic activity) (15). After exposure to antimicrobial agents, biofilms were washed with 200 µL of saline solution, then 200 µL of a solution containing 200 mg/L of XTT and 20 mg/L of phenazine methosulphate (PMS; Sigma, St Louis, USA) was added to each well. The microtiter plates were incubated for 3 h at 37°C in the dark. The absorbance was measured at 490 nm.

To assess the number of viable cells after treatment, 200 µL of saline solution were added to each well before removing the biofilm by scrapping. An aliquot of 1 mL of each sample was centrifuged (for 10 min at 9500*g* and 4°C) and the pellet ressuspended in 1 mL of saline solution. Next, the suspension was sonicated (20 s with 22% of amplitude; Ultrasonic Processor, Cole-Parmer, Illinois, USA) to promote biofilm disruption. The number of colony forming units (CFU) in biofilm for each *S. epidermidis* strain was determined by performing 10-fold serial dilutions in saline solution and plating in TSA in triplicate and incubating for 24–48 h.

Biofilm controls were not exposed to any antimicrobial agent tested. All experiments were carried out in triplicate and repeated three times.

Effect of NAC and rifampicin on planktonic cells

For each strain, 200 µL of a cell suspension adjusted to 1×10^9 cells/mL and 1.5 mL of that suspension was added to 15 mL of TSB until a cell density of 1×10^8 cells/mL was reached and were then dispensed in test tubes followed by the addition of the treating agents alone, NAC (4 mg/mL and 40 mg/mL) or rifampicin (10 mg/L), and in combination. All the tubes were incubated at 37°C with shaking at 130 rpm.

Cell susceptibility to the treatment agents was determined by the XTT colorimetric method. For that, 1 mL of each sample was collected and centrifuged (for 10 min at 9500*g* and 4°C) and the pellet was ressuspended in 1 mL of saline solution (0.9% NaCl; Merck). From each suspension, 200 µL were transferred to individual wells of a 96-well microtiter plate. Then, 50 µL of a solution containing 200 mg/L of XTT and 20 mg/L of PMS were added to each well and the microtiter plates were incubated for 3 h at 37°C in the dark. The absorbance was measured at 490 nm.

For CFU determination, 1 mL of each sample was collected and centrifuged (for 10 min at 9500*g* and 4°C) and the pellet ressuspended in 1 mL of saline solution. The suspension was sonicated (20 s with 22% of amplitude; Ultrasonic Processor, Cole-Parmer, Illinois, USA) and the viable planktonic cells were determined by performing 10-fold serial

dilutions in saline solution and plating in TSA. Colonies were counted after 24-48 h at 37°C.

Controls were planktonic cells not exposed to any treatment agent tested. All experiments were carried out in triplicate and repeated three times.

Extraction of the exopolymeric matrix

Biofilms were formed in 6 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 4 mL of a *S. epidermidis* cell suspension (1 x 10^6 cells/mL) in TSB supplemented with 0.25% of glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 h. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 4 mL of saline solution. The biofilms were incubated in fresh nutrient medium containing NAC (4 mg/mL and 40 mg/mL), rifampicin (10 mg/L) alone and NAC in combination with rifampicin. After 24 h of incubation, the biofilm was washed twice with 4 mL of saline solution. Next, 4 mL of phosphate buffered saline (pH 7.0) were added and the biofilm formed was removed from the well by scrapping with a cell scraper (zellschaber/24 cm). The samples were centrifuged for 5 min at 9500*g* and 4°C, and ressuspended in 5 mL of extraction buffer (2 mM Na3PO4; 4 mM NaH2PO4; 9 mM NaCl and 1 mM KCl, pH 7.0) and 1 g of resin (Dowex/50X8, Na^+ form, 20-50 mesh, Aldrich-Fluka 44445) was added. The samples were incubated at -

5°C for 2 h. After the incubation the samples were centrifuged for 20 min at 9500*g* and 4°C.

The polysaccharide content in the biofilm matrix was quantified by the method of Dubois et al. (16). The protein content of the biofilm matrix was measured using the BCA^{tm} Protein Assay Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, USA).

Controls were biofilms not exposed to any treatment agent tested. All experiments were carried out in triplicate and repeated three times.

Scanning electron microscopy (SEM)

Biofilms formed in 12 well tissue culture plates (Sarstedt, Newton, NC, USA) were dehydrated by 15 min immersion in increasing ethanol concentrations (70, 95 and 100% [v/v]), and then placed in a sealed desiccator. The samples were mounted on aluminum stubs with carbon tape, sputter coated with gold and observed with a Leica Cambridge S-360 scanning electron microscope (Leo, Cambridge, UK).

Controls were biofilms not exposed to any treatment agent tested. All experiments were carried out in triplicate.

Statistical Analysis

The data from all assays were compared using one-way analysis of variance (ANOVA) by applying Tukey's test with all calculations carried out using SPSS software (Statistical Package for the Social Sciences).

Differences achieving a confidence level of 95% were considered significant.

RESULTS

Figure 1 presents the number of viable cells of the two *S. epidermidis* strains tested, expressed as log CFU, remaining after the treatment of either biofilm or planktonic cells. It can be observed that NAC alone at 4 mg/mL (1x MIC) had a slight inhibitory effect on planktonic cells but almost no effect on biofilms, while at 10x MIC it showed a notorious killing effect on planktonic cells (almost total eradication) and a significant bactericidal effect on biofilms, in this case promoting CFU reductions of about 4 $\log_{10}$ ($p<0.05$). Rifampicin alone, showed an effect on biofilm cells promoting CFU reduction of about 3 $\log_{10}$ ($p<0.05$). Although the reduction promoted by rifampicin in planktonic cells was less than 3 log. The NAC-rifampicin combination consistently decreased the number of viable biofilm associated bacteria by 3-4 $\log_{10}$ independently of NAC concentration used. In fact, NAC at 40 mg/ml (10x MIC), rifampicin alone or NAC-rifampicin combinations showed significant but similar bactericidal effect against biofilms ($p<0.05$). The combination of NAC (10x MIC) with rifampicin in planktonic cells promoted a killing effect ($p<0.05$).

