Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings

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ABSTRACT

With an epidemic increase in obesity combined with an ageing population, chronic wounds such as diabetic foot ulcers, pressure ulcers and venous leg ulcers are an increasing clinical concern. Recent studies have shown that bacterial biofilms are a major contributor to wound bioburden and interfere with the normal wound healing process; therefore, rational design of wound therapies should include analysis of anti-biofilm characteristics. Studies using the combined treatment of bacterial biofilms with the innate immune molecule lactoferrin and the rare sugar-alcohol xylitol have demonstrated an antimicrobial capacity against a clinical wound isolate. Studies presented here used a colony-drip-flow reactor biofilm model to assess the anti-biofilm efficacy of a lactoferrin/xylitol hydrogel used in combination with commercially available silver-based wound dressings. Log reductions in biofilm viability are compared with a commercially available wound hydrogel used in combination with the silver-based wound dressings. For both a single species biofilm and a dual species biofilm, the lactoferrin/xylitol hydrogel in combination with the silver wound dressing Acticoat™ had a statistically significant reduction in biofilm viability relative to the commercially available wound hydrogel. This study also demonstrated a statistical interaction between the lactoferrin/xylitol hydrogel and the silver wound dressing.

Key words: Biofilm ● Lactoferrin ● Silver ● Wound Dressing ● Xylitol

Key Points

- with the increase in diabetic patients comes the increase in diabetes-associated pathologies including chronic wounds such as diabetic ulcers
- over 2% of the US population suffers from such chronic, non healing wounds and with only transiently effective antimicrobials, the current standard of care is amputation with up to 24% of diabetic patients undergoing amputation surgery in their lifetime
- clearly, novel tools in the treatment of chronic wounds need to be added to the medical arsenal

INTRODUCTION

The combined epidemic of obesity and diabetes has crept into the developed world resulting in a significant drop in overall quality of life and a disturbing rise in premature death. With a doubling of obesity rates in the USA to a rate of 32% (1) combined with 20 million diagnosed diabetics, 6 million undiagnosed diabetics and 60 million prediabetics (2), the socioeconomic impacts of this epidemic cannot be over estimated. Indeed, estimates of the worldwide incidence of diabetes will top 350 million people by 2030 (3).

With the increase in diabetic patients comes the increase in diabetes-associated pathologies including chronic wounds such as diabetic ulcers. Other chronic wounds are associated with an increasingly aged and immobile population such as pressure ulcers and venous leg ulcers. Over 2% of the US population suffers from such chronic, non healing wounds (4) and with only transiently effective antimicrobials, the current standard of care is amputation with up to 24% of diabetic patients undergoing amputation surgery in their lifetime (5). Clearly, novel tools in the treatment of chronic wounds need to be added to the medical arsenal.

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Although chronic and acute wounds progress through similar stages of healing, chronic wounds appear to stall in the inflammatory stage of wound healing, likely because of persistent colonisation by bacteria (6,7). Colonisation by bacteria contributes to non healing of the wound; however, recent evidence suggests that development of a chronic wound is dependent on contaminating bacteria forming biofilms (8,9). Biofilms consist of structured communities of cells, adhered to a surface, and phenotypically diverse (10). Because of the correlation between chronicity in wounds and bacterial biofilm contamination, a number of biofilm-targeted antimicrobials have emerged including the iron-binding innate immune molecule lactoferrin (11).

Because adaptation by biofilm communities has resulted in the failure of multiple antimicrobials, synergistically acting antimicrobials have the greatest likelihood of remaining efficacious in the clinic. Recently, it has been demonstrated that lactoferrin can act synergistically with the rare sugar-alcohol xylitol to inhibit growth of established biofilms of a clinical wound isolate *Pseudomonas aeruginosa 215* (12). To derive further antimicrobial efficacy, synergistic relationships between lactoferrin and xylitol and other antimicrobials should be explored, including synergism with silver.

Medicinal use of silver has been recognised for centuries; however, only recently has silver been used in wound care, specifically ionic silver wound dressings (13). The efficacy of silver-impregnated dressings for treatment of wounds has remained inconclusive with some meta-analyses supportive and some not (14–16). Although silver has been demonstrated to be efficacious alone against biofilm growth (17), over the course of treatment, bacterial biofilms have exhibited a remarkable ability to overcome single-treatment antimicrobials. Indeed, silver resistance has been demonstrated in methicillin-resistant *Staphylococcus aureus* (18). Therefore, establishment of novel antimicrobials that enhance the efficacy of silver and demonstrate statistical interaction with silver may have significant therapeutic value.

