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Antimicrobial effectiveness of wound matrices containing native extracellular matrix with polyhexamethylene biguanide

Stephen C. Davis[1] | **Joel Gil**[1] | **Michael Solis**[1] | **Alexander Higa**[1] | **Allyson Mills**[2] | **Colin Simms**[1] | **Pilar Valencia Pena**[1] | **Jie Li**[1] | **Vivek Raut**[2]

[1]Dr. Phillip Frost Department of Dermatology and Cutaneous Surgery, Miller School of Medicine, University of Miami, Miami, Florida

[2]Organogenesis Inc., Canton, Massachusetts

Correspondence
Stephen C. Davis, Dr. Phillip Frost Department of Dermatology and Cutaneous Surgery, Miller School of Medicine, University of Miami, Miami, Florida, USA.
Email: sdavis@med.miami.edu

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Organogenesis, Inc.

Abstract

A variety of wound matrix materials that are designed to help heal both acute and chronic wounds are currently available. Because wounds often encounter opportunistic microbes that can delay healing, the effectiveness of these materials is often suboptimal, resulting in delayed or compromised wound healing. The importance of reducing and controlling wound microbes is well recognised and there are several antimicrobial options available to address this unmet clinical need. This study compares the antimicrobial and wound healing capabilities, both in vivo and in vitro against methicillin-resistant *Staphylococcus aureus* (MRSA) USA 300, for the following compounds: Collagen Wound Matrix-Anti Microbial (CWM-AM); Collagen Wound Matrix-Anti Microbial XT (CWM-AM XT); Antimicrobial Hydrofiber Wound Dressing (AHWD); Dermal Scaffold with Silver (DRSAg); Collagen Extracellular Matrix (CEM); Collagen Wound Matrix (CWM); Matrix Wound Dressing with Silver (MWDAg); Cadexomer Iodine Gel (CIG); Triple Antibiotic Ointment (TAO); and Antimicrobial Wound Gel (AWG). For the in vitro zone of inhibition assay, AWG and CIG had the largest diffused areas, followed by CWM-AM and CWM-AM XT. Furthermore, CWM-AM, CWM-AM XT, AWG, and CIG exhibited a persistent antimicrobial activity for up to 10 days after incubation. However, in the cytotoxicity studies performed using human fibroblasts, CWM-AM and CWM-AM XT had no detrimental effects in cell proliferation and viability, while AWG and CIG were cytotoxic and prohibitive for cell proliferation. Treatments were then assessed for microbiology and wound healing efficacy using an in vivo porcine deep reticular dermal wound model. CWM-AM XT displayed the greatest in vivo antimicrobial activity against MRSA USA300 and expedited the reepithelialisation at a faster rate than other treatment groups. This study shows that a novel collagen matrix containing an antimicrobial agent can reduce the bacterial load and support healing.

KEYWORDS

acute wounds, antimicrobial, collagen, infection, pig

1 | INTRODUCTION

There is need for advanced products to address both acute and chronic wounds because of their impact on health and economic concerns.[1] One of the many modalities that could address these challenges are dressings that contain bioactive components required for dermal and epidermal reconstruction. Collagen has been clinically proven to be safe and effective wound matrix for wound healing applications.[2] There are numerous types of collagen products, ranging from Types I to V and XI collagen, which contain tertiary structures or more complex three-dimensional quaternary protein structures.[3-5] Current literature shows collagen dressings to be efficient and safe. A recent study conducted by Sevki et al[6] found a collagen matrix to induce diabetic ulcers to heal faster when compared against standard wound care modalities. While a panel of health care professionals in a separate study[7] found that collagen resulted in a lower pH level within the wound environment, resulting in bacteriostasis. The collagen-based matrix can provide a structural framework for new cells to migrate through newly developed tissue supporting tissue repair and replacement.[8] Furthermore, previous preliminary studies and clinical trials have demonstrated that the collagen-based dressings effectively encouraged wound healing.[9-11] The concept behind using collagen dressings is to provide biomaterial components that are required for various phases of healing.[12,13] During chronic wounding, many of these native ECM proteins and cellular components are broken down and degraded by the surrounding inflammatory process.[14] While such novel modalities can be beneficial, another challenge is the presence of pathogenic organisms in the wound bed,[15] many commonly found in both acute and chronic wounds.[16,17]

However, collagen-based wound matrices by themselves do not offer antimicrobial resistance. Therefore, currently-available collagen-derived products fail to provide the adequate native collagen structure and a protective layer against pathogenic agents, which significantly compromises the wound healing process without an external intervention for effective antimicrobial control. Some of the common bacterial (and fungal) wound infections are caused by both gram-positive and gram-negative organisms, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[18] Pathogenic microorganisms are able to proliferate and express biofilm growth, which makes wound infections much more difficult to treat, resulting in chronic wounds that are unable to continue through the wound healing phases.[19] It is crucial to prevent and/or treat infections in the early stages to avoid biofilm formation so that acute wounds are prevented from becoming chronic.

Key Messages

- Ideal wound matrices should enhance healing while inhibiting proliferation of pathogenic bacteria.
- CWM-AM and CWM-AM XT were not cytotoxic to fibroblasts
- CWM-AM and CWM-AM XT maintained antimicrobial activity for 10 days.
- CWM-AM and CWM-AM XT reduced the bacterial burden (3-4 logs CFU/g) while supporting the healing process

Polyhexamethylene biguanide (PHMB) has been used in many wound care products, and has been proven to be beneficial for wound healing and infection.[20] Current literature shows beneficial effects by PHMB against bacteria (both gram-positive, gram-negative)[6] and yeast, such as *Candida albicans*.[21] Using a collagen-based scaffold that can place PHMB as a barrier on an infected wound could have the potential to effectively reduce and prevent bioburden and biofilm formation, while simultaneously supporting the process of wound healing.

The following studies evaluate multiple collagen matrix-based dressings and other antimicrobial agents in controlled in vitro and in vivo settings. Several methods are commonly used in microbiology to determine the efficacy of antibiotics, such as the zone of inhibition method. In our in vitro studies, we measured the zones of inhibition (ZOI) using a modified Kirby-Bauer method. This test provides adequate data to determine the testing agent's potency by measuring the diffusion within the medium against the pathogen.[22,23] The treatments were challenged under two different concentration levels of the inoculum MRSA USA300 in the in vitro analysis to simulate wounds that may be slightly or highly colonised. Further, the persistence of the antimicrobial activity of

the testing agent was tested by incubating the test agent in sterile PBS at 37°C for up to 10 days before testing the effectiveness in the zone of inhibition assay.

