Effects of acellular equine amniotic allografts on the healing of experimentally induced full-thickness distal limb wounds in horses

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Funding information
AniCell Biotech; Fund for Orthopedic Research in honor of Gus and Equine athletes (F.O.R.G.E)

Abstract
Objective: To characterize the growth factors contained in equine amniotic membrane allograft (eAM; StemWrap scaffold and StemWrap+ injection) and to evaluate the effect of eAM on equine distal limb wound healing.

Study design: Prospective experimental controlled study.

Sample population: Eight adult horses.

Methods: Transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), epidermal growth factor, platelet-derived growth factor-BB, and prostaglandin E2 (PGE2) concentrations in StemWrap+ were assessed with enzyme-linked immunosorbent assay. Two full-thickness 6.25-cm² skin wounds were created on each metacarpus. On one forelimb, one wound was treated with eAM, and the other was left untreated (eAM control). On the contralateral limb, one wound was treated with a silicone dressing, and the other served as negative control. Three-dimensional images were obtained to determine wound circumference and surface area analyses at each bandage change until healed. Excessive granulation tissue was debrided once weekly for 4 weeks. Biopsy samples were taken to evaluate quality of wound healing via histologic and immunohistochemistry assays.

Results: StemWrap+ contained moderate concentrations of TGF-β1 (494.10 pg/mL), VEGF (212.52 pg/mL), and PGE2 (1811.61 pg/mL). Treatment of wounds with eAM did not affect time to healing or histologic quality of the healing compared with other groups but was associated with increased granulation tissue production early in the study, particularly on day 7.

Conclusion: Application of eAM resulted in increased granulation tissue production while maintaining appropriate healing of experimental wounds.

Clinical significance: Use of eAM is likely most beneficial for substantial wounds in which expedient production of large amounts of granulation tissue is desirable.
1 | INTRODUCTION

Horses are known to display a dampened and delayed inflammatory response to wounding, thereby affecting subsequent stages of healing and potentially resulting in a chronic wound state.1,4 Distal limb wounds are particularly common in horses, often extensive, and heavily contaminated by entrainment of the limb in fencing or other material. These wounds must rely on second intention healing and can impose substantial financial and management burdens on owners. These factors explain why wounds are among the leading reasons for euthanasia of horses.5 Current strategies to manage wounds seek to enhance the environment and the inflammatory response to promote appropriate wound healing.6-14 Therapeutic options include administration of regenerative biological products, treatment with extracorporeal shockwave, and application of specialized wound dressings and ointments during specific stages of the wound healing process.6-14 However, additional research is required to improve the treatment of extensive and/or chronic distal limb wounds in horses.

The triad concept of tissue engineering relies on cells, scaffolds, and signaling molecules that enhance cellular growth.15,16 Synthetic and natural scaffolds are designed to provide support for cells to attach, proliferate, and differentiate, ultimately striving for tissue regeneration.16-18 Amniotic membranes provide extracellular matrix components and cytokines that are integral for wound healing. They have consequently been investigated as a biological source for scaffolds.19-22 Amniotic scaffolds have been produced after extensive processing and decellularization techniques to address concerns regarding transmissible diseases or management burdens on owners. These factors explain why wounds are among the leading reasons for euthanasia of horses.5 Current strategies to manage wounds seek to enhance the environment and the inflammatory response to promote appropriate wound healing.6-14 Therapeutic options include administration of regenerative biological products, treatment with extracorporeal shockwave, and application of specialized wound dressings and ointments during specific stages of the wound healing process.6-14 However, additional research is required to improve the treatment of extensive and/or chronic distal limb wounds in horses.

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Human amniotic membrane scaffold products have been developed to contain quantifiable levels of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor (TGF)-β1, basic fibroblastic growth factor, and prostaglandin E2 (PGE2),21,28-30 each of which is known to play a role in wound healing.3,6,31-41 In addition, various cell types including fibroblasts and keratinocytes have been found to adhere to, proliferate, and function in the presence of amniotic scaffolds.42-45 In vivo studies in man have substantiated these in vitro findings that these products improve the rate of wound healing.24,25,46,47 Furthermore, several case series have documented the use of decellularized amniotic membrane scaffolds facilitating the healing of pharyngocutaneous fistulas, burns, bed sores, and chronic wounds that were nonresponsive to standard-of-care treatments in human patients.23,48-50 Human amniotic membrane products have been commercially available and used clinically over the past decade in human medicine.21,24,47,50 Similar equine products have recently become commercially available, including a decellularized and dehydrated equine amniotic membrane scaffold (StemWrap; AniCell Biotech, Chandler, Arizona) and an injectable acellular liquid morcellized amniotic membrane product (2 mL/vial) reported to contain growth factors and extracellular matrix particles (StemWrap+; AniCell Biotech). These allogeneic products are collectively referred to as an equine amniotic membrane allograft (eAM) by the company and can be terminally sterilized for enhanced safety. eAM provides two components of the tissue engineering triad while relying on endogenous cells to migrate across the scaffold during wound healing.

