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Bactericidal Activity of *N*-Chlorotaurine against Biofilm-Forming Bacteria Grown on Metal Disks

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Many orthopedic surgeons consider surgical irrigation and debridement with prosthesis retention as a treatment option for postoperative infections. Usually, saline solution with no added antimicrobial agent is used for irrigation. We investigated the activity of *N*-chlorotaurine (NCT) against various biofilm-forming bacteria *in vitro* and thereby gained significant information on its usability as a soluble and well-tolerated active chlorine compound in orthopedic surgery. Biofilms of *Staphylococcus aureus* were grown on metal alloy disks and in polystyrene dishes for 48 h. Subsequently, they were incubated for 15 min to 7 h in buffered solutions containing therapeutically applicable concentrations of NCT (1%, 0.5%, and 0.1%; 5.5 to 55 mM) at 37°C. NCT inactivated the biofilm in a time- and dose-dependent manner. Scanning electron microscopy revealed disturbance of the biofilm architecture by rupture of the extracellular matrix. Assays with reduction of carboxanilide (XTT) showed inhibition of the metabolism of the bacteria in biofilms. Quantitative cultures confirmed killing of *S. aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* biofilms on metal alloy disks by NCT. Clinical isolates were slightly more resistant than ATCC type strains, but counts of CFU were reduced at least 10-fold by 1% NCT within 15 min in all cases. NCT showed microbicidal activity against various bacterial strains in biofilms. Whether this can be transferred to the clinical situation should be the aim of future studies.

Metals are widely used in biomedical devices such as dental and orthopedic implants. For example, titanium alloys and metal-on-metal bearings are becoming more popular in total hip arthroplasties. However, they can be colonized by bacteria, mainly *Staphylococcus epidermidis* and *Staphylococcus aureus*, leading to implant-related infections (1). These species can form biofilms, an organized and long-lived colony form well protected from fluctuations in nutrition and immune attack. Further, bacteria in biofilms are highly resistant to antibiotics (2). A chronic periprosthetic joint infection can be fatal to the patient, and prolonged stays in the hospital and consecutive surgeries can cause a major financial burden.

A remedy against biofilms resulting from surgery-related infections is highly desirable. A substance of interest is *N*-chlorotaurine (NCT), the main representative of long-lived oxidants produced by human granulocytes and monocytes (3). It can be synthesized chemically (4) and displays broad-spectrum microbicidal properties against bacteria, viruses, fungi, and protozoa (5). Despite that, its oxidative reactivity is mild and its tolerability upon topical application to skin, eye, and body cavities, such as the urinary bladder and joints, is high (5–7; M. Nagl, unpublished data). Previous studies on its antimicrobial activity have been performed against planktonic forms of bacteria (8–10), and very recently, the first study on its activity against biofilm formation of *Pseudomonas aeruginosa* was published (11). Micromolar concentrations of NCT and its analog *N*-bromotaurine inhibited the formation of the biofilm but were not active against a mature biofilm (11). However, *N*,*N*-dichlorodimethyltaurine (0.1% to 0.5%), another analog, was active against *S. aureus* biofilm in the frontal sinus of sheep (12).

Since NCT can be considered as an irrigation substance during joint replacement surgeries, it was of interest to know if its millimolar concentrations, which are applied clinically, kill bacteria in biofilms. Furthermore, the aim of this study was to investigate *in vitro* its activity against biofilms on titanium alloy material used for implants in orthopedic surgery.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *P. aeruginosa* ATCC 27853 were grown overnight at 37°C on Mueller-Hinton (MH) agar plates (Sigma-Aldrich, Hamburg, Germany). Discrete colonies were suspended in MH broth to a McFarland turbidity of 0.5 (1.5×10^8 bacteria/ml). Clinical strains of *S. aureus* and *S. epidermidis* (extricated from an excised graft by sonication) and *P. aeruginosa* (from sputum) were grown as described above.

***N*-Chlorotaurine.** Pure NCT was prepared as crystalline sodium salt (Cl-HN-CH_2-CH_2-SO_3Na; molecular weight, 181.57 [4]) and dissolved in 0.1 M phosphate buffer (pH 7.1).