METHODS

Dressings

A selection of commercially available silver-impregnated dressings were obtained from the manufacturers: Acticoat™ Absorbent (Smith and Nephew, London, England) with 105 mg of nanocrystalline silver per 100 cm^2 of polyethylene mesh dressing, Aquacel® Ag (ConvaTec, Skillman, NJ) with 8·3 mg of ionic silver per 100 cm^2 of a sodium caboxymethyl cellulose dressing and Tegaderm™ Ag (3M, St. Paul, MN) with 8 mg of silver sulfate per gram of absorbent mesh dressing. For a conventional dressing, 12-ply, 100% cotton sterile gauze was used (Fisher Scientific, Pittsburg, PA). Sterile samples of the silver-impregnated dressings and gauze dressing were pre-cut under sterile conditions into 2·5 × 2·5 cm squares for testing. For synergy assays, a hydrogel base containing 2% lactoferrin and 5% xylitol (LfX) was obtained from Glanbia Research and Development (Twin Falls, ID). For the conventional hydrogel, a commercially available product, Elta Wound Gel, was obtained from the manufacturer (Swiss-American, Carrollton, TX).

Challenge organisms

P. aeruginosa 215 and methicillin-resistant *S. aureus* (MRSA) 10943 were originally isolated from a chronic wound debridement samples from patients at Southwest Regional Wound Clinic (Lubbuk, TX) as previously described (5).

Inoculation and bacterial growth

Bacteria were cultured in 10% strength brain–-heart infusion (BHI) broth at 37°C for 24 hours prior to dilution in 10% BHI to an optical density of 0·05 at 600 nm.

Bacterial biofilms were grown and treated in an *in vitro* wound model as previously described with some methological deviations (19). This model is based on the colony biofilm model (20) and an ASTM-approved (E2647) drip-flow model (21) and mimics the environmental conditions of the chronic wound by growing the biofilms at a liquid/solid/air interface. Briefly, 10 µl of inoculum was dripped into a 0·22-mm porous membrane (GE Water & Process Technologies, Trevose, PA) and allowed to establish biofilms for 72 hours with a drip-flow rate of 5 ml/hour with 10% BHI at 25°C. Under sterile conditions, 0·5 ml of hydrogel was added to each dressing and placed directly over the established biofilms. These bacteria were allowed to grow for another 72 hours at 25°C prior to harvest and viability analysis.

Key Points

- colonisation by bacteria contributes to non healing of the wound; however, recent evidence suggests that development of a chronic wound is dependent on contaminating bacteria forming biofilms
- recently, it has been demonstrated that lactoferrin can act synergistically with the rare sugar-alcohol xylitol to inhibit growth of established biofilms of a clinical wound isolate *Pseudomonas aeruginosa 215*
- to derive further antimicrobial efficacy, synergistic relationships between lactoferrin and xylitol and other antimicrobials should be explored, including synergism with silver
- a selection of commercially available silver impregnated dressings were obtained from the manufacturers
- *P. aeruginosa 215* and methicillin-resistant *S. aureus* (MRSA) 10943 were originally isolated from a chronic wound debridement samples from patients at Southwest Regional Wound Clinic (Lubbuk, TX)
- bacterial biofilms were grown and treated in an *in vitro* wound model as previously described with some methological deviations

Quantification of bacterial growth

After the second 72-hour growth period, the samples were removed from the chambers and placed in 10 ml of sterile phosphate buffered saline (PBS). The bacteria were disaggregated from the membrane and dressing by vortexing the samples for 10 seconds and sonicating the samples for 5 minutes, and finally repeating the vortexing for an additional 10 seconds. Serial tenfold dilutions in sterile PBS were then plated on 100% tryptic soy agar. For two species biofilms, culturing on selective media plates with 100% Difco Pseudomonas isolation agar (BD Diagnostic Systems, Detroit, MI) or 100% Difco Staphylococcus Medium 110 agar (BD Diagnostic Systems) separated *P. aeruginosa* and MRSA, respectively. Recovery plates were allowed to incubate overnight at 37°C prior to counting the colony forming units (CFU) per membrane. CFU were converted to log densities for analysis.

Statistical analysis

Log density for each membrane sample was $\log_{10}$-transformed and converted to log reduction (LR) relative to gauze control. Statistical analysis was performed using one-way analysis of variance performed on indicated data sets. Post-test analysis used Tukey's pairwise comparisons. Differences with $P < 0{\cdot}05$ were considered statistically significant.

