Evaluation of Lyophilized Human Amnion for Equine Wound Management

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Evaluation of Lyophilized Human Amnion for Equine Wound Management

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ABSTRACT

Objective: To assess the safety and efficacy of lyophilized milled human amnion as a wound dressing of experimentally created equine distal limb wounds.

Animals: Four clinically normal adult horses (3 Thoroughbred and 1 Paint, median age 11 years) obtained via donation.

Procedures: One forelimb of each horse was randomly assigned to the treatment group, and the contralateral limb was assigned as the control. Full-thickness skin wounds were created on each metacarpus. Treatment limb wounds were dressed with lyophilized, milled, human-derived amnion material delivered under triple antibiotic ointment. Control wounds were dressed with triple antibiotic ointment. All wounds were covered in non-adherent dressings and distal limb bandages were applied. Digital photographs were taken of the wounds at each bandage change, performed every 2-4 days throughout a 98-day study period. Biopsies were collected at days 7, 21, 35, and 84.
**Results:** One horse developed unilateral cellulitis that resolved with additional treatment. All treatment limbs exhibited an inflammatory response characterized by focal edema and discharge from the wounds. Wounds were completely epithelialized in control limbs sooner than treatment limbs in all horses, although there was no statistical difference between control (mean 46.8 days) and treatment (mean 51.8 days) wounds. Histologic scores were better in control wounds than in amnion-treated wounds at all time points.

**Conclusions and Clinical Relevance:** Because wounds treated with amnion material in this study exhibited an inappropriate inflammatory response that resulted in delayed time to wound closure, human lyophilized milled amnion is not recommended for use in equine wound management.
Poorly healing wounds can be debilitating in horses. Amnion-derived treatments have been used in the horse and other species and been shown to improve wound healing. The purpose of this project was to evaluate a specific human-derived amnion material in the horse to evaluate its safety and efficacy. After creation of standardized skin wounds, the test product was applied to one limb and compared to untreated wounds on the horse's opposite limb. We found that the amnion material in this study caused swelling and longer wound healing times in horses. Therefore, this particular material is not recommended for use on horse wounds.
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INTRODUCTION

Amniotic membrane has been used for decades in man and animals to promote healing for many different conditions. Delivery of growth factors provides desirable effects on proliferation of fibroblasts, keratinocytes, and blood vessels, leading to scientific interest in its use for healing of tissues cornea, skin, and other tissues.\textsuperscript{1-6} Amnion has shown promise due to its antimicrobial factors, promotion of epithelialization, and provision of a wide variety of growth factors.\textsuperscript{5,7-10} Despite multiple studies demonstrating improved healing in wounds treated with amnion, the clinical use of amnion remains limited, primarily due to challenges in collection, processing, and storage. The use of a lyophilized and processed amnion product could improve convenience and accessibility, enabling more widespread use if positive effects on wound healing are preserved.

Distal limb wounds in horses present clinical challenges, as they exhibit poor healing characteristics compared to wounds in other areas of the body or in different species.\textsuperscript{11-13} Development of exuberant granulation tissue, lack of redundant skin, ease of contamination, slow epithelialization and low vascularity are some of the challenges faced in equine distal limb wound healing. Therefore, the equine distal limb wound healing model represents a more challenging trial than models in other species such as rodents or swine. Identification of products that improve wound healing in the distal limb of the horse not only improves horse welfare, comfort, and function, but also may be applicable to treatment of challenging wounds in other animals and man.
LITERATURE REVIEW

The Physiology of Wound Healing

Wound healing progresses through four phases, which overlap in temporal and physiological characteristics. These phases are hemostasis, inflammation, proliferation, and maturation. Hemostasis and inflammation begin at the time of wounding. Hemostasis is characterized by bleeding at the site of the wound and retention of platelets. The inflammatory phase consists of influx of polymorphonuclear cells and mononuclear cells for wound debridement. In the proliferative phase, proliferation of mesenchymal cells occurs with repair of the wound through deposition of extracellular matrix components such as collagen and endothelial cells. The proliferative phase is concluded once intact epithelium covers the wound and the maturation phase remains. In the maturation phase, cross-linking of collagen and organization of the matrix occurs to remodel the wound bed and restore varying degrees of strength.

Hemostasis is complete within hours and starts at the time of wounding with disruption of blood vessels and bleeding at the site of the wound. The coagulation cascade is initiated and a clot forms at the site of the wound, resulting in cessation of hemorrhage and providing a scaffold for repair during subsequent phases of healing. Many signaling pathways are activated during the hemostasis phase, including release of factors by damaged blood vessels such as phospholipids initiating the arachidonic acid cascade, and factors released by platelets such as platelet derived growth factor (PDGF). PDGF and others are chemoattractants for influx of cells involved in the subsequent phase of wound healing, the inflammatory phase.\(^{14,15}\)