Biofilm formation and scanning electron microscopy (SEM). Titanium alloy ($TiMo_{12}Zr_6Fe_2$ [TMZF]) disks (disk area, 157 mm^2; Stryker GmbH & Co KG, Duisburg, Germany) were cleaned with soap, washed with 75% ethanol, and then autoclaved. Sterile disks were placed in 15-ml Falcon tubes (Becton Dickinson) containing 2 ml MH broth inoculated with 2×10^5 CFU/ml of the corresponding bacterial strain. The tubes were incubated at 37°C for 48 h on a shaker. Subsequently, the disks were rinsed in saline solution for 1 min to discard planktonic cells, added to tubes containing 0.1% or 0.5% NCT solution, and incubated at 37°C for 1 h, 5 h, and 7 h. Negative controls in phosphate-buffered saline (PBS) were performed in parallel.

Biofilm-covered TMZF disks treated with NCT or buffer were rinsed

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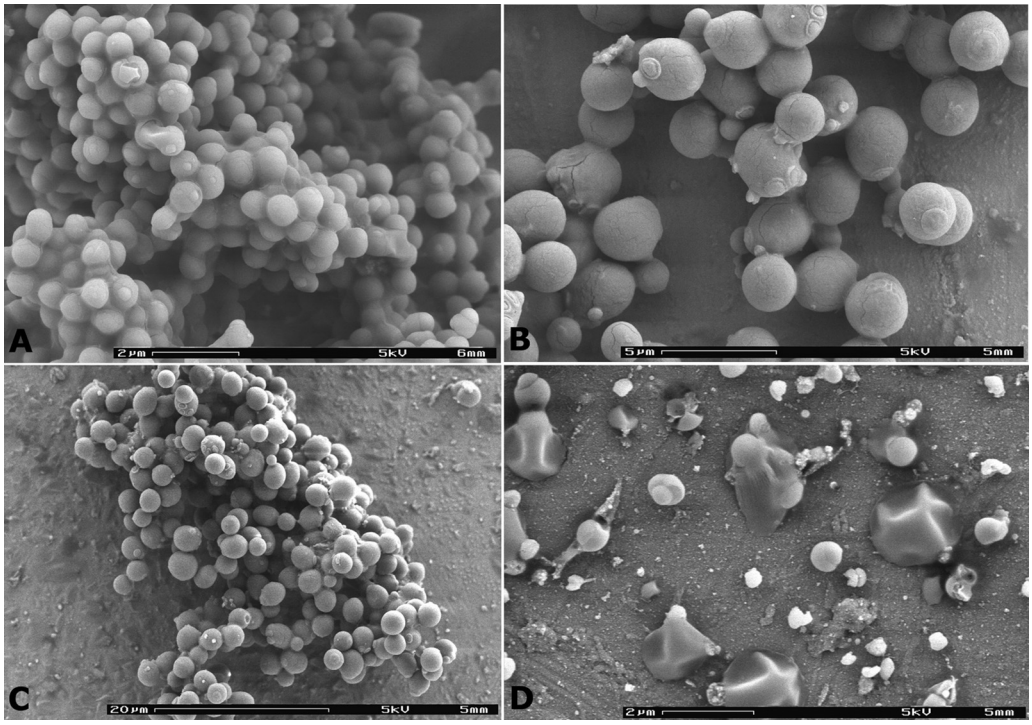

FIG 1 Disruption of biofilm by NCT. *S. aureus* biofilms were formed on a TMZF surface, incubated for 1 h with buffer solution (magnification, ×10,000) (A), 1 h with 0.1% NCT (magnification, ×20,000) (B), 5 h with 0.5% NCT (magnification, ×2,000) (C), and 7 h with 0.5% NCT (magnification, ×10,000) (D).

in saline solution for 1 min. They were fixed with 2.5% glutaraldehyde (BioChemika Fluka, Buchs, Switzerland) in 0.1 M phosphate buffer (pH 7.4). After a brief wash in phosphate buffer, followed by postfixation for 1 h with 1% aqueous osmium tetroxide (ReagentPlus; Sigma-Aldrich, Hamburg, Germany), the samples were gradually dehydrated with ethanol. After critical-point drying (critical-point drier [CPD] 030; Bal-Tec), the specimens were mounted on aluminum stubs with a double-sided adhesive tape, sputter coated with 10-nm Au/Pd (Bal-Tec), and examined with a field emission scanning electron microscope (Gemini 982; Zeiss, Goettingen, Germany). Magnification was set to 10,000 to examine the biofilm as a multicellular organization and to 20,000 to investigate at a single-cell level.

