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Original Paper

The Interaction of *N*-Acetylcysteine and Serum Transferrin Promotes Bacterial Biofilm Formation

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Key Words
Biofilm • *N*-acetylcysteine • Serum • *Staphylococcus aureus* • *Pseudomonas aeruginosa* • *Staphylococcus epidermidis*

Abstract
Background/Aims: *N*-acetylcysteine (NAC) is a novel and promising agent with activity against bacterial biofilms. Human serum also inhibits biofilm formation by some bacteria. We tested whether the combination of NAC and human serum offers greater anti-biofilm activity than either agent alone. ***Methods:*** Microtiter plate assays and confocal laser scanning microscopy were used to evaluate bacterial biofilm formation in the presence of NAC and human serum. qPCR was used to examine expression of selected biofilm-associated genes. Extracellular matrix (ECM) was observed by transmission electron microscopy. The antioxidants GSH or ascorbic acid were used to replace NAC, and human transferrin, lactoferrin, or bovine serum albumin were used to replace serum proteins in biofilm formation assays. A rat central venous catheter model was developed to evaluate the effect of NAC on biofilm formation *in vivo*. ***Results:*** NAC and serum together increased biofilm formation by seven different bacterial strains. In *Staphylococcus aureus*, expression of genes for some global regulators and for genes in the *ica*-dependent pathway increased markedly. In *Pseudomonas aeruginosa*, transcription of *las*, the PQS quorum sensing (QS) systems, and the two-component system GacS/GacA increased significantly. ECM production by *S. aureus* and *P. aeruginosa* was also enhanced. The potentiation of biofilm formation is due mainly to interaction between NAC and transferrin. Intravenous administration of NAC increased colonization by *S. aureus* and *P. aeruginosa* on implanted catheters. ***Conclusions:*** NAC used intravenously or in the presence of blood increases bacterial biofilm formation rather than inhibits it.

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Introduction

Biofilms are organized microbial communities that contain a group of microorganisms localized within a self-produced extracellular matrix. Biofilms often form on living or non-living surfaces and are prevalent in natural, industrial, and medical settings. In hospitals, biofilms occur on implanted medical devices such as urinary or intravenous catheters, prosthetic cardiac valves, orthopedic implants, and contact lenses [1]. They can also be found on soft tissues, with notable examples including the *Pseudomonas aeruginosa* biofilms in cystic fibrosis (CF) patients and the bacterial biofilms that develop on chronic wound surfaces such as diabetic foot ulcers and burns [2-5]. Because biofilms can effectively shield pathogens from host immune responses and antimicrobial treatment, and may lead to refractory infections or biofilm-associated diseases, they present a great health risk [6, 7].

Traditional antibiotics are often ineffective against biofilm infections because of their low penetration into the deeper layers of the biofilm biomass [6]. Recent studies show that *N*-acetylcysteine (NAC), which is widely used as an antioxidant and a mucolytic agent in conventional clinical treatment, also exhibits antibacterial properties and can inhibit biofilm formation [8, 9]. NAC used alone or in combination with certain antibiotics can reduce biofilm formation by a variety of bacteria that are commonly isolated in hospitals, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* [10-15]. NAC not only affects bacterial growth, but also inhibits extracellular polysaccharide production, reduces bacterial adherence to epithelial cells and solid surfaces, and promotes dispersal of mature biofilms [9]. These properties suggest that NAC is a promising candidate for the treatment of biofilm-associated infections.

Human serum also has been reported to inhibit biofilm formation by some bacteria [16, 17]. Serum proteins such as transferrin, lactoferrin, albumin, and other unknown components, have important roles in preventing biofilm formation [16, 18-21]. Because NAC has also been reported to reduce biofilm formation on vascular catheters *in vitro* [22], we speculated that a combination of NAC and human serum might inhibit biofilm formation to an even greater extent. Unexpectedly, we found that NAC increases biofilm formation in broth containing human serum. The combination of NAC and human serum increases expression of some biofilm-associated genes and enhances extracellular matrix (ECM) production by *S. aureus* ATCC25923 and *P. aeruginosa* PAO1. Additional experiments demonstrated that the potentiation of biofilm formation occurs primarily due to interaction between NAC and the serum protein transferrin. Experiments using an animal infection model showed that intravenous administration of NAC also increases colonization of ATCC25923 and PAO1 on implanted catheters. Taken together, these results suggest that NAC may be not suitable as an anti-biofilm agent when used intravenously or in the presence of blood.

