

## ORIGINAL ARTICLE

# The increased killing of biofilms in vitro by combining topical silver dressings with topical negative pressure in chronic wounds

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## Key words

Biofilms; Chronic wounds; Silver dressings;  
Topical negative pressure therapy

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## Abstract

Chronic wounds remain a significant medical and financial burden in hospitals of today. A major factor in the transition from an acute to a chronic wound is its bacterial bioburden. Developments in molecular techniques have shown that chronic wounds remain colonised by many species of bacteria and that the bacteria within these chronic wounds exist in two forms. Treatments of chronic wounds have maintained a challenging field and significant ongoing research is being conducted. With the development of an in vitro wound model, we applied topical negative pressure (TNP) dressings to a spectrum of common bacterial biofilms found in chronic wounds and studied the synergistic efficacy between the application of TNP and silver-impregnated foam against these biofilms. This synergistic response was seen within the laboratory strains of staphylococcal biofilms over a 3-day treatment period but lost following the 5 days of treatment. However, combining topical pressure dressings and silver foam lead to a synergistic inactivation in *Pseudomonas* species over both 3-day and 5-day treatments.

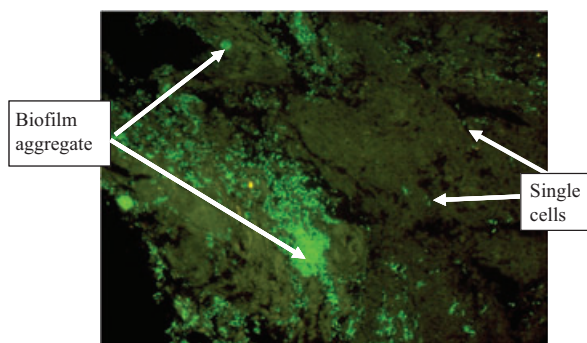
## Introduction

Chronic wounds or ulcers represent a significant cost in both financial and human terms. Up to 1% of people in developed countries develop chronic venous ulcers (1), whereas 15% of diabetic patients will present with non-healing foot ulceration (2). The prevalence of pressure ulcers in acute care patients is around 16% (3). However, around 29% of patients in long-term care will develop pressure sores (4). One Australian study found that 24% of patients had their ulcer for more than 12 months and of these patients 35% had ulceration for greater than 5 years (5).

The cost of treating grade IV pressure ulcers has been estimated to be over \$124 000 per case (6). As the prevalence of diabetes and obesity increases, and the world's population age increases, it is expected that the incidence of chronic wounds will also increase (4,7). Therefore, effective treatment

## Key Messages

- chronic wounds place a significant financial and medical burden on healthcare budgets
- bacterial biofilms are thought to significantly contribute to lack of healing of these wounds
- we used our in vitro wound model to test the efficacy of topical negative pressure (TNP) and silver dressings on *Staphylococcus spp* and *Pseudomonas aeruginosa*, species commonly isolated from wounds and known to form biofilms in chronic wounds. Testing in vitro allowed us to determine effect of treatment on biofilm in the absence of confounding patient factors
- silver impregnated dressings and TNP acted synergistically to kill *Paeruginosa* biofilm within 3 days and this



**Figure 1** Fluorescent in situ hybridisation (FISH) image of a biopsy taken from a patient with a chronic wound. Bacteria are detected with 16S rRNA fluorescein-labelled EUB537 universal probe that detects all species of bacteria. This FISH slides show multiple biofilm aggregates as well as single bacterial cells, which are present throughout the wound environment (60x objectives). Bacterial cells are in bright green colour.

effect was maintained. There was a variable effect of combined therapy on *Staphylococcal spp* with both silver dressing alone and combined with TNP reducing biofilm numbers

of chronic wounds has the potential to greatly reduce our future health care burden and increase the quality of life of many people.

A major factor in the transition from acute to chronic wound is bacterial infection (8–10). The severity of the infection is related to the absolute number of infecting bacteria (11). Conversely, healing wounds are characterised by a reduction in planktonic bacterial counts and markers of inflammation (12).

Newly developed molecular techniques have shown that chronic wounds are colonised by many species of bacteria, not just the few easy-to-culture organisms previously identified (13,14). The bacteria within chronic wounds also exist in two forms, planktonic or free swimming, single cells and biofilm or sessile cells fused together in biofilm aggregates (15–18) (Figure 1). A biofilm is a dynamic self-organised aggregation of sessile micro-organisms attached to a surface and to each other. Within biofilms, bacteria are encased in extracellular polymeric substances (EPSs), principally polysaccharides, which are produced by the bacteria themselves.