In the inflammatory phase, the population of neutrophils peaks at 1-2 days, followed shortly thereafter by the peak influx of mononuclear cells at 5-10 days.\(^{16}\) Neutrophils are the body’s
primary defense against microorganisms and provide chemoattractants for mononuclear cells which are also important in the proliferative phase and are responsible for some phagocytosis of fibrin in the normal remodeling of the provisional fibrin clot in the wound.\textsuperscript{13} Once debris is cleared from the normal wound by neutrophils and macrophages, neutrophils are phagocytosed by macrophages or become entrapped in the fibrin clot. This dessicates, adheres to the wound surface, and is eventually sloughed as a scab. Monocytes are the main orchestrator cells in wound repair, differentiating into tissue macrophages and releasing many cell signaling molecules and growth factors including transforming growth factor-alpha (TGF-\textalpha{}), transforming growth factor-beta 1 (TGF-\textbeta{}1), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and fibroblastic growth factor (FGF). These stimulate blood vessel growth and fibroblast proliferation in the subsequent proliferative phase.\textsuperscript{17} Platelets also remain active in this phase of wound healing, continuing to secrete growth factors such as PDGF and VEGF, and therefore aiding in stimulation of revascularization of the wound.\textsuperscript{15}

The proliferative phase begins around day 4 and is characterized by migration and proliferation of fibroblasts, endothelial cells (angiogenesis), and epithelial cells.\textsuperscript{14,18} Wound closure ultimately occurs through a combination of epithelialization and contraction, mediated by myofibroblasts. Fibroblasts enter the wound environment in response to previously described chemoattractants, including platelet derived growth factor (PDGF) released by platelets and macrophages.\textsuperscript{17} Once in the wound environment, they may differentiate into myofibroblasts in response to TGF-\textbeta{} for wound contraction, or they may begin synthesis of collagen to build the wound’s new extracellular matrix.\textsuperscript{19} Granulation tissue formation, or production of type 3 collagen by fibroblasts in extracellular matrix, is dependent on a variety of cytokines and growth factors,
with the transforming growth factor beta family playing a key role via upregulation of proteins such as fibronectin and collagen, as well as glycosaminoglycans.\textsuperscript{14,20}

Epithelialization proceeds from the wound periphery, and relies on stem cell keratinocytes from 3 main sources: the bulge of the hair follicle, the base of the sebaceous gland, and the basal layer of the interfollicular epidermis.\textsuperscript{14} Additional keratinocytes may be provided by therapies such as skin grafting techniques.\textsuperscript{21,22} Proliferating keratinocytes spread to cover the new granulation tissue and subsequently adhere to the underlying basement membrane via hemidesmosomes. The basement membrane consists of Type IV collagen. Type VII collagen may also be found in the skin, anchoring the keratinocytes to the cytoskeleton of the dermis.\textsuperscript{14} The rate of epithelialization is highly dependent on species and anatomic location of the wound, with horses having slower epithelialization than ponies and distal limb wounds slower than wound of the body or trunk.\textsuperscript{11} This will be discussed further in later sections.

Finally, the maturation phase begins 3 weeks after wounding and may last up to one year. The provisional matrix formed in the initial stages of wound healing is degraded by matrix metalloproteinases. Type III collagen is largely replaced by type I collagen, and the collagen fibers become organized and aligned with lines of tension. The greatest rate of gain in strength coincides with maximum collagen deposition between days 7-14, but the wound remains weak and susceptible to re-injury until later, when collagen fibers are organized and cross-linked.\textsuperscript{14} Maximal wound strength even after remodeling remains weaker than uninjured intact skin, reaching about 80\% of normal skin strength.\textsuperscript{23}
Specific Roles of Growth Factors

Platelet derived growth factor (PDGF) has multiple effects, playing a role in all phases of wound healing. After release from platelet alpha granules, PDGF stimulates cell division and chemotaxis of neutrophils, macrophages, fibroblasts, smooth muscle cells and stem cells. It is also released by other cell types, including macrophages, endothelial cells, fibroblasts and keratinocytes. PDGF stimulates macrophages to secrete other growth factors to prepare the wound for remodeling. Although PDGF is not the most potent angiogenic growth factor (see VEGF and FGF), it also plays a role in promoting angiogenesis. Its effect promotes the
expression of VEGF in ischemic environments and also via improving structural integrity of newly formed blood vessels through recruitment of pericytes and smooth muscle cells. PDGF is downregulated in chronic wounds and its application is FDA-approved as a drug for chronic wound treatment in humans.\textsuperscript{24}

Vascular endothelial growth factor (VEGF) is produced by many cell types, but exerts its action predominantly by binding vascular endothelial cells. Its main effects are promoting endothelial cell proliferation and cell migration, especially in the early stages of wound healing. It also binds to monocytes and may be important for their migration.\textsuperscript{25} It is released in platelets and macrophages, as well as from keratinocytes and fibroblasts in response to TNF-\(\alpha\). Both the expression of VEGF and its receptors are upregulated in hypoxia, leading to a mechanism to restore oxygen tension and homeostasis in the wound environment.\textsuperscript{24}