Materials and Methods

Bacterial strains and growth conditions

S. aureus ATCC25923 (MSSA) and N315 (MRSA), *S. epidermidis* ATCC35984, *P. aeruginosa* PAO1, *A. baumannii* ATCC19606, and two clinical strains of *E. faecalis* and *K. pneumoniae,* isolated from our institute (Institute of Burn Research, Southwest Hospital, Chongqing, China), were used in this study. Typically, *S. aureus* and *S. epidermidis* were cultured in tryptic soy broth (TSB), and *P. aeruginosa*, *A. baumannii*, *E. faecalis*, and *K. pneumoniae* were grown in Luria Bertani (LB) broth. Cultures were incubated at 37 °C with shaking at 200 rpm.

Human serum isolation

Human whole blood was obtained from 10 healthy volunteers by venipuncture, using a protocol approved by the Laboratory Animal Welfare and Ethics Committee of Southwest Hospital, Third Military Medical University. Blood samples were incubated at room temperature for 2 h and then centrifuged at 500

g for 5 min to separate the serum. Serum samples were stored at –80 °C until use. Just prior to use, serum was incubated at 56 °C for 30 min then filtered through a 0.22 μm membrane.

Microtiter plate assay

Biofilm formation was determined by microtiter plate assay as described previously [23]. Briefly, overnight bacterial cultures were diluted 1:100 in the broth and aliquoted into wells of a 96-well plate and incubated for 24 h on a rocker platform at 37 °C. When required, NAC (5 mM, 10 mM, 20 mM, pH 7.2, daily fresh preparation, Sigma-Aldrich, powder, CAS 616-91-1), GSH (20 mM, daily fresh preparation, Sigma-Aldrich, CAS 70-18-8), or ascorbic acid (20 mM, daily fresh preparation, Sigma-Aldrich, CAS 50-81-7) and human serum (20% vol/vol), transferrin (2 mg/ml, apo-transferrin, Sigma-Aldrich, CAS 11096-37-0), lactoferrin (500 ng/ml, Partially iron saturated, Sigma-Aldrich, L4040), or BSA (2 mg/ml, Sigma-Aldrich, CAS 9048-46-8) were added to the broth. Following 24 h incubation, planktonic bacteria were discarded and the plate was washed gently with water three times. The attached biomass was fixed by baking at 60 °C for 1 h. After staining with 100 μl 0.3% crystal violet for 15 min at room temperature, the plate was washed with running tap water to remove excess stain. The plate was then dried and the biomass associated stain was extracted in a 100 μl 70% ethanol–10% methanol mixture. Absorbance at 590 nm (A_{590}) was measured.

Confocal laser scanning microscopy (CLSM)

Overnight cultures of *S. aureus* ATCC25923 and N315, *S. epidermidis* ATCC35984, and *P. aeruginosa* PAO1 were diluted 1:100 in TSB or LB media in the presence of 10 mM NAC, or 20% serum, or a combination of NAC and serum. Bacteria were cultured in 15 mm glass-bottom cell culture dishes (polystyrene) on a rocker platform at 37 °C. After incubation for 24 h, the dishes were washed with water three times and fixed with 4% formaldehyde. Two ml of a 1:1000 dilution of the nucleic acid stain SYTO 61 (Invitrogen) was added to the dishes, followed by incubation in the dark at room temperature for 30 min. The dishes were then washed and 2 ml of 50 μg/ml FITC-labelled concanavalin A type IV (Sigma-Aldrich) was added. After incubation in the dark for 5 min at 37 °C, the dishes were rinsed with water three times and air-dried. Visualization of biofilms was performed using a laser scanning confocal microscope (LSM780, Carl Zeiss, Plan-Apochromat 63×/1.40 Oil M27 objective lens) with 561 nm excitation and 640 nm emission wavelengths for SYTO61, and 488 nm and 537 nm for FITC-ConA. Images were acquired from at least three distinct regions of each cell culture dish and processed using the ZEN image analysis package (Carl Zeiss). Biofilm thickness was measured by Z-stack images [15]. Three independent replicas were conducted for each experiment.

Quantitative real-time PCR

S. aureus ATCC25923 and *P. aeruginosa* PAO1 were grown to late exponential phase in the presence of 20% human serum alone or in combination with 10 mM NAC. Total RNA was extracted and transcribed to cDNA as described