The authors have been applying eAM on horses with distal limb wounds that are chronic and/or in areas of high tension with substantial tissue loss and exposed underlying anatomic structures such as bone or tendon. The observed outcomes prompted this study, in which we seek to characterize the growth factors contained in eAM and to evaluate the effect of eAM on the healing of distal limb wounds, including the production of granulation tissue. An established acute wound healing model was used7,8,10-12 because there is no universal definition or model of chronic wound healing, and the use of clinical trauma cases would have introduced a substantial number of uncontrollable variables for an initial efficacy study. We hypothesized that eAM would contain growth factors and cytokines involved in wound healing, which would accelerate healing and improve histological healing quality compared with other treatments evaluated. In addition, we hypothesized that eAM would be associated with more granulation tissue compared with other treatments because of the scaffold contained in eAM and on the basis of our clinical observations.

2 | MATERIALS AND METHODS

2.1 | Growth factor and cytokine analyses of StemWrap+

Transforming growth factor β1, VEGF, EGF, PDGF-BB, and PGE2 concentrations were assessed in duplicate aliquots of the StemWrap+ lot used in the current study as well as three independent lots (manufactured from independent amnion harvests). Samples were centrifuged at 10000 g at room temperature for 1 minute to pellet the extracellular matrix particles contained in the product, and the supernatant was analyzed by using the commercially available human TGF-β1 Emax enzyme-linked immunosorbent assay (ELISA; Promega, Madison, Wisconsin), equine VEGF-A ELISA (Kingfisher Biotech, St Paul, Minnesota), human EGF ELISA (R&D Systems, Minneapolis, Minnesota), human PDGF-BB ELISA (R&D Systems), and PGE2 ELISA (Enzo Life Sciences, Farmingdale, New York) kits. The supernatant was diluted 1:5 for the
TGF-β1 ELISA kit only as required by the acidification step of that kit according to the manufacturer's directions. These kits were chosen because they have been previously validated for use on equine samples and used by our group. Standards in each kit were used to generate standard curves, and samples were analyzed for optical density on a multiple detection plate reader (Synergy 2; BioTek Instrument, Winooski, Vermont) at 450 nm with wavelength correction set at 540 nm.

2.2 | Animals

This study was conducted in a manner consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/olaw.htm), the Animal Welfare Acts (US PL 89–544;91–579;94–279), and the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (http://www.fass.org/page.asp?pageID=216). The North Carolina State University Institutional Animal Care and Use Committee approved the use of horses in this study (Protocol 16–253; PI: L.V.S.).

Eight adult horses (five geldings and three mares) of various breeds (five thoroughbreds, one Dutch warmblood, one quarter horse, and one haflinger) between the ages of 2 and 10 years were used. All horses received a full physical examination and were determined to be clinically healthy and free of dermatological disease prior to study inclusion. Throughout the duration of the study, horses were housed in indoor temperature-controlled box stalls.

2.3 | Surgical procedure (experimental wounds)

Each horse was sedated with detomidine hydrochloride (0.01 mg/kg intravenously) and butorphanol tartrate (0.01 mg/kg intravenously) for the standing surgical procedure. The dorsolateral aspect of both metacarpi was clipped and aseptically prepared, after which a horizontal line block was administered by infusing 2% lidocaine hydrochloride subcutaneously at the proximal most aspect of the metacarpus. A 6.25-cm² square template (2.5-cm width × 2.5-cm height) was used to outline the surgical sites on the metacarpus with a sterile marker. By following the outlined templates and guided by a ruler, two full-thickness skin wounds were created, leaving the subcutaneous tissue intact on the dorsolateral aspect of each metacarpus with a No. 10 scalpel blade (Figure 1A). Wounds were positioned in line from proximal to distal with a 2-cm distance between wounds. In total, 32 wounds were created for the study (four per horse). All wounds were bandaged with a nonadherent dressing and a compression distal limb bandage to facilitate hemostasis. Bandages were kept in place for 3 days, at which time wounds were administered respective treatments.

