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Lyopreserved amniotic membrane is cellularly and clinically similar to cryopreserved construct for treating foot ulcers

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Abstract

We compared cellular viability between cryopreserved and lyopreserved amniotic membranes and clinical outcomes of the lyopreserved construct in a prospective cohort study of 40 patients with neuropathic foot ulcers. Patients received weekly application of lyopreserved membrane for 12 weeks with standard weekly debridement and offloading. We evaluated the proportion of foot ulcers that closed, time to closure, closure trajectories, and infection during therapy. We used chi-square tests for dichotomous variables and independent t-tests for continuous variables with an alpha of $\alpha = .10$. Cellular viability was equivalent between cryo- and lyopreserved amniotic tissues. Clinically, 48% of subjects' wounds closed in an average of 40.0 days. Those that did not close were older (63 vs 59 years, $P = .011$) and larger ulcers at baseline (7.8 vs 1.6 cm^2, $P = .012$). Significantly more patients who achieved closure reached a 50% wound area reduction in 4 weeks compared with non-closed wounds (73.7% vs 47.6%, $P = .093$). There was no difference in the slope of the wound closure trajectories between closed and non-closed wounds (0.124 and 0.159, $P = .85$), indicating the rate of closure was similar. The rate of closure was 0.60 mm/day (SD = 0.47) for wounds that closed and 0.50 mm/day (SD = 0.58) for wounds that did not close ($P = .89$).

KEYWORDS

amniotic membrane, diabetic foot ulcer, infection, neuropathy

1 | INTRODUCTION

Failure of a wound to heal in an organised and timely fashion is complex and multifactorial. Chronic wounds are characterised by a persistent inflammatory state resulting from local factors such as necrotic tissue, high microbial burden, low oxygen, repetitive injury, and systemic disease processes like peripheral arterial disease

and hyperglycemia. As part of a comprehensive treatment, human amniotic membrane (AM) has been widely applied in the management of diabetic foot ulcers (DFUs), burns, dermatological defects, and ocular surface reconstruction.

The preparation of most amniotic tissue products uses dehydration methods that do not require the product to be stored in a freezer or thawed before application in clinic. The biological activity of AM tissue has been thought to be dependent on the preservation of its components, including extracellular matrix (ECM), growth factors, and viable cells.[1] Cryopreserved AM retains these viable components and has greater anti-inflammatory, antioxidant, angiogenic, and chemoattractive activities compared with devitalized AM.[2-4] Laboratory comparisons of cryopreserved and dehydrated amniotic tissue have reported differences in structure and cell viability that suggest cryopreserved tissue has more similarities to native tissue than dehydrated products.[5-7]

There are currently many amniotic tissue products commercially available. In the past decade, this genre of product has shown a dramatic increase in the commercial marketplace. Providing evidence that these products are effective has been the impetus for many randomised clinical trials (RCTs).[8] One of the commercially available cryopreserved AM products (Grafix PRIME®, Smith +Nephew, Columbia, Maryland) is comprised of an ECM rich in collagen, growth factors, fibroblasts, mesenchymal stem cells, and epithelial cells native to the tissue. A lyopreservation technique to preserve living tissues was developed based on accumulated data on cell preservative agents, lyophilization processes, and preliminary protocols for mammalian cell drying.[9-15] This process allows for shelf-stable storage at room temperature, removing the barrier of having a medical quality freezer.[8] RCTs using cryopreserved and lyopreserved AM have shown a higher proportion of closure in the treatment of DFUs and faster closure compared with standard treatments in RCTs.[16] Using the lyopreserved construct, Ananian showed wound closure rates of 65.8% in DFU that had been present for <12 months with median time to closure of 63 days.[17]

In this paper, we report the results of a bench study of cellular viability of cryo- and lyopreserved amniotic tissue samples and the clinical outcomes of a 12-week cohort study. The primary outcome of the study was the proportion of ulcers that achieved closure during treatment. Secondary outcomes included the time to closure, adverse events (AEs) (foot-related infection, all hospitalizations, foot-related hospitalizations and amputations). In addition to complete closure, we evaluated 50% wound area reduction (WAR) by 4 weeks and wound closure trajectories.

