

More effective cell-based therapy through biofilm suppression

- **Objective:** To determine if targeted biofilm suppression of a specific wound improves the efficacy of cell-based therapy (CBT).
- **Method:** A retrospective study designed to compare a cohort of patients who received CBT in conjunction with biofilm-based wound management, published outcomes from randomised controlled trials in the literature, utilising standard of care and CBT. Biofilm-based wound management is the use of multiple simultaneous strategies to suppress wound biofilm, specifically targeting the individual wound's bioburden with agents and methods to suppress the wound biofilm and improve wound-bed preparation.
- **Results:** A Kaplan-Meier analysis shows that all wound aetiologies receiving biofilm-based wound care and CBT showed better healing than published outcomes at 3 months and 6 months. Overall wound closure for all wound types combined was 79% at 3 months (versus 56% for the literature) and 92% at 6 months, compared with 63–78% in the literature. All wounds that failed to heal showed some improvement with therapy.
- **Conclusion:** With adequate biofilm suppression, the entire graft material was observed to remain intact on the surface of the wound and wounds healed faster than what is reported in the literature. The relationship between improvement of wound biofilm suppression and less degradation of the graft suggests that the wound microbiota plays a significant role in the degradation of applied bioengineered skin.
- **Declaration of interest:** There were no external sources of funding for this study. The author has no financial or commercial conflicts of interest to declare.

cell-based therapy; biofilm; suppression

Cell-based therapies (CBT), such as Apligraf (Organogenesis) and Dermagraft (Shire Regenerative Medicine), have been shown to improve wound-healing outcomes.¹⁻³ While the exact mechanisms for enhanced wound healing have yet to be fully determined, they are thought to include promotion of cytokine signalling by the applied cells. The existing literature suggests that only the cytokines, not the cells, are active agents;⁴ however, given the complex signalling processes involved in wound healing,⁵⁻⁸ it seems reasonable that intact cells may contribute an element of coordination to the diverse molecules necessary for healing.

Early galvanotaxis of fibroblasts within a wound is thought to promote wound closure;⁹ therefore, the applied intact fibroblasts themselves may orchestrate at least some portion of healing within the wound. However, growth factor-induced fibroblast migration and the intracellular signalling mechanisms of fibroblast migration are poorly understood. A cytokine signal, including growth factors, which is not specific in terms of strength, time or location, has a greatly diminished effect on any biologic system.^{10,11}

An important principle of biologic systems is that signalling pathways are nonlinear. A linear signalling response occurs when an increasing stimulus produces a consistently increasing receptor result. However, biologic receptors protect the integrity of their

function by responding only to a narrow range of signalling molecules; too little or too much stimulus will produce no response. There is a very discreet, optimal range for the concentration of signalling molecules to produce the intended effect on their respective receptors.¹² Cells may have the ability to evaluate an environment and respond in a physiologically appropriate manner; however, CBT in a hostile inflammatory environment, such as a chronic wound, may be ineffective due to the destruction of cytokines that the cells release in a controlled fashion.

The environment of the chronic wound is highly proteolytic and oxidative, presenting significant challenges for any proactive therapy used to promote wound healing, whether those therapies are cell or cytokine-based. Understanding of the specific barriers within the chronic wound that limit the effectiveness of CBTs is just emerging.

One possible barrier is chronic wound biofilm. Biofilm has demonstrated a number of mechanisms associated with impaired host healing.¹³ In the wound bed, biofilms firmly adhere to the host extracellular matrix, yet do not destroy the host structures (otherwise the biofilm would lose its attachment).¹⁴ Rather, biofilms are suggested to obtain nutrition by inflaming host tissues and causing highly oxidative and proteolytic processes to produce plasma exudate, which nourishes the biofilm.¹⁵ Wound biofilm may play an important role in producing the chronic

R.D. Wolcott,¹ MD,
Medical Director;
S. Cox,² PhD, Director
and Bioinformatics;
1 Southwest Regional
Wound Care Centre,
Texas, USA;
2 Research and Testing
Laboratory, Texas, USA.
Email: randy@
randallwolcott.com

Table 1. Cohort baseline demographics

	Total	DFU	VLU	DU	NHSW	Tr/A
No. of patients	97	55	17	13	4	8
Age (years)	64 ± 17 (27–91)	60 (30–91)	68 (38–91)	65 (43–89)	60 (48–78)	69 (27–84)
Gender (M/F)	54/43	32/23	10/7	6/7	1/3	5/3
Diabetes (%)	68 (70%)	55 (100%)	6 (35%)	3 (13%)	2 (50%)	2 (25%)
Race						
• White	48	27	7	8	2	4
• Hispanic	31	20	5	3	1	2
• Black	14	8	2	1	1	2
• Other	4	0	3	1	0	0
Wound area (cm ²)	2.7 ± 3.2	2.75 ± 2.9	2.9 ± 3.5	2.3 ± 3.8	2.6 ± 1.3	1.8 ± 1.1
ABPI*						
• ABPI > 1.0	16	14	2	0	0	0
• ABPI 0.7–1.0	43	23	14	3	1	2
• ABPI < 0.7	19	17	1	0	0	1

