Human cryopreserved viable amniotic membrane inhibits the growth of bacteria associated with chronic wounds

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Abstract:

Chronic wounds are challenging to treat and are often associated with an increased risk of soft tissue and bone infection leading to amputation. Therefore, modalities that accelerate wound closure should reduce morbidity and prevent infection. In a recent, large, multicenter, randomized controlled clinical trial, the safety and efficacy of a commercially available human cryopreserved viable amniotic membrane (hCVAM) was evaluated for treatment of diabetic foot ulcers (DFUs). A significantly higher proportion of patients achieved complete wound closure through treatment with hCVAM as compared to the standard of care. In addition, treatment with hCVAM also resulted in significantly fewer wound-related infections. One of the properties of placental membranes *in utero* is to protect the fetus from infections. Therefore, we hypothesized that the antimicrobial properties of fresh placental membranes are retained in hCVAM. To test this hypothesis, we evaluated the antimicrobial activities of hCVAM *in vitro* against bacteria associated with chronic wounds. The antimicrobial activity of hCVAM against clinical isolates of *S. aureus* and *P. aeruginosa* was evaluated using two standard methods. We observed that using the AATCC Method 100, the growth of *S. aureus* and *P. aeruginosa* in the presence of hCVAM was significantly reduced, compared with that of the negative control. Furthermore, the activity of hCVAM against 6 pathogens associated with chronic wounds – *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and *E. aerogenes* (ESKAPE) – were tested using a liquid culture assay. We demonstrated that hCVAM inhibited the growth of all ESKAPE bacteria. Our results confirm that hCVAM retains the antimicrobial properties of fresh amnion.

Key words: Amnion, Antimicrobial, Cryopreserved Viable Amniotic Membrane, Diabetic Foot Ulcer, Wound-Associated Bacteria, Wound Infection

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INTRODUCTION

Chronic wounds are wounds that failed to progress through the normal healing process in a timely manner. They represent a huge financial burden to the patient and to the healthcare system.[1,2,3] Among different types of chronic wounds, the prevalence of DFUs is rising. Currently in the US, DFUs affect 25% of the more than 25 million patients with diabetes, with an annual cost of $9-13 billion.[4,5,6] Non-healing DFUs place patients at a high risk for wound-related morbidities and mortality. DFU-related infection is a serious complication which increases the risk of hospitalization by 55.7, times and the risk of amputation by 154.5, times than if the infection was not DFU-related.[7] More than half of non-healing DFUs become infected, and one out of five of those will require an amputation.[4]

The use of fresh human amniotic membrane (hAM) for wound treatment was first reported more than a century ago.[8,9] However, limited availability and short shelf-life precluded the widespread use of fresh hAM. A novel hCVAM was recently developed and is now commercially available.[10,11,12] Several studies have demonstrated the clinical benefits of hCVAM for treating different types of chronic wounds including a multicenter, randomized, controlled clinical trial for chronic DFUs.[13,14,15] Compared to standard of care (SOC), treatment with hCVAM

resulted in complete wound closure by week 12 in 62% of patients versus only 21% in the SOC arm, and a significantly faster median time to complete wound closure compared with the SOC group (42 versus 69.5 days, respectively). In addition, patients treated with hCVAM experienced significantly fewer wound-related infections (18%) compared with SOC (36.2%).[13] The lower incidence of wound-related infections may be explained by the faster healing rate and the higher proportion of patients achieving wound closure. It may also be associated with the antimicrobial activity of hCVAM. It has been well established that the amnion has inherent antimicrobial properties.[16,17] However, different processing methods can impair structural and functional integrity of the tissue.[18,19] This study was designed to evaluate the antimicrobial activity of hCVAM against the panel of microorganisms that are most often associated with chronic wound infection.

MATERIALS AND METHODS

Preparation of Samples

Multiple lots of hCVAM derived from different donors were prepared as previously described.[10] Prepared samples were stored at -80°C. Prior to the experiments, the hCVAM was thawed in a room temperature water bath for 3-5 minutes. The cryobag containing hCVAM was then transferred to a sterile biosafety cabinet. The tissue was removed from the cryobag and transferred into a rinse basin containing sterile Phosphate Buffered Saline (PBS). 3 cm^2 pieces of hCVAM were used in the following experiments.