Key Messages

- there is no difference in cellular viability between cryo- and lyopreserved constructs
- trajectories for wound closure were similar between groups who achieved closure and those that did not
- wounds that were larger and had been present longer took longer to close

2 | MATERIALS AND METHODS

2.1 | Laboratory studies

We evaluated a lyopreserved viable AM sample (GrafixPL PRIME®, Smith+Nephew, Columbia, Maryland) for cell viability and compared it with the cryopreserved viable AM (Grafix PRIME®, Smith+Nephew, Columbia, Maryland). For this study, three individual samples of cryopreserved and lyopreserved AM derived from three different donors were assessed for cell viability. Each 3 cm^2 piece was cut into four sections. Each section was stained with SYTO 24 green fluorescent nucleic acid stain (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts) staining viable cells and Ethidium Homodimer-1 red fluorescent dye (Invitrogen, Thermo Fisher, Waltham, Massachusetts) staining dead cells. Stained samples were analysed using fluorescent microscopy. Imaging was performed for 10 microscopic fields for each section at 5× and 10× magnification for viable and non-viable cells (in green and red fluorescent channels, respectively). Images of viable and non-viable cells were overlapped and blindly assessed using a semiquantitative scale: non-viable, <50% viable, ~50% viable, or >50% viable; representative images shown in Figure 1.

2.2 | Clinical trial

The prospective cohort study was approved by the University of Texas Southwestern Medical Center Institution Review Board (STU 022018-035) and reported in clinicaltrials.gov (NCT03742440). This prospective cohort study included 40 patients that were treated between December 2018 and August 2019 with lyopreserved amniotic membrane (LAM) once weekly for 12 weeks.

Study inclusion criteria included patients between ages 18 and 89, able to provide informed consent, ulceration below the ankle for 30 days or longer, and an ankle

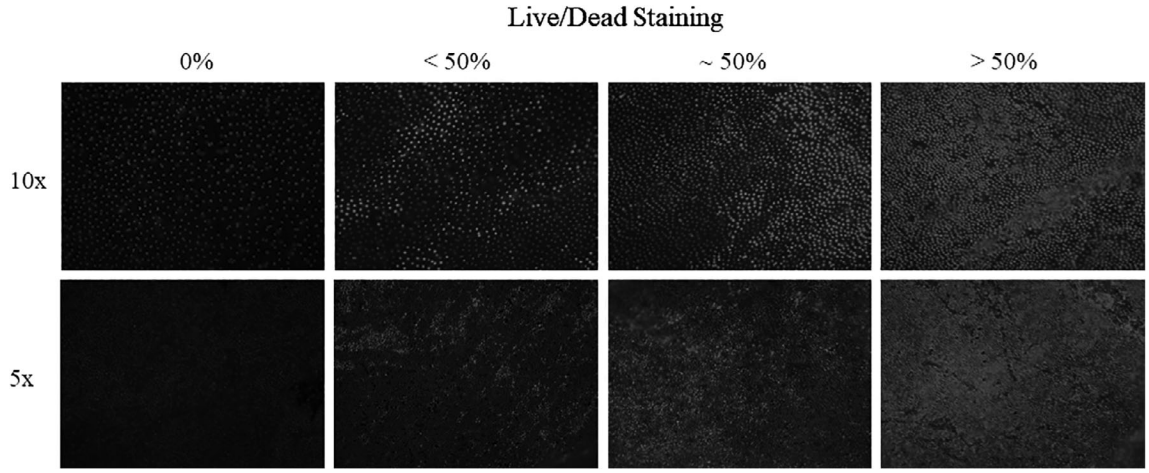

FIGURE 1 Live/dead staining of cryopreserved (above) and lyopreserved (below) amniotic tissue at 5× and 10× magnification. Green are viable cells, and red are non-viable

brachial index (ABI) >0.5. The study excluded patients with a history of poor compliance with follow-up visits, gangrene, untreated osteomyelitis, widespread malignancy, active alcohol or substance abuse such as cocaine, heroin, or methamphetamines, currently pregnant or planning pregnancy during the course of intended participation in the study, nursing or actively lactating.