Statistical interaction was calculated based on LR from multiple experiments as previously described (22). Briefly, a quantitative measure of interaction was calculated from LR as follows: interaction = (LR for the LfX hydrogel and silver wound dressing) − (LR for LfX hydrogel alone) − (LR for silver wound dressing alone). A positive statistical interaction indicates synergism and a negative statistical interaction indicates antagonism. A null value or zero interaction indicates neither synergy nor antagonism.

RESULTS

Quantification of *P. aeruginosa* biofilms treated with silver wound dressings and hydrogels

The efficacy of the hydrogel with 2% lactoferrin and 5% xylitol added was compared with a commercially available wound dressing with the same hydrogel base. Hydrogels were applied directly to established *P. aeruginosa* biofilms (Figure 1, lanes 1–3) and standard gauze dressing was applied on top of the hydrogels (Figure 1, lanes 4–6). As can be seen in Figure 1, lane 3, the addition of lactoferrin to the hydrogel gives the hydrogel a slight red tint. In Figure 1, lanes 1 and 4 are biofilms treated with the gauze dressing only. The efficacy of the lactoferrin/xylitol hydrogel was evident when compared with the commercially available hydrogel. Relative to the gauze only control, the lactoferrin/xylitol hydrogel had a $2{\cdot}1 \pm 0{\cdot}4$ LR in viability, whereas the commercially available wound hydrogel had a $0{\cdot}6 \pm 0{\cdot}5$ LR in viability, demonstrating a statistically significant difference in LR between the two hydrogels (Figure 2).

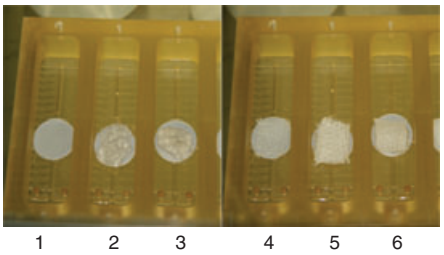

Figure 1. Images of a colony-drip-flow reactor. Established biofilms are treated with 0·5 ml hydrogel (lanes 2 and 3) prior to application of wound dressing (lanes 4–6).

To assess the efficacy of the hydrogels when used in combination with silver-based wound dressings, established *P. aeruginosa* biofilms were treated as above substituting the standard

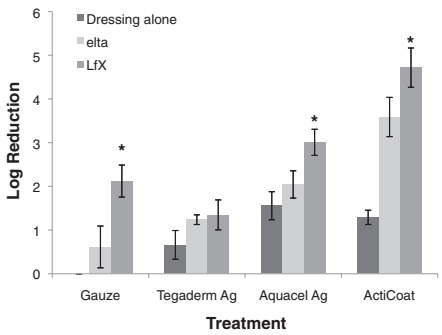

Figure 2. Log reductions (LRs) for *Pseudomonas aeruginosa* biofilms treated without or with silver-based wound dressings and with commercial hydrogel or lactoferrin/xylitol hydrogel. LRs for wound dressing alone are also shown. Combined hydrogel and wound dressing treatments assayed viability for biofilms treated with Tegaderm™ Ag, Aquacel® Ag and Acticoat™. All LRs are normalised to the gauze control. Data are presented as mean ± standard error. Asterisk denotes statistically significant difference between hydrogel samples.

gauze dressing with an equally sized, commercially available, antimicrobial silver wound dressing. The least effective treatments were with the wound dressing Tegaderm™ Ag. Although there was a statistically significant difference between the LR in viability of the samples treated with the silver dressing alone and both the samples treated with the hydrogels used in combination with the silver dressings (not indicated), there was not a statistically significant difference between the two hydrogels when used in combination with Tegaderm™ Ag. The average LR in viability of the dressing alone was $0{\cdot}7 \pm 0{\cdot}3$. The average LRs of the commercially available hydrogel and the lactoferrin/xylitol hydrogel used in combination with Tegaderm™ Ag were $1{\cdot}2 \pm 0{\cdot}1$ and $1{\cdot}4 \pm 0{\cdot}3$, respectively (Figure 2).