We also performed in vitro cytotoxicity and cell proliferation analysis. Many cytotoxicity investigations have found that some antimicrobials can interfere with fibroblasts, keratinocytes, and other protein factors present during the wound healing process.[24-26] Despite the fact that collagen has been found to be biodegradable,[27,28] other chemical components present within the testing materials needed to be further investigated for any potential cytotoxicity that could inhibit the reepithelialisation and granulation processes.[29]

We then evaluated these treatments in a well-established porcine model.[29-32] Pigs were used because of their skin's similarity to humans.[33,34] The wounds were infected with MRSA USA300 bacterium and were allowed to form biofilm. The wounds were then debrided before the application of testing agent. Microbiology analysis was performed to quantify and compare the amount of MRSA colonies in each wound at various stages of wound healing. Histological analysis analysing several wound healing factors was performed to determine the effect of PHMB combined with a collagen-based scaffold when compared against other collagen-based treatment modalities.

2 | MATERIALS AND METHODS

2.1 | Test materials

The treatment modalities tested in this study were Collagen Wound Matrix-Anti Microbial, CWM-AM (PuraPly Antimicrobial, Organogenesis, Canton, Massachusetts); Collagen Wound Matrix-Anti Microbial XT, CWM-AM XT (PuraPly Antimicrobial XT, Organogenesis, Canton, Massachusetts); Antimicrobial Hydrofiber Wound Dressing, AHWD (Aquacel Ag, Convatec Inc., Bridgewater, New Jersey); Dermal Scaffold with Silver, DRSAg, (PriMatrix Ag, Integra LifeSciences, Plainsboro, New Jersey); Collagen Extracellular Matrix, CEM (Endoform, Hollister Inc., Libertyville, Illinois); Collagen Wound Matrix, CWM (Promogran, Systagenix, San Antonio, Texas); Matrix Wound Dressing with Silver, MWDAg (Promogran Prisma, Systagenix, San Antonio, Texas); Cadexomer Iodine Gel, CIG (Iodosorb, Smith & Nephew, Andover, Massachusetts); Triple Antibiotic Ointment (TAO, Actavis Generics, Parsippany, New Jersey); Antimicrobial Wound Gel, AWG (BlastX Wound Gel, Next Science, Jacksonville, Florida); Polyhexamethylene biguanide solution (PHMB-Cosmocil CQ, Arch Chemical Inc., Rochester, New York); and Benzalkonium Chloride (BKCL, Millipore Sigma, St. Louis, Missouri) as shown in Table 1, with their corresponding components and active ingredients. Each testing material was prepared and used in accordance to their respective manufacturers' instructions for treatment application. Additionally, pursuant to the research laboratory's standard operating procedures, the groups were blinded to prevent any unintentional biased data analysis prior, during, and after the study.

2.2 | In vitro zone of inhibition assay

2.2.1 | Inoculum preparation

A fresh culture of methicillin-resistant *S aureus* (USA300) were used for in vitro zone of inhibition assays. Freeze-dried bacterial cultures were recovered per standard recovering protocol. Challenge inoculum suspensions were prepared by swabbing an area 3 cm in diameter from a freshly grown culture plate. The collected swab is placed in 4.5 mL of sterile phosphate buffer saline (PBS OmniPur, Millipore Corporation, Billerica, Massachusetts), resulting in a suspension of approximately 10^{10} colony forming units/mL (CFU/mL). Serial dilutions were made until concentrations of 10^8 and 10^4 CFU/mL were achieved. Concentrations were confirmed using historical optical density measurements. Additionally, serial dilutions of the suspensions were plated onto microorganism-specific media using an Autoplate 4000 Spiral Plater System (Spiral Biotech, Advanced Instruments, Norwood, Massachusetts). This system deposits 50 μL of the suspension over the surface of the rotating culture plate to quantitate the exact concentration of viable organisms prior to beginning the experiment. Concentrations of 10^4 and 10^8 CFU/mL were used for zone of inhibition assays.

2.2.2 | Kirby-Bauer method

All treatments used were cut into 10 mm discs, except for CIG and AWG, which were topical treatments. Discs were moistened by placing each disc into individual wells on a sterile 12-well plate. Three millilitres (3 mL) of sterile 1X PBS was added to each well that contained a disc treatment. For topical treatments, a sterile 10 mm disc (Whatman Cellulose Filter Paper, Millipore Sigma, St. Louis, Missouri) was cut and placed in a sterile 12-well plate with sterile forceps. Each disc received 200 μL of topical treatment in addition to the 3 mL of sterile 1X PBS added to each well. The testing materials inside the plates were each labelled to their respective time point, from days 0, 1, 4, 7, and 10. Plates were sealed with parafilm (Parafilm, Bemis, Oshkosh, Wisconsin) to prevent moisture loss and incubated at 37°C. Plates were

TABLE 1 Test materials

Treatment	Nomenclature	Components/active ingredients
CWM-AM	Collagen Wound Matrix-Anti Microbial[a]	Type I ECM and PHMB
CWM-AM XT	Collagen Wound Matrix-Anti Microbial XT[b]	Type I ECM and PHMB
AHWD	Antimicrobial Hydrofiber Wound Dressing[c]	Sodium carboxymethylcellulose and silver
DRSAg	Dermal Scaffold with Silver[d]	Fetal bovine (Type III collagen) and silver
CEM	Collagen Extracellular Matrix[e]	Ovine forestomach matrix (85% collagen) and glycosaminoglycans
CWM	Collagen Wound Matrix[f]	55% collagen and 45% oxidised regenerated cellulose
MWDAg	Matrix Wound Dressing with Silver[g]	Collagen, oxidised regenerated cellulose, and silver
CIG	Cadexomer Iodine Gel[h]	Cadexomer Iodine
TAO	Triple Antibiotic Ointment[i]	Bacitracin Zinc, Neomycin Sulfate, and Polymyxin B Sulfate
AWG	Antimicrobial Wound Gel[j]	Benzalkonium chloride, polyethylene glycols (400 and 3350), sodium citrate, and citric acid

Note: Each of the treatment groups used for this study had a variety of active ingredients ranging from PHMB, silver, different types of collagen and chemicals.
[a]PuraPly AM, Organogenesis, Canton, Massachusetts.
[b]PuraPly AM XT, Organogenesis, Canton, Massachusetts.
[c]Aquacel Ag, Convatec Inc., Bridgewater, New Jersey.
[d]PriMatrix Ag, Integra LifeSciences, Plainsboro, New Jersey.
[e]Endoform, Hollister Inc., Libertyville, Illinois.
[f]Promogran, Systagenix, San Antonio, Texas.
[g]Promogran Prisma, Systagenix, San Antonio, Texas.
[h]Iodosorb, Smith & Nephew, Andover, Massachusetts.
[i]Triple Antibiotic Ointment, Actavis Generics, Parsippany, New Jersey.
[j]Wound Gel, Next Science, Jacksonville, Florida.