After informed consent was obtained, study subjects all received treatment with LAM once weekly for the 12-week evaluation period. Wounds were sharply debrided at each visit and offloading of post-op shoe (Med-Surg Post-Operative Shoe, Darco, Huntington, West Virginia), removable cast boot (DH Offloading Walker, Össur, Reykjavík, Iceland), and total contact cast was provided based on the location of the ulcer and the postural stability or fall risk of the subject. We evaluated patients in clinic every 7 days for a total of 84 days. Data collected during the study included the following: demographics, comorbidities, history of drug, alcohol, tobacco use, wound location and aetiology, and wound duration. Sensory neuropathy was evaluated with a 10-g Semmes Weinstein monofilament and Vibration Perception Threshold Testing (VPT) (Vibration Perception Threshold Meter, Xilas Medical Inc., San Antonio, Texas) at the great toe and medial malleolus. Sensory neuropathy was defined as either VPT >25 or any site missed with 10-g monofilament. Tissue oxygenation was evaluated with hyperspectral imaging (SnapshotNIR, Kent Medical, Calgary, California) and perfusion with ABI. The lowest systolic pressure from dorsalis pedis or posterior tibial arteries was used to define ABI. Wound size was recorded using a 3D measurement device to evaluate area (inSight, eKare, Fairfax, Virginia).

Study variables were summarised as median, means, and SDs for continuous variables and proportions or percentages for categorical variables. An analysis of variance test was used for differences in continuous variables (SPSS, IBM, Chicago, Illinois). For categorical variables, we used Chi square and Fisher's exact test to compare the proportion of outcomes. We used a regression model to compare the wound closure trajectories between patients that closed and those who did not. Because this was an exploratory pilot study, we used an alpha of $\alpha = .10$.

3 | RESULTS

Each sample of cryo and lyopreserved amniotic tissue was sectioned into 40 individual sections and categorised as non-viable, less than 50% viable, ~50% viable, or >50% viable. The number of samples in each viability category is averaged for each tissue sample. The viability assessment distribution was equivalent between cryopreserved AM and LAM samples in each assessment category (Figure 2). Non-viable (cryo 0.67 ± 1.15 vs lyo 2.67 ± 4.62), <50% (5.67 ± 7.37 vs 8.39 ± 6.33), ~50% (12.67 ± 5.86 vs 2.31 ± 6.33), and >50% (21.00 ± 12.53 vs 12.10 ± 24.67).

In the prospective cohort study, there were no differences in patient demographics or comorbidities among patients who achieved closure and those who did not (Table 1). After 12 weeks of therapy, 48% (n = 19) subjects achieved closure. The average time to closure was 40.0 (SD = 20.1) days. In addition to the proportion of closed ulcers, we used a 50% WAR as a surrogate for ulcer closure after 4 weeks of therapy and wound closure trajectories to compare patients who achieved closure and those who did not. Overall, 60% of patients had a 50% WAR. Significantly more patients who achieved closure reached a 50% WAR in 4 weeks compared with