* 19 patients did not have ABPI evaluation;
DFU=diabetic foot ulcer; VLU=venous leg ulcer; DU=decubitus ulcer; NHSW=non-healing surgical wound; Tr/A=trauma/abscess; ABPI=ankle brachial pressure index

inflammation present in the wound bed; therefore, reducing wound biofilm could limit the oxidative and proteolytic environment.¹⁵ Once biofilm is controlled, the host resources available, nurtured by appropriate physiologic stimulation with CBT, could improve host healing.

The objective of this non-comparative, retrospective study was to investigate whether CBTs were more effective if wound biofilm is adequately suppressed and the characteristic hyperinflammatory milieu is normalised, compared with the published literature.

Methods

This retrospective, non-comparative study was conducted in an outpatient wound-care centre serving a million person region in West Texas. Medical notes from patients who initiated CBT during a 3-month period, from 1 May 2009 through 31 July 2009, were retrospectively analysed and evaluated regarding the use and efficacy of CBT.

Patients were considered eligible for inclusion in the study if they received between one and five applications of CBT during the length of the study, which was admission which ended November 30th for all patients. There was no exclusion criteria based on age, comorbidities, vascular status of other host factors. Also the patients were not excluded for factors such as previous treatment, immunosuppression, or duration

of wound, in an effort to keep the patients included in the study the same as patients encountered on a day-to-day basis in any wound care centre. There was only one outcome evaluated, complete healing, which was defined as 100% re-epithelialisation.

The study received ethical approval from the Western Institutional Review Board (#20062347).

Treatment protocol

All patients received the same generalised treatment protocol, regardless of the aetiology of the wound. This consisted of management of comorbidities, addressing perpetuating factors (repetitive pressure/trauma, oedema, poor perfusion) and suppressing wound biofilm.

In order to prepare the wound bed for CBT, it was debrided weekly, in conjunction with precise diagnosis of the wound bioburden. Suppression of wound biofilm was dependent on rapid diagnosis of the microbial constituents of the wound bioburden, utilising different molecular methods, such as PCR and sequencing technologies.

• **Diagnosis of wound bioburden** Sharp debridement was used to remove a tissue sample from the surface of the wound bed, as evidenced by a small amount of bleeding. PCR evaluation was then conducted in a CAP-certified clinical laboratory, utilising proprietary primers¹⁶ to identify universal 16S (quantitating all bacteria), as well as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Candida albicans*, universal mecA cassette and van A. No efforts were made to determine if the detected microorganism were viable.

Sequencing was carried out using a validated primer set, 28F–519R,¹⁷ as well as proprietary primers on the Roche 454, and was performed at a CAP-certified molecular laboratory. Valid sequences meeting criteria for length (350bp) were compared with the National Center for Biotechnology Information (NCBI) database for identification. The process of denoising was used to correct errors in reads from next-generation sequencing technologies.^{18–20} Once this process was completed, all reads that failed to have a similar or exact match elsewhere in the region were removed, to correct base-pair errors generated during sequencing.

Formation of chimeric sequences occurs when an aborted sequence extension is misidentified as a primer and is extended upon incorrectly in subsequent PCR cycles. The laboratory's data analysis performs chimera detection and removal by executing UCHIME in de novo mode on the clustered data that was output by the denoising methods.^{21,22} By using this method, chimeras can be determined across entire region of data, even after accounting for noise and removing low quality sequences.

• **Suppression of wound biofilm** A personalised gel was created for each patient, following diagnosis

of the wound bioburden, this consisted of a nano-lipid gel (Sanguitec) with appropriate antibiofilm agents, such as hammamelatannin (*Staphylococcus* quorum-sensing inhibitor), lactoferrin (attachment blocker) and xylitol (false metabolite), added, along with antibiotics, indicated to target the specific microorganisms identified in the wound biofilm.

If the wound showed steady progression towards closure, no reevaluation was considered necessary; however, if the wound stalled or showed any deterioration, such as increased exudate, pain or erythema, a sample was taken for reevaluation of the wound biofilm. If the microbial constituents of the wound biofilm changed significantly, then reformulation of the personalised gel was carried out.