The results expressing the decrease in metabolic activity measured by the XTT reduction assay after treatment with the tested agents are showed in figure 2.

In addition, total biofilm biomass, assessed by CV staining, also confirms the effect of the agents tested as can be seen in figure 3.

The matrix composition showed a generally significant increase ($p<0.05$) in the amount of proteins and polysaccharides after treatment, presented in figure 4. The amount of proteins was very high after treatment with the combination NAC 40 mg/mL with rifampicin ($p<0.05$). However, the amount of polysaccharides was high after treatment with NAC (10x MIC) alone or the combination NAC-rifampicin, independently of NAC concentration used ($p<0.05$).

Scanning electron microscope images of the biofilms are presented in figure 5. The SEM shows representative images of *S. epidermidis* (strain 1457) biofilms after treatment with the agents tested alone and in combination. As can be seen (Figure 5C and F), after treatment with NAC at 40 mg/mL alone or combined with rifampicin the amount of matrix present is very small and this might be due to an easy removal of the matrix during the desiccation procedure by successively washing with increasing ethanol concentrations.

DISCUSSION

Modern medicine is facing the challenge to control the increasing incidence of biofilm infections and this situation has boosted the search of new therapeutic strategies able to evade the intrinsic tolerance of biofilms to antimicrobial agents. Accordingly, the rationale of this study stems from the reported effect of NAC in disrupting mature biofilms (4) and the high efficacy of rifampicin against *S. epidermidis* biofilms when compared with other common antibiotics (9-12). Thus, we hypothesized that in combination they could have a synergistic effect due to their different modes of action.

The great efficacy of NAC against planktonic cells was also confirmed in experiments with other gram-positive and gram-negative strains, showing that NAC did indeed reduce the growth of all strains tested (8). Rifampicin alone, as we could expect from previous studies (9-11), showed higher efficacy against biofilm cells than planktonic cells. As demonstrated by viable cells reduction (Figure 1) and in opposition to our hypothesis the combinations tested were not synergistic or additive in controlling *S. epidermidis* biofilms. In a previous study (13), reported a synergistic effect of NAC in combination with tigecycline on *S. epidermidis* biofilms using NAC at 20x MIC (80 mg/mL) and tigecycline at 1000x MIC (1 mg/mL) with the MIC values reporting to planktonic cells. Nevertheless,

their results in viable cells reduction of this species were less than 3 $\log_{10}$. Regarding the NAC-rifampicin combination tested herein the values obtained where higher, corresponding to a bactericidal action, even if not corresponding to a synergy between the two agents.

In this work, the biofilm formation capability was as well evaluated through crystal violet, allowing the quantification of the total biomass. The metabolic activity of biofilms was evaluated through XTT reduction assays. The results shown are in very good agreement with those obtained in terms of cell viability.

Interestingly, the matrix composition showed a generally significant increase in the amount of proteins and polysaccharides after treatment (Figure 4). Notoriously, the amount of proteins was very high after treatment, which is consistent with a higher degree of cell lysis. The increase in polysaccharides content is probably due in greater extent to the loosening effect of NAC on the matrix structure and thus making its extraction more efficacious. As shown previously the strain 1457 produces more extracellular polysaccharides than 9142 (17). Moreover, NAC is a thiol containing molecule that is described to disrupt disulfide bonds in mucus (18). In fact, SEM observations support this hypothesis.

It should be stressed that it is now generally accepted that a combination of antimicrobial agents is a strategy to evade the development of bacterial resistance to antibiotics. In fact, it is not advisable to use rifampicin as a single agent to treat infections because of

the rapid selection of resistant mutants (19). Zheng and Stewart (20) detected rifampicin-resistant mutants, when colony biofilms were exposed to rifampicin for periods longer than 48 h. In the present case, the combination 40 mg/mL NAC- 10 mg/L rifampicin showed a higher and real bactericidal effect on biofilms (above 3 $\log_{10}$), which probably opens the way for an efficient action of the immune system in the eradication of *in vivo* biofilms.

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LEGEND TO FIGURES

Figure 1. Number of viable cells (expressed as $\log_{10}$ CFU) of *S. epidermidis* recovered from biofilm cells (A) and planktonic cells (B) after treatment with NAC and rifampicin (RIF) alone or in combination.

Figure 2. Cellular activity expressed as XTT absorbance of *S. epidermidis* biofilm cells (A) and planktonic cells (B) after treatment with NAC and rifampicin (RIF) alone or in combination.

Figure 3. *S. epidermidis* total biofilm biomass expressed as crystal violet absorbance after treatment with NAC and rifampicin (RIF) alone or in combination on biofilm cells.

Figure 4. Quantification of protein (A) and polysaccharides (B) of the exopolymeric matrix of *S. epidermidis* (9142 and 1457) biofilms.

Figure 5. Scanning electron microscopy photographs of 24 h *S. epidermidis* (strain 1457) biofilms: control (A), NAC 4 mg/mL (B), NAC 40 mg/mL (C), rifampicin (D), NAC (4 mg/mL) with rifampicin (E) and NAC

(40 mg/mL) with rifampicin (F). The bar in the images corresponds to 5µm. Magnification x 5000.