Metabolic turnover (XTT test). To investigate the NCT-mediated impact on bacterial metabolism in biofilms, we applied the XTT assay (Sigma-Aldrich, Hamburg, Germany), measuring the reduction of 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide to the orange formazan product. Biofilms were grown at 37°C in polystyrene dishes of a 96-well plate and washed with PBS. They were treated at 37°C with 3 different concentrations of NCT (0.1%, 0.5%, and 1%) for 15 min, 30 min, 1 h, or 3 h each. Subsequently, they were washed twice with PBS, XTT was added, and the plates were incubated at 37°C in the dark for 2 h. The supernatants were transferred to fresh 96-well plates, and the absorbance was measured at 450 nm. All values were calculated as percentage of the negative control (PBS).

Quantitative killing tests on metal alloy disks. Biofilm-covered TMZF disks, prepared as described above, were added to tubes containing 0.1%, 0.5%, or 1% NCT solution and incubated at 37°C for 15 min, 30 min, 1 h, or 3 h. Negative controls in phosphate-buffered saline (PBS) and positive controls in 0.2% chlorhexidine (Sigma-Aldrich, Hamburg, Germany) were performed in parallel.

After each interval, disks were sonicated for 1 min in an ultrasound water bath (40 kHz; BactoSonic; Bandelin Electronic, Berlin, Germany) to detach the remaining live bacteria from the disk. Subsequently, 10-μl aliquots of these solutions were spread on Mueller-Hinton agar plates with a sterile inoculation loop, undiluted and after 10-fold and 100-fold dilution in saline, leading to a detection limit of 100 CFU/ml. NCT was inactivated on the plates within 3 min by reaction with the agar components, as verified by addition of potassium iodide in separate experiments. Plates were incubated at 37°C, and CFU were counted after 24 and 48 h.

Statistical analysis. All experiments were performed independently at least three times. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison test were applied for calculations using GraphPad Prism 5 software. *P* values of <0.05 were considered statistically significant.

RESULTS

Visual characterization of NCT-mediated effects on *S. aureus* biofilms grown on metal disks by SEM. Scanning electron microscopy (SEM) revealed several biofilm areas distributed on the surface of the disks treated with phosphate buffer. Amorphous material covering the bacteria like a film can be observed in these areas (Fig. 1A). In contrast, SEM images of biofilms after 1 h of incubation with 0.1% NCT showed disturbances of the biofilm architecture, absence of covering amorphous material, and an increase in the size of bacteria (Fig. 1B). On disks incubated for 5 h with 0.5% NCT, granule-like elements and total eradication of the extracellular matrix were observed (Fig. 1C). On disks incubated for 7 h with 0.5% NCT, only amorphous bacteria lying on the metal surface and no intact biofilms were found (Fig. 1D).

Effects of NCT on the metabolic turnover of *S. aureus* organized in biofilm on metal disks. The metabolic turnover rate after incubation with 3 different concentrations of NCT (0.1%, 0.5%, and 1%) at 4 different time points ranging from 15 min to 3 h was measured by a standard XTT assay. All concentrations of NCT significantly reduced the metabolic turnover. While 1% NCT and 0.2% chlorhexidine reached similarly high values already after 15