The significance of the identification of biofilms in chronic wounds is the increased resilience of bacterial aggregates to environmental insults, antimicrobial therapy and the host immune response, compared to planktonic communities. Biofilm bacteria can typically survive 100–250 times the amount of an antibiotic needed to kill the same bacteria growing in liquid culture. This increased resistance of biofilm bacteria to biocide inactivation is due to a combination of several factors [reviewed in Refs (19,20)] including (a) lack of biocide penetrating the whole thickness of the biofilm due to binding and inactivation by the EPS; (b) nutrient deficiency in the deeper layers of the biofilm results in bacteria that are metabolically inactive and, therefore, more resistant to antibiotics and biocides; (c) a concentration gradient of oxygen

and nutrients promoting the growth of different types of bacteria with different intrinsic susceptibilities; (d) increased genetic exchange within the biofilm resulting in acquisition of new resistance determinants and (e) phenotypic polymorphism brought about by surface attachment causing a change in gene expression resulting in a distinct biofilm phenotype 'dormant or persister cell' with increased biocide resistance.

Recently, limited research, using fluorescent in situ hybridisation (FISH) technology, suggests that within the wound, aggregates of biofilm may be composed of single species of bacteria; *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been identified (17,21).

Treatment of chronic wounds is highly challenging and there is a significant ongoing research being conducted. Debridement remains the gold standard of chronic wound care. A process that involves the removal of exudate, slough, eschar and bacterial biofilms from a wound bed using a scalpel, scissors and curettes is referred to as sharp debridement (22). The benefits of sharp debridement are multifactorial but the primary aim is to remove non-viable tissue until well-vascularised tissue becomes visible. This process sets off an inflammatory reaction with neutrophils and macrophages that aid in wound healing. Other forms of debridement such as wet-to-dry dressings remove non-viable tissue through a process of decussation and adherence. However, this is not without significant discomfort to the patient.

A newer disrupting technology, topical negative pressure (TNP) dressing, has been shown in some clinical situations to improve wound healing (23–25). In a French study, TNP was associated with fewer amputations in diabetic ulcers and lower costs (26). TNP places the wound bed under negative pressure of between 125 and 150 mmHg and its mechanisms of action maintains a moist wound environment, removes wound exudate, reduces bacterial loads, increases local blood flows and formation of granulation tissue.

We have recently developed an in vitro wound model that incorporates biofilm-covered coupons into an agar base and allows for the application of TNP (15). Using this model, we have applied TNP dressings to *P. aeruginosa* biofilm. We found that TNP caused structural changes in treated biofilm, compressing the biofilm, reducing the size of the channels, decreasing the average and maximum diffusion distances and perhaps fragmenting the biofilm. We found that this structural change in *P. aeruginosa* was associated with increased sensitivity to silver-impregnated foam and resulted in complete killing of the biofilm (15). In this study, we confirm the synergistic efficacy between the application of TNP and silver-impregnated foam against the Gram-negative organism *P. aeruginosa* and investigate the effect of combination therapy on the Gram-positive organisms *Staphylococcus epidermidis*, *S. aureus* and methicillin-resistant *S. aureus* (MRSA) (a laboratory and a clinical strain).

## Methods

### The wound model

*S. epidermidis* ATCC 35984, *S. aureus* ATCC 25923, MRSA ATCC 43300 (laboratory strain), a clinical strain of MRSA

obtained from an infected catheter tip, and *P. aeruginosa* (ATCC 25619) were tested in pure culture. Biofilm was grown on removable teflon discs in the CDC biofilm reactor (Bio-Surface Technologies Corporation, Bozeman, MT) in 10% tryptone soya broth (TSB) under shear at 37°C as previously described (15).

Each wound chamber contained six biofilm-covered coupons incorporated into 3% bacteriological agar base representing a low nutrient and moist organic wound surface. To maintain biofilm viability and mimic wound exudate, 10% TSB was flowed at 40 ml/hour across the agar surface. Excess fluid was drained via a gravity drainage tube for chambers not subjected to TNP or via a specialised TNP drainage tube (TRAC pad) for chambers subjected to TNP (15).

### Test parameters

The chambers were overlaid with either V.A.C.<sup>®</sup> GranuFoam<sup>®</sup> Dressing (black foam), black polyurethane foam or V.A.C.<sup>®</sup> GranuFoam Silver<sup>®</sup> Dressing (silver foam) which contains micro-bonded metallic silver uniformly distributed throughout a polyurethane foam dressing. Previous experimental work by KCI (27) has shown a consistent release of non-ionic silver particles from the silver foam for the first 72 hours.