Puracol® Plus, a collagen dressing (Medline), and Acticoat 7, an antimicrobial silver-containing dressing (Smith & Nephew), were used as negative and positive controls for each experiment, respectively. These dressings were removed from their packages, cut into 3 cm^2 pieces, and rehydrated by a brief soaking in sterile PBS before the experiment.

Cell Viability Testing

The LIVE/DEAD® Viability/Cytotoxicity Kit (ThermoFisher Cat# L3224) was used to analyze the cell viability of hCVAM. Tissue samples were incubated in tryptic soy broth (TSB) (BD Difco) or in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals) (the assay medium) for 24 hours. After incubation, the samples were rinsed with PBS twice and stained with 4 µM calcein AM and 2 µM ethidium homodimer-1 according to the LIVE/DEAD® Viability/Cytotoxicity Kit protocol. Stained samples were then analyzed using a Zeiss fluorescent microscope. Viable cells were green fluorescent, whereas dead cells were red fluorescent. Digital images were taken for both green and red fluorescence channels, and corresponding “red” and “green” images were merged using ImageJ software (NIH). Freshly thawed hCVAM that had not been cultured for 24 hours was used as a cell viability baseline control. The numbers of viable and non-viable cells were counted manually using two lots of hCVAM (three images/lot). The percent of viable cells in the tissue was calculated and expressed as the average percentage of viable cells [(total number of green cells)/(total number of green and red cells)] x 100%.

Bacterial Culture and Preparation of Inoculums

Clinical isolates of ESKAPE bacterial strains were purchased from ATCC: *E. faecium* ATCC® 51559™, *S. aureus* ATCC® 25923™, *K. pneumoniae* ATCC® 700603, *A. baumannii* ATCC® 49466™, *P. aeruginosa* ATCC® 15692™, and *E. raerogenes* ATCC® 49469™. All bacterial strains were cultured and maintained following their manufacturing instructions. The growth media for *S. aureus, P. aeruginosa, E. aerogenes, or A. baumannii* is TSB, for *E. faecium* it is brain heart infusion (BHI), and for *K. pneumoniae* it is nutrient broth. Tryptic soy agar (TSA) plates were streaked with *S. aureus, P. aeruginosa, E. aerogenes,* and *A. baumannii*; BHI agar plates were streaked with *E. faecium*; and nutrient agar plates were streaked with *K. pneumoniae*. All plates were incubated at 37°C overnight. One single colony from each bacterial

strain plated was used to inoculate 1 mL of its respective growth medium, then inoculated bacteria were grown in suspension overnight in a 37°C shaker. After overnight growth, the bacteria were diluted 1:100 using fresh growth medium and cultured for an additional 2-4 hours until the absorbance optical densities of the culture measured in the range of 0.2 to 0.6 at a wavelength of 600 nm. The number of colony forming units (CFUs) for each strain was estimated based on an Optical Density $(OD)_{600}$ = 1.0, which corresponds to 10^9 CFU/mL. To prepare the inoculum for antimicrobial testing of hCVAM, approximately 100 CFU/mL of bacteria was further diluted using the assay medium. The actual CFU of each inoculum was determined by serial dilutions and colony counting on agar plates for each experiment.

Antimicrobial Activity of hCVAM Using a Modified American Association of Textile Chemists and Colorists (AATCC) Method 100

3 cm^2 of hCVAM, or control samples were placed onto assay medium agar plates. Samples of hCVAM were placed on an agar plate with the stromal layer of hCVAM facing up for inoculation of bacteria. 50 µl of bacteria (an inoculum of approximately 100 CFU) was then spread evenly across the entire surface of each sample, and the inoculated samples were incubated in a 37ºC incubator with 100% humidity for 18 hours. After incubation the samples were each transferred to 1 mL of TSB and vortexed to elute bacteria, and the OD_{600} of the eluted bacteria was measured using a spectrophotometer.