examined for moisture content on a daily basis; additional PBS was added every day to a beaker containing PBS in the incubator. Twelve Tryptic Soy Agar plates with 5% sheep's blood (TSA II, Becton Dickinson, Franklin Lakes, New Jersey) were challenged with 100 μL of each inoculum (10^4 and 10^8 CFU/mL) and was spread using glass beads. Three discs from each treatment group were removed from the respective 12-well plates and placed onto inoculated plates with a sterile spatula. Three TSAII plates were used for each treatment to obtain an $n = 9$. Treatments were allowed to diffuse into agar for 2 to 3 hours at room temperature before incubating at 37°C for 24 hours. After the incubation period, zones of inhibition were imaged using a planimetry measurement software (ImageJ 1.41o, National Institute of Health, Bethesda, Maryland) to analyse areas of inhibition. The areas from which the testing material was diffused within the media against the pathogen were applied once and challenged after the five time-points to be analysed.

2.2.3 | Cytotoxicity analysis

The purpose of this assay was to compare the in vitro cytotoxicity of CWM-AM, CWM-AM XT, among other products using human dermal fibroblasts (HDF). This assay measured cell growth over an incubation period with media conditioned with the test materials. TAO was used as a positive control and primary normal HDFs were used for this study.

Stock solutions of materials and controls were prepared with aliquots of 50 mL of DMEM with 1x antibiotics-antimycotics (see cell culture below) per condition into a 50 mL conical centrifuge tube and placed in a shaking water-bath at 37°C for 72 hours. Each test material and controls were added into each 50 mL medium following the instruction:

- Physical bandage CWM-AM, 5 units of 8-mm punch
- Physical bandage CWM-AM XT, 5 units of 8-mm punch
- PHMB, 4.1 μL of 0.1% PHMB solution
- Benzalkonium Chloride (BKCL, Millipore Sigma, St. Louis, Missouri), 41 mg
- AWG, 1 g
- Positive Control-TAO, 1 g
- Untreated negative control: none

After 72 hours in the shaker, materials and controls stock solutions were supplemented with 5% fetal bovine

serum (FBS) (HyClone, GE Healthcare, Chicago, Illinois) for cell culture.

2.2.4 | Cell culture

Primary normal human dermal fibroblasts (HDF) were supplied by University of Miami. Cells were grown in growth media of Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and 584 mg/L of L-glutamine (Lonza Walkersville Inc., Walkersville, Maryland) supplemented with 8% FBS with antibiotic-antimycotic of 100 IU/mL of Penicillin, 100 ug/mL of Streptomycin, and 0.25 μg/mL of Amphotericin B (Mediatech Inc., Manassas, Virginia) at 37°C in a Thermo humidified culture incubator containing 5% CO_2. At 90% confluence, the cells were detached from the dishes using 0.05% trypsin/0.53 mM EDTA, counted, split 1:4, and plated into a 12 well plate.

2.2.5 | Cell proliferation and viability analysis

HDF of 1×10^5 were plated in each well of a 12-well cell culture plate (Corning Inc., Corning, New York). Cells were divided into seven groups for each condition, in triplicate, incubated in normal growth medium (DMEM media supplemented with 5% FBS), 1 mL/well, in an incubator, at 37°C, and 5% CO_2 for overnight. After 16 hours of incubation, the growth medium in each well was replaced by a conditioned medium from each group. After 24 and 48 hours of treatment, cells were washed with PBS, treated with 0.05% trypsin/0.53 mM EDTA solution for 5 minutes, and detached from the wells. The Trypan Blue Dye-Exclusion haemocytometer technique was used to calculate cell proliferation and viability analysis. The principle of this method is that viable cells clear the dye and appear shining, while dead cells cannot clear the dye and turn blue. The cell proliferation (total cell numbers) and viability (percentage of viable cells) results were determined and graphed.

3 | IN VIVO DEEP DERMAL WOUND INFECTION MODEL

3.1 | Experimental animals

The following study and protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). The study was performed according to the University of Miami's Department of Dermatology and Cutaneous Surgery's Standard Operating Procedures. Swine were used as the research animal because of the morphological similarities between porcine and human skin.[16] The swine model assimilates many of the same characteristics as human skin in morphological factors when facing an infection and biochemical mechanisms for the epidermis and dermis to engage when receiving treatment, such a erythema and wound exudates. Pig skin is relatively similar to human skin in sparse hair coat, epidermal turnover time, a well-differentiated papillary body, and elastic tissue content, which makes it the best platform to test treatment when applied to partial-thickness wounds.[35] The swine model has been extensively tested with current medical interventions, which makes this platform the optimal translational model prior to clinical trials. Six female Yorkshire pigs that were specific pathogen-free (SPF; Looper Farms, Granite Falls, North Carolina) weighing 35 to 40 kg were housed to acclimate to the vivarium for approximately 3 weeks preceding the experiment. Animals were fed a non-antibiotic feed ad libitum and housed individually in our animal care facilities (American Association for the Accreditation of Laboratory Animals accredited) with a controlled temperature (19°C-21°C) and light schedule (12 hours/12 hours LD).

3.2 | Animal preparation

Animals were anaesthetised and hair on the backs and flanks of the animals were trimmed with standard animal clippers. The shaved skin on both sides of each animal was washed with a non-antibiotic soap (Neutrogena, Johnson & Johnson Consumer Inc., New Brunswick, New Jersey) and sterile water.

3.3 | Wounding technique

A specialised electrokeratome was used to make a total number of 126 deep reticular dermal wounds (each measuring 22 mm × 22 mm × 3 mm deep) on the paravertebral and thoracic areas in six animals (21 wounds per animal). All wounds were randomly divided into six treatment groups. Each animal had three additional wounds designated for quantifying baseline bacterial counts.

