Peroxy Pyruvic Acid-Containing Topical Anti-Infective: A Potential Candidate for a Wound Instillation Solution

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Objective: Evaluate the therapeutic properties of a peroxy pyruvic acid (PPA)-containing topical anti-infective in a human *ex-vivo* model that replicates the natural conditions of a human chronic wound.

Approach: Wound material was extracted from patients with nonhealing diabetic ulcers, venous stasis ulcers, and arterial wounds. Microbial species were identified, and wound colonization was quantified. Extracted samples were then exposed to a PPA-containing topical anti-infective as an instillation solution with negative pressure wound therapy NPWT at concentrations of 1,000, 1,500, or 2,500 ppm for a period of 1, 5, or 10 min to determine the effect of exposure on isolated pathogens, including effect on proteins.

Results: A total of 32 samples were collected from patients. Samples presented with a range of bacteria and fungi representing 14 genera and 22 species, many of which are or are evolving to be resistant to many, if not most, current systemic antibiotics. Thirteen of twenty-three samples (57%) from chronic wounds had bacteria counts $\geq 10^5$ and most were 6 logs or more. Seven of 10 samples (70%) from acute wounds had bacteria counts $\leq 10^5$ and most were much lower. Exposure to PPA-containing topical anti-infective at 1,000 ppm killed all bacteria and fungi in all samples within 1 min of exposure.

Innovation: PPA-containing topical anti-infective is a potentially valuable clinical option for NPWT.

Conclusion: PPA-containing topical anti-infective is a potential candidate for use as an NPWT instillation solution for the treatment of wound infections caused by susceptible pathogens.

Keywords: peroxy-pyruvic acid, PPA, wound instillation, topical anti-infective, chronic wound, microbial biofilm

INTRODUCTION

CHRONIC NONHEALING WOUNDS affect ~3–6 million people in the United States yearly, with an estimated annual cost of \$5–\$10 billion.^{1,2} Impacted most are elderly adults ≥ 65 years old, who comprise 85% of the chronic wound population.² All wound types have the potential to become chronic.¹

Microbial biofilms are known to be present in chronic wounds, and the role of biofilms as a causative agent of wound chronicity is being debated.³ Certainly it is known that microbial biofilms delay wound healing by prolonging the inflammatory phase. In addition, the bacteria residing in biofilms are significantly more resis-

tant to antibiotic treatment, which may explain why antibiotics provide limited value in treating chronic wounds.³

CLINICAL PROBLEM ADDRESSED

There are large numbers of therapies approved for treating acute and chronic wounds. These include debridement, off-loading, negative pressure wound therapy (NPWT), various dressings, topical therapies, hyperbaric oxygen therapy, growth factors, bioengineered skin substitutes, electrophysical therapy, and alternative therapies. With the exception of off-loading and NPWT, evidence of success for many of these therapies is lacking.⁴ The use of topical antibiotics is not recommended for treatment of infected wounds; in fact, topical antibiotics can provoke delayed hypersensitivity reaction, contact allergy, and may encourage colonization of resistant organisms.⁵ Therefore, in recent years, NPWT has been used as an aspirator of dead tissue and biofilm and to increase blood flow to nonhealing wounds.

Topical antibiotic treatment is not considered effective in most cases; therefore, instillation solutions have been included with NPWT treatment to enhance wound healing effectiveness. These solutions have included iodine or normal saline, biguanides (polyhexanide), hypochlorite-based solutions, sulfur-based compounds (sulfonamides), cationic solutions (benzalkonium chloride), acetic acid, octenidine dihydrochloride, povidone-iodine, and silver compounds (*e.g.*, silver nitrate).^{4,6-9} These solutions have been perceived as agents that can facilitate the use of NPWT. Saline alone has demonstrated to be an effective instillation solution. However, microbial bioburden is not necessarily eliminated with NPWT,¹⁰ and therefore, the inclusion of a microcidal instillation solution is an advantage.

Although some of the antiseptic instillation solutions mentioned above have been shown to kill bacteria, others have low microcidal effectiveness such as polyhexamethylene biguanide (PHMB) and saline.⁹ Two solutions that appear to be the most effective in chronic infected wounds are polyhexamide (0.1%) and acetic acid.⁸ Most NPWT solutions will not be effective in killing colonized bacteria present in biofilms unless they have an acid-oxidizing capability.¹¹ In addition, most instillation solutions have limited or no ability to expediently break down wound exudate, which typically also requires an oxidation process. Finally, in some instances, repeated use of these instillation reagents can be toxic to tissues and perhaps complicate the healing response.¹²

The following *ex-vivo* clinical trial in a human model designed to replicate the natural conditions of a human chronic wound was conducted to evaluate the potential therapeutic properties of a relatively new peroxy pyruvic acid (PPA)-containing topical anti-infective for use as an NPWT (VERIOX™; CHD Bioscience, Inc., Fort Collins, CO).

MATERIALS AND METHODS

Approval for the study was granted by the Investigational Review Board at the University of Colorado North Medical Center of the Rockies. Patients at Poudre Valley Hospital Wound Healing Clinic, Fort Collins, CO, and the Medical Center of the Rockies, Loveland, CO, participated in the study. Patients with chronic wounds and acute wounds who had received NPWT were identified and their informed consent to participate in the study was obtained before initiation of any study procedures.

