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Influence of poly-N-acetylglucosamine in the extracellular matrix on N-chlorotaurine mediated killing of *Staphylococcus epidermidis*

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SUMMARY

N–chlorotaurine (NCT) has recently been shown to have bactericidal activity against bacterial biofilm on metal discs (Coraca-Huber *et al.,* 2014). In a biofilm, *Staphylococcus epidermidis* polymerizes poly-N-acetylglucosamine (PNAG) to form an extracellular matrix (ECM). *Pseudomonas aeruginosa* does not express this PNAG and has been shown to be highly susceptible to NCT.

We compared the action of NCT on *S. epidermidis* 1457, a PNAG positive strain (SE1457) and *S. epidermidis* 1457-M10 an isogenic PNAG negative mutant (SE1457 M10). NCT-mediated killing was more effective and quicker on the PNAG negative strain SE1457 M10. Bacteria hidden in biofilms for prolonged periods of time were generally more susceptible than freshly formed biofilms.

The differences in NCT-mediated killing might not be direct effects since NCT did not react with the monomeric N-acetylglucosamine, but might be explained by denser growth in the PNAG-containing biofilm produced by the wild type strain, which results in delayed penetration of NCT. The higher susceptibility of older biofilms to NCT-mediated killing could be explained by more pronounced 3D architecture and subsequent larger surface area for interactions with NCT.

KEY WORDS: Biomedical device-related infection, Biofilm, Active chlorine compound, Poly-N-acetylglucosamine, N-chlorotaurine.

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In a recent publication we showed that NCT is highly effective against Staphylococcus (S.) and Pseudomonas strains, showing significant bactericidal activity within 15 min and eradicating biofilms within 3 h at a concentration of 1% (Coraca-Huber *et al.,* 2014). We found that the ATCC 27853 strain of *Pseudomonas aeruginosa*, which does not express PNAG in the ECM, proved especially susceptible to NCT-mediated killing. We thus sought to further investigate the interaction of NCT with the Staphylococcus extracellular matrix (ECM).

We grew biofilms on titanium alloy discs paralleling the material widely used in orthopedic grafts (D'Antonio *et al.,* 2001) as described in Coraça-Huber *et al.,* (Coraca-Huber *et al.,* 2012). This represents a well-established model of prosthetic joint infection. Such infections typically arise from *Staphylococcus aureus* or *S. epidermidis* strains (Costerton *et al.,* 1999; Costerton *et al.,* 1995). Both form biofilms on the grafted metal surface. The initial colonization of a graft will be the result of only a few bacteria, which proliferate to a certain count before shielding themselves with an ECM. In this phase bacteria resemble a biofilm that

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lacks an ECM, while later the fully mature biofilm will express such a protective matter during the chronic phase of infection (reviewed in Branda *et al.,* 2005). A total eradication of an established biofilm on a joint graft during the chronic phase with intravenously applied antibiotics is made virtually impossible due to the low saturation of blood vessels in the manipulated joint space and the resulting poor delivery (Konttinen *et al.,* 2001). Further, it has been shown that bacteria in a biofilm are approximately 1000 fold more resistant to antibiotics compared to planktonic counterparts (Lewis, 2001). Traditionally, during orthopedic surgery the joint is flushed with saline solution for cleaning. We think that the use of a bactericidal NCT solution could prevent the initial colonization and would significantly increase the chance of biofilm eradication if a biofilm is already established.

We utilized the SE 1457 M10 bacterial strain which has been shown to form multicellular structures, but due to a mutated *ica* operon does not polymerize PNAG to form the ECM (Rupp *et al.,* 1999). Its parental strain (SE 1457) was originally isolated from an infected central venous catheter and polymerizes PNAG.

We grew biofilms of each bacterial strain on titanium alloy discs for 24h and 7 days by incubating the discs with a 0.5 McFarland bacterial solution in Müller-Hinton medium at 37°C on a shaking platform. Planktonic cells were washed away with PBS, and only the bacteria attached to the metal surface were subjected to our tests. The SE1457 M10 strain grew attached to the titanium surface, but CFU counts did not reach levels as high as detected for the parental 1457 strain (Figure 1, PBS control). The differences were up to two logs in CFU count in the 24h biofilms but closely resembled each other af-