Control chambers were not subjected to TNP, whereas test chambers were subjected to 125 mmHg negative pressure, supplied by a V.A.C. Instill<sup>®</sup> machine (KCI Medical, San Antonio, TX). Biofilm viability was assessed at 72 hours (3 days) to determine the effect of silver impregnation and at 5 days to measure if there was any residual effect following the leaching of silver particle from the foam.

Biofilm viability was determined by counting colony forming units (CFUs) for five coupons per wound chamber. Upon removal, the coupons were individually placed in 5 ml of phosphate buffer solution (PBS) and sonicated in an ultrasonic bath (Soniclean; JMR, Sydney, Australia) with a sweeping frequency of 42–47 kHz for 5 minutes. Bacterial number for each coupon was determined by standard serial dilution and plate counting. Log reduction in titre was calculated by subtracting bacterial numbers recovered from coupons covered with V.A.C.<sup>®</sup> GranuFoam Silver<sup>®</sup> Dressing (silver foam) from coupons covered with V.A.C.<sup>®</sup> GranuFoam<sup>®</sup> Dressing (black foam) without the application of TNP.

One coupon from each chamber was subjected to scanning electron microscopy (SEM). The coupon was fixed in 3% glutaraldehyde, dehydrated through ethanol, immersed in hexamethyldisilazane (Polysciences, Inc., Warrington, PA) for 3 min

before being aspirated dry, mounted on specimen stubs and sputter coated with gold prior to examination in an electron microscope. Each coupon was scanned at low power magnification, and four random sections, at each quadrant of the coupon, were then examined at higher magnification.

### Statistical analysis

All statistical analyses were performed using SigmaPlot 11 statistical programme. The Mann–Whitney Rank Sum Test was used to compare bacterial numbers on coupons overlaid with black foam or silver foam, independently of TNP treatment, at days 3 and 5. The Mann–Whitney Rank Sum Test was used to assess change in bacterial numbers on coupons between days 3 and 5. To check for significant differences in bacterial viability between treatment groups, a one-way analysis of variance (ANOVA) was performed. If significant, the Holm–Sidak method of all pair-wise multiple comparisons was conducted to determine the treatment groups that were significantly different from each other. The data had to be transformed to ensure normality. For non-normally distributed data, a Kruskal–Wallis one-way ANOVA on ranks was performed, and if significant, the Tukey test for all pair-wise multiple comparisons were conducted to determine the treatment groups that were significantly different from each other.

### Results

The mean number of bacteria per coupon ( $n = 5$ ) for individual wound chambers, harvested on days 3 and 5, are detailed in Tables 1 and 2, respectively. For wound models overlaid with black foam, there was no significant difference in bacterial number harvested from coupons from chambers, at 3 (Table 1) or 5 days (Table 2) whether or not they were subjected to TNP ( $P > 0.05$ ). At both time points, less bacteria were recovered from coupons overlaid with silver foam dressings when compared with bacterial numbers recovered from coupons covered in black foam ( $P < 0.001$ ) for all bacterial species tested. However, individual bacterial species varied in their responses. Significant differences are shown in Tables 1 and 2.

#### Effect of silver foam without TNP after 3 days of treatment

The log<sub>10</sub> reduction in bacterial numbers recovered from coupons overlaid with silver dressing without the application of TNP, varied with the type of bacterial species tested,

**Table 1** Mean number of bacteria per coupon ( $n = 5$ ) harvested on day 3 from wound models overlaid with V.A.C.<sup>®</sup> GranuFoam<sup>®</sup> Dressing (black foam) or V.A.C.<sup>®</sup> GranuFoam Silver<sup>®</sup> Dressing (silver foam)  $\pm$  application of TNP

Foam type	Treatment	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i>	MRSA ATCC	MRSA clinical strain	<i>Pseudomonas aeruginosa</i>
Black foam	Control	$6.1 \times 10^7^*$	$5.5 \times 10^7^*$	$2.7 \times 10^8^\#$	$3.9 \times 10^8^*$	$6.3 \times 10^{8**}$
	TNP	$3.8 \times 10^7^*$	$3.4 \times 10^{6*}$	$2.6 \times 10^8^\#$	$2.5 \times 10^8^*$	$5.5 \times 10^{8**}$
Silver foam	Control	$9.9 \times 10^6$	$4.8 \times 10^{2**,\wedge}$	$1.5 \times 10^5$	$1.6 \times 10^6$	$4.0 \times 10^7$
	TNP	$1.8 \times 10^{5**}$	$3.8 \times 10^{5**,\wedge\wedge}$	$5.9 \times 10^{5\#\#}$	$9.1 \times 10^{4**}$	0*

MRSA, methicillin-resistant *S. aureus*; TNP, topical negative pressure.