![FIGURE 1](image-url) A. Example of surgically created 6.25-cm² proximal and distal wounds on the left forelimb of one of the study horses. The distal most aspect of each distal wound was at the level of the distal end of the fourth metacarpal bone. The distal most aspect of each proximal wound was 2 cm proximal to the proximal most aspect of each distal wound. Templates and rulers were used during wound creation to ensure consistent wound sizing. B. Schematic of the experimental design with partial randomization consisting of paired eAM/eAM control and silicone dressing/negative control wounds with equal distribution of left and right limbs as well as proximal and distal position on the limb. eAM, equine amniotic membrane allograft
2.4 | Experimental design

We evaluated four different treatment/control groups: (a) eAM, consisting of StemWrap and StemWrap+ products; (b) eAM control, which was potentially subject to the local subcutaneous injection effects of the StemWrap+; (c) commercially available occlusive silicone gel dressings (CICA-CARE; Smith & Nephew, Andover, Massachusetts) known for their ability to reduce the formation of granulation tissue on the distal limbs of horses as a comparison; and (d) commercially available nonadherent dressings (Telfa; Medtronic, Minneapolis, Minnesota) as a negative control. The ability of small volumes (2 mL) of subcutaneous injectate to diffuse both proximally and distally in the distal limbs of horses for total distances as far as 11 cm has been documented in many local anesthetic and contrast medium studies. For this reason, we chose not to treat the wound on the same limb as the eAM-treated wound because it was well within this distance range, and we speculated that it could be influenced by the injection of the eAM-treated wound. Therefore, it served as a control that was subject to growth factors from the StemWrap+ injection without the added effect of the StemWrap scaffold. As such, wounds were only partially randomized in that a coin toss was used to decide which wound would receive eAM until an equal number of left and right and proximal and distal wounds were obtained. After that decision, the remaining wound on the same limb was assigned as the eAM control. On the contralateral limb, the wound directly opposite the eAM wound was assigned treatment with a silicone dressing, while the wound directly opposite the eAM control wound was assigned treatment with a nonadherent control dressing (Figure 1B).

Equine amniotic membrane allograft therapy consisted of application of a 6.25-cm² square of decellularized and dehydrated equine amniotic membrane (StemWrap) topically to the wound according to the manufacturer’s directions. The StemWrap can be cut to size as required, and either side of the scaffold can be applied to the wound. In addition, a total of 1 mL of injectable decellularized liquid morselized amniotic membrane as a growth factor source (StemWrap+) was injected subcutaneously along each of the four sides of the wound (0.25 mL per side) approximately 5 mm from the wound edges, according to the manufacturer’s directions. Injections were performed with 22-gauge, 1-inch needles and one needle site per side by advancing the needle to the hub and then slowly injecting the StemWrap+ as the needle was withdrawn for even distribution of the product along the entire skin edge. Both eAM and eAM control wounds were covered with the same nonadherent dressing that were used for the negative control wounds as a primary bandaging layer. Silicone dressings were cut to size, and the correct side of the dressing was applied to the wound according to the manufacturer's directions. All dressings were held in place with conforming stretch gauze bandage. A half limb bandage consisting of 16-in cotton roll with an outer elastic roll that provided support to the surgical site was applied to each forelimb. Bandages were maintained and changed twice weekly for the duration of the study. At every bandage change, wound discharge and debris were gently removed from the skin surrounding the wound with 0.9% saline soaked gauze. Silicone dressings were manually cleaned with 0.9% saline at each bandage change and reapplied. They were discarded after 14 days, and new dressings were applied according to the manufacturer’s recommendations. The granulation tissue of each wound was debrided every 7 days to the level of the skin for the first 28 days with a No. 10 scalpel blade. All wounds were debrided down to the level of the skin regardless of the amount of granulation tissue present to standardize the effect of debridement on each wound. After 28 days, no additional debridement was required because no granulation tissue protruded above the level of the skin beyond this point of the study in any of the wounds.

2.5 | Macroscopic assessment of wound healing

At each bandage change prior to wound debridement, a three-dimensional camera (Fuel3D, Greenville, North Carolina) was used to obtain three-dimensional images by a single investigator (A.W.F.) who was blinded to the treatment. Each image was measured in associated imaging software for wound circumference (WC; in mm) and wound surface area (WSA; in mm²; Figure 2). Because the images were processed in three dimensions, evaluation of the wound surface area allowed a quantitation of granulation tissue present. The study was continued for 59 days until all wounds had healed. Progress notes on each wound were made in each horse’s record at every bandage to note any changes in gross appearance of the wounds or any issues with bandage displacement.