Hypoxia is expected in the wound environment during early stages of wound repair, and provides a stimulus for growth of new blood vessels, as well as increases the proliferation of equine dermal fibroblasts, synthesis of TGF-\(\beta1\), and collagen.\textsuperscript{26} As new blood vessels form, the degree of hypoxia should improve and allow regenerative processes, which require oxygen metabolism. In equine wounds, oxygen saturation vary depending on wound location, and may be related to poor healing in distal limb wounds. Oxygen saturation values in unbandaged distal limb wounds were inferior to those of body wounds in the inflammatory phases of wound healing. Prolonged hypoxia in distal limb wounds is associated with increased levels of Hypoxia Inducible Factor 1 (HIF1), which stimulates proliferation of equine dermal fibroblasts. Thus, hypoperfusion may be the primary mechanism of exuberant granulation tissue formation in equine distal limb wounds.\textsuperscript{27}
Basic fibroblastic growth factor (bFGF, or FGF-2) originates from inflammatory cells as well as fibroblasts, keratinocytes, and endothelial cells. It regulates the synthesis of granulation tissue, epithelialization and tissue remodeling. It also stimulates migration of fibroblasts. bFGF levels are increased in acute wounds and decreased in chronic wounds. Other members of the fibroblast growth factor family are also important in wound healing, including FGF-7, also known as keratinocyte growth factor-1. The FGF-7 is stimulates keratinocyte proliferation, and thus is important for epithelialization.24

The most relevant members of the TGF-β family in wound healing are TGF-β1 and TGF-β3. These factors work alongside one another to balance the production of new extracellular matrix. TGF-β1 induces fibroblast proliferation and type III collagen synthesis, and is prominent in healing wounds and absent in normal skin.28 TGF-β3, on the other hand, decreases collagen deposition. TGF-β1 and TGF-β3 have an inverse temporal relationship, and as time proceeds, TGF-β1 decreases and TGF-β3 increases, with a peak of TGF-β3 at 10-14 days.16 High levels of TGF-β3 are associated with reduced scarring in wound healing and high levels of TGF-β1 are present in excessive collagen deposition, as seen in keloids in man.29,30. Fibroblasts proliferating in the wound bed are responsible for collagen type III production, which is the major type of collagen in immature wounds. This collagen is later remodeled and replaced with organized, regular bundles of Type I collagen in the maturation phase, which gives the wound its optimal strength.14

**Table 1.** Growth factors important for wound healing, their cellular origins, and roles in various phases of wound healing.
Unique Characteristics of Equine Wound Healing
Differences in wound healing have been demonstrated between horses and ponies, between horses and other species, and between body wounds and distal limb wounds of horses, with distal limb wounds of horses having the poorest healing rate and quality of healing. Horses have repeatedly been shown to have a weak, prolonged inflammatory phase of wound healing with decreased numbers but persistence of inflammatory cells, which plays a key role in inhibiting progression of the normal healing processes. Specifically, wounds on the distal limb suffer from prolonged healing times when compared to limbs of the body (thoracic skin or over the biceps femoris muscle). The profound difference in healing of the equine distal limb has led many researchers to further characterize the molecular events in equine wound healing, such as the role of neutrophils, degree of inflammatory response, character of granulation tissue and characteristics of myofibroblasts, with the intent of identifying a therapeutic target for intervention.

The inflammatory phase of normal wound healing is characterized by a robust, but short-lived influx of neutrophils. When comparing horses to ponies, ponies exhibited an expected strong acute inflammatory response with infiltration of a large number of polymorphonuclear (PMN) cells during the first 3 weeks of wound healing, then decreased, and were absent by week 5. Horses, however, had roughly half the number of polymorphonuclear cells infiltrate both body and metatarsal wounds. The low-grade inflammatory response persisted, and PMNs were still present in the wound by week 5. Importantly, monocytes, the cell type responsible for orchestrating wound repair, they were present in far fewer numbers than PMNs in all wounds of horses.

Other characteristics observed in equine wounds include poor quality of granulation tissue. Granulation tissue appears in horse wounds earlier than in ponies, covering exposed bone at 1
week in horses versus 3 weeks in ponies, but the quality of the tissue is irregular and disorganized, with clefts in the tissue. The granulation tissue also contains fibrinopurulent debris persisting until 6 weeks, which is longer compared to wounds in ponies. Formation of exuberant granulation tissue requiring intervention is more common in horse metatarsal wounds. Granulation tissue is graded on a scale of 1-4 and grade 4 granulation tissue, or “exuberant” granulation tissue, protrudes superficial to the advancing epithelial margin. This type of granulation tissue inhibits further epithelialization and must be removed to allow progression of healing. Although pony wounds take longer to become covered in granulation tissue, the quality of the granulation tissue is superior to that of horses, and provides a better substrate for subsequent epithelialization. The collagen content in the wound bed was similar in horse and pony wounds, across body and metatarsal locations, demonstrating that the quantity of collagen present is not responsible for the difference in wound healing. However, improved collagen organization and crimp occurred earlier in ponies than in horses.