Outcomes measurement

Study outcomes included identification of microbial genera and species extracted from the wounds, quantification of wound colonization based on microbial counts and extracted protein weight, and effect of exposure to a PPA-containing topical anti-infective on isolated pathogens, including effect on proteins.

Patient selection criteria

Patients were selected based on the criteria of having a chronic wound in one of three wound classes: nonhealing diabetic ulcers, nonhealing venous stasis ulcers, or nonhealing wounds from arterial insufficiency. The goal was to enroll more than 10 patients in each of these wound classes, with a target minimum of 32 patients/32 wound samples.

Wound sample procurement and handling

Vacuum Assisted Closure® (V.A.C.®) Granu-Foam™ (Kinetic Concepts, Inc. San Antonio, TX) foam dressings were used to obtain patient wound samples for the study. These dressings were selected because the dressing extract mimics the environment of human wounds. Extracted microbes, eschar, and slough are absorbed onto the foam dressing by NPWT, thereby providing an optimal *ex-vivo* testing environment.

Foam dressings were obtained from patients who presented to the outpatient wound care clinic or who were in the inpatient setting. The dressings cannot be stored in a manner that preserves the sample in its organic/live state, so samples were collected and delivered for testing on the same day

they were removed from the wound. Each foam dressing was placed in its own barcode-labeled hazardous material bag and transported in a cooler kept at 4°C to Microbial Research Incorporated (MRI) for testing. Deidentified patient data regarding wound etiology, date of first appearance, antibiotic usage, debridement techniques used, and wound measurement were obtained at the time of specimen collection, linked to the barcode on the patient sample bag, and listed on a corresponding case report form that was submitted separate from the sample. Upon delivery of the samples to MRI, photos were taken before and after each treatment and labeled with an identification number and the date and time of processing.

Wound sample processing and analysis

Each wound sample was combined with 100 mL tryptic soy broth (TSB) media, placed in a Stomacher® bag, and processed into slurry through a Stomacher® instrument to extract the wound matter from the foam dressing. A 10 mL volume of the extract was placed into a preweighed centrifuge tube. To precipitate out the proteins, 0.2 mL of concentrated nitric (or phosphoric) acid was added. The tubes were centrifuged at $\geq 3,000$ g for 15 min, the supernatant poured off, and the mass of the tissue extract measured to the nearest 0.0001 g.

The remaining extraction aliquots were removed from the TSB and dispensed into sterile test tubes. Wound samples were labeled numerically by patient for testing (e.g., 1, 2, 3, and so on). Labels identified the PPA-containing topical anti-infective treatment group according to concentration (A=control [H_2O], B=PPA-containing topical anti-infective 1,000 ppm, C=PPA-containing topical anti-infective 1,500 ppm, and D=PPA-containing topical anti-infective 2,500 ppm) and incubation period (1, 5, 10 min) to which the samples were assigned. One milliliter of the appropriate PPA-containing topical anti-infective concentration, or water for the control, was added to 9 mL of the homogenized extract solution in the appropriate labeled test tubes.

At the end of the designated incubation periods, activity of the PPA-containing topical anti-infective was neutralized by the addition of TSB and the test tube contents were streaked onto 5% sheep blood agar plates for subsequent microbial counts and species identification. In addition, time of flight mass spectrometry (TOFMS) of each treated sample was performed and compared with the control sample to assess the degradation effects of PPA-containing topical anti-infective on the foam dressing extract proteins. TOFMS analysis was conducted by adding 1 mL of wound extract and

10 mL of PPA-containing topical anti-infective at a concentration of 1,500 ppm to each dressing sample, followed by neutralization at the end of the designated incubation time. The TOFMS analysis was performed on all dressing extracts before and after exposure to PPA-containing topical anti-infective at all incubation times.

RESULTS

A total of 32 foam dressings were collected from 19 male and 13 female patients who ranged in age from 33 to >89 years old; 23 patients (72%) were >50 years old. Thirty-one percent of dressings collected were from acute wounds and were collected within 30 days of surgery. Most (69%) were collected from later-stage chronic wounds, all of which were at least 2 months old. One patient had been undergoing wound treatment for more than 2 years. It is unclear whether they had received other treatment options besides NPWT, which is most likely the case.

Table 1 shows the percentages of microbial species found in the wound samples that tested positive for bacteria. The most common bacterial species found were as follows: *Staphylococcus epidermidis* (55.2%), *Staphylococcus aureus* (31.0%), *Pseudomonas aeruginosa* (20.7%), *Corynebacterium striatum* (17.2%), *Enterococcus faecalis* (13.8%),