Table 1. Primers used for quantitative real-time PCR

Primers	Sequence (5'-3')	Use
16S-F	GCGCTGCATTAGCTAGTTGGT	qPCR in S. aureus
16S-R	TGGCCGATCACCCTCTCA	qPCR in S. aureus
fnbA-F	CCAGGTGGTGGTCAGGTTAC	qPCR in S. aureus
fnbA-R	TGTGCTTGACCATGCTCTTC	qPCR in S. aureus
fnbB-F	ACCTGCTAAAGAAGAACC	qPCR in S. aureus
fnbB-R	CGTAATAACGCTAAACCTA	qPCR in S. aureus
clfA-F	TTTCAACAACGCAAGATA	qPCR in S. aureus
clfA-R	GCTACTGCCGCTAAACTA	qPCR in S. aureus
clfB-F	TTTGGGATAGGCAATCATCA	qPCR in S. aureus
clfB-R	TCATTTGTTGAAGCTGGCTC	qPCR in S. aureus
icaA-F	TACTATTTCGGGTGTCTTCA	qPCR in S. aureus
icaA-R	CAAAGACCTCCCAATGTT	qPCR in S. aureus
rbf-F	ACGCGTTGCCAAGATGGCATAGTCTT	qPCR in S. aureus
rbf-R	AGCCTAATTCCGCAAACCAATCGCTA	qPCR in S. aureus
sarA-F	GCACAACAACGTAAAAAAATCGAA	qPCR in S. aureus
sarA-R	TTCGTTGTTTGCTTCAGTGATTC	qPCR in S. aureus
saeS-F	AATCCAGAACCACCCGTTTT	qPCR in S. aureus
saeS-R	ACGCCACTTGAGCGTATTTT	qPCR in S. aureus
rot-F	TCGCTTTCAATCTCGCTGAA	qPCR in S. aureus
rot-R	CGACACTGTATTTGGAATTTTGCA	qPCR in S. aureus
sigB-F	CCTACTGTAATCGGTGAAATC	qPCR in S. aureus
sigB-R	GTCCCATTTCCATTGCTTC	qPCR in S. aureus
agrC-F	ACCCGATGAAGTAAGTAGCA	qPCR in S. aureus
agrC-R	TAGACCTAAACCACGACCTT	qPCR in S. aureus
cidA-F	AATTTCGGAAGCAACATCCA	qPCR in S. aureus
cidA-R	CTTCCCTTAGCCGGCAGTAT	qPCR in S. aureus
atl-F	TTTGGTTTCCAGAGCCAGAC	qPCR in S. aureus
atl-R	TTGGGTTAAAGAAGGCGATG	qPCR in S. aureus
clpC-F	TCAGGTGCCACAGGAAG	qPCR in S. aureus
clpC-R	ATTTCAACAGTATCTTTAGCG	qPCR in S. aureus
clpX-F	TGTAGCAGGAAGTGGTGT	qPCR in S. aureus
clpX-R	CATCATCTTCTTTTGGTCC	qPCR in S. aureus
rpsL-F	GCTGCAAAACTGCCCGCAACG	qPCR in P. aeruginosa
rpsL-R	ACCCGAGGTGTCCAGCGAACC	qPCR in P. aeruginosa
lasI-F	CGCACATCTGGGAACTCA	qPCR in P. aeruginosa
lasI-R	CGGCACGGATCATCATCT	qPCR in P. aeruginosa
lasR-F	CTGTGGATGCTCAAGGACTAC	qPCR in P. aeruginosa
lasR-R	AACTGGTCTTGCCGATGG	qPCR in P. aeruginosa
rhlI-F	GTAGCGGGTTTGCGGATG	qPCR in P. aeruginosa
rhlI-R	CGGCATCAGGTCTTCATCG	qPCR in P. aeruginosa
rhlR-F	GCCAGCGTCTTGTTCGG	qPCR in P. aeruginosa
rhlR-R	CGGTCTGCCTGAGCCATC	qPCR in P. aeruginosa
pqsA-F	GACCGGCTGTATTCGATTC	qPCR in P. aeruginosa
pqsA-R	GCTGAACCAGGGAAAGAAC	qPCR in P. aeruginosa
pqsR-F	CTGATCTGCCGGTAATTGG	qPCR in P. aeruginosa
pqsR-R	ATCGACGAGGAACTGAAGA	qPCR in P. aeruginosa
algC-F	CTACTTCAAGCAGATCCGC	qPCR in P. aeruginosa
algC-R	AGGTCCTTCAGGTTCTCC	qPCR in P. aeruginosa
flgK-F	GAGGCGACCACCAACAAC	qPCR in P. aeruginosa
flgK-R	CGCTCAGAGGCAGACCAT	qPCR in P. aeruginosa
gacA-F	CGGGGCTGGAGGAAATG	qPCR in P. aeruginosa
gacA-R	TGCTGCGGCTGGAAGGA	qPCR in P. aeruginosa
gacS-F	GCTTGGCGGTTACTTCAC	qPCR in P. aeruginosa
gacS-R	AGCACGGCGGTATCCTTT	qPCR in P. aeruginosa
pilF-F	TACGGCGGTTTCCTCTACG	qPCR in P. aeruginosa
pilF-R	CTGGGCTGGTTACGGTTCA	qPCR in P. aeruginosa
pslA-F	ACCGACACCCTGCACAAG	qPCR in P. aeruginosa
pslA-R	CGAACCACACCGACCACT	qPCR in P. aeruginosa