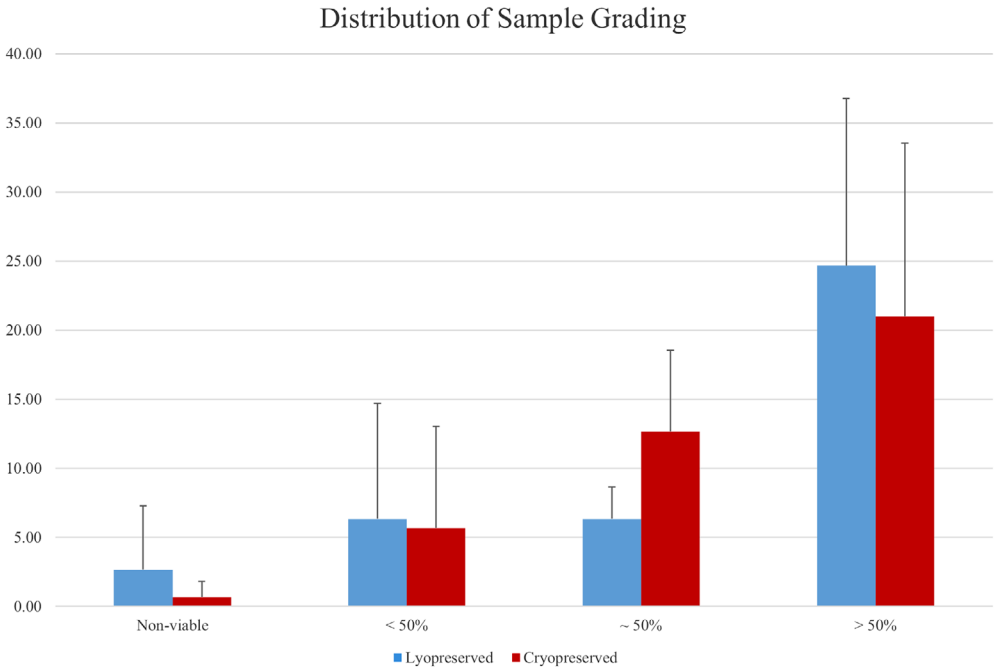

FIGURE 2 The viability assessment distribution was equivalent between cryo- and lyopreserved amniotic membrane samples in each assessment category

those who did not (73.7% vs 47.6%, $P = .093$). Regression on average wound size for the 12 weeks of the study using a standard regression model with an indicator variable for closure {yes, no} and an ordinal variable for each week, showed no statistical evidence for an interaction term ($P = .85$). Wound closure trajectories were shown for the change in ulcer size from baseline (Figure 3A) and the percent WAR from baseline (Figure 3B). There was no significant difference between the slopes of the two wound closure trajectories in Figure 3A (−0.124 and −0.131, respectively), indicating that the rate of closure was similar in both groups. The rate of closure was 0.60 mm/day (SD = 0.47) for patients who achieved closure and 0.50 mm/day (SD = 0.58) for patients who did not achieve closure ($P = .98$).

Among people that did not close during the 12-week study, five subjects were identified to have no response to treatment, defined as a percent WAR of <30% at the end of the study. The percent WAR for these five subjects averaged a 5% increase and ranged from a 29.0% decrease to a 45% increase. Figure 4A shows the five subjects with a nearly flat closure trajectory (slope = 0.018), as well as trajectories for patients who achieved closure and those who did not achieve closure excluding the five outliers. The trajectories for patients who closed and those who did not close (excluding outliers) were very similar (−0.124 and −0.159, respectively). The rate of closure was 0.60 mm/day (SD = 0.47) for patients who achieved closure, 0.58 mm/day (SD = 0.59) for patients who did not achieve closure, and an increase of 0.18 mm/day (SD = 0.44) for wounds with no progress. A comparison of percent WAR per week is shown in Figure 4B.