3.4 | Wound inoculation

For the microbiology analysis, a fresh culture of methicillin-resistant *S aureus* (USA300) was used to inoculate each wound bed. The challenge inoculum was prepared from a

culture plate by swabbing a 3-cm diameter area grown overnight. The scraping was placed in 4.5 mL of sterile water, resulting in a suspension concentration of approximately 10^{10} colony forming units/mL (CFU/mL). Serial dilutions were made until a concentration of 10^4 CFU/mL was achieved, as determined by optical density measurements. Serial dilutions of the suspension were also plated onto selective media to quantify the exact number of viable organisms used in each experiment. The inoculum was then vortexed, and each wound was inoculated with a 25 μL aliquot of the inoculum suspension, deposited into a glass cylinder (22 mm in diameter) in the centre of each wound. The aliquoted suspension was then lightly scrubbed into the wound site for 10 seconds using a sterile Teflon spatula. Each wound was individually covered with a polyurethane film dressing (Tegaderm, 3 M, St. Paul, Minnesota) for 72 hours to allow biofilm formation.[36]

3.5 | Treatment regimen

After 72-hours to allow biofilm formation, three wounds were recovered to analyse baselines for microbiology counts and histological parameters. After biofilm formation, but before treatment, surgical debridement was performed on each wound to remove the newly formed biofilm layer, using a sterile 4 mm curette (Disposable Dermal Curette, Integra LifeSciences, Princeton, New Jersey). The wounds were assigned randomly to six groups—CWM-AM; CWM-AM XT; AHWD; DRSAg; AWG; and Untreated Control. Wounds were treated once on the first day (except for wounds treated with AWG gel where 200 mg of the topical ointment was reapplied every 4 days as per manufacturer instructions for use). Dressings from wounds that received treatments CWM-AM and DRSAg required hydration with 200 μL of sterile 1X PBS. While those treated with CWM-AM XT did not require any hydration when dressings were placed on their designated wound sites. Dressings covering wounds treated with AHWD were hydrated with 700 μL of sterile 1X PBS. All wounds were covered with a polyurethane dressing and were secured in place with surgical tape and wrapped with self-adhering bandages (Coban, 3 M, St. Paul, Minnesota).

4 | MICROBIOLOGY ASSESSMENT

Three wounds were cultured 72 hours after inoculation to obtain baseline bacteria counts prior to treatment. In addition, three wounds per treatment group were biopsied with a 6 mm punch biopsy on days 4, 8, and 11 post-treatment application. Each punch biopsy was taken at the centre of the wound site and deep enough to remove subcutaneous tissue. This enabled evaluation of bacteria around the wound edges, bed, and surface. Biopsies were weighed and immediately placed in a homogenisation tube (Tenbroeck Glass Tissue Grinder, Omni International, Kennesaw, Georgia) with 1 mL of cold, sterile 1X PBS, homogenised, and combined with an additional 4 mL of 1X PBS. Serial dilutions were made and quantified using the Autoplate Spiral Plater System which deposits a defined amount (50 μL) of the suspension onto the surface of a rotating agar plate. Oxacillin Resistance Screening Agar Base (ORSAB, Remel Products—Thermo Fisher Scientific, Lenexa, Kansas) was used to isolate MRSA USA 300, excluding counts for any other microorganism present on site. After plating, plates were incubated aerobically at 37°C for 48 hours. After incubating, colonies were counted, and the Log CFU/g was calculated.

5 | HISTOLOGICAL ASSESSMENT

On days 4, 8, and 11, four excisional biopsies were taken from each treatment group for histological assessment. Biopsies were taken passing through the centre of the wound, including healthy tissue at each end of the sample. Excised biopsies were immediately placed in formalin, then processed and stained with haematoxylin and eosin (H&E). To determine the wound healing effects of each treatment group, samples were analysed and evaluated by a trained dermatopathologist for the following parameters: percent of wound epithelialised: length of the wound surface that has been covered by newly formed epithelium, which is expressed as a percentage of total length; epithelial thickness: thickness (cell layers; μm) of the epithelium, which is averaged from five points equidistant from each other in the wound; white cell infiltrate: presence and amount of subepithelial mixed leukocyte infiltrates, which is graded as mean score- 1 = absent, 2 = mild, 3 = moderate, 4 = marked, 5 = exuberant; granulation tissue formation: approximate amount of newly formed granulation tissue (dermis), which is graded as follows: 0: 0, 0.5: 1% to 10%, 1: 11% to 30%, 2: 31% to 50%, 3: 51% to 70%, 4: 71% to 90%, 5: >90%; angiogenesis: measured by the degrees of newly formed blood microvasculature, which is graded as mean score—1 = absent, 2 = mild, 3 = moderate, 4 = marked, 5 = exuberant.

6 | STATISTICAL ANALYSIS

For data results involving the cytotoxicity analysis, data processing was performed using Microsoft Excel 2010 (Microsoft Office, Microsoft, Redmond,

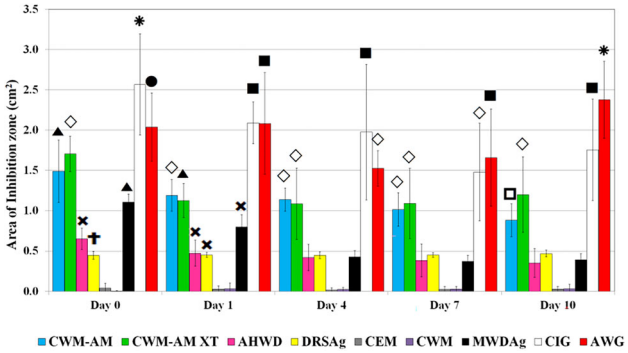

FIGURE 1 MRSA 10^4 CFU/mL zone of inhibition assay. Mean areas of inhibitory areas of various treatments against MRSA USA300 at a bacterial concentration of 10^4 CFU/mL for days 0, 1 4, 7, and 10. Significant differences are defined as $*P < .05$ compared with all treatments; ●$P < .05$ compared with CWM-AM, AHWD, DRSAg, CEM, CWM, MWDAg; ◊$P < .05$ compared with AHWD and DRSAg, CEM, CWM, and MWDAg;× $P < .05$ compared with CEM and CWM; ▲$P < .05$ compared with AHWD, DRSAg, CEM, and CWM; □$P < .05$ compared with AHWD, CEM, CWM, and MWDAg; ■$P < .05$ compared with CWM-AM, CWM-AM XT, AHWD, DRSAg, CEM, CWM, and MWDAg; † $P < .05$ compared with CWM; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CEM, Collagen Extracellular Matrix (Endoform); CIG, Cadexomer Iodine Gel (Iodosorb); CWM, Collagen Wound Matrix (Promogran); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag); MWDAg, Matrix Wound Dressing with Silver (Promogran Prisma)