• **Cell-based therapy** Once the wound bed was adequately prepared as evidenced by no exudate, no devitalised tissue and 100% granulation tissue and clinical and/or laboratory analysis indicated that wound biofilm was adequately suppressed, CBT was begun. Apligraf was applied according to standard methods previously reported.¹⁻³ CBT was conducted on a biweekly basis, until full-healing was achieved 100% re-epithelialisation.

Results

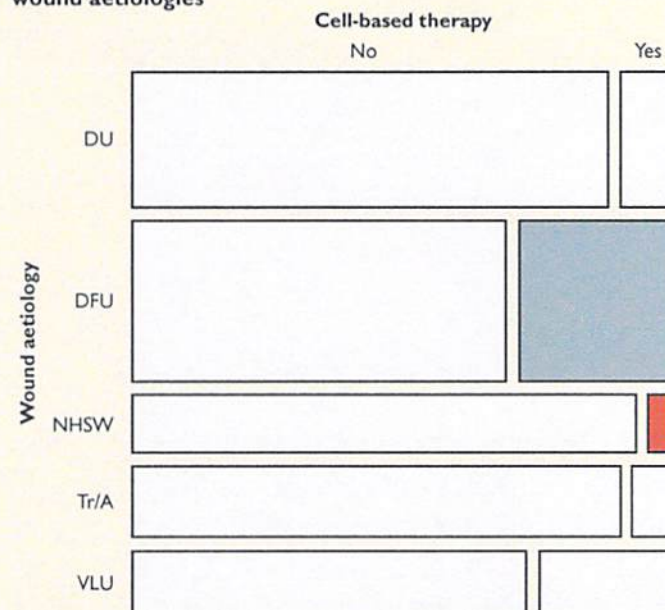
In total, 97 patients were evaluated in the study with a mean age of 64 years old (range 27–91 years). The patients presented with decubitus ulcer (DBU; n=13), diabetic foot ulcer (DFU; n=55), non-healing surgical wound (NSW; n=8), traumatic/abscess (TA; n=3) and venous leg ulcer (VLU; n=17; Fig 1 and Table 1). The median healing time varied depending on wound aetiology (Fig 2).

Comparison with published outcomes

Given the non-comparative, retrospective nature of this study, direct comparison of healing times for biofilm suppression combined with CBT-treated and non CBT-treated patients, is not possible. However, CBTs, such as Apligraf and Dermagraft, have been previously shown to improve wound-healing outcomes;^{1-3,23} therefore, it is possible to compare the results from this retrospective analysis with healing outcomes for similar cohorts published in the literature (Fig 3), suggesting that CBT is more effective when combined with biofilm suppression.

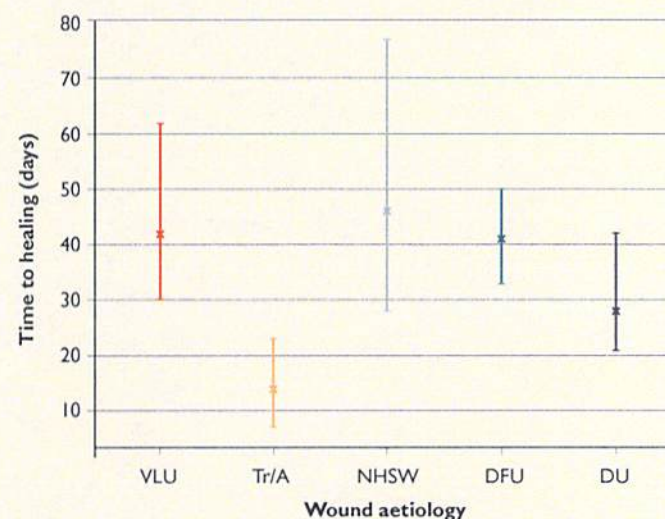
Comparator studies were matched for reported patient characteristics, including age, ABPI and wound size, and all were based on best local practice (Table 2),^{1-3,23} without specifically targeting biofilm suppression. For example, in the Falanga study,² the mean age was 60.2 years (28–84 years), very similar to our mean age of 64 years (27–91 years). In the Veves study,³ the mean age was 58±10 years, with comparable ankle brachial pressure indices (ABPIs) and average wound size of 3cm², slightly larger than our average wound size of 2.7cm². For the Brem

Fig 1. Mosaic plot of the use of cell-based therapy in different wound aetiologies



Each tile in the plot is proportional to the number of observations within a category, and shading denotes Pearson residuals. The proportion of individuals receiving CBT differed among wound types ($p < 0.001$), with DFUs receiving more, and non-healing surgical wounds (NSWs) receiving less CBT than expected, assuming an equal likelihood of use.