From baseline demographics, patients that did not close were older (63 vs 59 years, $P = .011$), had larger ulcers at baseline (7.8 vs 1.6 cm^2, $P = .012$), and had ulcers of longer duration (60.0 vs 130.0, $P = .062$). During the course of the study, patients who did not close were more likely to develop a new ulcer (38.1% vs 10.5%, $P = .044$), take an antibiotic (36.8% vs 66.7%, $P = .059$), and experience an AE (81.0% vs 31.6% $P = .002$; Table 2) compared with patients that closed. Fifty-eight percent of patients had at least one AE during treatment. AEs included 25.0% (n = 10) foot infection, 5% (n = 2) hospitalisation for infection, 10.0% (n = 4) hospitalisation for non-foot related disease processes, and 2.5% (n = 1) for amputation or foot surgery. More patients who did not close received antibiotics at any time during treatment (66.7% vs 36.8%, $P = .059$). Several patients received antibiotics the entire duration of the study. There was a trend that was not statistically significant that these patients were less likely to close (23.8% vs 5.3%, $P = .10$).

4 | DISCUSSION

When the cryopreserved product used in this study was compared with other dehydrated products, the cellular activity was greater in the cryopreserved tissue. For instance, Duan-Arnold and colleagues[3] compared cytokine expression between cryopreserved AM (GrafixPRIME®) and a dehydrated product (Epifix®, MiMedx, Marietta, Georgia) and reported significantly higher levels of viable cells (80% vs 0%, $P < .001$) and downregulation of TNF-α and IL-1α and upregulation of PGE2 and IL-10 in the

TABLE 1 Patient demographics, comorbidities, and past medical history

	Closed n = 19	Not closed n = 21	*P*-Value
Male	15 (78.9%)	17 (81.0%)	.874
Age	59.0 (22.0)	63.0 (14.0)	.011
BMI (kg/m^3)	30.2 (8.6)	28.2 (9.8)	.406
Race			
Caucasian	7 (36.8%)	18 (85.7%)	.001
African American	5 (26.3%)	2 (9.5%)	.162
Hispanic	7 (36.8%)	1 (4.8%)	.010
Substance use history			
Tobacco	5 (26.3%)	7 (33.3%)	.629
Alcohol	7 (36.8%)	8 (38.1%)	.935
Illicit drugs	2 (10.5%)	1 (4.8%)	.478
Foot ulcer history on study foot	12 (63.1%)	14 (66.7%)	.816
Amputation history	10 (52.6%)	13 (61.9%)	.554
Offloading			
Boot	9 (47.4%)	8 (38.1%)	.553
Shoe/sandal	10 (52.6%)	13 (61.9%)	.553
Type II diabetes	12 (63.2%)	17 (89.5%)	.208
Diabetes duration (years)	16.0 (28.0)	17.0 (17.5)	.982
Coronary artery disease	3 (15.8%)	5 (23.8%)	.527
Congestive heart failure	2 (15.8)%	4 (14.3%)	.894
Chronic kidney disease	14 (73.7%)	13 (61.9%)	.427
End stage renal disease	1 (5.3%)	0 (0%)	.475
Index wound area (cm^2)	1.5 (1.6)	3.7 (7.8)	.012
Wound duration (days)	60.0 (179.0)	130.0 (382.0)	.062
Osteomyelitis history in study foot	7 (36.8%)	3 (14.3%)	.100
Wound location			
Plantar	14 (73.7%)	15 (71.4%)	.873
Dorsal	5 (26.3%)	4 (19.0%)	.583
Glycated haemoglobin (%)	6.9 (1.7)	7.1 (2.2)	.630
Albumin (g/dL)	3.7 (0.7)	3.5 (0.7)	.320
Sensory neuropathy	16 (84.2%)	21 (100%)	.098
Abnormal 10-g monofilament	15 (78.9%)	18 (87.7%)	.574
Vibration perception—ankle (volt)	44.2 (41.0)	64.2 (42.2)	.481
Vibration perception—forefoot (volt)	50.1 (55.8)	80.6 (55.5)	.026
Ankle brachial index	1.1 (0.5)	1.1 (0.3)	.941
Hyperspectral imaging			
Dorsal oxygen saturation	66.0 (40.0)	67.5 (21.0)	.477
Dorsal oxygenated haemoglobin	39.0 (50.0)	49.5 (58.0)	.035
Dorsal deoxygenated haemoglobin	19.0 (23.0)	28.5 (12.0)	.700
Plantar oxygen saturation	82.0 (13.0)	85.0 (7.0)	.45
Plantar oxygenated haemoglobin	89.0 (97.0)	114.0 (46.0)	.089
Plantar deoxygenated haemoglobin	21.0 (12.0)	19.0 (8.0)	.122