2.6 | Histological and immunohistochemical assessment of healing

Three days after completion of the study (day 62), an 8-mm diameter, full-thickness biopsy sample was obtained from the center of each wound (with the same sedation and local anesthesia protocol for the previously described surgical) to evaluate the quality of healing via histology and immunohistochemistry assays. Dermal biopsies were embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin. Additional unstained sections were prepared for immunohistochemistry for factor VIII-related antigen (Cat. No. A0082; Dako North America, Carpinteria, California) and for C1cas/cleaved caspase 3 (Cat. No. 9661; Cell Signaling Technology, Danvers, Massachusetts) to highlight vascularization and apoptosis, respectively. Immunohistochemistry automated steps were performed with the Biocare Intellipath System (Biocare Medica, Pacheco, California). Antibody for
factor VIII-related antigen was diluted 1:500 and incubated for 30 minutes at room temperature. Antibody for C1cas/cleaved caspase 3 antigen was diluted 1:200 and incubated for 30 minutes at room temperature. For both immunohistochemistry protocols, primary antibodies were raised in the rabbit, and the ImmPRESS HRP anti-rabbit immunoglobulin G (peroxidase) polymer was used as a secondary antibody (Cat. No. MP 7401; Vector Laboratories, Burlingame, California). Sections were rinsed, incubated with chromagen, counterstained with hematoxylin, dehydrated, and visualized with a light microscope. Sections of ileum, as a positive control for apoptosis, were included on each slide for immunohistochemistry staining for C1cas/cleaved caspase 3.

Features of histology and immunohistochemistry were scored by a board-certified anatomic pathologist (V.E.W.) unaware of the treatment and horse. Categories of healing included epidermal healing (degree of epithelialization), presence of cutaneous adnexa, abundance of dermal collagen, inflammation, and vascularity. Degree of epithelialization was evaluated with the following scoring system: 0 = absent or present at edge only, 1 = surface spanned by loosely attached epithelium, 2 = focal separation between epidermis and dermis or epidermal cell degeneration, and 3 = surface spanned by epidermis. The presence of cutaneous adnexa was evaluated with the following scoring system: 0 = absent, 1 = present at edge only, 2 = up to 50% of the section; and 3 = spanning >50% of the section. Abundance of dermal collagen was evaluated with the following scoring system: 0 = absent, 1 = mild, 2 = moderate, and 3 = marked. Degree of inflammation was evaluated with the following scoring system: 0 = severe, 1 = moderate, 2 = mild, and 3 = rare to absent. Sections immunohistochemically stained for factor VIII-related antigen were evaluated with a scoring system in which 0 = normally distributed vessels, 1 = vessels increased slightly, 2 = vessels increased moderately, 3 = vessels increased markedly. This overall scoring system was adapted from previously published scoring systems to provide a resultant cumulative score from 0 (inappropriate healing) to 15 (most appropriate healing). In addition to this scoring system, length of epidermal/dermal defects and depth of the viable epidermis alone as well as the epidermis and hyperkeratosis were measured (in μm). Epidermal and dermal defects were reported as a total length and were distinguished from possible sectioning artifacts by the presence of underlying inflammation, hemorrhage, or granulation tissue in the dermis. Depth was reported as the average obtained from measuring the depth of three random areas of epidermis per section.

2.7 Statistical analyses

Values of WC and WSA for three separate images per wound were averaged, and the coefficient of variation (CV) determined to estimate relative measurement error. For WC and WSA CV, separate linear mixed models were fit with fixed effects for treatment and random effects for horse. Because of the randomized complete block design with repeated measures in which four treatments were randomized to four wounds on each of eight horses, linear mixed models were fit to the WC and WSA data in SAS PROC MIXED (SAS Institute, Cary, North Carolina) to investigate possible treatment effects. These models included factorial effects for treatment, day, and their interaction and random effects for horse and horse-by-treatment interaction. These random effects impose a structure
that allows for intrawound and intrahorse correlations among repeated measures. The initial size of the wound (at day 0) was included in the model as a covariate in an analysis of covariance.59 Because the responses become deterministically zero after a wound has healed, any observations for a given wound subsequent in time to the first observed 0 for that wound were treated as missing. Any infected wounds were right-censored after the first detectable signs of infection, and all subsequent observations were treated as missing. For days until the wounds had healed, a linear mixed model was fit with fixed effects for treatment and random effects for horse.

To investigate the possibility of treatment effects on the single time point histologic outcome measures that included both measurements and scores, the assumption of normality was first checked by using goodness-of-fit tests on residuals from an additive model with horse and treatment effects. Individual score categories and the cumulative score were assessed. Because these tests gave some evidence of non-normality for the majority of the outcome measures, Friedman's test was performed in SAS PROC FREQ (SAS Institute). Data from infected wounds or biopsy samples damaged during histologic processing were eliminated from the analyses and treated as missing. For any outcome measure determined to be significant with the Friedman's test, pairwise comparisons among the treatments were carried out using rank sums. The least significant difference for pairwise comparisons was computed with a Bonferroni-type adjustment for multiplicity. All analyses described above were performed in SAS 9.4 (SAS Institute).

\[ P \leq .05 \] was considered significant.