In contrast, a statistically significant difference between the lactoferrin/xylitol hydrogel and the commercially available hydrogel was observed when used in combination with the sodium caboxymethyl cellulose wound dressing Aquacel® Ag and the nanocrystalline silver mesh wound dressing Acticoat™ (Figure 2). The Aquacel® Ag dressing and the Acticoat™ dressing alone had average LRs in viability of $1{\cdot}6 \pm 0{\cdot}3$ and $1{\cdot}3 \pm 0{\cdot}2$, respectively. Average LRs in viability for the commercial hydrogel used in combination with Aquacel® Ag and Acticoat™ were $2{\cdot}0 \pm 0{\cdot}3$ and $3{\cdot}6 \pm 0{\cdot}4$, respectively. In combination with Aquacel® Ag, the lactoferrin/xylitol hydrogel resulted in an average LR of $3{\cdot}0 \pm 0{\cdot}3$. Combined use of the lactoferrin/xylitol hydrogel with Acticoat™ was the most efficacious treatment with an average LR in viability of $4{\cdot}7 \pm 0{\cdot}5$ relative to the standard gauze control.

Quantification of bacterial viability in dual species biofilms

As the Acticoat™ dressing was most efficacious against established *P. aeruginosa* biofilms, this dressing was chosen for further analysis in a dual species biofilm model using clinical wound isolates (5). In all treatments, the MRSA strain was found to be more sensitive than the *P. aeruginosa* strain when average LRs in viability were compared (Figure 3). Indeed, with the gauze dressing, it appeared that the *P. aeruginosa* was more resistant to treatment with the hydrogels alone when cultured in combination with MRSA. For example, the average LRs of *P. aeruginosa* in the dual species model were $0{\cdot}3 \pm 0{\cdot}4$ and $0{\cdot}2 \pm 0{\cdot}2$ when treated with the commercial hydrogel and the lactoferrin/xylitol hydrogel, respectively. This contrasted with the around two-LR observed in the single species model (Figure 2). The average LRs for MRSA in the dual species model were $1{\cdot}5 \pm 0{\cdot}8$ and $1{\cdot}8 \pm 0{\cdot}4$ for the commercial hydrogel and the lactoferrin/xylitol hydrogel, respectively.

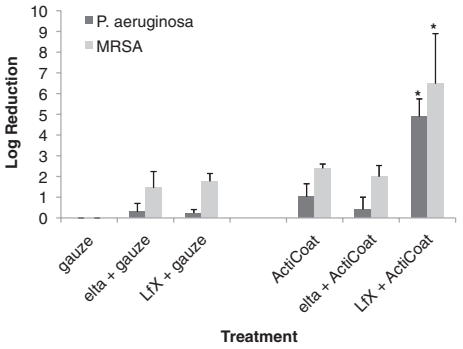

Figure 3. Log reduction (LR) in viability of dual species biofilms treated with either commercial hydrogel or lactoferrin/xylitol hydrogel in combination with either gauze dressing or Acticoat™. LRs for dressings alone are also shown. Data are presented as average ± standard deviation. Asterisk denotes statistically significant difference between hydrogel samples ($P \leq 0{\cdot}05$).

When Acticoat™ was used alone in the dual species biofilm model, the average LRs in viability were slightly better than with the hydrogels used alone. The average LR in viability for MRSA and *P. aeruginosa* in these samples were $2{\cdot}4 \pm 0{\cdot}2$ and $1{\cdot}0 \pm 0{\cdot}6$, respectively (Figure 3). For both MRSA and *P. aeruginosa*, the combined treatment of lactoferrin/xylitol hydrogel with Acticoat™ was statistically significantly more effective than the commercial hydrogel in combination with Acticoat™ (Figure 3). Although the average LRs in viability of MRSA and *P. aeruginosa* treated with the commercial hydrogel in combination with Acticoat™ were $1{\cdot}995 \pm 0{\cdot}535$ and $0{\cdot}4 \pm 0{\cdot}6$, respectively, the average LRs in viability of MRSA and *P. aeruginosa* treated with the lactoferrin/xylitol hydrogel in combination with Acticoat™ were $6{\cdot}5 \pm 2{\cdot}4$ and $4{\cdot}9 \pm 0{\cdot}9$, respectively.