Note: Dichotomous variables are presented as N (%). Continuous variables are presented as median (interquartile range).

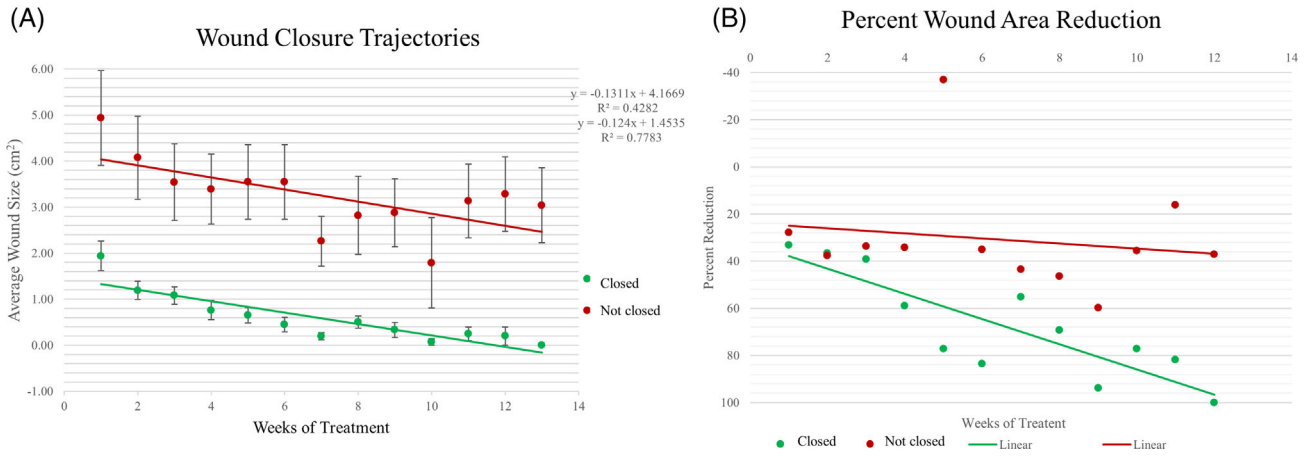

FIGURE 3 A, Graph of wound size of healers compared with non-healers at each visit. There was no difference between the slope of healers (−0.13) and slope of non-healers (−0.12). B, Graph of percent wound area reduction at each visit