## RESULTS

### 3.1 Growth factor and cytokine analyses of StemWrap+

The lot of StemWrap+ used in this study contained detectable concentrations of TGF-β1 (494.10 pg/mL), VEGF (212.52 pg/mL), and PGE2 (1811.61 pg/mL). Transforming growth factor β1 and PGE2 concentrations were comparable to the averages obtained from the additional three independent lots reported here as the average ± SD (TGF-β1, 363.63 ± 46.93 pg/mL; PGE2, 1460.93 ± 740.97 pg/mL), while the VEGF concentration was lower than the average obtained from the additional three independent lots (800.00 ± 536.78 pg/mL). Concentrations of EGF and PDGF-BB were below the lower limits of quantitation for the ELISA kits used for all lots tested.

### 3.2 Adherence to study design

Overall, the horses tolerated the procedures and 59 days of bilateral distal forelimb bandaging very well. Only one horse (horse 4) was prone to chewing on bandages and was found once on the morning of day 25 without an outer bandage on the eAM-treated limb. The inner conforming stretch gauze bandage was intact. After that episode, horse 4 was placed in a neck cradle, and no additional issues were experienced. On rare occasions, silicone dressings were found to have shifted slightly, but were noted to be covering at least 75% of the wound in the progress notes.

Twenty-nine of the 32 total wounds healed without complication by 59 days postcreation, while three of the wounds became infected (Table 1). Infected wounds included one

### Table 1 Time to healing for each wound categorized by horse and group

<table>
<thead>
<tr>
<th>Horse</th>
<th>eAM, n = 8</th>
<th>eAM control, n = 8</th>
<th>Silicone dressing, n = 6</th>
<th>Negative control, n = 7</th>
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<tr>
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<td>56</td>
<td>56</td>
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<td>Horse 2</td>
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<td>(Q1, Q3)</td>
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<td>(49.50, 59.00)</td>
<td>(53.00, 56.00)</td>
<td>(53.00, 59.00)</td>
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</table>

Note: Values are days. No differences were found between groups for time to healing (linear mixed model with fixed effects for treatment and random effects for horse; \( P \leq .05 \)).

Abbreviations: eAM, equine amniotic membrane allograft; Q1, 25th percentile; Q3, 75th percentile.

*aInfected wounds (n = 3); values are the last data points used for analyses.

*bSummary data for each group are for healed wounds only.
wound treated with a silicone dressing on the limb of horse 3 that also had a healed negative control wound and two wounds on the same limb of horse 7 that were treated with silicone and negative control dressings. The infected wound on horse 3 had a small amount of purulent exudate first observed on day 32, and all data points past day 28 were excluded from additional analyses because the wound began to slowly increase in size on day 32 measurements. This wound was gently cleaned at each bandage change with saline and went on to heal after conclusion of the study after bandaging was discontinued. The infected wounds on horse 7 had purulent discharge as well as associated mild cellulitis of the limb first observed on day 35, and all data points past day 32 were excluded from additional analyses when the first wound on that limb began to slowly increase in size on day 35 measurements. Treatment with trimethoprim sulfadiazine (30 mg/kg by mouth twice daily) was instituted at that time, and the silicone dressing was discontinued. The infected wounds were gently cleaned at each bandage change with saline until the end of the study, at which time this particular horse was euthanized for an unrelated postmortem study. Biopsies of the three infected wounds were excluded from histologic analyses.

### 3.3 | Macroscopic wound healing assessment

Overall, the CV remained low for WC and WSA measurements during the course study but did increase as the wounds decreased in size. The average CV for the WC of all 32 wounds over all time points measured was 2.01% (median, 1.15%; range, 0.09%–9.99%). The average CV for the WSA of all 32 wounds for all time points measured was 3.04% (median, 2.20%; range, 0.09%–9.91%). Neither treatment nor horse effects on CV were significant for either WC ($P = .95$ and $P = .83$, respectively) or WSA ($P = .67$ and $P = .22$, respectively).

No differences in time to healing were detected between any of the groups evaluated (Table 1, Figure 3A,B). Granulation tissue formation was more pronounced in wounds treated with eAM from days 4 to 14 compared with other wounds as determined by differences in WSA without a corresponding difference in WC (Figure 3D,C, respectively). While WC increased slightly in all wounds initially, WSA increased only in wounds treated with eAM compared with wounds treated with silicone dressings on days 4 ($P < .01$), 7 ($P < .01$), 11 ($P < .01$), and 14 ($P = .02$). In addition, wounds treated with eAM had increased WSA compared with eAM control wounds on days 7 and 11 ($P = .01$ and $P = .04$, respectively) and compared with negative control wounds on day 7 only ($P < .01$). On day

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**FIGURE 3**  A,C, Evaluation of wound circumference (A) and wound surface area (C) data during the course of the study for each wound (mean ± SD). B,D, Box and whisker plots illustrate days 4 to 14 data and illustrate differences between the groups. In these plots, each box extends from Q1 to Q3; the median is the line dividing each box, and the whiskers extend to the 10th and 90th percentiles. *$P \leq .05$, **$P \leq .01$. eAM, equine amniotic membrane allograft. Q1, 25th percentile; Q3, 75th percentile.
11, negative control wounds also had increased WSA compared with wounds treated with silicone dressings ($P = .05$).