Table 1. Bacterial species found in wounds and percentage of bacteria extracted

Bacterial Species	Wound Cases ^a	Wound Percentage ^b (%)	Wound Type	
			Acute (%)	Chronic (%)
<i>Bacillus cereus</i>	1	3.4	100.0	0.0
<i>Corynebacterium striatum</i>	5	17.2	0.0	100.0
<i>Corynebacterium amycolatum</i>	1	3.4	100.0	0.0
<i>Corynebacterium jeikeium</i>	1	3.4	0.0	100.0
<i>Corynebacterium species</i>	1	3.4	0.0	100.0
<i>Dermabacter hominis</i>	1	3.4	0.0	100.0
<i>Enterococcus faecalis</i>	4	13.8	0.0	100.0
<i>Enterococcus faecium</i>	3	10.3	0.0	100.0
<i>Gemella morbillorum</i>	2	6.9	0.0	100.0
<i>Lactobacillus gasseri</i>	1	3.4	0.0	100.0
<i>Staphylococcus epidermidis</i>	16	55.2	31.3	68.8
<i>Staphylococcus aureus</i>	9	31.0	20.0	70.0
<i>Staphylococcus simulans</i>	4	13.8	25.0	75.0
<i>Streptococcus gordonii</i>	1	3.4	0.0	100.0
<i>Streptococcus peroris</i>	1	3.4	0.0	100.0
<i>Streptococcus salivarius</i>	1	3.4	0.0	100.0
<i>Citrobacter freundii</i>	2	6.9	0.0	100.0
<i>Enterobacter cloacae</i>	2	6.9	50.0	50.0
<i>Klebsiella pneumonia</i>	3	10.3	33.3	66.7
<i>Morganella morganii</i>	1	3.4	0.0	100.0
<i>Proteus mirabilis</i>	2	6.9	50.0	50.0
<i>Pseudomonas aeruginosa</i>	6	20.7	50.0	50.0
<i>Serratia marcescens</i>	1	3.4	0.0	100.0
Total bacteria	29	90.6	65.5	31.0

^aNumber of wounds testing positive for the bacterial species.

^bPercentage of bacteria testing positive for the bacterial species.

Enterococcus faecium (10.3%), and *Klebsiella pneumoniae* (10.3%). Chronic wounds were twice as likely to harbor bacterial species compared to acute wounds (65.5% vs. 31.0% of wounds studied, respectively). In the bacteria-positive wounds, *Corynebacterium striatum*, *E. faecalis*, and *E. faecium* were only found in chronic wound samples.

The dysfunctional healing of the wounds in this study can be characterized by inhibition of the inflammatory response, dysfunctional macrophages leading to an inability to combat infection, impaired angiogenesis and vasculogenesis, accumulation of fibrous tissue, and aberrant extracellular matrix accumulation.¹³ There is no consensus definition of a chronic wound, but most consider a wound to be chronic if it has not healed in 4–6 weeks.¹⁴

The recorded number of NPWT treatments patients received before dressing collection for this study ranged from 1 (one patient) to 14 (one patient). The average number of NPWT treatments received by the patients prior to dressing collection for this study was five (24 patients). In seven patients, no data were available to ascertain the number of previous treatments, including one patient for whom the number of prior treatments was not known because of unavailable treatment history.

Microbial extractions from the foam dressing samples resulted in identification of 14 genera and 22 species of bacteria (Fig. 1). The bacterial genera

isolated from the dressings included *Staphylococcus*, *Pseudomonas*, *Dermabacter*, *Enterococcus*, *Citrobacter*, *Corynebacterium*, *Gemella*, *Streptococcus*, *Proteus*, *Klebsiella*, *Enterobacter*, *Bacillus*, *Lactobacillus*, and *Candida*. Only two wounds (6%) were not infected; 30 wounds (94%) were infected, and 22 (73%) of the infected wounds had polymicrobial colonization.

The highest microbial count extracted was 6.7 logs (Table 2). This was collected from patient 7 following the eighth NPWT treatment of a 6-month-old wound. Seven of ten dressings (70%) collected from acute wounds had microbial counts $\leq 10^5$, and most microbial counts were much lower. In comparison, 13 of 23 dressings (57%) collected from chronic wounds had microbial counts $\geq 10^5$, and most of these were 6 logs or higher. Thus, the microbial presence in acute wounds was found to be lower than that in chronic wounds.

No microbial colonies were detected in any of the 10, 20, and 30 mg samples following treatment with PPA-containing topical anti-infective. As shown in Table 2, colony forming unit (CFU)/mL is reported as a minimum of $2.00E + 02$; therefore, the theoretical value of no detected colonies is a CFU/mL of 0 to $\leq 2.00E + 02$. Furthermore, all bacterial and fungal extracts from the NPWT foam dressings were killed within 1 min of exposure to 10 mg (1,000 ppm) of PPA-containing topical anti-infective (Table 3). This included 16 species of gram-positive bacteria, five species of gram-negative bacteria, and one species of fungus. The average weight of protein extracted from the dressings was 147.8 mg (range: 77.9–224.8 mg).

Through paired *t*-tests of CFU/mL, mean differences between control groups and 10 mg PPA-containing topical anti-infective test groups were performed at corresponding 1-, 5-, and 10-min intervals (Table 4). Differences between control and PPA-containing topical anti-infective treatment groups were statistically significant; with alpha set at 0.05, *p*-values were 0.014, 0.012, and 0.009 for the 10 mg 1-min, 10 mg 5-min, and 10 mg 10-min groups, respectively. Comparable *t*-values were calculated for all PPA-containing topical anti-infective test groups, as there were no statistical differences between treatment groups. These results support that the kill rate of PPA-containing topical anti-infective against the tested bacteria was significant in comparison to the control (no-treatment) groups.