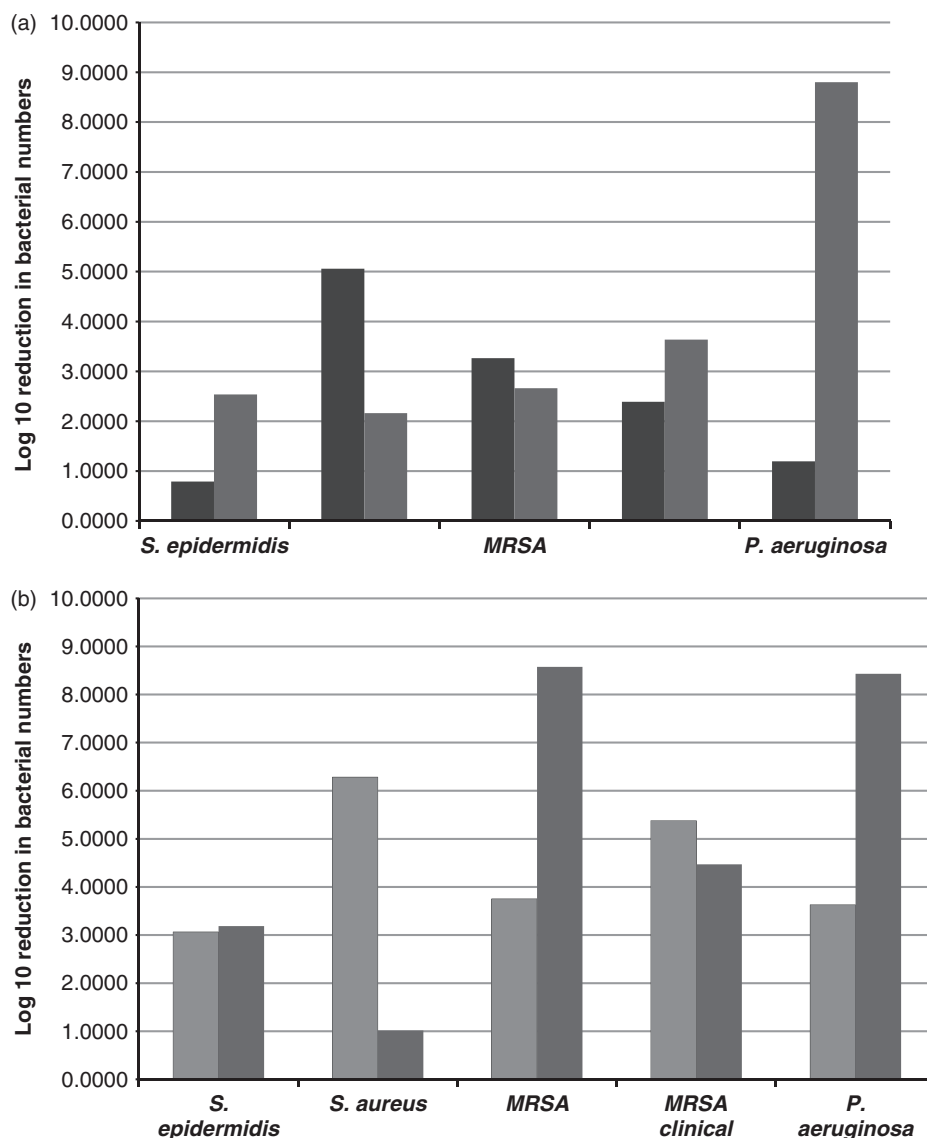
Column wound models significantly different from each other are denoted by unlike symbols: \*, \*\* and  $\wedge\wedge$   $P = 0.001$ ;  $\#\#\#$   $P = 0.002$ .