### 3.4 Histological and immunohistochemistry wound healing assessment

In addition to the three infected wounds, a negative control wound was excluded from histologic analyses because of a flaw in processing that lead to removal of the epidermis. No differences in histological scores were found between any of the groups for any of the individual parameters examined or for the cumulative score (Table 2). Cutaneous adnexa scores were uniformly low. Inflammation was uniformly composed of perivascular macrophages, lymphocytes, and plasma cells, and the macrophages often contained hemosiderin. Representative photomicrographs are displayed in Figure 4A-C. While C1ca/cleaved caspase 3 staining was apparent on the positive control sections of ileum, staining was not evident on wound sections in sufficient quantities to allow for comparison between treatment groups. Finally (and not included

<table>
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<th>Histological parameter</th>
<th>eAM, n = 8</th>
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<th>Negative control, n = 6</th>
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<td>Vascularity</td>
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<td>9.5 (8.5, 10.5)</td>
<td>8.5 (8, 9)</td>
<td>8 (4, 11)</td>
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Note: Infected wounds (n = 3) and biopsies damaged during processing (n = 1) were excluded from histological assessment as indicated by the lower n values for the silicone dressing and negative control groups. Each parameter was scored from 0 (inappropriate healing) to 3 (most appropriate healing); the resultant cumulative score is from 0 (inappropriate healing) to 15 (most appropriate healing). Data are median (Q1, Q3). No differences were found between groups for any of the individual parameters assessed or for the cumulative score (Friedman’s test with LSD; $P \leq .05$).

Abbreviations: eAM, equine amniotic membrane allograft; LSD, least significant difference; Q1, 25th percentile; Q3, 75th percentile.

**FIGURE 4** Representative photomicrographs of day 62 biopsies illustrate the histopathology associated with a low (less appropriate) healing score (A), high (more appropriate) healing score (B), inflammatory cells common to healing in these horses (C), and an epidermal-dermal defect (D).

A. Photomicrograph of a wound with a low healing score of 5 illustrating loosely attached epidermis, absence of adnexa, and hemorrhage.
B. Photomicrograph of a wound with a high healing score of 12 illustrating epidermal healing and also the presence of cutaneous adnexa (arrows).
C. Predominant inflammatory cell types: macrophages, often laden with hemosiderin (short black arrows), mixed with lymphocytes (open arrowheads) and plasma cells (long thin black arrows). D. Photomicrograph of a wound with an epidermal-dermal defect. Arrows indicate edema in the areas of separation between the epidermis and dermis. The epidermis on both sides is hyperplastic. Scale bars = 1 mm in A,B; 50 μm in C; and 200 μm in D
in the data analyses), histologic assessment of the three wounds determined to be infected by gross examination and increasing WC and WSA measurements were confirmed to have ulcerated and/or absent epidermis with neutrophils predominating within the dermis.

Figure 4D illustrates a representative epidermal-dermal defect. eAM control wounds had fewer defects in the epidermis or dermis compared with the negative control group only \( (P = .04; \text{Figure 5A}) \). No differences were found between any of the groups in terms of viable epidermal depth or epidermal and hyperkeratosis depth (Figure 5B,C).

4 | DISCUSSION

The objective of this study was to characterize and examine the use of new commercially available equine amniotic membrane products collectively referred to as eAM for their effect on distal limb wound healing, including the production of granulation tissue. In accordance with our hypothesis, eAM does contain growth factors and cytokines known to be important for wound healing in the StemWrap+ product and did lead to the production of significantly more granulation tissue compared with all other treatment groups on day 7. In addition, eAM led to the production of significantly more granulation tissue compared with the eAM control on day 11 and compared with silicone dressings on days 4, 11, and 14. Contrary to our hypothesis, however, treatment with eAM did not affect time to healing or the histologic quality of healing compared with any of the other groups in this acute wound healing model.