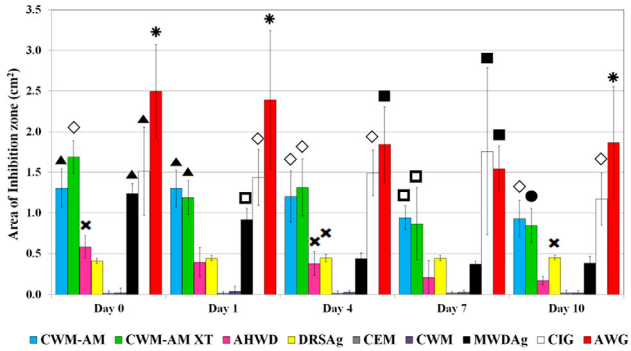

FIGURE 2 MRSA 10^8 CFU/mL zone of inhibition assay. Mean areas of inhibitory areas of various treatments against MRSA USA300 at a bacterial concentration of 108 CFU/mL for days 0, 1 4, 7, and 10. Significant differences are defined as $*P < .05$ compared with all treatments; ●$P < .05$ compared with AHWD, CEM, CWM, and MWDAg; ◊$P < .05$ compared with AHWD, DRSAg, CEM, CWM, and MWDAg; □$P < .05$ compared with AHWD, CEM, and CWM; ▲$P < .05$ compared with AHWD, DRSAg, CEM, and CWM; × $P < .05$ compared with CEM and CWM; ■$P < .05$ compared with CWM-AM, CWM-AM XT, AHWD, DRSAg, CEM, CWM, and MWDAg; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CEM, Collagen Extracellular Matrix (Endoform); CIG, Cadexomer Iodine Gel (Iodosorb); CWM, Collagen Wound Matrix (Promogran); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag); MWDAg, Matrix Wound Dressing with Silver (Promogran Prisma)

Washington) and GraphPad Prism v7. (GraphPad Software, San Diego, California) for Windows. Statistical analysis was calculated using one-way Analysis of Variance (ANOVA) followed by Student's *t*-test. For the remaining data for both in vitro and in vivo analysis, statistical analysis was performed using one-way ANOVA test (SPSS Statistics 25, IBM, Armonk, New York) for the mean Log CFU/g. *P* values of less than .05 were considered to be statistically significant.

7 | RESULTS

7.1 | In vitro: Areas of inhibition

At bacterial concentrations of 10^4 and 10^8 CFU/mL, AWG showed significantly higher areas of inhibition when compared against all other treatments, including CIG, by the end of the study on day 10 as shown in Figures 1 and 2. On Day 0, AWG showed the greatest zone of inhibition, followed by CWM-AM XT. AHWD showed one of the smallest areas of inhibition by the end of the study. As with assay results against MRSA at 10^4 CFU/mL, CWM-AM, and CWM-AM XT exhibited a consistent potency against MRSA throughout the study but were not as effective as AWG or CIG. However, these results were not unexpected, as the delivery mechanisms for ointments or gels (such as AWG and CIG) is based on a burst release of the active ingredients, which is substantially different than collagen-based testing materials (such as CWM-AM and CWM-AM XT), which are bound to the surface and persistently present the corresponding active ingredients, thereby providing antimicrobial activity after 10 days from being incubated.

7.2 | Cytotoxicity

7.2.1 | Fibroblast proliferation analysis

The cells were incubated with the different conditioned media for 24 and 48 hours, and cell proliferation was

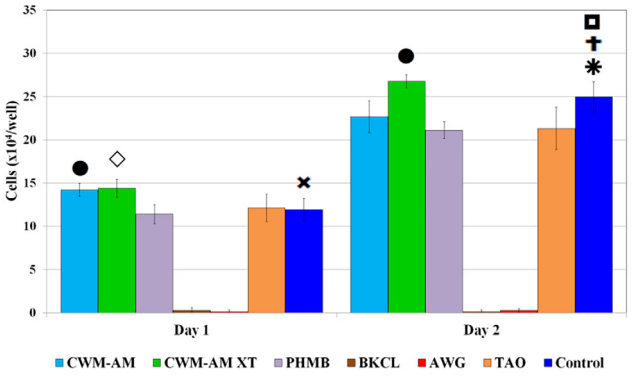

FIGURE 3 Fibroblast proliferation analysis after 1 and 2 days of treatments. Mean values of cell count ($\times 10^4$/well) were graphed for CWM-AM, CWM-AM XT, PHMB, BKCL, AWG, TAO, and untreated negative control (Control). Significant differences are defined as follows: ●$P < .05$ compared with Control; □ $P < .05$ compared with CWM-AM; *$P < .01$ compared with TAO; ◇$P < .01$ compared with Control; × $P < .001$ compared with BKCL and AWG; and †$P < .001$ compared with PHMB, BKCL, and AWG; error bars represent standard deviation. AWG, Antimicrobial Wound Gel (BlastX Wound Gel); BKCL, Benzalkonium Chloride; CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); PHMB, Polyhexamethylene biguanide solution (Cosmocil CQ); TAO, Triple Antibiotic Ointment (Triple Antibiotic Ointment)

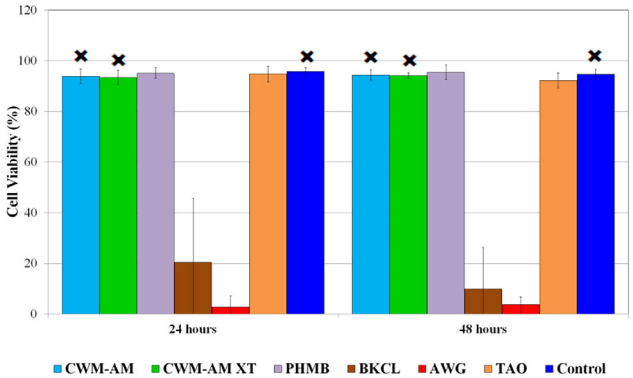

FIGURE 4 Cell viability analysis after 24 and 48 hours treatment. Mean values of percentage of viable cells were graphed for the treatments for CWM-AM, CWM-AM XT, PHMB, BKCL, AWG, TAO, and untreated negative control (Control). Significant differences are defined as follows: × $P < .001$ compared with BKCL and AWG; error bars represent standard deviation. AWG, Antimicrobial Wound Gel (BlastX Wound Gel); BKCL, Benzalkonium Chloride; CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); PHMB, Polyhexamethylene biguanide solution (Cosmocil CQ); TAO, Triple Antibiotic Ointment (Triple Antibiotic Ointment)