previously [23]. qRT-PCR was conducted in a CFX Connect Real-Time PCR System (BIO-RAD) using SYBR green real-time PCR master mix (TOYOBO). The 16S rRNA gene in *S. aureus* or *rpsL* in *P. aeruginosa* were used as an internal reference. Primers are shown in Table 1. The comparative threshold cycle ($\Delta\Delta C_T$) method was used to quantitate gene transcript levels. Results are shown as relative fold changes in transcript levels in bacteria challenged with NAC and serum in combination, compared to the values observed for bacteria cultured in 20% serum alone, with the latter normalized to a value of 1.

Transmission electron microscopy (TEM)

Bacteria were cultured in broth containing 20% human serum alone or in combination with 10 mM NAC. Overnight cultures were centrifuged and resuspended in PBS. *S. aureus* samples were prepared as previously described [24], and *P. aeruginosa* was stained with potassium phosphotungstate (2%, pH 7.0). Bacteria were then observed using a TECNAI 10 transmission electron microscope (Philips, Netherlands).

Interaction between NAC and transferrin

Serum protein transferrin (2 mg/ml) was incubated in the presence or absence of 20 mM NAC, GSH, or ascorbic acid at 37 °C for 24 h. After 5× non-reducing loading buffer was added to each sample, they were immersed in a boiling water bath for 10 min and then subjected to SDS-PAGE analysis. Protein bands were stained with Coomassie Brilliant Blue R250 dye overnight with gentle agitation, and gels were destained in methanol-acetic acid-H_2O (5:1:4) for 1 h.

Rat central venous catheter model

The rat central venous catheter model was developed as previously described [23]. Twenty-four hours after catheters were implanted, male SD rats (n=48, 250-300 g) were challenged with 5×10^6 CFU of *S. aureus* ATCC25923 or *P. aeruginosa* PAO1 via the tail vein (n=24 per group). Half of the rats in each group (n=12) were selected randomly to receive NAC (150 mg/kg/day) [25, 26] solution via tail vein injection for 7 days. Control rats were injected the same volume of saline. At the end of treatment, all rats were sacrificed and the catheters removed. Catheter segments were washed three times with sterile PBS and then subjected to ultrasonic agitation (45 kHz, 100% Power) in 1 ml PBS for 5 min. Detached bacteria were titered using the drop plate method. The protocol for animal experiments was approved by the Laboratory Animal Welfare and Ethics Committee of Southwest Hospital, Third Military Medical University.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA) or Student's *t* test as appropriate. A *P* value less than 0.05 was considered statistically significant.

Results

The combination of NAC and serum increases biofilm formation in vitro.

To determine the effect of NAC and serum on biofilm formation, 6 different species of bacteria were exposed to various concentration of NAC in broth containing 20% human serum or deionized water, biofilm formation was quantified using a microtiter plate assay. As shown in Fig. 1, consistent with previous studies, 20 mM NAC or 20%

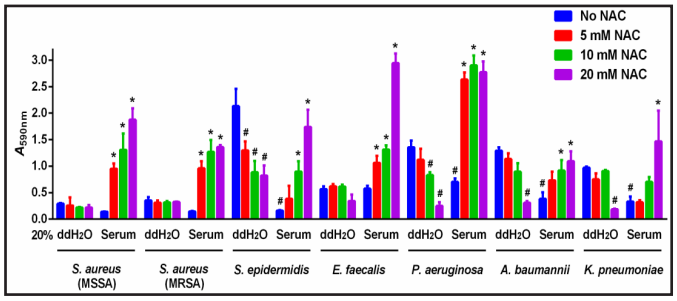

Fig. 1. Combination of NAC and serum increases biofilm formation. The indicated strains were exposed to various concentration of NAC (0, 5, 10, and 20 mM) in broth containing 20% human serum or deionized water. Biofilm formation was quantified using a microtiter plate assay in which absorbance of the extracted crystal violet was determined at 590 nm. Data are shown as means±standard deviations from three independent trials. **P*<0.05 indicates significant differences compared to samples exposed to 20% human serum alone. #*P*<0.05 indicates significant differences compared to controls without any exposure to NAC or serum.

human serum alone significantly decreased biofilm formation by *S. epidermidis*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*. However, the combination of NAC and serum increased biofilm formation in all bacteria tested. This effect was dose-dependent, increasing as the concentration of NAC increased from 0 to 20 mM.