**Table 2** Mean number of bacteria per coupon ( $n=5$ ) harvested on day 5 from wound models overlaid with V.A.C.<sup>®</sup> GranuFoam<sup>®</sup> Dressing (black foam) or V.A.C.<sup>®</sup> GranuFoam Silver<sup>®</sup> Dressing (silver foam)  $\pm$  application of TNP

Foam type	Treatment	<i>Staphylococcus epidermidis</i>	<i>Staphylococcus aureus</i>	MRSA ATCC	MRSA clinical strain	<i>Pseudomonas aeruginosa</i>
Black foam	Control	$8.1 \times 10^8^*$	$1.9 \times 10^6$	$3.8 \times 10^8^*$	$1.7 \times 10^8$	$2.7 \times 10^8^{\wedge}$
	TNP	$1.6 \times 10^8^*$	$3.5 \times 10^8^*$	$3.3 \times 10^8^*$	$8.3 \times 10^8^{\#}$	$1.0 \times 10^9^*$
Silver foam	Control	$6.9 \times 10^5^{**}$	0 <sup>**</sup>	$6.6 \times 10^8$	$6.9 \times 10^2^{\#\#}$	$6.3 \times 10^4^{**}$
	TNP	$5.2 \times 10^5^{**}$	$1.9 \times 10^5^{**}$	0 <sup>**</sup>	$5.6 \times 10^3^{\#\#}$	0 <sup>**^{\wedge\wedge}</sup>

MRSA, methicillin-resistant *S. aureus*; TNP, topical negative pressure.

Column wound models significantly different from each other are denoted by unlike symbols: \*, \*\* and  $^{\wedge\wedge}P=0.001$ ;  $^{\#\#}P=0.002$ .

**Figure 2** Log<sub>10</sub> reduction in bacterial numbers recovered from coupons overlaid V.A.C.<sup>®</sup> GranuFoam Silver<sup>®</sup> Dressing without (blue bars) or with (red bars) the application of topical negative pressure (TNP). After (A) 3 days and (B) 5 days of treatment.

even for closely related species such as *S. aureus* and MRSA (Figure 2A). Following 3 days of treatment, silver foam alone had a minimal, non-significant effect on *S. epidermidis* and *P. aeruginosa*. However, the numbers of bacteria recovered from *S. aureus* biofilm overlaid with silver foam were reduced over 5 log<sub>10</sub> or 100 000 times ( $P=0.001$ ) with three of the five

coupons showing no growth. There was a difference in the response between the laboratory and clinical strains of MRSA with the clinical strain being more resistant to silver alone. Even so, the numbers of MRSA (clinical strain) were reduced over a 100-fold, whereas the numbers of the laboratory strain were reduced 3.3 log<sub>10</sub> or more than 1000-fold (Figure 2A).



### Effect of silver foam in conjunction with TNP after 3 days of treatment

The number of bacteria recovered from *P. aeruginosa* biofilm was significantly reduced with the simultaneous application of silver foam and TNP ( $P < 0.001$ ) with the occurrence of complete bacterial inactivation. The combined effect of TNP and silver foam produced reduction in bacterial numbers for the staphylococcal biofilms (Figure 2A), *S. epidermidis*, *S. aureus*, MRSA clinical strain ( $P = 0.001$ ) and MRSA laboratory strain ( $P = 0.002$ ), between 2 and 3.7 log<sub>10</sub> and this reduction was significant. For staphylococcal biofilms, a synergistic effect between silver foam and TNP was evident for *S. epidermidis* and the clinical strain of MRSA where a 10-fold more bacteria were killed using combination therapy compared with silver foam alone.

### Residual effect of silver foam after 5 days of treatment

Silver foam alone: contact with silver foam for 5 days further reduced the mean number of viable biofilm bacteria for most species of bacteria (Table 2). All five coupons covered with *S. aureus* biofilm were culture negative, suggesting the complete inactivation of bacteria by the silver foam.