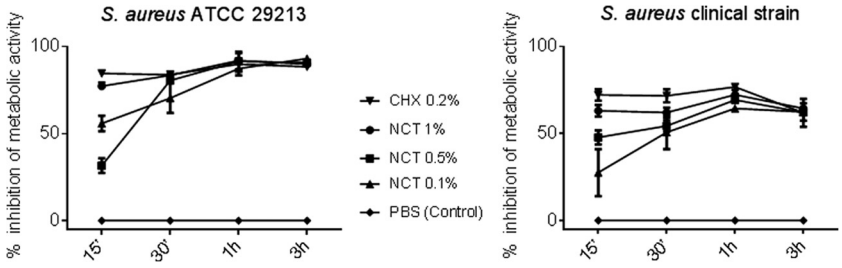

FIG 2 XTT metabolic turnover assay. *S. aureus* ATCC 29213 (left) and clinical strain (right) biofilms were grown for 48 h and then incubated with 1% (circles), 0.5% (squares), and 0.1% (triangles) NCT for 15 min, 30 min, 1 h, and 3 h. PBS (diamonds) and 0.2% chlorhexidine (CHX) (inverted triangles) served as negative and positive controls, respectively. Metabolic turnover was measured by standard XTT assay. Mean values ± standard deviations of three independent experiments. *P* was <0.01 for NCT and chlorhexidine versus PBS at all incubation times; *P* was <0.01 between chlorhexidine and 0.1% and 0.5% NCT at 15 min; *P* was >0.05 between chlorhexidine and 1% NCT at all incubation times; these values apply to both strains.

min, the lower NCT concentrations showed a reduction of 30% to 60% after 15 min and between 60 and 80% after 30 min (Fig. 2, left). The clinical *S. aureus* strain showed higher resistance with a maximum inhibition of 75% for both 1% NCT and 0.2% chlorhexidine. A dependence on time and concentration was observed for 0.1% and 0.5% NCT (Fig. 2, right).

Killing activity of NCT against *S. aureus*, *S. epidermidis*, and *P. aeruginosa* organized in biofilms on metal disks. Next, we sought to test for the actual killing of bacteria in the biofilm, since that is the ultimate goal of antimicrobial treatment of metal grafts. All tested bacterial strains were completely inactivated after 15 min by 0.2% chlorhexidine, the positive-control agent, while no killing and, after 3 h, even some growth could be detected using PBS as the negative control (Fig. 3). With NCT, we found significant killing of all strains in a time- and dose-dependent manner. All species were reduced by at least 1 $\log_{10}$ within 15 min by 1% NCT ($P < 0.01$). The detection limit of 2 $\log_{10}$ was reached after 1 h with staphylococci (3 h with the *S. epidermidis* clinical isolate) and already after 30 min with *P. aeruginosa* (Fig. 3). The lower concentrations of 0.5% and 0.1% NCT showed a delayed effect. However, the 0.5% NCT solution still eradicated the biofilm after 3 h at the latest, and 0.1% induced significant killing of all test strains at this time. In biofilm, *P. aeruginosa* was inactivated by 1% NCT within 15 min (by 0.5% NCT within 30 min) and therefore appeared to be more susceptible to NCT than were staphylococci (Fig. 3).

Notably, the biofilms formed by clinical isolates contained a significantly higher CFU count by 1 $\log_{10}$ compared to the ATCC strains ($P < 0.01$) (Fig. 3). Eradication of viable counts by 1% and 0.5% NCT needed more time for clinical isolates of *S. epidermidis* and *P. aeruginosa* than for the ATCC strains ($P < 0.01$; Fig. 3, middle and bottom).