Using CLSM, we measured the thickness of biofilms formed by *S. aureus* (ATCC25923 and N315), *S. epidermidis* (ATCC35984), and *P. aeruginosa* (PAO1) in the presence of NAC alone, serum alone, or in combination. As shown in Fig. 2a, bacterial cells were stained red with SYTO 61 and extracellular polysaccharide was stained green with FITC-labelled concanavalin A. Consistent with the results of the microtiter plate assay, the biomass was much denser when bacteria were exposed to NAC and serum together (Fig. 2a). Biofilm thickness was also significantly increased (Fig. 2b). This supports the conclusion that the combination of NAC and human serum enhances biofilm formation.

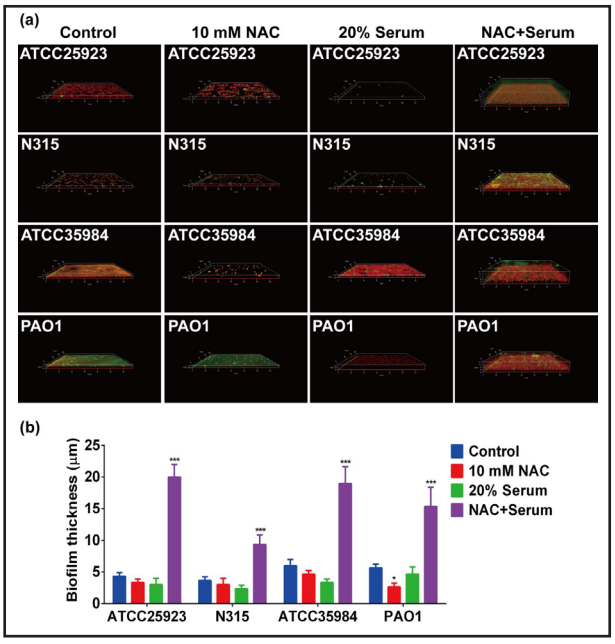

Fig. 2. Thickness of biofilms formed by bacteria treated with NAC and serum. The indicated strains were grown in the presence of 10 mM NAC alone, 20% serum alone, or in combination. The red fluorescent nucleic acid stain SYTO61 was used to identify bacteria in the biofilms, and the green fluorescent stain FITC-ConA was used to identify extracellular polysaccharide. (a) Representative CLSM Z-stack images (× 630) acquired from three independent experiments. (b) Biofilm thicknesses are shown as means±standard deviations. **P*<0.05 and ****P*<0.001 indicate differences are significant compared to controls.

It's worth noting that in the microtiter plate assay, biofilm formation of *S. epidermidis* ATCC35984 in the control group showed no significant difference in comparison to the NAC and serum combination group, while when observed by CLSM, biofilm thickness of *S. epidermidis* ATCC35984 in the presence of NAC and serum was much denser than the control group. This discordance may attribute to the strong biofilm formation ability of *S. epidermidis* ATCC35984 in the basal culture medium and the limitation of optical density (OD) measurement in the microtiter plate assay. It is not very accurate when the OD reaches higher readings (especially greater than 1.5). So, it is more precise when

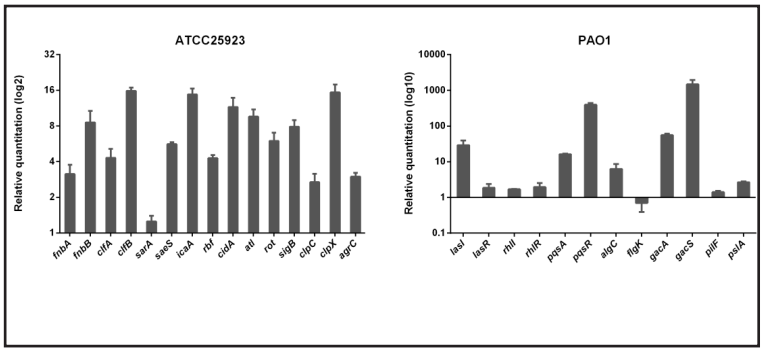

Fig. 3. Exposure to NAC and serum alters transcript levels for some biofilm-associated genes. Transcript levels of genes relevant to biofilm formation in S. aureus ATCC25923 and P. aeruginosa PAO1 were evaluated by qPCR after exposure to serum alone or in combination with NAC. Bars represent relative fold changes for genes in strains treated with both NAC and serum, compared to their serum-only-treated counterparts (normalized as 1). Data are from three independent experiments.

observed by CLSM. Overall, these results indicate that NAC combined with serum induces biofilm formation of all the tested strains.