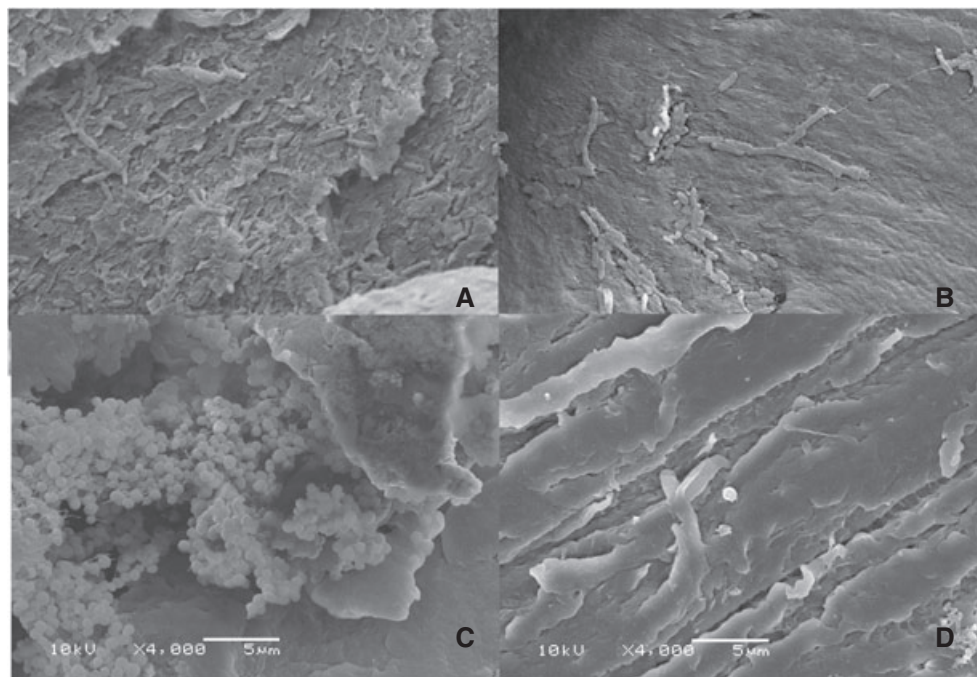
There was a synergistic effect when silver foam was combined with TNP for treating *P. aeruginosa* biofilm and the laboratory strain of MRSA with both bacteria being completely inactivated ( $P < 0.001$ ). The efficacy of silver foam alone or with TNP was similar for *S. epidermidis* and the clinical strain of MRSA reducing bacterial numbers a minimum

of 1000-fold. In contrast, silver foam alone was the more efficacious treatment for *S. aureus* reducing bacterial numbers of 6 log<sub>10</sub> ( $P < 0.001$ ) (Table 2).

SEM confirmed the viability of these results. All control coupons subjected to SEM were uniformly covered with higher numbers of bacteria and EPS (Figure 3). For treatments that resulted in a large log reduction in CFU, coupons were covered in scanty biofilm or only residual bacterial cells (Figure 3). However, SEM still remains insensitive in differentiating biofilm counts between 10<sup>4</sup> and 10<sup>6</sup> CFU.

### Discussion

Chronic wounds are usually colonised by a number of bacterial species (13,14). However, *P. aeruginosa*, *S. aureus* and haemolytic *Streptococci* are most commonly cultured, and in this study we focused on *P. aeruginosa* and *S. aureus*. Both *P. aeruginosa* and *S. aureus* have been classified by Infectious Diseases Society of America as ESKAPE organisms because of their multiple antibiotic resistance profiles. However, this antibiotic resistance is compounded by the ability of organisms to form biofilms, which increases their tolerance even more to antibiotics, antiseptics, disinfectants and the host immune response (28). Although planktonic cultures of MRSA (8 clinical strains) and multi-resistant *P. aeruginosa* (8 strains) are very sensitive to benzalkonium chloride, chlorhexidine and triclosan, their biofilm counterparts survive even when much higher concentration of biocides are used (Smith and Hunter, 2008). The presence of biofilm in a wound provides an ongoing



**Figure 3** Scanning electron microscopy of a 5-day wound model with high magnification (4000x): (A) *Pseudomonas aeruginosa* control coupon treated with black foam and no topical negative pressure (TNP) showing a thick multi-layered biofilm; (B) *P. aeruginosa* coupon treated with silver foam and subjected to TNP treatment showing a few scattered residual bacterial cells; (C) *Staphylococcus aureus* coupon treated with black foam and no TNP (control) showing a multi-layered biofilm; (D) *S. aureus* coupon treated with silver foam and subjected to TNP treatment showing no biofilm and only occasional isolated cocci.

nidus of inflammation and there is a need of reliable, targeted therapy capable of disrupting the biofilm. In the literature, there is little evidence for the development of silver resistance; so we investigated the potential of combining a biofilm disrupting technology with a chemical silver treatment.

Experimental work by KCI (27) has shown a consistent release of non-ionic silver particles from the silver foam for the first 72 hours (29). We, therefore, compared the efficacy of silver foam and black foam with and without concurrent TNP treatment at 3 days. We then investigated whether there was any residual bacteriocidal effect of silver foam if left in a wound for 5 days. Combining TNP and silver foam lead to a synergistic response, completely inactivating *P. aeruginosa* biofilm by day 3 and this effect was maintained until day 5. This is consistent with previous results showing that combined therapy inactivated *P. aeruginosa* biofilm following 7 days of in vitro treatment (15).