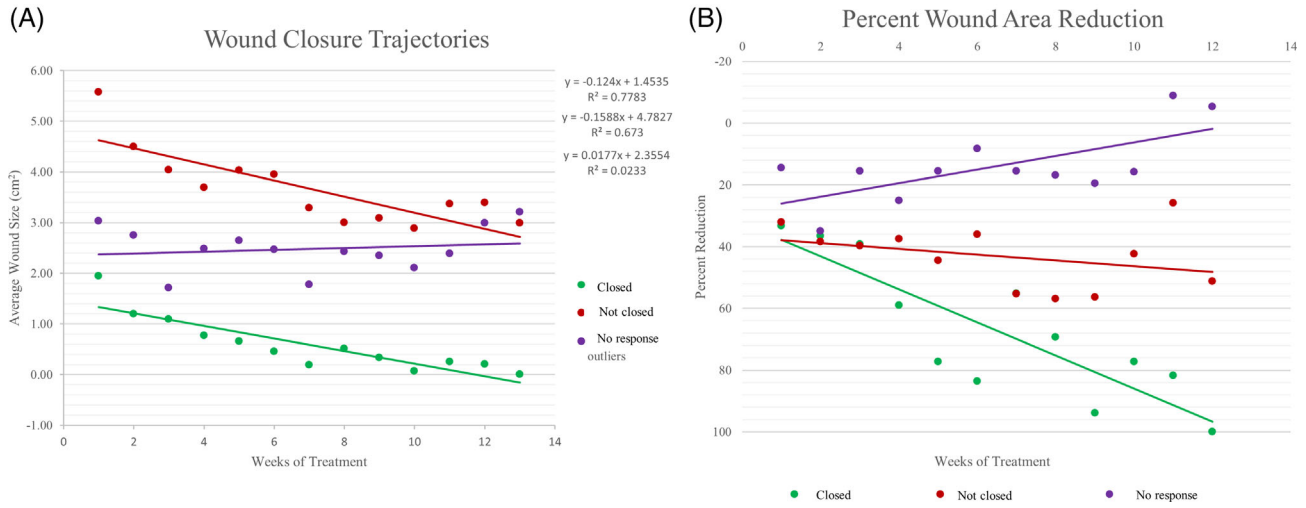

FIGURE 4 A, Graph of healers, non-healers, and non-healer outliers. Purple line represents the five non-healing patients who had less than 12% wound area reduction at the end of study. Slope of their trajectory was 0.018. B, Graph of percent wound area reduction of the same three groups

cryopreserved product as compared with a dehydrated amniotic tissue product. However, these findings are likely due to the difference in the viability of different amniotic products. To this end, our study demonstrated similar viability when lyopreserved and cryopreserved versions of the same product were compared. Our independent results were similar to other published findings that compared cryopreserved and lyopreserved amniotic tissue from this construct.[18]

The primary outcome of the prospective cohort study was to evaluate the proportion of ulcers that achieved complete closure in 12 weeks in patients treated with LAM. The proportion of patients who achieved complete closure was 48%. Interestingly, the ulcer size was significantly larger in patients who did not achieve closure. Wound closure trajectories and 50% WAR after 4 weeks were evaluated in order to adjust for the difference in baseline wound area. The wound closure trajectories were similar among patients who achieved closure and those who did not, suggesting that wound size is a factor in wound closure within a specified timeframe.

Several studies have identified that larger ulcers are less likely to heal within a defined time period[19] and advocate for using wound closure trajectories to provide more data on the continuum of the non-linear closure process as a "moving picture" as opposed to a "snapshot" of traditional dichotomous endpoints in chronic wounds.[20-23] Evaluation of wound closure trajectories and the time to close per week would allow a better format to compare the results of clinical trials, especially because most studies use very similar inclusion and exclusion criteria.

TABLE 2 Wound closure and outcome measures

	Closed n = 19	Not Closed n = 21	*P*-Value
50% wound area reduction in 4 weeks	14 (73.7%)	10 (47.6%)	.093
Subjects who received antibiotics at any time	7 (36.8%)	14 (66.7%)	.059
Subjects on antibiotics therapy entire treatment	1 (5.3%)	5 (23.8%)	.101
Adverse events			
Subjects with at least one adverse event	6 (31.6%)	17 (81.0%)	.002
Subjects developed foot infection	3 (15.8%)	7 (33.3%)	.201
Subject developed new foot ulcer	2 (10.5%)	8 (38.1%)	.044
Subject hospitalised for foot related issue	0 (0.0%)	2 (9.5%)	.489
Subject hospitalised for non-foot related issue	1 (5.3%)	3 (14.3%)	.342
Subject required foot surgery for infection	0 (0.0%)	1 (4.8%)	1.0
Subject required partial foot amputation	0 (0.0%)	1 (4.8%)	1.0

Note: Dichotomous variables are presented as N (%). Continuous variables are presented as median, mean (SD).