Examples of sources of foam dressing extracts analyzed by TOFMS are shown in Fig. 2. TOFMS analysis found that dressing extract proteins had been degraded after exposure to PPA-containing topical anti-infective at 1,500 ppm for an exposure

Figure 1. Identified microbes extracted from the 32 foam dressing samples. Foam dressings from negative pressure wound therapy treatment of 32 patients. The dressings were processed and analyzed for microbial populations on each dressing. Gram Positive: B-*Bacillus*, C-*Corynebacterium*, D-*Dermabacter*, E-*Enterococcus*, G-*Gemella*, L-*Lactobacillus*, S1-*Staphylococcus*, S2-*Streptococcus*; gram Negative: C-*Citrobacter*, E-*Enterobacter*, K-*Klebsiella*, P-*Proteus*, P-*Pseudomonas*; Fungus: C-*Candida*.

Table 2. Exposure of microbes extracted from negative pressure wound therapy dressings with 10 mg peroxy pyruvic acid-containing topical anti-infective versus control

Patient no.	Microbes	Control (CFU/mL)	10 mg PPA-Containing Topical Anti-Infective ^a (CFU/mL)
1	<i>Staphylococcus epidermidis</i> <i>Dermabacter hominis</i>	2.30E + 06	2.00E + 02
2	<i>Staphylococcus epidermidis</i> <i>Pseudomonas aeruginosa</i>	1.30E + 06	2.00E + 02
3	<i>Staphylococcus aureus</i> <i>Enterococcus faecium</i> <i>Pseudomonas aeruginosa</i> <i>Proteus mirabilis</i>	2.70E + 04	2.00E + 02
4	<i>Staphylococcus epidermidis</i> <i>Staphylococcus simulans</i> <i>Citrobacter freundii</i>	4.40E + 05	2.00E + 02
5 ^b	<i>Staphylococcus aureus</i>	2.00E + 02	2.00E + 02
6	<i>Staphylococcus epidermidis</i> <i>Staphylococcus simulans</i> <i>Enterococcus faecalis</i>	9.60E + 05	2.00E + 02
7	<i>Corynebacterium striatum</i> <i>Gemella morbillorum</i> <i>Streptococcus gordonii</i>	5.00E + 06	2.00E + 02
8	<i>Proteus mirabilis</i> <i>Staphylococcus simulans</i> <i>Corynebacterium amycolatum</i>	5.00E + 06	2.00E + 02
9	<i>Staphylococcus epidermidis</i> <i>Enterococcus faecalis</i> <i>Citrobacter freundii</i>	3.00E + 05	2.00E + 02
10	<i>Klebsiella pneumoniae</i> <i>Enterobacter cloacae</i>	5.00E + 06	2.00E + 02
11	<i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Corynebacterium striatum</i>	1.00E + 06	2.00E + 02
12	<i>Enterobacter cloacae</i> <i>Staphylococcus epidermidis</i> <i>Corynebacterium jeikeium</i>	5.00E + 06	2.00E + 02
13	<i>Staphylococcus epidermidis</i>	5.00E + 06	2.00E + 02
14	<i>Staphylococcus aureus</i>	5.00E + 06	2.00E + 02
15	<i>Corynebacterium striatum</i>	7.20E + 03	2.00E + 02
16 ^b	None	2.00E + 02	2.00E + 02
17	<i>Bacillus cereus</i>	1.50E + 04	2.00E + 02
18	<i>Enterococcus faecium</i> <i>Enterococcus faecalis</i> <i>Staphylococcus epidermidis</i>	9.20E + 04	2.00E + 02
19 ^b	None	2.00E + 02	2.00E + 02
20	<i>Pseudomonas aeruginosa</i> <i>Staphylococcus epidermidis</i>	5.50E + 06	2.00E + 02
21	<i>Enterococcus faecalis</i> <i>Staphylococcus aureus</i> <i>Streptococcus peroris</i> <i>Lactobacillus gasseri</i> <i>Klebsiella pneumoniae</i>	9.80E + 05	2.00E + 02
22	<i>Staphylococcus epidermidis</i>	5.40E + 03	2.00E + 02
23	<i>Staphylococcus epidermidis</i> <i>Streptococcus salivarius</i> <i>Candida albicans</i>	4.00E + 02	2.00E + 02
24	<i>Klebsiella pneumoniae</i> <i>Staphylococcus epidermidis</i> <i>Corynebacterium species</i>	3.80E + 06	2.00E + 02
25	<i>Staphylococcus epidermidis</i>	4.00E + 02	2.00E + 02
26	<i>Staphylococcus epidermidis</i> <i>Pseudomonas aeruginosa</i>	1.30E + 05	2.00E + 02

(continued)

Table 2. (continued)

Patient no.	Microbes	Control (CFU/mL)	10 mg PPA-Containing Topical Anti-Infective ^a (CFU/mL)
27	<i>Enterococcus faecium</i> <i>Staphylococcus aureus</i>	2.70E + 04	2.00E + 02
28	<i>Pseudomonas aeruginosa</i>	6.20E + 03	2.00E + 02
29	<i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i>	3.80E + 04	2.00E + 02
30	<i>Staphylococcus aureus</i> <i>Corynebacterium striatum</i>	3.00E + 07	2.00E + 02
31	<i>Staphylococcus aureus</i> <i>Pseudomonas aeruginosa</i>	2.00E + 06	2.00E + 02
32	<i>Serratia marcescens</i> <i>Morganella morganii</i> <i>Corynebacterium striatum</i> <i>Gemella morbillorum</i>	1.00E + 06	2.00E + 02

^aThe three PPA-containing topical anti-infective test groups (10, 20, and 30 mg) tested identical at 1-, 5-, and 10-min time points. No postexposure bacterial colonies detected. Theoretical range for no colonies found is indicated as 0 ≤ to ≤ 2.00E + 02 microbial CFU/mL.