More important than poor granulation tissue quality is poor myofibroblast function. Both myofibroblast numbers and organization are decreased in horse distal limb wounds, although the content of contractile α-smooth muscle actin (SMA) is the same as in ponies. In pony wounds, myofibroblasts transformed within 2 weeks to become regularly organized, parallel with the wound surface and perpendicular to blood vessels. Horse wounds of the body showed delayed organization at 3 weeks, but myofibroblasts in wounds of the distal limb remained chaotic throughout wound healing. Poor organization of myofibroblasts leads to poor ability to function, as haphazardly arranged cells with contractile filaments lacking organization contract in all different direction, so contraction, therefore, contributes little to distal limb wound healing in the horse. Contraction contributes to wound closure in body wounds to a greater extent than
metatarsal wounds in both horses and ponies (responsible for 76% of body wound closure in ponies versus only 49% in metatarsal wounds). Although tension in surrounding tissue plays a role in rate of contraction, this does not appear to be the cause of decreased contraction in metatarsal wounds, as body wounds retracted (enlarged) more than metatarsal wounds. This suggests that contractile forces of the myofibroblasts were even greater in body wounds, as they achieved closure despite the wounds enlarging more. Horse distal limb wounds contract very little, with only 12% of wound closure attributed to contraction by 9 weeks of healing. Epithelialization, therefore, accounts for the majority of wound closure in the horse’s distal limb. This is an undesirable outcome, as the neoepeithelium in healed wounds is fragile and prone to reinjury. Healing by epithelialization results in increased scarring with thick, hyperplastic epithelium, fibrosis and lack of normal adnexal structures such as sweat glands and hair follicles.

Wound healing therapies in horses may, therefore, be directed at decreasing chronic inflammation and improving organization and function of myofibroblasts. Many novel therapies have been studied to improve wound healing in the equine distal limb, and regenerative therapies such as dressings derived from fetal membranes warrant further investigation.

**Treatment of Equine Wounds**

Wounds of horses are managed differently based on location of the body. Wounds of the body cannot be covered with a bandage due to a horse’s size; however, wounds of the distal limb are often bandaged to prevent contamination with dirt and debris. The effect of bandaging has been studied and results in an increased frequency of granulation tissue formation as well as increased biofilm presence of biofilm on the wound surface over the first 2 weeks of healing.
Local hypoxia in the wound environment attributed to bandage application is thought to be partially responsible for excessive granulation tissue formation in distal limbs.\textsuperscript{12,34} Corticosteroids may be employed for topical treatment of exuberant granulation tissue and result in inhibition of excessive granulation tissue formation, but also result in decreased epithelialization and contraction, so use should be limited or avoided in favor of sharp excision.\textsuperscript{12} Topical application of antimicrobials such as triple antibiotic ointment, povidone iodine ointment or silver sulfadiazine do not have an impact on time to wound closure in surgically created wounds, but triple antibiotic ointment seems to mitigate the development of exuberant granulation tissue when distal limb wounds are bandaged.\textsuperscript{34,35} While a moist wound environment is desirable, fully occlusive dressings are generally contraindicated in equine distal limb skin wounds, as they result in decreased epithelialization and delayed time to wound closure.\textsuperscript{36} The use of non-adherent dressings in distal limb wounds resulted in faster time to wound closure than occlusive or semi-occlusive dressings in horses.\textsuperscript{36}

**Biologic wound dressings**

Many products have been investigated for use as biologic scaffolds in wound healing. Of greatest interest include collagen products, small intestinal submucosa (SIS), urinary bladder submucosa, and amniotic membrane products. Collagen products are desirable because collagen is a biocompatible polymer which is biodegradable and only weakly antigenic.\textsuperscript{37} Collagen sponges have been used in wound healing as a mechanical scaffold to support ingrowth of new cells and as a delivery vehicle for cells and growth factors.\textsuperscript{37,38} The scaffold not only provides a vehicle for exogenous treatments, but also stimulates influx of autogenous fibroblasts within the wound bed and the sponge is degraded by collagenases as the wound remodels within 8-10 weeks.\textsuperscript{22} Collagen products may be cost prohibitive for use in large equine wounds and carries
risk of immunogenicity. Porcine small intestinal submucosa and urinary bladder submucosa have also been investigated in wound healing, and equine studies describe their use of in corneal disease. The acellular membranes are used not only as sources of sheets of organized collagen, but also for provision of other matrix molecules such as glycoproteins and proteoglycans. Porcine SIS originates from the submucosa of porcine jejunum, which is processed, any remaining cells are lysed, lyophilized and sterilized with ethylene oxide. The product is commercially available as sheets or discs and has been used in multiple veterinary species. Small intestinal submucosal grafts were successful in treatment of corneal ulcers, corneal abscesses and corneal perforations in cats, dogs and horses. Porcine urinary bladder matrix has many similar properties and is also commercially available as a powder, lyophilized sheet, or corneal disc from (ACell). Advantages of the urinary bladder matrix include provision of growth factors including fibroblast growth factor-2 and transforming growth factor-β. The matrix is acellular, which is desirable in corneal healing for absence of immunogenicity and corneal transparency. The manufacturer’s recommendations include withholding topical medications 48 hours prior to the use of the membrane, but successful results have been achieved when disregarding this recommendation. Postoperative uveitis has been documented after use of ACell, but the direct association of uveitis with the membrane cannot be confirmed.