Antimicrobial Activity of hCVAM Using a Liquid Culture Assay

For each bacterial strain, hCVAM or control samples were placed into tubes with 1 mL of assay medium containing approximately 100 CFU of bacteria and were incubated at 37ºC with shaking for 24 hours. After incubation the bacterial cultures were serially diluted and plated onto TSB agar plates. The CFUs were counted after overnight incubation at 37ºC. For each bacterial strain, hCVAM from different lots (n ≥ 3 per lot) were used. The efficiency of bacterial growth inhibition by hCVAM was expressed in log reductions, which was calculated as the log_{10}CFU (negative control) - log_{10}CFU (hCVAM). Data from independent experiments (n≥ 2) were used to calculate the mean and standard deviation.

RESULTS

Optimization of the Culture Condition

Conventionally, nutrient medium such as TSB is used to culture bacteria, which provides all the elements that bacteria need for growth. However, the effect of TSB on mammalian viable cells in tissue grafts was unknown. To select the optimal culture medium, we evaluated the effect of TSB and of DMEM with 10% FBS on viable cells in hCVAM. For these experiments, hCVAM was incubated in either TSB or in DMEM/10% FBS (assay medium) for 24 hours, and the cell viability was subsequently examined. TSB is detrmintal to cells in hCVAM ***(Figures 1b and e)*** - the viability of epithelial and stromal layer of hCVAM was 3% and 6%, respectively. In contrast, 86% (epithelial layer) and 78% (stromal layer) of cells in hCVAM remained viable after culturing in the assay medium ***(Figures 1c and f)***. A positive control, hCVAM stained immediately post-thaw, confirmed high cell viability (90% on average) of the starting material ***(Figures 1a and d)***, which is

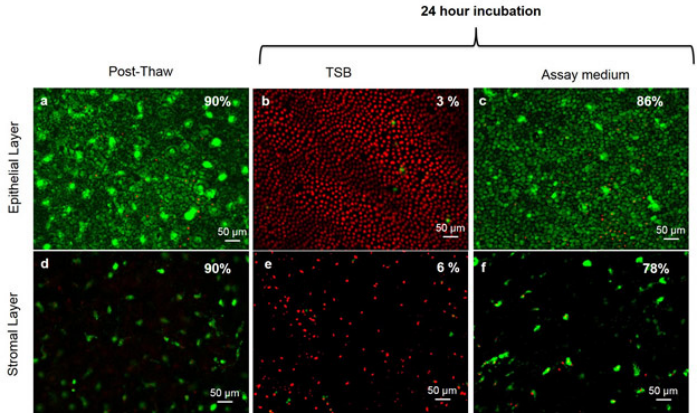


Figure 1. Effect of the medium on the cell viability of hCVAM (human cryopreserved viable amniotic membrane). The cell viability of hCVAM was evaluated using a LIVE/DEAD® Viability/ Cytotoxicity Kit after 24 hours of culturing hCVAM in TSB (b and e) or DMEM containing 10% FBS (assay medium) (c, f). Cell viability of hCVAM post-thaw without culturing (a, d) was used as a control. The numbers of viable and dead cells were counted using a fluorescent microscope, and the percent of viability from 2 lots of tissue (3 images/lot) were averaged. They are shown in the top right corner of each image. Live cells stained by Calcein AM are green fluorescent, and dead cells stained by ethidium homodimer-1 are red fluorescent. Scale bar = 50 µm.

consistent with previous reports.[10,11,12] Therefore, the assay medium (DMEM+10% FBS) was chosen for the antimicrobial testing of hCVAM.