^bOutliers removed for failure to test positive for bacteria.

CFU, colony forming unit; PPA, peroxy pyruvic acid.

period of 10 min (Fig. 3) versus 5 min or 1 min. However, visual inspection of the color difference between the nontreated and PPA-containing topical anti-infective-treated dressing extracts indicated that protein degradation occurred at all times and at all concentrations in all wound samples. The average weight of protein extracted from the dressings was 147.8 mg (range: 77.9–224.8 mg).

DISCUSSION

This *ex-vivo* study involved the analysis of NPWT wound dressing extracts from a wide array of acute and chronic human wound types for microbial identification and quantification and evaluation of treatment using a PPA-containing topical anti-infective to determine the compound's potential for killing the bacteria and perhaps debriding the dead tissue.

In a recent study of wound swabs from 51 human patients with chronic wounds, the primary bacteria isolated were *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, and *Enterococcus*, with 10 other genera also included.¹⁵ In another study of chronic leg ulcer microbial swabs, the primary bacteria obtained from 75 patients studied were *Pseudomonas*, *Proteus*, and *Escherichia coli*, with 18 other geneses identified.¹⁶ In a recent review article, Dana and Bauman stated that the most common organisms found in pressure ulcers are *S. aureus*, *Proteus mirabilis*, *P. aeruginosa*, and *E. faecalis*.¹⁷ With the exception of *E. coli*, all of the microbes isolated in the reviewed wound studies, excluding *E. coli*, were also found within the foam dressing extracts in this

Table 3. Colony forming unit/milliliter analysis of tested samples

	Control			10 mg PPA-Containing Topical Anti-Infective ^a		
	1 min	5 min	10 min	1 min	5 min	10 min
n = 29 ^b	29	29	29	29	29	29
Mean	2.76E + 06	2.64E + 06	2.69E + 06	2.00E + 02	2.00E + 02	2.00E + 02 ^c
SE	1.05E + 06	9.81E + 05	9.51E + 05	0.00	0.00	0.00
SD	5.64E + 06	5.28E + 06	5.12E + 06	0.00	0.00	0.00
Range	3.00E + 07	2.80E + 07	2.70E + 07	0.00E + 00	0.00E + 00	0.00E + 00
Minimum	4.00E + 02	2.00E + 02	6.00E + 02	2.00E + 02	2.00E + 02	2.00E + 02
Maximum	3.00E + 07	2.80E + 07	2.70E + 07	2.00E + 02	2.00E + 02	2.00E + 02

^aAll PPA-containing topical anti-infective test groups (10, 20, and 30 mg) tested negative for bacterial colonies.

^bNumber of samples adjusted for no bacteria detected on dressing.

^cAll plates with zero growth detected were assumed to have 0 to $\leq 2.00E + 02$ microbial CFU/mL. This conservative assumption was made due to neutralization dilution, that is, there was one colony remaining (0.05 mL plated from the 1:10 dilution required for neutralization: $1/0.05 \times 10 = 2 \times 10^2$).

SE, standard error; SD, standard deviation.

study. The most commonly identified microbes in these wounds were *Staphylococcus* (27), *Corynebacterium* (7), *Enterococcus* (7), *Pseudomonas* (5) *Streptococcus* (3), and *Klebsiella*. All others were identified either once or twice within the patient dressings. Many bacteria identified in this study have been found in critically ill patients with sores and/or wounds that are resistant to a multitude of antibiotics. For example, of 1,418 patients admitted to the ICU in a cancer care center, 266 microorganisms were isolated from their wounds. Of these microbes, 39.5% were found to be multidrug-resistant

hospital-acquired infections.¹⁸ Patients in this study had been treated with multiple systemic antibiotics.

Staphylococcus spp. was extracted from 84% (27) of all patient dressings in the current study. *Staphylococci* are gram-positive bacteria associated with many disease epidemiologies. *S. epidermis* is a common external skin bacterium that indwells skin penetrating wounds and was extracted from 15 dressings. It has been associated with enhancing biofilm formation and virulence,¹⁹ and with the pathogenicity of certain hospital strains.²⁰ *S. simulans* is found on skin, is not generally known as a

Table 4. Mean difference of colony forming unit/milliliter of control versus 10 mg peroxy pyruvic acid-containing topical anti-infective treatment groups

	Mean	95% Confidence Interval		t ^a	df ^b	p-Value (Two-Tailed) ^c
		Lower	Upper			
1 min control vs. 1 min 10 mg PPA-containing topical anti-infective	2.76E + 06	6.12E + 05	4.90E + 06	2.633	28	0.014
5 min control vs. 5 min 10 mg PPA-containing topical anti-infective	2.64E + 06	6.29E + 05	4.65E + 06	2.690	28	0.012
10 min control vs. 10 min 10 mg PPA-containing topical anti-infective	2.69E + 06	7.41E + 05	4.64E + 06	2.827	28	0.009

^aMatched t-test analysis.