The Amniotic Membrane in Wound Management

The amniotic membrane is the membrane directly surrounding the fetus in utero. It consists of an epithelial cell layer, a thick basement membrane, and a mesenchymal layer. This structure is generally well-preserved across species (Figure 2). It is a source of stem cells and numerous growth factors for the fetus. It has been used extensively to improve wound healing of the eye and other organs for over 100 years in human and veterinary medicine with good success.
The exact mechanisms of improved wound healing are still being fully characterized, and are multifactorial. The greatest benefits of amniotic membrane treatment are likely due to provision of soluble growth factors and a biomechanical scaffold for influx of cells, and are partially dependent on amnion processing and application. Both fibroblasts and keratinocytes have improved proliferation and migration in the presence of amnion, and amnion results in a more robust angiogenic response.

**Figure 2.** Histologic structure of the amniotic membrane in various species. A) human, B) rabbit, C) dog, D) cat, E) equine, F) bovine. EL – epithelial layer. BL – basal layer. ML – mesenchymal layer.

Cryopreservation is the most common form of amnion processing after sterilization. Briefly, processing involves collection of an amniotic membrane at birth, rinsing the membrane repeatedly in sterile fluids, and freezing the membrane in solution at -80°C. A cryoprotectant such as glycerol may be added to aide in preservation of structure of proteins, but survival of epithelial cells in glycerol is poor. Approximately 50% of human amnion epithelial cells remain viable after cryopreservation. When a cryopreserved sample is to be used, it is simply thawed, rinsed and applied.

Lyophilization is another common technique for preservation of the amnion, and is favored due to longer shelf life of the tissue. In lyophilization, the membrane is harvested and rinsed in a similar manner, then frozen. In a lyophilization chamber, the water molecules are extracted from
the tissue by sublimation, transforming the molecules from a solid to gas state for complete dehydration. The membrane is then rehydrated prior to clinical use, and has similar physical properties as cryopreserved membranes. Lyophilized samples are easier to store, as they do not require storage at -80°C, and can be kept at room temperature. 

The structural retention of basement membrane, epithelial cells and growth factors have been evaluated in fresh, cryopreserved, and lyophilized amniotic membrane samples. Each preservation technique has good maintenance of structural properties. Histologically, both cryopreserved and lyophilized samples maintain the basement membrane covered in cuboidal epithelium and stains positively for type IV collagen, laminin, and fibronectin. The basement membrane is slightly thinner in cryopreserved samples than it is in fresh amnion, and can be intact or interrupted in lyophilized preparations. Epithelial cells are often present after lyophilization, but are non-viable or may be missing, with presence of cellular debris on the epithelial side of the basement membrane. Exposure of the basement membrane in the absence of adhered epithelial cells may improve the ability of growing cells to use the membrane as a scaffold. In vitro, endothelial cells grown in culture have improved adhesion and viability on lyophilized amniotic membranes than on cryopreserved membranes.

The role of amnion in promoting or inhibiting angiogenesis has been debated. Depending on the clinical application, either process may be desired. Avascularity, for example, may be preferable in corneal applications so that scarring does not impede vision. In contrast, increased vascularity is beneficial in skin wounds to promote blood supply to healing tissues. It has been found that amnion can have either effect depending on its application technique. Amniotic epithelial cells have been documented to have an anti-angiogenic effect, and do not have to be viable to exert this effect. The anti-angiogenic mechanism is via thrombospondin-1, endostatin
and heparan sulphate and tissue inhibitors of metalloproteinases (TIMPs). Fresh amniotic membrane, in contrast, also has a pro-angiogenic effect via presence of Interleukin-8 (IL-8), growth related oncogene (GRO), monocyte chemoattractant protein-1 (MCP-1), and angiogenin. After cryopreservation, IL-8 and MCP-1 remain elevated. The net effect of these factors depends partially on how the membrane is applied. With the amniotic membrane placed with the epithelial cells against the wound or tissue to be treated, it has a net anti-angiogenic effect. When amnion is placed with the mesenchymal side against the tissue to be treated or if epithelial cells are removed, it has a pro-angiogenic effect. This has been documented by a greater number of vessel sprouts and longer length of blood vessels when amnion is placed with the epithelial side up, and the mesenchymal side against tissue. If intact amnion is applied for dermal wound healing, then, it would be most beneficial if applied with the mesenchymal surface in contact with the wound.