For the staphylococcal biofilms, this synergistic response was seen with the laboratory strain of MRSA biofilm where combined silver foam and TNP resulted in complete inactivation with over an 8 log<sub>10</sub> reduction in CFUs ( $P < 0.001$ ). Although not resulting in complete inactivation, the synergistic effect between silver and TNP was also seen when testing a recently isolated clinical strain of MRSA, attaining over a 4000-fold decrease in bacterial numbers. Where possible clinical strains should be included in test protocols; however, the similar trends we obtained with laboratory biofilm-producing strains and the clinical isolate confirm the validity of using laboratory strains for initial testing. The world-wide availability of laboratory strains ensures that other researchers can reproduce our findings or test new wound care products without the additional confounder of strain differences.

A synergistic effect between silver foam and TNP was also evident for *S. epidermidis* following 3 days of treatment with more than 10-fold greater reduction in bacterial titre occurring with combined therapy over silver foam alone (Table 1). This synergistic effect was lost following 5 days of treatment (Table 2) probably reflecting increased leaching of silver ions due to TNP application. Silver ions bound to individual bacteria and the surrounding EPS demonstrated interference within the intermolecular forces found in a biofilm. The efficacy of these ions differed between biofilm colonies.

However, for *S. aureus*, although 5 days of combined therapy resulted in a significant 10% decrease in bacterial numbers, silver treatment alone resulted in complete inactivation and more than a 6-log<sub>10</sub> reduction in bacterial numbers (Figure 2). Thus, for this species, combined therapy offered no advantage over the use of silver foam alone.

The clinical implications of this research is that if a difficult to manage chronic wound is colonized with *P. aeruginosa*, combining TNP and silver may offer a better clinical outcome than either TNP or silver treatment alone. A variety of silver forms exist are powders, foams, hydrocolloids, hydrogels, polymeric films and meshes. When in solution, reactive silver ions (Ag<sup>+</sup>) are formed and induce toxicity to many of the structural components of the bacterial cell wall, and biochemical pathways such as the respiratory cytochromes, and DNA and RNA alterations to prevent transcription and cellular division (30). The results are more complex for the members of the staphylococcal

group that remain a significant contributor to chronic wounds. Additional work needs to be carried out on investigating other potential synergisms.

This in vitro study used our established wound model to study the effects of silver dressings with or without TNP dressings on the most commonly cultured bacteria in chronic wounds. The extrapolation of these results still maintains a conundrum for the health professional dealing with chronic wound in clinical practice because it still fails to assess many important unsolved issues: for example, do all silver dressings show the same efficacy at different concentrations to the common bacterial bioload found in the clinical wound, should we utilise these dressings over a different timeframe for different wounds on different sites, and what is the likelihood of silver resistance developing in these bacteria?

## Conclusion

The continued need for knowledge and understanding of chronic wounds and its polymicrobial nature has aided in the appropriate analysis of these biofilms and its key central role in the formation of these infections. Furthermore, the emerging use of silver dressings in clinical practice, with or without TNP, in chronic wounds has shown some degree of variability between these biofilm species. It is therefore critical to define the wound and efficaciously balance the use of silver dressings as an appropriate antimicrobial without leading to toxicity or resistance if we are to identify cost-effective and clinical relevant approaches to chronic non-healing wounds.

## References

1. Tognazzo S, Gemmati D, Palazzo A, Catozzi L, Carandina S, Legnaro A, Tacconi G, Scapoli GL, Zamboni P. Prognostic role of factor XIII gene variants in nonhealing venous leg ulcers. *J Vasc Surg* 2006;**44**:815–9.
2. Ramsey SD, Newton K, Blough D, McCulloch DK, Sandhu N, Reiber GE, Wagner EH. Incidence, outcomes, and cost of foot ulcers in patients with diabetes. *Diabetes Care* 1999;**22**:382–7.
3. Shahin ESM, Dassen T, Halfens RJG. Pressure ulcer prevalence in intensive care patients: a cross-sectional study. *J Eval Clin Pract* 2008;**14**:563–8.
4. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy: perspective article. *Wound Repair Regen* 2009;**17**:763–71.
5. Baker SR, Stacey MC. Epidemiology of chronic leg ulcers in Australia. *Aust N Z J Surg* 1994;**64**:258–61.
6. Brem H, Maggi J, Nierman D, Rolnitzky L, Bell D, Rennett R, Golinko M, Yan A, Lyder C, Vladeck B. High cost of stage IV pressure ulcers. *Am J Surg* 2010;**200**:473–7.
7. Greun RLCS, MacLellan DG. Optimising the hospital management of leg ulcers. *Aust N Z J Surg* 1996;**66**:171–4.
8. Robson MC. Wound infection: a failure of wound healing caused by an imbalance of bacteria. *Surg Clin North Ame* 1997;**77**:637–50.
9. Schultz A. Predicting and preventing pressure ulcers in surgical patients. *AORN J* 2005;**81**:986–1006; quiz 9–12.
10. Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. *Plast Reconstr Surg* 2006;**117**(7 Suppl):35S–41S.
11. Bowler PG. Wound pathophysiology, infection and therapeutic options. *Ann Med* 2002;**34**:419–27.