Fig 2. Median time to healing (with 95% confidence interval) for all patients in the study

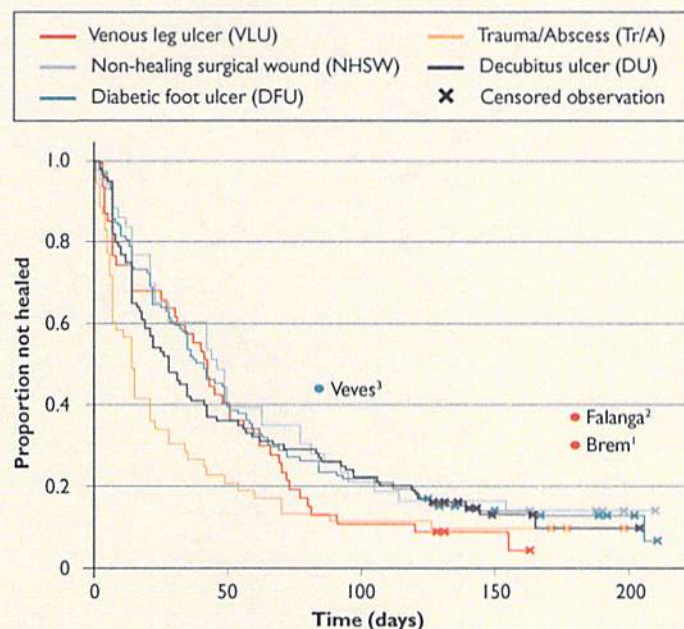


study,¹ the ages reported were similar to ours (centre A 67 years and centre B 65 years), but the initial wound size was much larger with means of 12cm² and 9cm² for centres A and B, respectively.

Table 2. Comparison of study groups

	Current	Brem ¹		Falanga ²	Veves ³
		Centre A	Centre B		
No. of patients	97	16	17	146	112
Age (years)	64±17 (27–91)	67±13 —	65±21 —	60.2±14.7 (28–84)	58±10 —
Wound size (cm ²)	2.7±3.2	39±68	24±42	1.33±2.69	2.97±3.10
• Median	—	12	9	—	—
Gender (M/F)	54/43	—	—	53.4/46.6	88/24
Diabetes (n)	68 (70%)	—	—	—	112 (100%)
Healing (%)					
• 3 months	77(79%)	—	—	—	56%
• 6 months	89(92%)	78%	72%	63%	—

Fig 3. The proportion of wounds not healed, as predicted from Kaplan-Meier survival analysis, plotted as a function of time



For reference, the proportion of wounds not healed in comparable studies are designated as closed circles (Brem,¹ Falanga² and Veves³)

Importantly, each study showed statistically significant improvement of wounds with the use of CBT.

At each time point reported, the biofilm-based wound care cohort showed a higher percentage of completely healed wounds (Fig 3). At 12 weeks, complete wound healing in DFUs was significantly higher in patients using biofilm-based wound care

(77%; 95%CI 67.5; 83.0) than it was in patients under standard care plus CBT (56%).³ Similarly for VLUs, biofilm-based wound care demonstrated a significantly higher rate of complete healing (>96%; 95%CI 77.3; 99.2) by 180 days, than the two other studies (70%¹ and 63%,² respectively). The improved wound healing demonstrated with biofilm suppression suggests that the wound microbiota may impair the full efficiency of the applied cells.

It is important to note that decubitus ulcers demonstrate a similar healing pattern as the patterns for DFUs and VLUs. This outcome suggests that, regardless of the aetiology, all wounds follow the same molecular, biochemical and cellular pathways to healing. Also, all wounds seem to suffer equally from the presence of biofilm, and it seems CBT faces the same competition, which is the presence of biofilm in each of these wounds. This suggests that the wound microbiota of decubitus ulcers could be an important barrier that needs to be addressed to allow CBT to be more effective.

The included case histories document the clinical change over time in wounds that were treated with CBT. Without biofilm suppression what we observed clinically was the collagen substrate, along with the cells, was degraded forming a semi-liquid mass, which incorporates host plasma proteins, making it look much like slough (Fig 4). When the inflammatory status of the wound was controlled, the CBT material acts differently. As seen in Fig 4, the applied cells appear more intact, and adhered on the surface of the wound. By suppressing wound biofilm, there anecdotally appears to be less degradation of the scaffolding and possibly less damage to the cells.