Unlike cryopreservation, lyophilization precludes survival of epithelial cells. However, the membrane retains the ability to improve healing even without viable cells. Growth factors have been evaluated to further characterize the mechanism in which amnion improves healing even in the absence of viable cells. The most abundant growth factor is hepatocyte growth factor (HGF) in wound environments with fresh, cryopreserved and lyophilized amnion. Lyophilized samples had lower total protein concentrations in vitro when compared to fresh samples and a similar content to cryopreserved samples. However, the levels of growth factors were similar among amnion types with regards to epidermal growth factor (EGF), HGF, keratinocyte growth factor (KGF), and transforming growth factor beta-1 (TGF-β1). Basic-fibroblastic growth factor (bFGF) was lowest in lyophilized amnion, and fresh non-preserved samples had the lowest levels of EGF.
Although slight decreases in certain growth factors have been documented after lyophilization, further processing into a powdered form improved availability of the growth factors. After powdering the lyophilized amnion, increased levels of hepatocyte growth factor (HGF), fibroblastic growth factor (FGF), and transforming growth factor beta-1 (TGF-β1) were present in the surrounding area. Levels of TGF-β1 in powdered amnion were higher than in cryopreserved amnion, demonstrating that powdering counteracts the decrease in growth factors that occurs in the lyophilization process. Powdered, lyophilized amnion materials, therefore, may be a highly desirable formulation due to ease of storage and potential for improved healing characteristics with higher levels of available soluble growth factors. A detergent based protocol has also been investigated for amnion processing, in order to remove all cellular components to render the material non-immunogenic, and the processed material retained its collagen type I and IV, laminin and fibronectin after processing.

Amniotic membrane carries antimicrobial properties, but the mechanisms are incompletely understood. It has been documented to inhibit bacterial growth in vitro, but is not effective for inhibiting growth of all bacterial species. In one study, growth of *Pseudomonas aeruginosa* and one strain of *Escherichia coli* were inhibited in vitro by the presence of amnion, but growth of *Staphylococcus aureus* and two other strains of *Escherichia coli* were uninhibited. The inhibitory antimicrobial properties of amnion remain consistent regardless of processing such as freezing and lyophilizing. Elafin is a known antibacterial peptide present in the amniotic membrane and it was not present in sufficient quantities in processed (frozen or lyophilized) amnion, so the antimicrobial properties must be due to other properties of the amniotic membrane.
Amnion has been previously investigated for use in equine distal limb wounds and has demonstrated favorable effects on wound healing. When 2.5cm distal limb wounds were lightly bandaged with non-adherent dressings, those treated with topical, frozen equine amnion had a significantly less extreme exudate and faster time to wound closure. All amnion-treated wounds healed within 50 days and 8/12 healed in less than 40 days, with a mean of 39 days. Control wounds healed in a mean of 47 days. The faster time to wound closure is not completely characterized, but lack of wound expansion in the first 14 days of wound management may be partially responsible. Amnion-treated wounds also had a faster time to complete epithelialization in a pony wound model when 2.5cm distal limb wounds were treated with pinch grafts with or without refrigerated equine amnion, with a median of 30 vs. 39 days to wound closure.

Development of a Human Amnion Material for Wound Healing

Preliminary studies were conducted at Wake Forest Institute of Regenerative Medicine to determine the ideal delivery formulation for processed amnion. A porcine wound healing model was selected with future intentions of developing a product that is easy to apply and suitable for commercial treatment of human burn wounds. Formulations investigated included ground amnion, amnion gel, and an amnion graft. The powder and hydrogel treated wounds retracted the least during the first 4 days after wound creation, and had the least wound remaining with the most epithelialization after 28 days. Untreated control wounds had the most contraction, while the gel treated wounds contracted moderately and the powder treated wounds contracted the least. On histologic evaluation of the wounds, the hydrogel and powder treated wounds had epidermal and dermal organization most similar to normal skin, organized, mature collagen, and decreased inflammatory cellular infiltrate when compared to the other treatments.
In further investigation, the powder-treated wounds had increased elastic fibers on pentachrome staining. The powder treated wounds contained mature collagen, while the hydrogel treated wounds had only immature collagen. Due to the desirable healing characteristics of this human powdered amnion material compared to other types of processed amnion materials, a study was designed to evaluate its suitability in healing the problematic equine distal limb wound.

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Xenogeneic Amnion Inhibits Healing in an Equine Distal Limb Wound Model

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ABSTRACT

Objective: To assess the safety and efficacy of lyophilized, milled, human amnion on wound healing in an equine distal limb wound model in a blinded controlled study.

Animals: Four clinically normal adult horses (3 Thoroughbred and 1 Paint, age 6-19 years) obtained via donation.

Procedures: One forelimb of each horse was randomly assigned to the treatment group, and the contralateral limb was assigned as the control. Full-thickness skin wounds were created on each
metacarpus. Treatment limb wounds received with lyophilized, milled, human-derived amnion material delivered on triple antibiotic ointment on non-adherent dressing. Control wounds received with triple antibiotic ointment on non-adherent dressing. Distal limb bandages were applied. Digital photographs of the wounds every 2-4 days throughout a 98-day study period were analyzed wound area and epithelialization. Histopathology was performed on samples from days 7, 21, 35, and 84.

Results: One horse developed unilateral cellulitis that resolved with additional treatment. All treatment limbs exhibited an inflammatory response characterized by focal edema and discharge from the wounds. Wounds were completely epithelialized in control limbs sooner than treatment limbs in all horses, although there was no statistical difference between control (mean 46.8 days) and treatment (mean 51.8 days) wounds ($p = 0.23$). Overall histologic scores were better in control wounds than in amnion material-treated wounds at all time points, only reaching significance on day 84 ($p = 0.039$).