Unfortunately, wound closure trajectories are usually not reported in DFU RCTs and when they are, data is not presented in a uniform way. Driver et al reported an ulcer healing rate of 7.2% per week in healers and 4.8% per week in non-healers.[20] Both Zelen and Cazzell show data in graphs but did not report specific rates.[21,22] Problems of comparing closure in multiple groups are averted with a trajectory analysis method if the relevant data is reported. Wounds are normalised by using the percentage closure in studies that include considerable variation in size.[24] *In our prospective cohort study, the proportion of ulcers that healed in 12 weeks was only 48%. At first glance, this seems unimpressive, although not unlike the rates reported in other DFU RCTs.*

There were five patients that had no response to treatment, defined as <30% change in WAR by the end of the study. When these five patient outliers were removed from analyses, the slopes for the patients that achieved closure and those who did not were very similar, suggesting the rate of closure would have been around 87.5% if the study had not been limited to 12 weeks of treatment.

To the best of our knowledge, this is the first study that compares wound closure outcomes in patients routinely treated with antibiotics for foot ulcers that are not clinically infected as compared with ulcers that receive treatment without antibiotics. The role of antibiotics and ulcer healing is often debated. The International Working Group on the Diabetic Foot[25] and Infectious Diseases Society of America[26] both recommend that antibiotics should not be used routinely for ulcers that do not have clinical signs of infection. In this study, one of the investigators routinely prescribes oral antibiotics throughout the course of ulcer treatment. The majority of subjects that received routine antibiotics (83%) did not heal, but the association was not significant ($P = .10$). The use of antibiotics in wound healing needs to be evaluated to determine the effect on the healing process.

There are several important limitations of this study. The most important limitations of the study were that it did not include a control arm with either cryopreserved product or standard of care, and the study was underpowered. While the results appear to be similar to other published results, at best we might consider the results "proof of concept." In this cohort study, all of the study subjects were from a single site, and larger RCTs recruit from multiple sites. Because this was a pilot study, we used an alpha of $\alpha = 10\%$ rather than the more traditional 5% value. The use of a *P*-value of 5% is a convention that has been widely adopted, but it is not set by a specified law of mathematics or science. The selected *P*-value has to do with the goal of the analysis and the tradeoff of type 1 and 2 errors. We believe the exploratory nature of this project warranted the use of a higher alpha value.

In conclusion, the viability of LAM is similar to that of the cryopreserved product. When evaluated clinically, 48% of the study subjects achieved closure in 12 weeks; however, most patients, excluding outliers, demonstrated very similar wound healing trajectories during the LAM treatment course, suggesting a high rate of closure in both groups. Results suggest that larger wounds require more time to achieve closure beyond the 12 weeks of treatment in the present study. Overall, these studies suggest that the LAM product is a clinically effective alternative to cryopreserved AM and reduces the storage and handling process that is required with cryopreserved product. These findings require further

investigation in a treatment controlled and statistically significant RCT.

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CONFLICT OF INTEREST

Kathryn E. Davis has received research funding from EO2 Concepts, Inc.; Smith+Nephew; Integra; Cardinal Health, Astra Zeneca, Avazzia, and Pluristem.

Amanda L. Killeen has received research funding from EO2 Concepts, Inc.; Smith+Nephew; Integra; Cardinal Health, Astra Zeneca, Avazzia, and Pluristem.

David Farrar and Zachary D. Berriman-Rozen declare no conflicts of interest.

Katherine M. Raspovic has received funding from Smith+Nephew and has consulted for Orthofix.

Matthew Malone has received funding from Medline Industries.

Lawrence A. Lavery has received research funding from EO2 Concepts, Inc.; Smith+Nephew; Integra; Cardinal Health, Astra Zeneca, Avazzia, and Pluristem.

DATA AVAILABILITY STATEMENT

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

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