The StemWrap+ samples analyzed for this study all contained detectable quantities of TGF-\( \beta \), VEGF, and PGE\(_2\) but not EGF or PDGF-BB. This lack of EGF and PDGF-BB was unexpected because of the reported presence of these growth factors in human amniotic products.\(^{21,28-30}\) In addition, the commercially available EGF and PDGF-BB ELISA kits used in this study have been used in numerous published studies for the successful characterization of equine protein-rich samples, including platelet-rich plasma (PRP) and stem cell conditioned media,\(^{51,52}\) providing confirmation that these growth factors were truly not present in the StemWrap+. A very recently published study examining the protein profile of equine amnion dressings via liquid chromatography-mass spectroscopy, however, also did not detect quantifiable levels of EGF or PDGF in their samples.\(^{60}\)

Although the amnion dressings in this protein profile study were processed very differently than the commercial products used in this current study, the results highlight the potential loss of growth factors that can occur through amnion processing\(^{60,61}\) as well as the inherent variability in amnion dressings due to their nature as biologic products such as PRP.\(^{62,63}\)

Unfortunately, available in vivo data regarding the use of locally applied growth factors and how they influence wound healing in the horse are limited. The topical application of PRP for the treatment of equine distal limb wounds in vivo by using an acute wound model comparable to the one used in this current study has been examined in two studies.\(^{6,7}\) While PRP contains a large number of growth factors and cytokines, these studies have generally attributed their main outcomes to the high concentrations of TGF-\( \beta \) in PRP. Transforming growth factor \( \beta \) is known to cause an influx of inflammatory cells critical to the wound healing cascade and to promote fibroblast proliferation, resulting in the production of granulation tissue.\(^{3,4,34-36}\) In addition, it has been shown that equine distal limb wounds have increased expression of TGF-\( \beta \) compared with thoracic wounds after the acute inflammatory phase.\(^{4,34}\)

While topical PRP treatment did not improve the quality or accelerate wound healing in either of the referenced equine studies, one of these studies reported an increase in the production of granulation tissue,\(^{7}\) while the other found improved collagen organization compared with control wounds.\(^{6}\) Another in vivo equine distal limb study in which the effects of topically and subcutaneously applied orf virus-derived interleukin (IL)-
10 and VEGF were assessed provided evidence that treatment significantly increased granulation tissue production at day 12 compared with controls. Vascular endothelial growth factor is known to stimulate angiogenesis and increase capillary permeability, which also causes an influx of inflammatory cells. The combinatorial effect of IL-10 in the referenced equine study is unknown, but the results still provide evidence that exogenous VEGF may stimulate the production of granulation tissue in equine distal limb wounds. Although it is well documented that PGE2 is important in the wound healing process by causing vasodilation and an increase in blood flow to the wound, no studies have been performed to assess the impact of topically applied PGE2 on equine distal limb wounds and its effects on healing and granulation tissue production, for such wounds are unknown. It is also important to acknowledge that the increased production of granulation tissue would be viewed positively only in the context of deep wounds with substantial tissue loss. In the context of acute and less severe wounds, it is known that excessive granulation tissue negatively impacts the wound environment by preventing epithelialization and ultimately slowing healing.

While StemWrap+ provides growth factors and cytokines, StemWrap provides a supportive physical structure for endogenous cells to adhere to and produce granulation tissue. In this study, eAM-treated wounds had significantly increased granulation tissue production compared with other treated and control wounds at certain early time points. Specifically, eAM treatment increased granulation tissue production compared with silicone dressings on days 4, 7, 11, and 14, while negative control wounds had increased granulation tissue production only compared with wounds treated with silicone dressings on day 11. This latter finding is in agreement with results of a previously published study that provided evidence that silicone dressings can reduce exuberant granulation tissue production in distal limb wounds of horses compared with negative control nonadherent dressings.

In addition, eAM-treated wounds had increased granulation tissue production compared with negative control wounds on day 7 and compared with eAM control wounds on days 7 and 11. The eAM control wounds, which should have been subject to the local effects of the growth factors contained in StemWrap+, did not have increased granulation tissue production compared with any other group at any time point. Although it is possible that eAM control wounds may not have actually been subject to growth factor effects, it is more likely that the combination of growth factors in StemWrap+ and the StemWrap scaffold is required for effective production of increased granulation tissue. We speculate that the growth factors in StemWrap+ increased the delivery of cells, particularly fibroblasts, to the wounds and that the presence of the StemWrap scaffold in the eAM-treated wounds then provided an appropriate substrate for cell adherence and proliferation, leading to enhanced tissue production. It has been shown in vitro that fibroblasts and keratinocytes remain viable and proliferate when attached to decellularized amniotic scaffolds, similarly to the StemWrap tested in this study. Several human clinical trials and case series have also used decellularized and, often, dehydrated amniotic membrane products with associated growth factors to treat chronic nonhealing wounds, including diabetic foot ulcers and venous leg ulcers.

In these studies, production of granulation tissue was not assessed or quantitated, but decreased time to healing was reported with these products as well as resolution of wounds that had previously failed with standard-of-care treatment.