Conclusions and Clinical Relevance: Because wounds treated with amnion material in this study exhibited an inappropriate inflammatory response that resulted in delayed time to wound closure, human lyophilized milled amnion is not recommended for use in equine wound management. Xenogeneic amnion may not be appropriate for wound healing in equines, and further study is needed to determine if other animal sources exhibit similar results.
Introduction

Wounds of the distal limb present a clinical challenge in horses, as they heal slowly and sometimes incompletely, leading to persistent lameness, limb swelling, extensive scars, and subsequent loss of use or euthanasia of the horse. Amnion-derived materials have been studied in the horse, man and other species, and have shown promise in acceleration of wound healing with good cosmetic outcomes.\textsuperscript{1-4} A processed, human-derived amnion material that may become commercially available could make amnion more widely available for use by practitioners and medical providers without the inconveniences of harvesting, processing and storing equine amnion. Previous research on this material in a porcine model showed accelerated healing times with no adverse effects.\textsuperscript{5,6}

The proposed effects of amnion as a wound dressing include acting as a scaffold and providing growth factors to accelerate healing. In the presence of amnion in vitro, both fibroblasts and keratinocytes have improved cellular proliferation and migration.\textsuperscript{1,4} Growth factors and cytokines essential for healing that have been demonstrated in amnion include hepatocyte growth factor, keratinocyte growth factor, transforming growth factor $\beta$1, and others. Further processing of amnion membranes into a powdered form increased the availability of these growth factors.\textsuperscript{5,49} Amnion membranes also have favorable antimicrobial effects, inhibiting the growth of some microorganisms in vitro including \textit{Pseudomonas spp.}\textsuperscript{10}

The primary purpose of this study was to assess the safety of a processed human amnion material for distal limb wound healing in the horse. Secondary objectives for the study were to assess the impact of the human amnion material on rate of wound healing, contraction and epithelialization, the quality of healed skin and the cosmetic appearance of wounds treated with the amnion
material. We hypothesized that the lyophilized human amnion material would be safe for use on wounds of the distal limb in the horse. We also hypothesized that the amnion would accelerate contraction and epithelialization of experimentally created distal limb wounds.

Materials and Methods

Study Design: A randomized, blinded, controlled research study was designed using an equine model of distal wound healing. Bilateral forelimbs of 4 healthy horses were used, with one limb randomly assigned as treatment, and the contralateral limbs assigned as control. Limbs were assigned to the treatment or control group randomly via random number generator, with odd numbers receiving the treatment on the left forelimb and even numbers receiving the treatment on the right forelimb. All wounds on the treatment limb received the amnion-material treatment protocol and all wounds on the control limb received the control protocol.

Animals: Four clinically healthy adult horses (3 Thoroughbred and 1 Paint Cross) with a median age of 11 years (ages 6, 10, 12 and 19 years) were obtained via donation. Inclusion criteria were distal forelimbs free from wounds or scars and horses with no known systemic or metabolic disease. Horses were evaluated for lameness evident at the walk and received physical examinations consisting of temperature, pulse rate, respiratory rate, as well as visual inspection and manual palpation of the limbs. All animal involvement was performed under the regulation and approval of the institution’s animal care and use committee.

Tested Material: A human-derived amnion material was used for amnion treated wounds. Briefly, the amnion was washed with sterile saline, frozen, lyophilized, milled, gamma irradiated at 1 megarad and stored at -80°C. The material was prepared at Wake Forest Institute of Regenerative Medicine, frozen, and shipped in 70 mg doses to the testing center.
**Wounding and Treatment Application:** Wounds were created on Day 0. Each horse was administered 1g phenylbutazone per os pre-operatively, then once daily for 5 days postoperatively. The horses were sedated with detomidine (0.01mg/kg IV) and butorphanol (0.01mg/kg IV) and the distal forelimbs were clipped and aseptically prepared. Mepivacaine hydrochloride 2% (30mL/horse) was infiltrated subcutaneously as a circumferential ring block at the level of the proximal metacarpus. Additional local anesthetic was provided as a high palmar nerve block in one horse that retained cutaneous sensation after the ring block. A sterile plastic template was used to create four evenly spaced, 2.5 cm x 2.5 cm, full-thickness skin wounds with a scalpel blade on each forelimb (Fig 1). Each wound was a minimum of 3cm in any direction from the adjacent wounds. The excised skin was placed in 10% buffered formalin and retained as a normal control on histopathology.

Either 70 mg of amnion material spread on 1mL of triple antibiotic ointment (treatment limb), or 1mL of triple antibiotic ointment alone (control limb) was applied to a non-adherent dressing on Day 0 using the sterile plastic template to ensure complete delivery of the treatment or control material to each wound. The non-adherent dressing was secured to the limb over each wound using conforming gauze immediately after skin excision. The gauze was held in place with elastic adhesive tape (Elastikon) and a standard distal limb bandage applied using cotton combine bandages secured with elastic compression wraps. The top and bottom of each bandage was sealed with elastic adhesive tape to prevent entry of dirt or debris. The treatment material was applied only on Day 0.