^bn adjusted for outliers that tested negative for bacterial colonies or pelleting.

^c α of 0.05.

df, degrees of freedom.

Figure 2. Wound sources of foam dressing extracts analyzed by TOFMS. TOFMS, time of flight mass spectrometry.

Figure 3. TOFMS analysis of foam dressing extract protein degradation following exposure to 10 mL of PPA-containing topical anti-infective 1,500 ppm.

pathogen, and was extracted from three dressings. It appears to be susceptible to rifampicin and levofloxacin and most other antibiotics.^{21,22} *S. aureus* was extracted from nine different dressings and, therefore, was present in almost 30% of the collected dressings. A study performed in a French hospital found *S. aureus* strains in 205 of 477 patients (43%). These strains were resistant to nine different antibiotics, including penicillin (86%), erythromycin (32%), ciprofloxacin (9.3%), tetracycline (5.8%), oxacillin (5.8%), fusidic acid (4.4%), clindamycin (3.4%), mupirocin (1%), and gentamicin (0.5%).²³ All tested *S. aureus* strains were sensitive to vancomycin and rifampicin. In this study, two patients whose dressings contained only *S. aureus* had been treated with vancomycin and cefazolin. Over 6 logs were isolated from one of those dressings. The remaining seven patients, who had other bacteria in the extraction, had been treated with a host of antibiotics, including cephalexin, doxycycline hyclate, piperacillin–tazobactam, levofloxacin, and ertapenem. *S. aureus* is known to be a human pathogen, which frequently forms biofilms in clinical settings and is therefore difficult to treat with antibiotics. This confirms the use of multiple antibiotics when treating polymicrobial infections.

Corynebacterium spp. was extracted from 25% (8) of all the patient dressings in the current study. *Corynebacteria* are gram-positive rods that are often regarded as normal skin inhabitants. However, emerging evidence implicates all three *Corynebacterium* spp. isolated in this study as being present in community acquired nosocomial infections, prosthetic joint infections, and chronic leg ulcers.^{24,25} *C. jeikeium* has been associated with prosthetic devices and indwelling catheters.²⁶ *C. jeikeium* was isolated with *E. cloacae* and *S. epidermidis* and had been treated with cefazolin. *C. amycolatum* has been found in multiple sites, such as catheters and surgical wounds, and has been pathogenic in some cases.²⁷ *C. amycolatum* strains have demonstrated antibiotic resistance to ampicillin (38.9%), cefazolin (38.9%), gentamicin (44.4%), erythromycin (94.4%), clindamycin (94.4%), ciprofloxacin (94.4%), and rifampin (94.4%). None of the strains was resistant to vancomycin or tetracycline.²⁴ In the current study, *C. amycolatum* was found in patient 8 in the presence of two other bacterium extracted with a log₁₀ of 6.7 CFU/mL. This wound had previously been treated with vancomycin and five other antibiotics. *C. striatum* was the only microbe isolated from patient 15 dressing. The antibiotic treatment for this patient included vancomycin, piperacillin, mupirocin, metronidazole, and clindamycin, and 3.9 log₁₀ of the *C. striatum* was still extracted. This might be inter-

preted two ways: first that other pathogenic microbes had been present in the wound and were subsequently killed by the mixed antibiotic treatment, or this isolate was a multiple antibiotic-resistant strain of *C. striatum*. In recent clinical studies, *C. striatum* has been implicated as a potential multidrug-resistant microbe occurring after implantation of devices.^{28–30} However, this microbe was extracted from a chronic gangrenous wound. A follow-up study of microbial resistance of this microbe revealed strong resistance to five antibiotics and intermediate resistance to five other antibiotics,³¹ which therefore might explain why it was not eliminated by the multiantibiotic treatments.

Enterococcus spp. was extracted from 21% (7) of all patient dressings in the current study. These bacteria are gram-positive cocci that often occur in pairs (diplococci) or short chains. It has been stated that *Enterococcus* spp. are among the leading cause of nosocomial infections, with *E. faecalis* and *E. faecium* being the most antibiotic resistant.²³ In the current study, *E. faecalis* and *E. faecium* were found in five of the seven patient wound extractions. A study conducted at the Hermanos Ameijeiras Hospital in Cuba identified *E. faecalis* and *E. faecium* in 50 wound infections, with antimicrobial resistance to ampicillin (76.0%), high-level gentamicin (66.0%), ciprofloxacin (52.0%), linezolid (8.0%), teicoplanin (34.0%), vancomycin (50.0%), minocycline (62.0%), tetracycline (68.0%), and nitrofurantoin (18.0%).³² In the current study, *Enterococci* had been treated with vancomycin, azithromycin, penicillin, ertapenem, bacitracin zinc, and piperacillin–tazobactam.

Pseudomonas aeruginosa was extracted from 18% (6) of all patient dressings in the current study and is one of the main microorganisms involved in nosocomial infections. It is known for its resistance to a broad range of antimicrobial agents. In a 10-year study of inpatient and outpatient antibiotic resistance of *P. aeruginosa* conducted at a 600-bed (3,778-patient) hospital network in Portugal, 39.9% of the isolates were considered multidrug resistant and 2.9% were considered extremely drug resistant. Antibiotics given to the six patients in whom *P. aeruginosa* was found included ciprofloxacin, augmentin, vancomycin, cefazolin, bacitracin zinc, mupirocin, levofloxacin, and ertapenem. *P. aeruginosa* was the only bacterium extracted from the foam dressing in patient 28. The amount extracted was ≤4 logs, and the patient had not received any antibiotic therapy. The wound etiology of this patient was an acute surgical wound. The patient had received six NPWT treatments. Therefore, this treatment routine may have been used to deter infection.