Exposure to NAC and serum together alters the expression of some biofilm-associated genes

To explore the molecular pathways of biofilm formation that may be involved in the response to NAC and serum exposure, we measured transcript levels for 15 representative genes in *S. aureus* and 12 genes in *P. aeruginosa* (Fig. 3). Compared with exposure to serum alone, addition of NAC to the broth containing serum increased transcript levels for most of the genes involved in biofilm formation. In *S. aureus* ATCC25923, the surface adhesins (*fnb* and *clf*), regulators involved in *ica*-dependent pathway (*icaA* and *rbf*), stress sensors (*clpC* and *clpX*), and some other *ica*-independent regulators (*saeS*, *cidA*, *atl*, *rot*, and *sigB*) showed remarkable increases. However, levels for the negative regulator *agrC* also increased approximately 2.98-fold. In *P. aeruginosa* PAO1, transcript levels for the regulators in quorum sensing (QS) pathways increased significantly, including for the *las* and PQS systems (*lasI*/*lasR* and *pqsA*/*pqsR*), and the two-component system GacS/GacA that acts as a super-regulator of the QS system. In addition, transcripts from genes controlling the production of extracellular

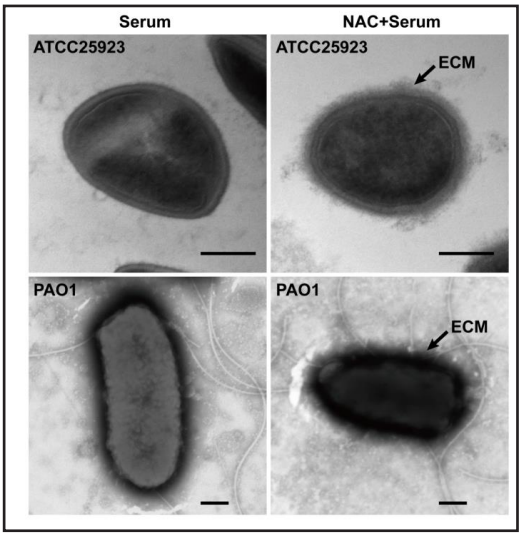

Fig. 4. The combination of NAC and serum promotes the production of extracellular matrix (ECM). *S. aureus* ATCC25923 and *P. aeruginosa* PAO1 were cultured overnight in broth containing 20% human serum alone or in combination with 10 mM NAC and then observed by TEM. Arrows indicate thick ECM layers around the cells treated with NAC and serum. Scale bars represent 200 nm.

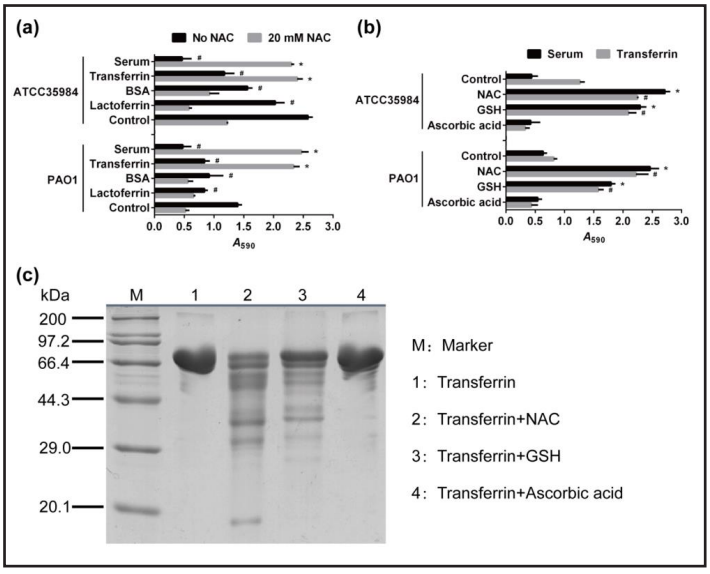

Fig. 5. NAC and serum-induced biofilm formation is due to interaction between NAC and transferrin. (a) *S. epidermidis* ATCC35984 and *P. aeruginosa* PAO1 were cultured in the presence or absence of NAC combined with human serum, transferrin, BSA, or lactoferrin. Biofilms were quantified using microtiter plate assays. Results are shown as means±standard deviations from three independent experiments. **P*<0.05 indicates significant differences between bacteria treated with or without NAC in the presence of serum or transferrin. #*P*<0.05 indicates significant differences compared to control (untreated) bacteria. (b) Bacteria were grown in broth containing serum or transferrin in combination with NAC, GSH, or ascorbic acid. **P*<0.05 or #*P*<0.05 indicates significant differences compared to controls treated with serum or transferrin alone, respectively. (c) SDS-PAGE analysis of the serum protein transferrin treated with NAC, GSH, or ascorbic acid.

polysaccharides (*algC* and *pslA*) also increased, while transcripts from motility genes (*flgK* and *pilF*) changed only slightly.