In our acute wound model study, eAM treatment did not affect time to healing or the histologic quality of the healed tissue. This is an important aspect of the study and provides evidence that, although granulation tissue production is enhanced with eAM treatment, it does not result in an overall delay in wound healing or the development of a chronic wound state when appropriately debrided. It has been the authors experience both in this study and in clinical cases in which they have used eAM that frequent debridement is required to maintain the granulation tissue bed at or below the level of the skin to allow for effective epithelial migration and normal healing. No statistical differences were found between groups for any of the individual wound healing histological scores or the cumulative score. Overall, the histology was consistent with secondary healing of acute surgical wounds. In addition, no differences were found in epidermal depth or epidermal and hyperkeratosis depth between the groups. The presence of smaller epidermal defects in the eAM control group compared with silicone dressing- or control-treated groups may have been due to the local growth factor effect. Indeed, the median length of epidermal-defect in the eAM-treated group was actually the lowest, but the eAM-treated group had one outlier with a large defect, likely preventing statistical significance in that group.

The acute surgical wound model is the main limitation of this study. A chronic or deep wound model would have been more clinically relevant, but no model has been validated for such wounds in horses. Inclusion of clinical trauma cases would have, alternatively, introduced a vast number of uncontrollable variables for an initial efficacy study on these equine amniotic membrane products. An associated limitation to using these products in an acute surgical wound model was the requirement for debridement of all wounds every 7 days for the first 28 days of the trial. Previous studies in which this model was used debrided granulation tissue only when it became exuberant. This approach was not used in this study to reduce variability between treatments and bias. Sharp debridement results in an acute inflammatory reaction that has been shown to improve healing rates in man and, therefore, could have influenced the outcome of the current study. To maintain consistency between treatment groups, all wounds were debrided.
at the same time point regardless of the presence and degree of exuberant granulation tissue. Another limitation was the development of infection associated with three wounds, which prevented the use of their data at later time points and in the histologic assessment. The cause of infection is unknown but may be related to the use of the occlusive silicone gel dressing. A previous study in which the same occlusive silicone gel dressing used in this current study was examined, however, did not detect any adverse effects when the gel dressing was used for 1 month and provided evidence for improved tissue quality at the completion of the study compared with wounds treated with nonadherent control dressings. In that study, wounds were treated 2 weeks after creation to first allow for exuberant granulation tissue production, and bandages were changed at least three times per week. It is possible that the wound treatment occurring at different stages of healing may have accounted for some differences in these results. In addition, the twice-weekly bandage changes in this study should be considered as a possible limitation. Although the silicone dressing was cut to fit the size of the wound, exudate that was produced accumulated on the skin at the edge of the dressing. With the bandage changes occurring only two times per week, this may have allowed the skin to become macerated, decreasing the natural barrier effect and predisposing these wounds to infection.

This outcome was not anticipated by the authors because it has been reported that occlusive dressings potentially decrease the risk for wound infection. Moreover, bandage changes in previous equine distal limb wound healing studies ranged from one to three times per week, and no evidence of wound infection was reported during these trials. The exact cause of infection in this study remains unknown. An additional flaw in histologic processing further eliminated a control wound, reducing our total number of wounds to be examined from 32 to 28.

In conclusion, the results of this study provide evidence to support the application of eAM to increase the production of granulation tissue in distal limb wounds of horses without altering the quality of wound healing. However, these data were obtained from experimental acute surgical wounds, and the response to treatment in chronic, nonhealing wounds may differ. In wounds with substantial tissue loss and complicating factors such as underlying exposed bone and/or tendons, the expedient production of large amounts of healthy granulation tissue is desirable to fill in the defect and cover underlying anatomical structures. Such wounds may, therefore, be the most relevant candidates for treatment with eAM. Our study provides evidence that eAM contains growth factors and cytokines important for wound healing and that eAM increased granulation tissue production in acute experimental wounds. A large-scale multicenter clinical trial is warranted to determine the success rate of these products and best use practices.

ACKNOWLEDGMENTS
The authors thank Julie Long BS and the members of the histology laboratory in the Department of Population Health and Pathobiology at North Carolina State University for their technical assistance and North Carolina State University Laboratory Animal Resources staff for their help with animal care and handling.

CONFLICT OF INTEREST
The authors declare that AniCell Biotech funded the majority of this study. The authors declare no other conflicts of interest related to this report.

AUTHOR CONTRIBUTIONS
Alexander W. Fowler, Jessica M. Gilbertie, Victoria E. Watson, Timo Prange, and Lauren V. Schnabel contributed to study conception, design, and execution. All authors contributed to the data analysis and interpretation and preparation of the manuscript. All authors approved of the final version of the manuscript.

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