hCVAM Inhibits Bacterial Growth *In Vitro*

S. aureus and *P. aeruginosa* are the most common bacteria present in chronic wounds and are often associated with wound-related infections.[20] These two pathogens are also well-known for their resistance to antibiotics.[21,22,23] For example, methicillin-resistant *S. aureus* (MRSA) alone accounts for more than 40% of hospital acquired infections.[24] Additionally, one third of *P. aeruginosa* clinical isolates are resistant to three or more antibiotics.[25] Moreover, secretion of exo- and endo-toxins and proteases by these two organisms have been associated with chronicity of the wound.[20] Therefore, we first examined the antimicrobial activity of hCVAM against *S. aureus* and *P. aeruginosa* using a modified AATCC Method 100 (a qualitative assay) and a liquid culture assay (a quantitative assay). Puracol Plus (a collagen dressing lacking antimicrobial activity) and Acticoat 7 (an antimicrobial silver dressing) served as negative and positive controls, respectively. In the modified Method 100 experiment, bacteria were inoculated directly onto the hCVAM or control samples, and after a 24-hour incubation, bacteria were eluted from samples. Measurements of the optical densities of solutions containing eluted bacteria resulted in a decrease in OD_{600} from 0.6 ± 0.03 to 0.1 ± 0.1 for *S. aureus* ($p<0.01$) and from 0.5 ± 0.1 to 0.07 ± 0.04 for *P. aeruginosa* ($p<0.01$) in the presence of hCVAM ***(Figures 2a and b)***.

Similarly, hCVAM demonstrated an inhibitory effect on both strains in a liquid culture assay ***(Figures 3a and b)***. The liquid culture assay is quantitative and enables calculations of bacterial growth inhibition in log reduction, which is a standard unit in microbiology expressing a magnitude of changes in bacterial cell numbers. The CFU counts for *S. aureus* and *P. aeruginosa* were 6.4 ± 1.5 and 5.3 ± 0.3, ***(Figures 3a and b)*** compared to their respective negative controls at 9.3 ± 0.3 and 11.2 ± 0.2. This results in statistically significant log reductions of *S. aureus*

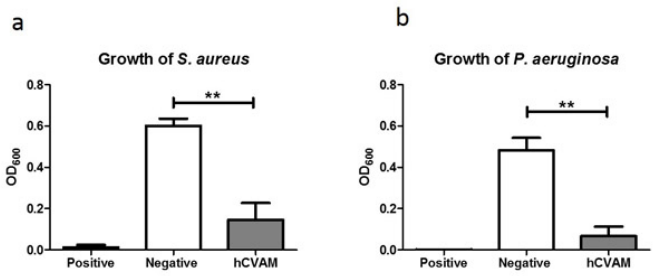


Figure 2. Growth inhibition of S. aureus and P. aeruginosa by hCVAM (human cryopreserved viable amniotic membrane) in a modified AATCC Method 100.
A 3 cm^2 piece of Acticoat 7 (positive control), Puracol plus (negative control), or hCVAM was placed onto agar plates. Approximately 100 CFU of *S. aureus* (a) or *P. aeruginosa* (b) were inoculated on the top surface of each test sample. After incubating for 18 hours, the samples were transferred to 1 mL of DMEM, and bacteria were eluted from the samples via vigorous vortexing. The Optical Density (OD) of the TSB-containing eluted bacteria was measured at 600 nm. Data are presented as mean ± SD of OD_{600} (n = 3 for controls and n = 6 for hCVAM). The student's t-test was used for statistical analysis comparing the negative and hCVAM. $^{**}p<0$.