12. Davies CE, Hill KE, Newcombe RG, Stephens P, Wilson MJ, Harding KG, Thomas DW. A prospective study of the microbiology of chronic venous leg ulcers to reevaluate the clinical predictive value of tissue biopsies and swabs. *Wound Repair Regen* 2007;**15**:17–22.
13. Dowd SE, Sun Y, Secor PR, Rhoads DD, Wolcott BM, James GA, Wolcott RD. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol* 2008;**8**:43.
14. Smith DM, Snow DE, Rees E, Zischkau AM, Hanson JD, Wolcott RD, Sun Y, White J, Kumar S, Dowd SE. Evaluation of the bacterial diversity of pressure ulcers using bTEFAP pyrosequencing. *BMC Med Genomics* 2010;**3**:41.
15. Ngo QD, Vickery K, Deva AK. The effect of topical negative pressure on wound biofilms using an in vitro wound model. *Wound Repair Regen* 2012;**20**:83–90.
16. James GA, Swogger E, Wolcott R, Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS. Biofilms in chronic wounds. *Wound Repair Regen* 2008;**16**:37–44.
17. Fazli M, Bjarnsholt T, Kirketerp-Moller K, Jorgensen B, Andersen AS, Krogfelt KA, Givskow M, Tolker-Nielsen T. Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J Clin Microbiol* 2009;**47**:4084–9.
18. Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridization (PNA FISH). *Microbiology* 2009;**155**(Pt 8):2603–11.
19. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002;**15**:167–93.
20. Fux CA, Costerton JW, Stewart PS, Stoodley P. Survival strategies of infectious biofilms. *Trends Microbiol* 2005;**13**:34–40.
21. Kirketerp-Moller K, Jensen PO, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Hoiby N, Givskov M, Bjarnsholt T. Distribution, organization, and ecology of bacteria in chronic wounds. *J Clin Microbiol* 2008;**46**:2717–22.
22. Fonder MA, Lazarus GS, Cowan DA, Aronson-Cook B, Kohli AR, Mamelak AJ. Treating the chronic wound: a practical approach to the care of nonhealing wounds and wound care dressings. *J Am Acad Dermatol* 2008;**58**:185–206.
23. Deva AK, Siu C, Nettle WJ. Vacuum-assisted closure of a sacral pressure sore. *J Wound Care* 1997;**6**:311–2.
24. Moisisidis E, Heath T, Boorer C, Ho K, Deva AK. A prospective, blinded, randomized, controlled clinical trial of topical negative pressure use in skin grafting. *Plast Reconstr Surg* 2004;**114**:917–22.
25. Argenta LC, Morykwas MJ, Marks MW, DeFranzo AJ, Molnar JA, David LR. Vacuum-assisted closure: state of clinic art. *Plast Reconstr Surg* 2006;**117**(7 Suppl):127S–42S.
26. Whitehead SJ, Forest-Bendien VL, Richard JL, Halimi S, Ha Van G, Trueman P. Economic evaluation of Vacuum Assisted Closure® Therapy for the treatment of diabetic foot ulcers in France. *Int Wound J* 2011;**8**:22–32.
27. Payne JL, Ambrosio AM. Evaluation of an antimicrobial silver foam dressing for use with a V.A.C therapy: morphological, mechanical, and antimicrobial properties. *J Biomed Mater Res B Appl Biomater* 2009;**89**:217–22.
28. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. *Wound Repair Regen* 2012;**20**:647–57.
29. Percival SL, Thomas JG, Slone W, Linton S, Corum L, Okel T. The efficacy of silver dressings and antibiotics on MRSA and MSSA isolated from burn patients. *Wound Repair Regen* 2011;**19**:767–74.
30. Toy LW, Macera L. Evidence-based review of silver dressing use on chronic wounds. *J Am Acad Nurse Pract* 2011;**23**:183–92.