Streptococcus spp. was extracted from 9% (3) of the patient dressings in the current study; *S. gordini*, *S. salivarius*, and *S. peroris*. *S. gordonii* is a bacterium involved in the initial colonization of tooth surfaces, contributes to dental biofilm formation, and is an important cause of infective endocarditis.³³ It was isolated with *Corynebacterium striatum* and *Gemella mobillorum*. *G. morbillorum* has been isolated in bacteremia, endocarditis, and patients with arthritis who are immunocompromised.³⁴ *S. salivarius* is a gram-positive microbe, which can cause bacteremia, endocarditis, sinusitis, and is rarely associated with meningitis.^{35,36} It has been shown to be susceptible to penicillin, amoxicillin, and cefotaxin.^{35,36} Because *S. salivarius* is not usually associated with wounds, it is possible that the infection occurred as a subset of endocarditis, bacteremia, or surface contamination. This patient had been treated with double-strength sulfamethoxazole and trimethoprim and maintained a 6.7 log₁₀ infection and had significant wound slough even after eight NPWT treatments.

Dermabacter hominis, *Citrobacter freundii*, *Bacillus cereus*, *Candida albicans*, *Serratia marcescens*, and *G. morbillorum* were each found in one dressing in the current study and account together for 15% (6) of all the patient dressings. Recently, *D. hominis* was extracted with *S. epidermis* and both have been implicated in the formation of biofilms.³⁷ This dressing extract contained 6.4 log₁₀ of bacteria and may have been protected by the presence of biofilm bacteria remaining in the wound. *C. freundii* was extracted from patient 4 in the current study along with *S. epidermis* and *S. simulans*. *Citrobacter* is a member of the Enterobacteriaceae family, which are common hospital-acquired pathogens known to cause urinary tract infections and dysentery, and they are well known for acquiring antibiotic resistance.³⁸ The patient in this study had been treated with amoxicillin, vancomycin, levofloxacin, doxycycline, and ceftriaxone, with all three bacteria surviving in the wound.

Bacillus cereus is a spore-forming saprophytic gram-positive bacterium associated with gastroenteritis.³⁹ *B. cereus* has also been associated with nosocomial outbreaks in immunocompromised patients.³⁹ *B. cereus* is generally resistant to penicillin, ampicillin, and cephalosporins.⁴⁰ In this study, the patient with *B. cereus* infection received prior antibiotic treatment with cefazolin.

Candida albicans was extracted along with *S. epidermis*, and *S. salivarius*. *C. albicans* has been observed to form biofilms that can contribute to microbial pathogenesis in the hospital setting

where there is significant use of medical devices with unsuspected contamination.⁴¹

Serratia marcescens is a species of rod-shaped gram-negative bacteria in the family Enterobacteriaceae. It was found in the foam dressing of a patient in the current study with *M. morganii*, *C. striatum*, and *G. morbillorum*. These four bacteria had a 6 log₁₀ presence remaining in the extract. *S. marcescens* is a facultative anaerobe and a human pathogen. *S. marcescens* has a high capacity for survival and consequently is involved in hospital-acquired nosocomial infections.⁴² There have been reports outside the United States that *S. marcescens* may have a wide spectrum of antibiotic resistance,⁴³ including to mupirocin, fusidic acid, and amoxicillin-clavulanate. *M. morganii* is mostly associated with urinary tract infections⁴⁴ and produces a chromosomally encoded AmpC β -lactamase that is responsible for resistance to aminopenicillins, amoxicillin-clavulanate, and first- and second-generation cephalosporins. In addition, *M. morganii* is resistant to tetracycline, polymyxins, and nitrofurantoin. In this study, the wound had been treated with cephalixin and ertapenem, and there remained a 6 log₁₀ presence in the dressing extract.

The predominant bacteria associated with skin and soft tissue infections in hospitalized patients include *S. aureus*, *E. coli*, *P. aeruginosa*, and *Enterococcus* spp.⁸ However, as this current study demonstrates, wounds that have become chronic are likely to be polymicrobial and require further broad-spectrum antibiotic treatment.^{6,9,45} Furthermore, increasing resistance among both gram-positive and gram-negative bacteria greatly complicates empirical treatment decision-making, and the number of currently available appropriate antimicrobial agents is limited.⁴⁶ Biofilm can form on the surface of a wound within 10 h and is difficult to eradicate due to its recalcitrant nature, including the relative lack of effectiveness of antibiotics due to penetration and the relatively quiescent bacterial colonies encased in a glycocalyx structure.⁴⁷

There is increasing need for effective antibiotics in the population of critically ill patients who are at a high risk of infection with multidrug-resistant pathogens. Based on the current medical literature and the range of antibiotics used to treat the patients from whom the foam dressings for this *ex-vivo* study were obtained, there is a clear indication that multiantibiotic resistance remains a common and considerable clinical challenge. The prospect that common bacteria will continue to become resistant to all antibiotics is an important concern in medicine today.⁴⁸ The large

number of bacteria counts that had remained in the wound samples despite prior NPWT indicates NPWT treatment with saline did not eradicate all the bacteria.