The combination of NAC and serum promotes the production of extracellular matrix (ECM)

Because the combination of NAC and serum increased the expression of some surface adhesins and genes encoding extracellular polysaccharides in *S. aureus* and *P. aeruginosa*, we speculated that the ECM surrounding the bacterial cells might also increase. ECM formation by *S. aureus* ATCC25923 and *P. aeruginosa* PAO1 was assessed using TEM. Bacteria cultured in broth containing both NAC and serum exhibited thick ECM layers surrounding the cells. In contrast, cells grown in medium containing serum alone were surrounded with markedly less ECM (Fig. 4). This result suggests that exposure to NAC and serum together promotes the production of ECM.

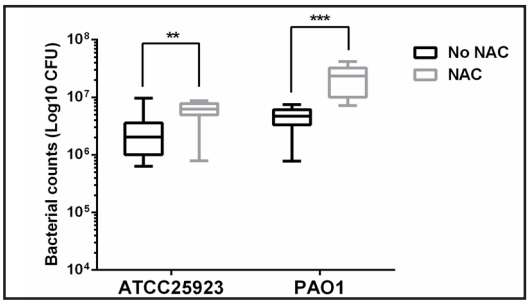

Fig. 6. Intravenous administration of NAC increases bacterial colonization on implanted central venous catheters in rats. Male SD rats (n=48) were implanted with central venous catheters and infected with 5×10^6 CFU of *S. aureus* ATCC25923 or *P. aeruginosa* PAO1 via the tail vein (n=24 per group). Half of the rats in each group (n=12) were selected randomly to receive NAC (150 mg/kg/day) solution or the same volume of saline intravenously for 7 days. Rats were then sacrificed and bacteria colonized on the catheters were counted. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences between animals with or without NAC treatment.

NAC and serum-induced biofilm formation involves the interaction between NAC and the serum protein transferrin

NAC and serum proteins such as transferrin, lactoferrin, and albumin have been reported to inhibit biofilm formation. We therefore hypothesized that NAC may interact with one of these proteins and eliminate their inhibitory activity. To test this, the 20% serum in bacterial culture medium was replaced with human transferrin, lactoferrin, and bovine serum albumin (BSA) at concentrations equivalent to those in the original preparation. As the ability of *S. aureus* ATCC25923 to form biofilms is weak in microtiter plate assay, it is not suitable for the observation of the inhibitory effect of NAC or serum alone on its biofilm formation. Thus we chose *S. epidermidis* ATCC35984 as a representative of gram-positive strain instead of *S. aureus* ATCC25923. We found that the combination of NAC and transferrin mimicked the effect of NAC and serum on biofilm formation in *S. epidermidis* ATCC35984 and *P. aeruginosa* PAO1 (Fig. 5a).

To explore if the sulfhydryl group in NAC is responsible for its interaction with serum and transferrin, we replaced NAC with glutathione (GSH) and ascorbic acid, two antioxidants with or without sulfhydryl groups, respectively. When combined with serum or transferrin, GSH promoted biofilm formation to the same extent as NAC, while ascorbic acid did not (Fig. 5b). This result suggests that the sulfhydryl group in NAC and GSH enables their interaction with transferrin. SDS-PAGE analysis of NAC-, GSH-, and ascorbic acid-treated transferrin further supports this conclusion (Fig. 5c), demonstrating that the sulfhydryl groups in NAC and GSH react and split disulfide bonds in transferrin. Taken together, these results suggest that NAC and serum-induced biofilm formation is due mainly to the interaction between the NAC sulfhydryl group and the serum protein transferrin.

Intravenous administration of NAC increases bacterial colonization on implanted central venous catheters in rats

To determine the effect of NAC on biofilm formation *in vivo*, we injected a high dose of NAC or saline intravenously to rats that had been implanted with central venous catheters and infected with *S. aureus* or *P. aeruginosa* (n=12 per group). After injection of NAC or saline daily for 7 days, the numbers of viable bacteria attached to the catheters were determined.

As shown in Fig. 6, bacteria that had colonized on the catheters in NAC-treated rats were significantly more abundant than in rats treated using saline alone for both *S. aureus* and *P. aeruginosa*. This strongly suggests that NAC also interacts with serum protein and leads to increased bacterial colonization on implanted catheters *in vivo*.