Lactoferrin and xylitol hydrogel has a statistical interaction with the nanocrystalline silver wound dressing acticoat™

'Synergy' and 'combined effect' are terms commonly used to express interaction between

Key Points

- in this study, the addition of lactoferrin and xylitol to a hydrogel wound dressing demonstrated better efficacy when compared to a commercially available hydrogel wound dressing
- this improvement in anti-biofilm efficacy of a silver wound dressing used in combination with the lactoferrin/xylitol hydrogel was further demonstrated in a dual species biofilm model using clinical wound isolates
- finally, a statistical interaction between the lactoferrin/xylitol hydrogel and the nanocrystaline silver wound dressing Acticoat™ was demonstrated for both the single species and dual species models
- in conclusion, this study demonstrates that a combined treatment including lactoferrin, xylitol and silver provides significant antimicrobial efficacy against established biofilms consisting of clinical wound isolates of MRSA and *P. aeruginosa*
- this is clinically relevant as chronic wounds are demonstrated to be inhabited by established biofilms and only treatments that address this chronic colonisation will prove promising in the growing field of chronic wound care

Table 1 Summary for statistical interaction between LfX hydrogel and silver wound dressings

Biofilm model/species	Treatment	Statistical interaction
Single species *Pseudomonas aeruginosa*	LfX hydrogel + Tegaderm™	−1·4 ± 0·4
Single species *P. aeruginosa*	LfX hydrogel + Aquacel®	−0·1 ± 0·5
Single species *P. aeruginosa*	LfX hydrogel + Acticoat™	1·3 ± 0·1
Dual species *P. aeruginosa*	LfX hydrogel + Acticoat™	4·9 ± 0·9
Dual species methicillin-resistant *Staphylococcus aureus*	LfX hydrogel + Acticoat™	6·5 ± 2·4

antimicrobials; however, these terms lack any statistical definition. To determine whether the lactoferrin/xylitol hydrogel had a statistical interaction with the commercially available silver wound dressings, the data across multiple experiments were analysed as previously described (22). As shown in Table 1, the lactoferrin/xylitol hydrogel demonstrated a statistical interaction with the nanocrystalline silver wound dressing Acticoat™ in both the single species and the dual species model.

DISCUSSION

Bacterial bioburden in the form of contaminating biofilms has been demonstrated to be a major factor contributing to non healing in chronic wounds (8,9,23,24); therefore, biofilm-targeted therapies in wound care are highly relevant. Treatment at the wound is managed primarily through mechanical manipulation such as debridement (25) and choice of wound dressing. Rationally designing wound dressings with effective anti-biofilm control has the potential to significantly improve wound therapy and lead to successful wound healing. The studies reported here demonstrate the combined use of commercially available silver wound dressings with an anti-biofilm hydrogel.

Combined use of lactoferrin and xylitol has been previously demonstrated as effective against *P. aeruginosa* biofilms (12). In this study, the addition of lactoferrin and xylitol to a hydrogel wound dressing demonstrated better efficacy when compared to a commercially available hydrogel wound dressing. This improved efficacy was demonstrated both independently and in combination with commercially available silver-based wound dressings (Figure 2). This improvement in anti-biofilm efficacy of a silver wound dressing used in combination with the lactoferrin/xylitol hydrogel was further demonstrated in a dual species biofilm model using clinical wound isolates (Figure 3). Finally, a statistical interaction between the lactoferrin/xylitol hydrogel and the nanocrystaline silver wound dressing Acticoat™ was demonstrated for both the single species and dual species models. Interestingly, the ionic silver and silver sulphate dressings had a negative statistical interaction suggesting that the hydrogel might inhibit the penetration of the antimicrobial silver into the biofilm. In support of this idea, the hydrogel without lactoferrin and xylitol also exhibited a negative statistical interaction when combined with the ionic silver and silver sulphate dressings (data not shown).

Although destabilisation of the bacterial membrane has been suggested as a mechanism for the anti-biofilm effect of lactoferrin and xylitol (12), it remains to be determined whether this mechanism contributes to the significant reduction in bacterial viability when a silver wound dressing is combined with a lactoferrin/xylitol hydrogel. In addition, biofilms in chronic wounds may have common colonisers such as *P. aeruginosa* and MRSA; however, with improved molecular analysis it has been shown that chronic wounds are populated by multiple species (5). In this study, growing *P. aeruginosa* and MRSA together appeared to reduce the sensitivity of *P. aeruginosa* to the silver containing dressing (Figures 2 and 3). Given this observation, it would be interesting to explore the potential for a change in bacterial sensitivity as the species composition of the biofilm changes.

In conclusion, this study demonstrates that a combined treatment including lactoferrin, xylitol and silver provides significant antimicrobial efficacy against established biofilms consisting of clinical wound isolates of MRSA and *P. aeruginosa*. This is clinically relevant as chronic wounds are demonstrated to be inhabited by established biofilms and only treatments that address this chronic colonisation

will prove promising in the growing field of chronic wound care.

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