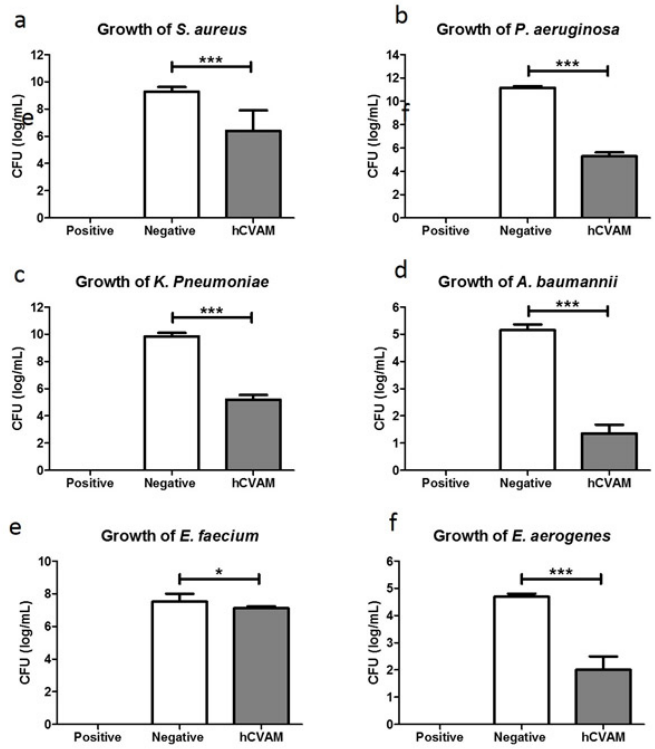


Figure 3. Growth inhibition of ESKAPE bacteria by hCVAM (human cryopreserved viable amniotic membrane) in a liquid culture assay.
A 3 cm^2 piece of Acticoat 7 (positive control), Puracol plus (negative control), or hCVAM was placed in tubes with 1 mL assay medium containing approximately 100 CFU of S. aureus (a), *P. aeruginosa* (b), *K. pneumoniae* (c), *A. baumannii* (d), *E. faecium* (e), or *E. aerogenes* (f). After incubation on a shaker at 37°C for 24 hours, the assay medium from each tube was serially diluted and plated onto TSB agar plates. The number of colony forming units (CFU) for each culture was calculated by using the colony count and dilution factor. Data are presented as mean±SD of one representative experiment out of two experiments for *E. faecium* and *E. aerogenes*, three experiment for *K. pneumoniae* and *A. baumannii,* and four experiments for *S. aureus* and *P. aeruginosa*. The student's *t*-test was used for statistical analysis comparing the negative control and hCVAM. $^{*}p<0.05$; $^{**}p<0.01$ and *** $p<0.001$.

and *P. aeruginosa* at 2.9 ± 1.5 and 5.8 ± 0.3, respectively ***(Figures 3a and b)*** p<0.001).

We also used the liquid culture assay to evaluate the effect of hCVAM on the growth of *E. faecium, K. pneumoniae, A. baumannii,* and *E. aerogenes*. These microorganisms have been known for rapid development of drug-resistance and evading the effects of conventional antibiotic treatment.[26] Results showed that hCVAM inhibited the growth of all tested microorganisms ***(Figure 3 and Table 1)***. Interestingly, the inhibitory effects of hCVAM on Gram-negative bacteria were more pronounced than on Gram-positive bacteria. More than a 5 log reduction of *K.pneumonia, A. baumannii,* and *P. aeruginosa* growth was observed in the presence of hCVAM, while hCVAM reduced the growth of Gram positive *E. faecium* by 0.6 ± 0.3 logs and *S. aureus* by 2.6 ± 1.5 logs. Our study demonstrated that hCVAM inhibits the growth of all ESKAPE pathogens.

ESKAPE	ATCC #	Gram stain	Clinical isolate	Growth reduction compared with control (log)
Enterococcus faecium	ATCC® 51559™	Positive	Yes	0.6 ± 0.3
Staphylococcus aureus	ATCC® 25923™	Positive	Yes	2.6 ± 1.5
Klebsiella pneumonia	ATCC® 700603™	Negative	Yes	5.1 ± 1.7
Acinetobacter baumannii	ATCC® 49466™	Negative	Yes	5.1 ± 0.9
Pseudomonas aeruginosa	ATCC® 15692™	Negative	Yes	6.6 ± 1.8
Enterobacter aerogenes	ATCC® 49469™	Negative	Yes	3.6 ± 1.4

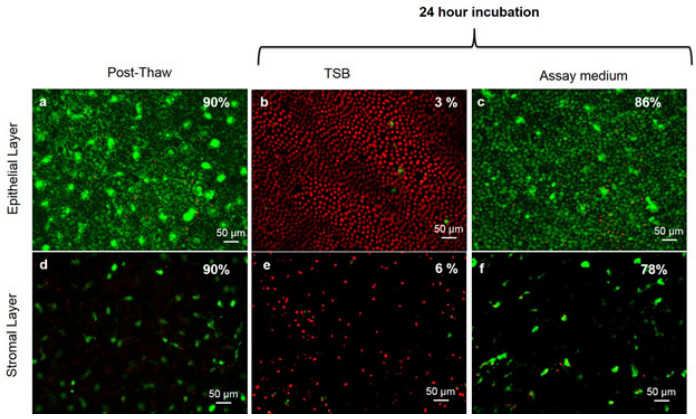
Table 1. Summary of the antimicrobial activity of hCVAM (human cryopreserved viable amniotic membrane) against ESKAPE bacteria.
For each bacterial strain, the effect of hCVAM on the growth of bacteria was evaluated in a liquid culture assay as described in "Materials and Methods". The log reduction of growth for each piece of hCVAM was calculated as the log10 CFU(negative control)- log10CFU(hCVAM)., and presented as mean±SD of two independent experiments for *E. faecium* and *E. aerogenes*, three experiment for *K. pneumoniae* and *A. baumannii,* and four experiments for *S. aureus* and *P. aeruginosa*.