Discussion

NAC, a widely used agent in the treatment of chronic bronchitis, paracetamol intoxication, cancer, and HIV infection, has also been reported to have antibacterial and anti-biofilm activities [9, 26-30]. Many *in vitro* studies have shown the ability of NAC to prevent biofilm formation by a variety of microorganisms, including a variety of Gram-positive and Gram-negative bacteria, as well as some yeasts [9]. In addition to its direct antibacterial effect, NAC also inhibits the production of extracellular polysaccharide, reduces bacterial adherence, and promotes dispersal of mature biofilms [10, 11, 14, 31]. However, the mechanisms by which NAC prevents biofilm formation are complex and largely unknown. Several possibilities have been suggested, including the reaction of the NAC sulfhydryl group with the disulfide bonds of bacterial or extracellular proteins, the competitive inhibition of bacterial enzymes involved in amino acid utilization, and the perturbation of cell metabolism or signal transduction pathways [10, 14].

Human serum and some serum components are also able to inhibit biofilm formation in certain bacteria. Ding *et al.* reported that human serum inhibits adhesion and biofilm formation by *Candida albicans* [17]. Hammond *et al.* demonstrated that the presence of human or bovine serum, and the serum component albumin (BSA), blocks biofilm formation by *P. aeruginosa* [16]. Abraham *et al.* proposed that a low molecular weight component of serum is responsible for inhibiting biofilm formation by *S. aureus* [21]. Two other studies found that transferrin and lactoferrin in serum or some human external secretions can prevent bacterial adhesion and biofilm formation [18, 20].

The sulfhydryl group in NAC makes it a reactive compound. It is usually used as a mucolytic agent and an antioxidant to react with mucous bronchial secretions and free radicals. NAC can affect the stability and compromise the activity of carbapenems [32]. It also decreases the activity of coagulation factors in plasma, and intravenous NAC may interfere with the sulfhydryl groups on these factors [25, 33]. In the current study, we found that the induction of biofilm formation by NAC and serum occurs primarily due to the interaction between NAC and transferrin. Further analysis indicates that the sulfhydryl group in NAC reacts with and splits disulfide bonds in transferrin. Although NAC and transferrin both exhibit inhibitory activities on biofilm formation, when combined this reaction may cause them to neutralize each other. Moreover, we also found that the combination of NAC and serum or transferrin actually increases biofilm formation by *S. aureus*, *E. faecalis*, and *P. aeruginosa* in comparison with untreated controls. Previous studies reported that NAC influences amino acid utilization in bacteria [34, 35], and its antioxidant activity may also perturb the redox status in the bacterial cell. In addition, the serum protein transferrin affects the bioavailability of iron in the culture medium [36]. Both factors are relevant to bacterial metabolism and biofilm formation. As a consequence, we speculate that the interaction of NAC with transferrin or other serum proteins may affect metabolism or signal transduction pathways in bacteria in complex ways. This hypothesis is supported by the observation that the expression of many biofilm-associated genes in *S. aureus* and *P. aeruginosa* increases upon exposure to NAC and serum, but further investigation will be required to understand the mechanism in detail.

Multiple molecular pathways are involved in the regulatory network of bacterial biofilm formation. In *S. aureus*, the *ica*-dependent pathway and some *ica*-independent regulators have important roles in the development of biofilms [37]. In cells treated using NAC and serum, the *ica*-dependent pathway (*icaA* and *rbf*) and some global regulators such as *saeS*, *cidA*, *atl*, *rot*, and *sigB* were activated and are likely to be responsible for increased biofilm formation. Regulators in QS systems are known to be the key factors in *P. aeruginosa* [38]. As

expected, we also found that expression of the *las* and PQS systems and their super-regulator GacS/GacA were significantly increased. By activating these global or key regulators, the combination of NAC and serum probably promotes the expression of adhesins, the release of eDNA, and even the production of extracellular polysaccharides, ultimately increasing biofilm formation.

Although most investigations about the anti-biofilm effects of NAC have been conducted *in vitro*, some studies used NAC to eradicate biofilms on vascular catheters [22], and even in one case as a catheter-lock solution for treatment of hemodialysis catheter-associated bacteremia [39]. This pilot trial of NAC in clinical patients was highly successful in preventing persistent or recurrent bacteremia. However, our *in vitro* and *in vivo* experiments showed the opposite result. The discrepancy may be explained by the fact that tigecycline and NAC were combined in the trial, or in a catheter-lock solution, much higher NAC concentrations can be achieved, suggesting a possible dose-dependent effect of NAC on biofilm formation. In contrast, our results show that in the absence of antibiotics, NAC used intravenously or in the presence of blood does not inhibit bacterial biofilm formation, but rather increases it.

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Disclosure Statement

No conflict of interests exists.

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