There are four major mechanisms that mediate bacterial resistance to antibiotics; enzymatic inactivation; bacteria synthesis of modified antibiotic targets, such as a mutant protein, which reduces the bacteria infection effect; reduced antibiotic permeability to the antibiotic and reduction of the intracellular antibiotic concentration to ineffectiveness; and active export of the antibiotic using resistance pumps, which pump the antibiotic back out of the bacteria.⁴⁹ There is a need for antimicrobials/antibiotics that do not use metabolic pathway inhibition to kill the bacteria because of the existence of continued genetic mutation to resistance.

The hypothesized mechanism of action of the PPA-containing topical anti-infective used in this study involves specific destruction of the bacteria cell wall and internal biochemistry. It includes a peracid, a very low concentration of hydrogen peroxide ($\geq 1.5\%$), and proprietary carboxylic acids. The hydrogen peroxide and peracid work synergistically to destroy the cell wall and then penetrate to the microbial internal structures, where carboxylic acids and low-level hydrogen peroxide are involved in signaling to the immune response during and after inflammation and angiogenesis.^{35,36,40–46,50–52} The resulting mode of action is not metabolic inhibition, but rather a nonspecific nuclear attack on the bacteria. Therefore, microbial evolution to resistance to this PPA-containing topical anti-infective is unexpected.

This *ex-vivo* study demonstrated that a PPA-containing topical anti-infective quickly kills bacteria that are significantly resistant to empiric therapies in nonhealing acute and chronic wounds. The PPA-containing topical anti-infective also degraded dressing extract proteins, which suggests it may also be effective in removing wound materials that could inhibit healing (slough). In addition, prior *in-vitro* studies indicate that the PPA-containing topical anti-infective might break down the existing biofilm and prevent the regrowth of biofilm at relatively low concentrations.^{35,36,40–46,50–52} These *ex-vivo* findings reinforce the results of *in-vitro* and *in-vivo* animal studies that demonstrate the ability of a PPA-containing topical anti-infective to remove exudate, possibly prevent wound biofilm, and, therefore, prophylactically enhance wound healing.

To investigate whether PPA-containing topical anti-infective is cytotoxic to healthy tissue, a nongood laboratory practice (non-GLP) *in-vivo* animal study was performed using a noninfected

wound swine model. Two noninfected wounds on four pigs were treated to negative pressure wound therapy with dwell time (NPWTi-d) using six 10-min instillations at a concentration of 400 ppm per day for 7 days. Afterward, two of the wounds were extracted and sent for histological analysis. Tissue response to the treatment procedure was evaluated by a board-certified veterinary pathologist blinded to the treatment therapy used. The pathologist summary indicated that PPA-containing topical anti-infective solution was not cytotoxic to the wounds and that wounds treated with PPA-containing topical anti-infective solution had granulation tissue formation and thickness indicative of healing (Fig. 4). Although this finding suggests that PPA-containing topical anti-infective at a much lower concentration may have had a positive effect on the wound, further study powered for statistical analysis is needed.

A limitation of the current study reported here is that no *ex-vivo* model can exactly replicate the natural conditions of the human chronic wound.

INNOVATION

Antimicrobial resistance remains a serious clinical challenge to the treatment of chronic wounds. Instillation solutions commonly used in NPWT have been shown to kill bacteria, but their low microbicidal properties and possible lack of efficiency in breaking down biofilms and removing exudate may allow resistant microbes to survive, proliferate, and complicate wound healing. This study demonstrates that the PPA-containing topical anti-infective, using a nonspecific nuclear mechanism of action, is effective at killing resistant pathogens and degrading wound proteins and suggests it may be a valuable tool for clinicians selecting an instillation solution for NPWT when treating chronic wounds.

Figure 4. Result of cytotoxic testing: PPA-containing topical anti-infective.

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AUTHOR DISCLOSURE AND GHOSTWRITING

E.D.N. discloses that he is an employee at the study sponsor. E.D.S. discloses that she is an employee at the study sponsor. A.M.C. discloses that she is an employee at the study sponsor. J.A.D., A.J., and G.J.L. have no conflict of interests to disclose. No competing financial interests exist.

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KEY FINDINGS

- Pathogens found in chronic wounds are resistant to empiric antibiotic therapies, and many are polymicrobial resistant.
- The kill rate of PPA-containing topical anti-infective as an instillation solution in NPWT was significantly superior to control at all dwell time periods against susceptible resistant gram-positive, gram-negative, and fungal pathogens.
- PPA-containing topical anti-infective may disrupt the biofilm in chronic wounds for subsequent microbial kill
- PPA-containing topical anti-infective degrades proteins that may inhibit wound healing

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Abbreviations and Acronyms

CFU	= colony forming unit
df	= degrees of freedom
MRI	= microbial research incorporated
NPWT	= negative pressure wound therapy
NPWTi-d	= negative pressure wound therapy with dwell time
PPA	= peroxy pyruvic acid
SD	= standard deviation
SE	= standard error
TOFMS	= time of flight mass spectrometry
TSB	= tryptic soy broth