Caprylic Acid and Glyceryl Trinitrate Combination for Eradication of Biofilm

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There is a growing need for biocompatible, broad-spectrum, nonantibiotic, antimicrobial treatments because of the frequent ineffectiveness of antibiotics against biofilms and the increasing incidence of antibiotic resistance. In this study, we demonstrate rapid and complete biofilm eradication in an *in vitro* model with synergistic combinations of glyceryl trinitrate and caprylic acid against resistant Gram-positive, Gram-negative, and fungal biofilms.

An estimated 400,000 patients in the United States annually require medical treatment for anal fissures or rectal tears (1, 2). Wounds in microbially contaminated environments (such as the colon and rectum) can be difficult to heal due to the presence of biofilms. Furthermore, surgical site infections associated with implanted devices pose significant medical problems, frequently complicated by microbial biofilm formation on device surfaces (3). This, coupled with the increasing emergence of antibiotic-resistant biofilms (4), adversely impacts the outcomes of antibiotic wound therapies.

Previously, we reported on the synergy of ethanol, citrate, and glyceryl trinitrate (GTN) as a novel nonantibiotic catheter lock solution (5). In other applications involving direct contact with tissues, the use of ethanol above trace concentrations can be undesirable due to potential irritation and cytotoxic responses from certain cell types. Additionally, in applications where bleeding occurs, chelator use can be undesirable, as it can interfere with blood coagulation, an essential step in initiating the healing response. Caprylic acid (CAP) is a medium-chain fatty acid naturally present in human breast milk. CAP has been used intravenously in some total parenteral nutrition formulations (6, 7). It is also readily absorbed following oral administration and yields significant blood concentrations (8). Protonated CAP has been reported to have antimicrobial properties (9, 10). Here, we studied biofilm eradication by the use of medical GTN plus CAP for potential use in medical applications where antimicrobial concentrations of ethanol might cause irritation and where the anticoagulant effects of citrate might be undesirable.

The *in vitro* antimicrobial efficacy of GTN plus CAP was assessed in a well-established biofilm colonization model testing the eradication of biofilm following 2 h of antimicrobial agent exposure. This exposure duration was selected because previous studies showed solutions exhibiting biofilm eradication within 2 h that were also clinically effective (11). Biofilm eradication was assessed for clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), multidrug-resistant *Pseudomonas aeruginosa*, and *Candida albicans*, representative of key Gram-positive, Gram-negative, and infectious fungal pathogens (12). Briefly, 1-cm silicone discs were placed in 1 ml of human plasma and incubated overnight at 37°C. The plasma was replaced with 1 ml of 5.5×10^5 CFU/ml of challenge organism inoculum and incubated for 24 h at 37°C. The inoculum was then removed, and the discs were washed to remove any planktonic organisms by

shaking for 30 min in 0.9% sterile saline. After they were washed, the discs were exposed to 1 ml of test solution at 37°C for 2 h. The discs were then removed, placed in 5 ml of 0.9% sterile saline, and sonicated for 15 min to disrupt any remaining biofilm. The resulting solution was then quantitatively cultured and plated on Trypticase soy agar plus 5% sheep blood (bacteria) or Sabouraud dextrose agar (yeast).

Antimicrobial solutions containing 0.04% GTN plus 0.05% CAP were prepared by adding CAP to GTN concentrate. GTN concentrate contains 5% dextrose, which carried over into the final solution tested. The pH was adjusted to 4.5 to 4.75 using HCl to protonate the CAP. Mueller-Hinton broth, 5% dextrose, and 0.05% caprylate ion (at neutral pH) were included as control solutions. The pH of each solution was adjusted to 4.5 to 4.75, except the Mueller-Hinton broth control and the 0.05% caprylate ion control (neutral pH).

At 0.05% concentrations, the CAP showed no visible signs of phase separation or immiscibility in any of the solutions tested. Figure 1 shows viable colony counts recovered from the biofilm (median of 6 replicates) following 2 h of treatment with different solutions. The 5% dextrose present in the GTN stock was tested as a control and had no antimicrobial effects against any of the organisms. The 0.05% caprylate ion (neutral pH) also had no antimicrobial effects against any of the organisms. Against MRSA and MRSE, 0.04% GTN alone showed no efficacy, while 0.05% CAP alone (protonated) reduced but did not eradicate the biofilm. The efficacy of 0.04% GTN plus 0.05% CAP in eradicating the biofilm was statistically significant relative to that of GTN alone ($P < 0.001$ for MRSA and MRSE) and 0.05% CAP alone ($P = 0.048$ for MRSA, $P = 0.017$ for MRSE). Against *C. albicans*, 0.05% CAP alone and 0.04% GTN alone had minimal effects, while complete eradication was seen with the combination of the two ($P = 0.01$).

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FIG 1 Results of 2-h exposure to 0.05% CAP plus 0.04% GTN solution against microbial biofilms. The combination of 0.04% GTN plus 0.05% CAP showed significant eradication compared to that of 0.04% GTN alone ($P < 0.001$ for MRSA and MRSE, $P = 0.01$ for *C. albicans*) or 0.05% CAP alone ($P = 0.048$ for MRSA, $P = 0.017$ for MRSE, $P = 0.01$ for *C. albicans*). Five-percent dextrose was present in the medical GTN employed in these studies and was also tested as a control to assess its effect on biofilm viability; 0.05% caprylate ion (deprotonated CAP at neutral pH) was tested to assess the effect of protonation of CAP on biofilm viability. All solutions were tested at pH 4.5 to 4.75, except the Mueller-Hinton broth and caprylate ion controls (neutral pH).

For *P. aeruginosa*, each agent alone eradicated the biofilm within 2 h, and no antagonism was seen with their combination.

High concentrations of protonated CAP and GTN have reported antimicrobial activities (9, 10, 13). In this study, we showed synergy of low concentrations of CAP plus GTN in fully eradicating MRSA and *C. albicans* biofilms within 2 h. CAP and GTN each alone were highly effective against *P. aeruginosa* biofilm.

CAP is a naturally occurring fatty acid; in the deprotonated state (neutral pH), caprylate ion has a well-established metabolic profile as a nutrient in mammals (14). Deprotonating CAP can be accomplished by raising the pH above 4.8 (15); hence, in most physiological environments, CAP will rapidly become deprotonated to a benign nutrient once the local pH increases. In a pharmaceutical formulation, the antimicrobial protonated CAP state can be maintained with a pH of less than 4.8. This pH falls within the range of natural skin pH values seen in humans (16). Other physiologic environments, such as the vaginal canal (17) and digestive tract (18), maintain an acidic pH. Caprylic acid has been reported to aid in wound healing (19, 20).

GTN has been reported to have antimicrobial activity against planktonic *Candida* at higher concentrations (0.15 to 0.3%) (13) than those used in this study (0.04%). GTN is currently FDA approved at ointment concentrations up to 0.4% for treating anal fissures, and doses of 0.2 to 0.4 mg/h are routinely administered transdermally to treat angina and hypertension. Side effects of repeated use of 0.4% GTN rectal ointment are reportedly headaches and hypotension (21). The synergistic combination of GTN with CAP in an ointment has the potential to reduce the GTN dose substantially and thereby reduce or eliminate these side effects. The mildly acidic pHs employed in this study did not impair the antimicrobial activity of GTN.

In conclusion, we found that GTN–CAP combinations can eradicate Gram-positive, Gram-negative, and fungal biofilms within 2 h. This synergistic, nonantibiotic, antimicrobial combination requires further testing for treating and eradicating pathogenic biofilms that colonize surgical wounds, device surfaces and insertion sites, fissures, and tears.

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