DISCUSSION

In this study we demonstrated that hCVAM retains the intrinsic antimicrobial activity of human AM. It inhibits the growth of ESKAPE pathogens, which are the predominant microorganisms associated with chronic wounds and are prone to developing drug-resistance.

In utero, placental membranes serve as the first line of defense against entry of microbial pathogens to limit both the degree and frequency of bacterial colonization within the placenta and to prevent pregnancy complications.[17,27,28] Previous in vitro studies using a zone of inhibition assay on nutrient agar plates have demonstrated that fresh hAM inhibits the growth of several strains of bacteria.[17] However, in the same study, the authors failed to replicate the results in a quantitative broth culture assay. One explanation is that the bacterial nutrient broth is detrimental to human cells and therefore is not suitable for

testing fresh hAM, the cellular viability of which may contribute to the antimicrobial activity of the tissue. Our results support that TSB is not suitable for culturing hCVAM ***(Figure 1)***. Culturing pieces of hCVAM in TSB resulted in cell death ***(Figures 1b and e)***. Switching from TSB to DMEM supplemented with 10% FBS (assay medium) supported cell viability ***(Figures 1c and f)***. Using the assay medium that supports cell viability, we demonstrated a pronounced inhibition of ESKAPE bacteria growth in the hCVAM group in comparison with the negative control.



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The mechanisms by which hCVAM inhibits bacterial growth remains unknown. However, it has been reported that hAM contains antimicrobial peptides (AMPs) that exhibit a wide range of antimicrobial activities.[28,29,30,31] Immunohistochemical analysis demonstrated that β-defensins 1-3, calprotectin, LL37, elafin, and bacterial/permeability-increasing protein are present in hAM.[32] Other studies have shown that amniotic cells can secrete AMPs.[33] Human AM contains several types of cells including epithelial and mesenchymal stem cells (MSCs).[34] Amniotic epithelial cells have been reported to release β-defensins when stimulated by bacteria or bacterial lipopolysaccharide (LPS),[33,35] and MSCs release LL-37 upon stimulation with LPS or bacteria.[36] The presence of AMPs in hCVAM and their regulation in the absence and presence of different bacterial strains or bacterial antigens, such as LPS, are currently under investigation.

Our results show differences in the magnitude of growth inhibition by hCVAM between Gram-positive and Gram-negative bacteria. In the presence of hCVAM, 4-7 logs of growth reduction were observed for Gram-negative bacteria, whereas 1-3 logs of growth reduction were recorded for Gram-positive bacteria. The preferential activity of hCVAM against Gram-negative strains may be associated with the presence of particular types of AMPs in hAM. For example, β-defensin 2 specifically targets Gram-negative bacteria and Candida, but not Gram-positive *S. aureus*.[37]

Studies of the bacterial distribution in non-healing wounds reveal that Gram-negative *P. aeruginosa* populates the deeper layer of wound tissues and is often involved in biofilm formation (a community of microorganisms covered by glycocalyx), which is resistant to antibiotic treatment.[38] The preferential activity against *P. aeruginosa* indicates that hCVAM is particularly suitable for chronic wounds.

In summary, hCVAM retains the antimicrobial activities of fresh hAM. Such properties are critical for treatment of chronic wounds that are characterized by the presence of low grade infections. In this study, we have demonstrated that hCVAM inhibits the growth of chronic wounds-associated bacteria in vitro. Our ongoing studies will investigate the role of retention of the viable cells in hCVAM and the expression of specific AMPs.

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