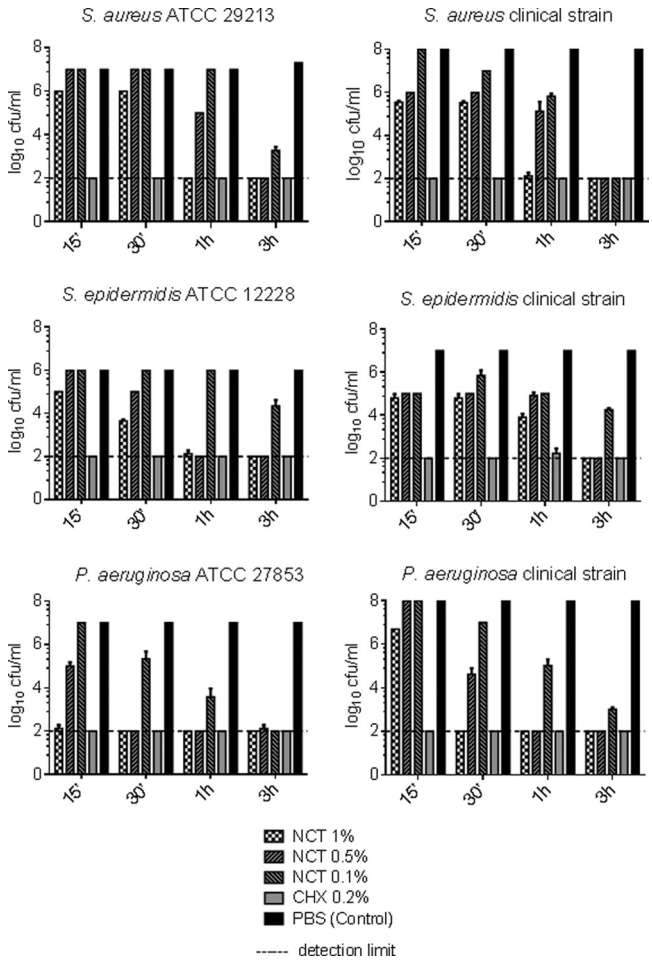

FIG 3 Killing of *S. aureus* by NCT. Biofilms of *S. aureus* ATCC 29213, an *S. aureus* clinical isolate, *S. epidermidis* ATCC 12228, an *S. epidermidis* clinical isolate, *P. aeruginosa* ATCC 27853, and a *P. aeruginosa* clinical isolate were grown on metal alloy disks for 48 h. Subsequently, disks were incubated with 1%, 0.5%, and 0.1% NCT for 15 min, 30 min, 1 h, and 3 h. PBS and 0.2% chlorhexidine (CHX) served as negative and positive controls, respectively. Surviving bacteria were detached by sonication, and 10-μl samples of the solution were spread on fresh agar plates. Mean values ± standard deviations of three independent experiments. *P* was <0.01 for all reductions in CFU compared to the PBS control; *P* was <0.01 between all NCT concentrations and 0.2% chlorhexidine, except for 1% NCT in *P. aeruginosa* ATCC 27853.

DISCUSSION

Titanium disks incubated with medium containing *S. aureus*, *S. epidermidis*, or *P. aeruginosa* proved to be a useful *in vitro* model for testing the active chlorine compound NCT against bacterial biofilms. The chosen metal alloys are major components of orthopedic implants (13), and the bacterial strains used are mainly responsible for implant-associated infections (14, 15). A biofilm was established by all tested bacteria within 48 h on the titanium alloy disks.

As can be derived from electron microscopy, NCT disrupts the biofilm matrix, which ultimately leads to detachment of the bio-

film. We visualized the disruption of the extracellular matrix during a 7-h course using a 0.5% NCT solution. On a single-cell basis, we observed swelling of bacteria early during treatment (Fig. 1B). This could be due to osmotic effects after membrane destabilization by NCT. After 5 h, no more extracellular matrix could be detected, and subsequently, bacteria were detached from the surface (Fig. 1C and D). Transferred to the *in vivo* situation, removal of biofilms from prosthetic material could render bacteria accessible to the defense system, which may lead to an immune response against germs not immediately killed by NCT. Adherence of bacteria to surfaces is mediated by proteins in *S. aureus* (16), *S. epidermidis* (2), and *P. aeruginosa* (17). Since NCT and analog substances recently have been shown to directly inactivate bacterial toxins (8, 18), it seems likely that the detachment of biofilms by NCT is due to an impact on these adherence proteins via oxidation of thiols and aromatic and amino groups (4, 5). To clarify the exact molecular sites of attack could be an interesting item in future studies.