quantified using Trypan Blue Dye-Exclusion haemocytometer technique. Compared with untreated negative control cells with cell counts at 11.93×10^4 cells/well and 25.0×10^4 cells/well at 24 and 48 hours, respectively, a slight increase in cell proliferation was noticed after 24 hours treatments of CWM-AM and CWM-AM XT with cell counts of 14.20×10^4 cells/well ($P < .05$) and 14.40×10^4 cells/well ($P < .01$), respectively, and after 48 hours treatment of CWM-AM XT with cell counts 26.8×10^4 cells/well ($P < .05$), respectively (Figure 3). There were no significant differences in cell counts at 24 hours when compared the negative control to the treatments of TAO and PHMB. After 48 hours, TAO and PHMB treatments exhibited lower cell proliferation than negative control with cell counts of 21.33×10^4/well ($P < .01$) and 21.13×10^4/well ($P < .001$), respectively (Figure 3). On the contrary, markedly, drop in cell proliferation was observed in cells treated with BKCL and AWG. After 24 hours, BKCL treated cells had 0.27×10^4 cells/well ($P < .001$) and AWG treated had 0.13×10^4 cells/well ($P < .001$). The similar effects were seen after 48 hours, when BKCL treated had 0.13×10^4 cells/well ($P < .001$) and AWG treated had 0.27×10^4 cells/well ($P < .001$), demonstrating severe cytotoxicity and adverse effects on cell proliferation.

7.2.2 | Fibroblast viability analysis

The effect of the different conditioned media on fibroblast viability was evaluated. When comparing the untreated negative control against the conditioned media of CWM-AM, CWM-AM XT, PHMB, as well as positive control TAO, there were no significant effects on cell viability after 24- and 48-hour treatments. However, a dramatic decrease in cell viability was observed in the treatments of BKCL and AWG, a decrease of 70% at 24 hours and 84% at 48 hours in BKCL treatment (both $P < .001$), and of 92% and 90% at 24 and 48 hours, respectively, in AWG treatment (both $P < .001$) (Figure 4).

Similar effects were observed when comparing CWM-AM or CWM-AM XT with the other conditioned media groups. The comparison between CWM-AM and BKCL showed a marked decrease in cell viability of 74% and 84% after 24 and 48 hours (both $P < .001$), respectively. A vast difference in cell viability was also found by comparing CWM-AM and AWG, the AWG group has 90% decrease in cell viability after 24 and 48 hours (both $P < .001$). There were marked differences of 73% and 84% in cell viability between CWM-AM XT and BKCL treatments at 24 and 48 hours (both $P < .001$), respectively. The differences between CWM-AM XT and AWG were 88% ($P < .001$) after 24 hours and 89% ($P < .001$) after 48 hours. It was also noted that the PHMB was not

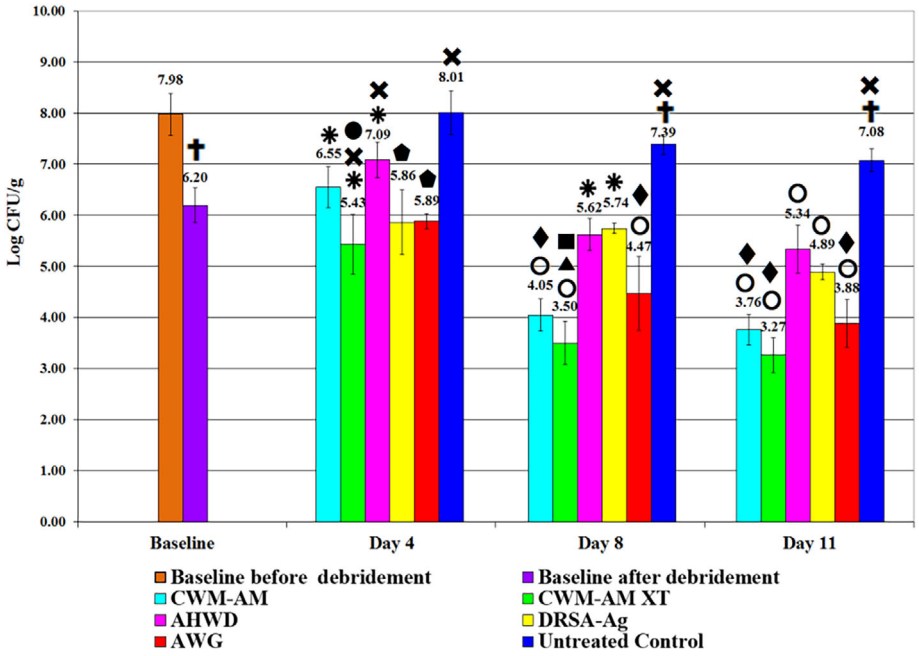

FIGURE 5 Growth of MRSA in vivo. Mean bacterial counts of methicillin-resistant *Staphylococcus aureus* USA300 for baseline (day 0) and days 4, 8, and 11 after treatment. Significant differences are defined as follows: ✳$P < .05$ compared with Baseline before Debridement and F-Untreated Control; †$P < .05$ compared with Baseline before Debridement; ⬟$P < .05$ compared with Baseline before Debridement, AHWD, and Untreated Control; ■$P < .05$ compared with DRSAg and AWG; ○$P < .05$ compared with Baseline before and after Debridement and Untreated Control; ×$P < .05$ compared with Baseline after Debridement; ●$P < .05$ compared with CWM-AM and AHWD; ◆$P < .05$ compared with AHWD and DRSAg; ▲$P < .05$ compared with AWG; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

cytotoxic while BKCL was, demonstrating the cytotoxicity of the antimicrobial agent itself.

7.3 | In vivo microbiology and histology analysis

As depicted in Figure 5, the baseline wounds recovered prior to debridement reached a MRSA count of 7.98 ± 0.53 Log CFU/g, which were significantly ($P < .05$) higher than baseline wounds recovered after debridement (6.20 ± 0.21 Log CFU/g), demonstrating the efficacy of debridement in bacterial control. On Day 4, wounds treated with CWM-AM XT showed the lowest MRSA counts of all treatments, showing significant reductions ($P < .05$) from baseline wounds before and after debridement, and at least a 99.0% reduction in bacteria. Wounds treated with CWM-AM XT were significantly ($P < .05$) lower than all other treatment groups, except DRSAg and AWG. DRSAg and AWG were both capable of reducing MRSA counts by over 99.0%. Untreated Tegaderm Control wounds showed MRSA levels comparable to baseline wounds before debridement and were significantly higher than all other treatment groups as shown in Figure 5, demonstrating that debridement alone is not sufficient to effectively control bacterial growth. CWM-AM XT showed the greatest ability to reduce MRSA counts in deep dermal wounds, with an overall reduction of MRSA counts greater than 99.99%, compared with baseline MRSA counts. While CWM-AM showed slightly higher bacterial counts than CWM-AM XT on days 8 and 11, both treatment groups were significantly ($P < .05$) lower than the baseline counts, and those of AHWD and DRSAg.