Discussion

There is a growing body of literature concerning biofilm and its possible role in chronic wound infections.^{16,24} Over the last several years, exciting revelations have emerged as to the cellular and biochemical mechanisms by which biofilm may impair healing. Microbes in biofilm phenotype have been demonstrated to impair host cellular functions, including host cytoskeleton,^{25,26} adhesion junctions between host cells, host-cell mitosis,²⁷ host-cell migration²⁸ and, most importantly, host-cell apoptosis.^{29–32} Microbes growing in biofilm phenotype have also been demonstrated to interfere with immune function of macrophages and neutrophils.³³

Chronic wounds are characterised by elevated proinflammatory cytokines,³⁴ excessive matrix metalloproteases,³⁵ and excessive neutrophils.³⁶ This hyper-inflammatory milieu, while providing plasma exudate to feed the biofilm community, can also produce a hostile environment that impairs host healing. This highly proteolytic and oxidative milieu also poses a problem for applied biologics. Naked peptides, such as growth factors and other cytokines, are strongly



Fig 4. With adequate biofilm suppression, the entire graft material often remains intact on the surface of the wound, as seen in these photographs (a–c), suggesting the wound microbiota plays a significant role in the degradation of applied bioengineered skin

challenged by a proteolytic environment;³⁷ however, it can also be assumed that, although an intact keratinocyte and/or fibroblast have some protection against the oxidative stress and proteolysis, such an environment could limit the efficacy of these cell types.

The first limitation encountered by these applied cells in a chronic wound would be just for their very survival given the presence of wound biofilm. The function of these cells would also be diminished, due to degradation of their receptors to sense the environment and then finally the degradation of any active cytokines that the cells secreted.³⁸ Biological systems protect their integrity by responding to only a limited range of a particular signal; it would be difficult for these cells to achieve this very specific range of signalling in a chronic-wound environment, unless biofilm and all its downstream effects were adequately suppressed. That is in a highly proteolytic environment signalling molecules from the cells can be rapidly degraded.

The results of this retrospective study suggest better efficacy of CBT when in conjunction with biofilm suppression (Fig 3). Previously, management of wound bioburden has been advocated in the form of wound bed preparation;^{39,40} however, whether the wound appears clinically infected or not, the wound microbiota is an important component that wound bed preparation addresses. By combining diagnostic tools with personalised gels, the wound biofilm could be better addressed in the wound-bed preparation paradigm. It is important to note, however, that molecular methods identify all microorganisms, regardless of phenotype. The strong probability is that both planktonic and biofilm phenotypes exist simultaneously in any chronic wound. Therefore, we treat for the more difficult biofilm phenotype, knowing this will also manage the planktonic cells present.

Why the CBTs were more effective is a more difficult question. It seems reasonable that the cells may be acting as an intact unit and that the resolution of the hyperinflammatory environment improves this function. However, it will be very difficult to assess whether the applied cells are working at a cellular level or only at a molecular (cytokine) level, as currently thought. It will require further research, in the form of molecular testing (transcriptome methods), to assess the messenger RNA changes within the tagged applied cells.

It is also interesting to note that all wound aetiologies in this study followed the same general healing curves as DFUs and VLU. The literature documents the efficacy of CBTs in DFUs,^{1,3,23,41} and VLUs,² but this does not preclude other wound types from also benefiting from CBTs. The healing patterns seen in Fig 3, especially for decubitus ulcers, which had a significant number of patients (n=13), suggest CBTs could show good clinical efficacy for other chronic wound type. As new advanced therapies emerge, it will be important to assess the potentials of these therapies within the framework of wound biofilm. Microbes, bacteria and/or fungus, existing as a community on the surface of the host wound, can negatively affect any advanced therapy. Wound biofilm produces host-cell senescence in the wound bed and in the periwound area for fibroblasts, keratinocytes, and immune cells.¹³ Just as importantly, wound biofilm produces the hyperinflammatory milieu that is the hallmark for chronic wounds. Only when these barriers are addressed will advanced technologies achieve their full potential.

Limitations

The main limitation of this study is its retrospective design. There is some variability between the three providers at our wound care centre providing the standard-of-care measures. Of course there is far more variability between our standard of care in 2009, and the techniques and materials present for the providers of the comparator studies, but not as much as could be expected. For example compression for VLUs was by a four-layer bandaging in all studies, although, offloading DFUs may have been less stringent in our patients. However, it should be noted that comparisons to the literature add more confounding variables that cannot be controlled and, therefore, should be treated with caution.

Conclusion

With adequate biofilm suppression, the entire graft material was observed to remain intact on the surface of the wound and wounds healed faster than what is reported in the literature. The relationship between improvement of wound biofilm suppression and less degradation of the graft suggests that the wound microbiota plays a significant role in the degradation of applied bioengineered skin. ■

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