In a recent study, low concentrations of NCT (300 and 1,000 μM) inhibited the *P. aeruginosa* biofilm formation but did not destroy the biofilm or kill hidden bacteria (11). In millimolar concentrations, however, we found a time- and concentration-dependent antibiofilm activity at concentrations between 0.1% and 1% (5.5 to 55 mM). Of note, 1% NCT, an approximately 1,000-fold excess over the physiological concentration (19), can be applied clinically to different body regions and cavities (for a review, see reference 5). Its bactericidal activity against *S. aureus* and *S. epidermidis* in biofilm (reduction of CFU to the detection limit by 1% NCT needed about 30 to 60 min [Fig. 3]) appears approximately 3 to 6 times slower than that against planktonic bacteria (10 min for a reduction to the detection limit [9, 10]) but is still sufficient. Remarkably, viable counts of all tested bacteria in biofilm were reduced by at least 1 power of 10 after 15 min of incubation in 1% NCT, so that an impact on biofilm by irrigations with NCT can be conceived *in vivo*. This remains to be confirmed in future clinical studies.

The absence of the antimicrobial activity of micromolar NCT against *P. aeruginosa* biofilm in the recent study (11) might be explained by consumption of oxidative capacity by reducing moieties of the proteinaceous matrix of the biofilm (chlorine consumption [4, 5]). However, the mild activity and high tolerability of *N*-chloro amino acids, particularly NCT, enable application of high concentrations to human tissue, which warrants a sufficient reserve of oxidation capacity despite some reduction (20). This can be of advantage compared with highly reactive oxidants that cause side effects at higher dosages (21). Accordingly, NCT was shown to be effective in topical therapy of infections where biofilms play a role, for instance, purulently coated crural ulcerations and external otitis in humans (22, 23) and joint infections in mice (7).

Comparing quantitative killing assays with metabolism (XTT tests), the expected result became obvious, i.e., that the bacterial metabolism is affected within minutes and therefore earlier than viability. Remarkably, as in killing tests, the clinical strains showed a slightly lower susceptibility to NCT than did the ATCC strains. In agreement with previous findings (24), clinical isolates formed a thicker biofilm with 10-fold-higher CFU counts, which obviously explains their higher resistance.

We used chlorhexidine as a positive control since it is one of the most widely used antiseptics on skin and mucous membranes (25). In agreement with previous studies (26, 27), we found activity of 0.2% chlorhexidine against our test strains in biofilm, so that it turned out to be a good positive control. Although the activity of NCT was lower than that of chlorhexidine, it has distinct advantages. NCT is an endogenous substance with mild activity connected with a very good tolerability even in highly sensitive body regions, an advantage compared to antiseptics (for a review, see references 5 and 21). As it is an active chlorine compound, the microbicidal spectrum is very broad without development of resistance, an advantage compared to antibiotics (5).

Use of antibiotics can further lead to development of bacterial persister cells, which are not growing and thus remain unaffected in the presence of antibiotics (28). However, these bacteria can regenerate and reestablish infection after termination of the antibiotic treatment. NCT completely eradicated the biofilm in our study, so it can be concluded that it kills bacteria independently of protein production, cell cycle, or proliferation. This is in accordance with its oxidative mechanism of action (5, 21) and with its efficacy in treatment of infections of different body sites in humans (for a review, see reference 5). For instance, successful application was demonstrated in purulently coated crural ulcerations and in external otitis in humans (22, 23). Based on these findings, we speculate that the substance could be effective also for irrigation of orthopedic joint implants in case of infection or for prophylaxis of infections, supported by the following arguments. There was a significant reduction of CFU by at least 1 $\log_{10}$ within 15 min in biofilms by 1% (55 mM) NCT (Fig. 2 and 3), the concentration used clinically in most indications (5). The contact time of NCT *in vivo* can be prolonged, for instance, by extended irrigation periods (22) or by clamping catheters (29). Moreover, in the mouse peritonitis model it has been shown that short, sublethal times of incubation in NCT (1 min for the 1% concentration) are connected with a lag of regrowth and loss of virulence of bacteria (30). Tolerability upon joint irrigation and efficacy in joint infections and collagen-induced arthritis have already been shown in mouse models (7, 19, 31, 32). Nevertheless, double-blind randomized clinical trials will be needed to demonstrate the efficacy of NCT in orthopedic infections in humans.

NCT in millimolar concentrations can efficiently kill bacteria in an active and established biofilm *in vitro*. It seems a promising task for future studies to determine whether NCT can be used in a clinical setting to combat bacterial colonization and biofilm formation in prosthetic joint surgery as well as to fight already-established infections.

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There are no conflicts of interest to be declared.

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