7.4 | Histology

Initially, those wounds treated with CWM-AM exhibited a higher reepithelialisation percentage than all other groups, with a statistically significant difference when compared against DRSAg ($P < .05$). By the end of the study, all wounds reached or exceeded 70% reepithelialised epidermis (Figure 6). There is no significant difference in epithelial thickness observed among all treatment groups, while DRSAg was the only treatment group to show a consistent

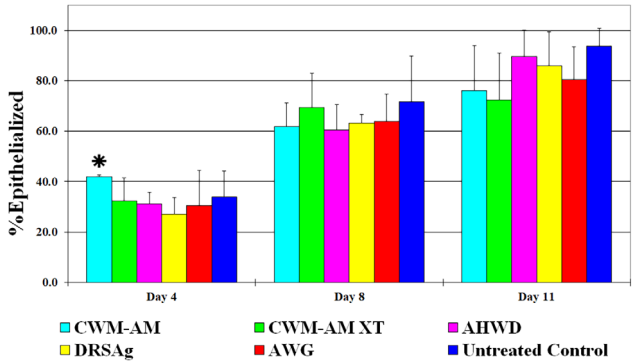

FIGURE 6 Reepithelialisation. Percentage of reepithelialised tissue for 4, 8, and 11 days after treatment. Percentages calculated as the mean of four samples; error bars represent standard deviation. *$P < .05$ compared with DRSAg; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

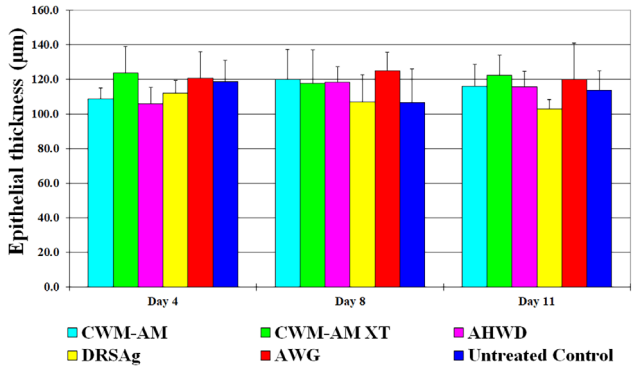

FIGURE 7 Epithelial thickness. Quantified length (µm) of newly formed epithelium for 4, 8, and 11 days after treatment. Percentages calculated as the mean of four samples; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

decrease in epithelial thickness throughout the study (Figure 7). Wounds treated with CWM-AM XT and AHWD showed a significantly ($P < .05$) lower WCI score on Day 4 (Figure 8). No differences were observed between any of the treatment groups on Day 8 or 11. Wounds treated with CWM-AM showed significantly ($P < .05$) higher granulation tissue formation on Day 4 compared with AHWD (Figure 9). No significant differences in

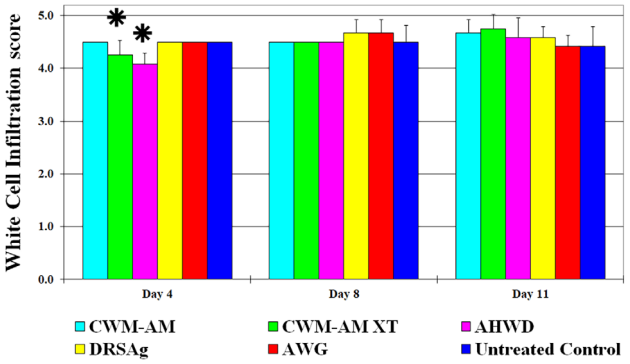

FIGURE 8 White cell infiltration. Mean scores of white cell infiltrates for 4, 8, and 11 days after treatment. Percentages calculated as the mean of four samples. Mean scores for white cell infiltration (WCI): 1, absent; 2, mild; 3, moderate; 4, marked; and 5, exuberant. Significant differences are defined as follows: *$P < .05$ compared with CWM-AM, DRSAg, AWG, and Untreated Control; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

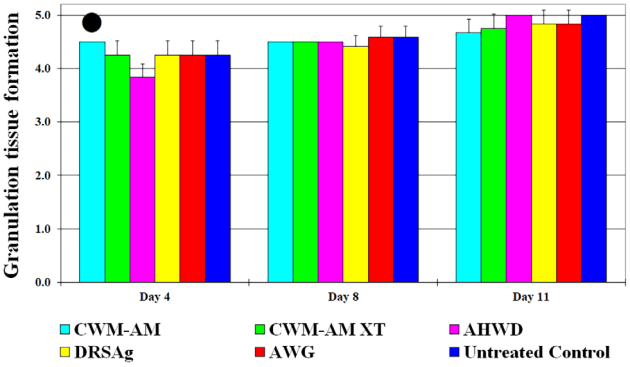

FIGURE 9 Granulation tissue formation. Mean scores of granulation tissue formation taken from deep dermal wounds for 4, 8, and 11 days after treatment. Percentages calculated as the mean of four samples. Mean scores for granulation tissue formation: 1 = ≤5%, 2 = 6% to 25%, 3 = 26% to 50%, 4 = 51% to 75%, 5 = 76% to 100%. Significant differences are defined as follows: ●$P < .05$ compared with AHWD; error bars represent standard deviation. AWG, Antimicrobial Wound Gel (BlastX Wound Gel); AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

angiogenesis were found when comparing treatments, or when comparing individual treatment progression throughout the entire study (Figure 10).

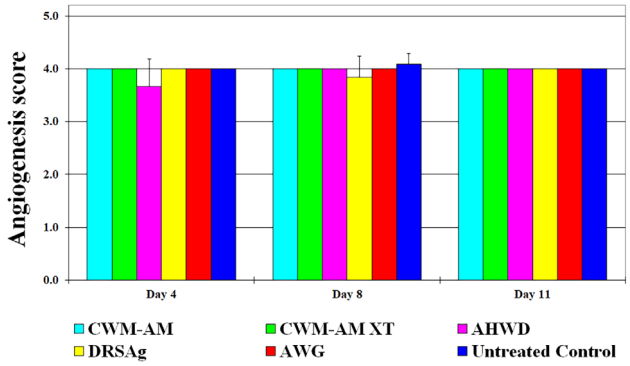

FIGURE 10 Angiogenesis. Mean scores of degrees of new microvascular blood vessel formation for 4, 8, and 11 days after treatment. Percentages calculated as the mean of four samples. Mean scores for angiogenesis: 1, absent; 2, mild; 3, moderate; 4, marked; and 5, exuberant; error bars represent standard deviation. AHWD, Antimicrobial Hydrofiber Wound Dressing (Aquacel); AWG, Antimicrobial Wound Gel (BlastX Wound Gel); CWM-AM, Collagen Wound Matrix-Anti Microbial (PuraPly AM); CWM-AM XT, Collagen Wound Matrix-Anti Microbial XT (PuraPly AM XT); DRSAg, Dermal Scaffold with Silver (PriMatrix Ag)