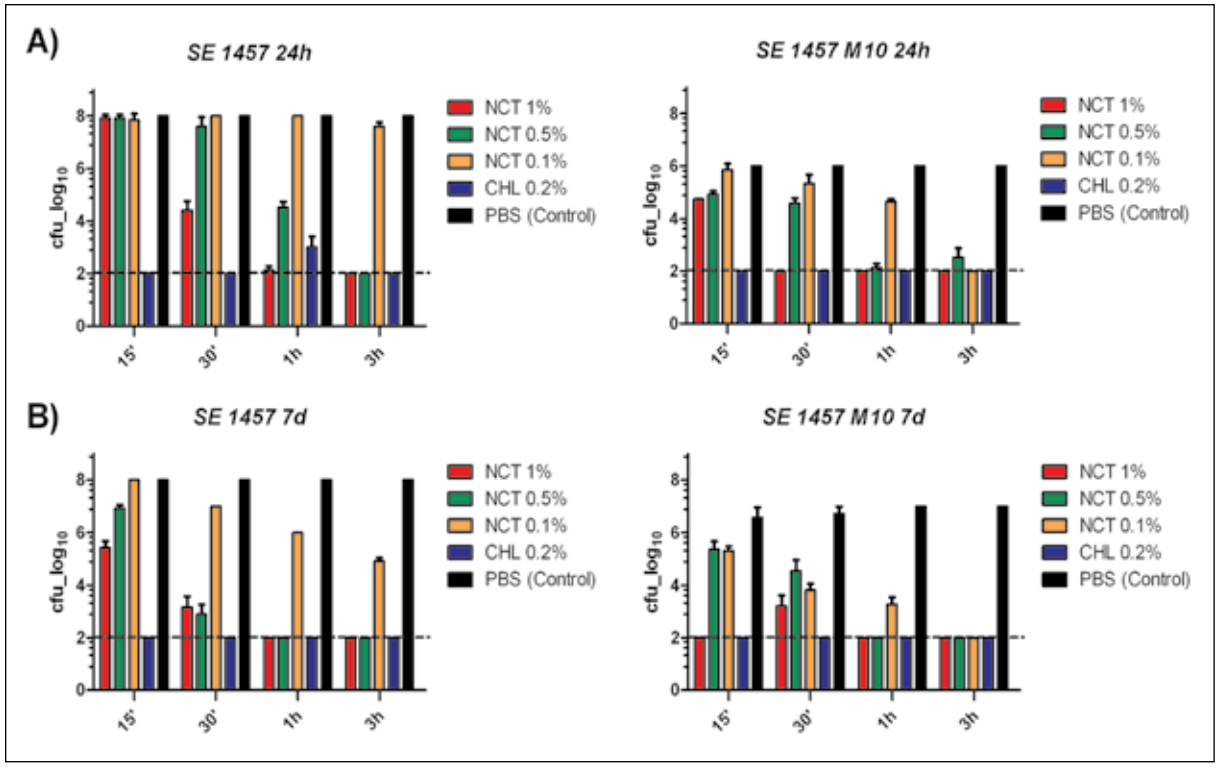

FIGURE 1 - *Bactericidal activity of NCT on Staphylococcus epidermidis biofilms. Staphylococcus epidermidis strains 1457 and 1457 M10 were grown on titanium alloy disks for 24h (A) and 7 days (B). After washing, discs were incubated with 1%, 0,5% and 0.1% NCT for 15 min, 30 min 1 h and 3 h respectively. PBS served as negative control, chlorhexidine as positive bactericidal control. Remaining bacteria were dislocated from the disc by sonication and 10 µl aliquots plotted onto agar plates. Mean values ± SD of three independent experiments are shown.*

ter longer culture. The difference in density in which the two strains grow could be attributed to adherence properties (Sousa *et al.,* 2009). We tested three concentrations of NCT previously shown to cause time and dose dependent effects on biofilm survival (Coraca-Huber *et al.,* 2014). The highest used concentration (1%, 55 mM) resembles an approximately 1000 fold excess over the physiological concentration (Marcinkiewicz *et al.,* 2012) and is well tolerated by patients (Gottradi *et al.,* 2010; Kontny *et al.,* 2007).

We detected a highly significant difference between the CFU count of PNAG expressing SE 1457 strain (approximately $1x10^8$ CFU/ml) and the knockout SE 1457 M10 strain (approximately $1x10^6$ CFU/ml) judging the 24 h biofilm data ($p<0.01$, Figure 1A). Killing by NCT was also slower in the wild type compared to the knockout strain ($p<0.01$, Figure 1A). The 1% (0.5%) NCT concentration completely killed SE 1457 after 60 min (3 h for 0.5% NCT), while the same effect occurred already after 30 min (1h for 0.5% NCT) with SE 1457 M10. NCT (0.1%) killed the knockout strain within 3h, while there was only a minimal reduction of CFU of the wild type strain after 3h. The control agent 0.2% chlorhexidine (CHX) had full activity against both strains (Figure 1A).

Surprisingly, the biofilms grown on metal discs in this study became more susceptible towards NCT-mediated killing with increasing growth time for 1 week. Even the SE 1457 strain was almost completely killed within 30 min by 1% and 0.5% NCT, and also 0.1% caused a significant reduction in CFU (Figure 1B). The PNAG negative strain was still more susceptible, and its CFU counts were still lower by one $\log_{10}$ compared to the wild type strain (10^7 versus 10^8, $p<0.01$).

These results indicate that PNAG-positive strains may form a more pronounced biofilm, which shows a higher resistance towards NCT-mediated killing. Since spectrophotometric tests demonstrated that NCT does not react with the monomeric N-acetylglucosamine (data not shown), we assume that the higher density of the biofilm produced by the wild type strain causes a delay in the penetration of NCT, resulting in longer killing times compared to the PNAG-knockout strain.

As a further yet surprising result we found that bacteria hidden in biofilms for prolonged periods of time were more vulnerable to NCT-mediated killing. The metabolic state of the bacterial culture has no effect on the bactericidal activity of active chlorine compounds, thus the relatively slow growth of an old biofilm does not explain this observation. We speculate that old biofilm containing PNAG in the ECM feature more pronounced 3D architecture with a denser network of tunnels that increases its surface area. We are currently investigating this hypothesis using scanning electron microscopy. The general vulnerability of all thus far tested bacterial biofilms and the high susceptibility of old biofilms towards NCT further highlights the usability of NCT as a bactericidal to fight bacterial infections during joint surgery. The use of NCT in the clinical setting thus provides a very promising outlook for future research.

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