8 | DISCUSSION

Preventing infection is important to all types of wounds, particularly the infections caused by drug-resistant bacteria, such as MRSA[37] and *P aeruginosa*,[38] that can prevent wound healing and/or cause further complications such as biofilm formation. Debridement methods have been established as an effective method to remove biofilm and thereby reduce infection levels.[39-41] However, using our porcine model we have shown that a number of common debridement methods cannot remove all of the bacteria once allowed to establish a biofilm (REF: Nusbaum AG, Gil J, Rippy MK, Warne B, Valdes J, Claro A, Davis SC. Effective Method to Remove Wound Bacteria: Comparison of Various Debridement Modalities in an In Vivo Porcine Model J Surg Res 2012, 176(2):701-7). Once the protective biofilm layer is mechanically removed, addressing the pathogenic infection is paramount to allow both acute and chronic wounds to commence proper wound healing mechanisms. The presence of planktonic infection in the wound bed remains a highly possible outcome after debridement, which would create a relapse in infection and ultimately hamper the wound healing phase.[42] Recent studies conducted by our team have extensively focused on addressing the prevention of infection while simultaneously enhancing the wound healing process.[20,43,44]

The importance of a treatment modality containing an antimicrobial component was confirmed through in vitro analysis. The in vitro analysis confirmed the risk of exposure to pathogenic microorganisms when using a treatment modality that does not have an antimicrobial component. In the ZOI assay, in the case of test articles with no antimicrobial material (CWM and CEM), there was zero inhibition observed. While CWM-AM and CWM-AM XT exhibited a consistent potency in their antimicrobial capabilities by maintaining similar levels for each of the time-points analysed. For both MRSA concentrations present into the plate wells, those collagen-enhanced treatment groups showed desirable areas of inhibitions. This in vitro analysis showed that collagen-based products can provide a continuous presence of antimicrobial agents for at least 10 days at physiological temperatures. The in vivo histological parameters of percentage of reepithelialised tissue and granulation tissue formation showed an initial enhancement on Day 4 for those wounds treated with CWM-AM. Overall, it appears that wounds treated with CWM-AM XT showed superior results compared with the other materials tested in this study when analysing the total number of bacteria present. CWM-AM has been shown to provide antimicrobial barrier effects that prevent MRSA bacterial growth and biofilm formation, allowing for more successful wound healing.[45,46] Combining the collagen-based wound matrix with the antimicrobial compound PHMB as an antimicrobial barrier has shown possible benefits to both the healing process and bioburden control. Collagen-based matrices have been shown to stimulate proteins related to collagen type I, II, and V, and dermal fibroblasts.[47-49] The effectiveness of collagen-based matrices can further be enhanced by tuning the amount of collagen, rate of degradation of collagen by chemical processes such as cross-linking. Reducing the rate of degradation of collagen-based matrices is likely to improve the efficacy of collagen-based matrices in wound healing.

In vitro studies analysing the antibacterial effects of each treatment showed that treatment with AWG provided the greatest overall inhibitory effect against MRSA USA300 grown at bacterial concentration of both 10^4 and 10^8 CFU/mL; however, AWG also demonstrated severe in vitro cytotoxicity. It should be noted that the AWG presents the antimicrobial agent in a gel form, facilitating a burst release of antimicrobial agent, whereas the collagen-based wound matrices present the antimicrobial agent in a matrix bound form, which facilitates sustained presentation of the antimicrobial agent. Consequently, AWG treatment may require a more frequent reapplication compared with the collagen-based materials, exposing the newly generated cells and tissues to a potentially severe cytotoxic signal at every dressing change. AWG is a treatment modality, manufactured to address biofilm present on a wound site, and does not have a collagen component. It should also be noted that results

from the in vivo study showed that CWM-AM XT and CWM-AM provided the greatest antimicrobial activity by the end of the study, further demonstrating the effectiveness of the sustained presence of PHMB. When analysing the in vitro results for this study, CWM-AM and CWM-AM XT persisted for 10 days, suggesting PHMB will ultimately provide the antimicrobial barrier efficacy necessary to control bioburden and biofilm reformation. CWM-AM XT was able to provide an area of inhibition for both MRSA concentrations that was comparable to other antibiotic treatments, providing evidence that the collagen matrix did not alter the antimicrobial agent present in the dressing. Further, the in vivo study demonstrated the capabilities for both CWM-AM and CWM-AM XT to reduce MRSA proliferation without being detrimental to the wound healing process. This study did not monitor pain management levels but previous studies have shown that collagen-based dressings reduced wound pain levels by over 66%, without any detrimental effects.[50] One of the limitations with this study, and all animal models used to assess the activity of wound healing and antimicrobial agents that are used for chronic wounds, is that they tend to be short-term studies without underlying comorbidities and do not necessarily replicate a low-grade chronic infection, with relevant clinical variables such as aetiology, size and depth of the wound, and increased protease levels.[51,52] Further, the pig wound healing models typically demonstrate a significantly faster rate of healing than typically observed clinically, particularly in the cases of acute and chronic wounds observed in the patients with comorbidities. Therefore, these animal models may not be optimal in assessing the wound healing potential of the test materials in infected wounds. Consequently, further clinical studies will be needed to evaluate the effectiveness of the use of collagen matrix dressings containing antimicrobial agents.

9 | CONCLUSIONS

The *in tandem* capabilities for CWM-AM and CWM-AM XT to control bioburden as an antimicrobial barrier with PHMB while simultaneously providing a collagen matrix that supports wound healing make these dressings desirable options when treating both acute and chronic wounds. This investigation analysed the potential use of native collagen that, when combined with an antimicrobial such as PHMB, may provide optimal results in the clinical settings to successfully combat chronic wounds.

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CONFLICTS OF INTEREST

The co-authors from the University of Miami certify not having any conflict of interest to declare related to the contents of the manuscript, the co-authors from the University of Miami received research funding from Organogenesis, Inc. to conduct the blinded study. The co-authors from Organogenesis, Inc. certify having a conflict of interest to declare related to the contents of the manuscript for being employed by the company funding this research study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Joel Gil https://orcid.org/0000-0001-8304-6722
Alexander Higa https://orcid.org/0000-0001-6880-3182

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