**Monitoring:** Horses were stall rested for 7 days prior to allowing confinement in a small paddock for the remainder of the study. Horses were examined daily for temperature, heart rate, respiratory rate, presence of swelling in the forelimbs and lameness at the walk for the first 7
days. Bandages were changed on all horses every 2-4 days throughout a 99-day study period. Triple antibiotic ointment was re-applied to all treatment and control wounds at each bandage change until day 9, then the wounds were bandaged without topical medication. The inner layer of the bandage was discontinued when the wounds were grossly epithelialized, and standing wraps were used for the remainder of the study period to protect the newly formed epithelium. The wounds were inspected for discharge, exuberant granulation tissue, edema and progression of healing (contraction and epithelialization).

Digital photographs (using Nikon Coolpix L340 20MP digital camera) were taken of each wound at each bandage change. A 5mm grid ruler was labeled with patient information, date and limb identifier, then placed immediately adjacent to each wound for photographic documentation and future blinded measurement from stored images. Three photographs were taken of each wound, centered and perpendicular to the wound, at a standardized distance of 20cm from the limb using the flash setting.

Wound Measurements: Two-dimensional digital planimetry was performed to calculate wound area and epithelialized area from the digital images using ImageJ software. The highest-quality photograph from each wound on each day was selected for measurement. The digital photograph was imported to ImageJ and magnified to 50%. A 20mm bar was measured on the photographed ruler and “set scale” software function used to standardize subsequent measurements for each photograph. The freehand selection tool was then used to measure the interface of normal skin with neoepidermis (“Outer - epidermal margin,” line A, Figure 2) as well as the junction of neoepidermis with non-epithelialized tissue (“Inner - wound margin,” line B, Figure 2). Each measurement was repeated in triplicate and the mean used for subsequent analysis. Epithelialized area was calculating by subtracting measurement B (“Inner”) from measurement
A (“Outer”). A single, blinded observer performed all measurements. Only measurements of proximal wounds were retained for statistical analysis to avoid confounding impact of biopsy procedures, which were performed on the distal wounds.

**Histopathology:** Pre-selected wounds were biopsied on days 7, 21, 35 and 84. The distolateral wounds were sampled on day 7 (distal abaxial margin) and 21 (distal axial margin), distomedial wounds on day 35 (distal axial margin), and proximolateral wounds on day 84 (proximal margin). The proximal wounds were left undisturbed until the final time point at day 84, so that influence of biopsy site did not confound wound measurements. To perform the biopsy, the adjacent skin was aseptically prepared with chlorhexidine and rinsed with sterile saline. Horses were administered 1g phenylbutazone per os and sedated with xylazine (0.3-0.4mg/kg IV) and detomidine (0.006-0.01 mg/kg IV) with or without butorphanol (0.01mg/kg IV), depending on the horse’s temperament. Local anesthesia was provided with subcutaneous infiltration of mepivacaine (5-10mL/wound) as an inverted “L” block adjacent to the wound to be sampled. A 6mm biopsy punch was used to obtain a sample of the wound margin, including 2-3mm of adjacent skin. The samples were immediately placed in 10% buffered formalin for histopathology.

The tissue samples were shipped to the secondary institution (WFIRM) and paraffin embedded, sectioned, and stained. Staining was performed with hematoxylin and eosin (H&E) and Masson’s trichrome. All prepared slides were evaluated and scored by a board-certified pathologist. All biopsied wounds and day 0 controls were scored according to a pre-determined scale for presence or degree of epidermal epithelialization (0-3), inflammatory cellular infiltrate (0-2), hair follicles (0-1), glands (0-1), smooth muscle (0-1), collagen orientation/density (0-2), fibroplasia (0-1), vascular proliferation (0-1), and hemorrhage (0-1), resulting in a maximum score of 13
representing normal skin (See Table 1). Only the wounded tissue in the biopsy sample was evaluated for scoring. Glands refer to the presence of either apocrine or sebaceous glands. Hemorrhage was defined as any evidence of chronic hemorrhage associated with the original wound or healing process including evidence of erythrophagocytosis, hemosiderosis, perivascular hemorrhage, or significantly more hemorrhage than in the day 0 control samples. Extravasated red blood cells without these changes were interpreted as acute hemorrhage associated with the biopsy procedure and were not counted in the scoring rubric.

**Statistical Analysis:** Student’s t tests were performed to determine differences between mean number of days to complete epithelialization in treatment and control groups. One-way ANOVA with repeated measures and post-hoc Tukey’s was performed to compare measurements (wound area and epithelialized area) after blocking by horse. Ordinal regression was used to evaluate histopathology scores. Significant difference was set to \( p < 0.05 \).

**Results**

**Safety and Complications:** No horses had serious adverse reactions to the wound model, nor to the treatment. One horse had complications develop on day 2 of the study, with cellulitis in the treatment limb. During creation of the wound on day 0, this horse retained sensation in the limb and underwent repeated injection of local anesthesia in both forelimbs followed by a high palmar nerve block in the treatment limb until desensitization was achieved. On day 2, the horse was reluctant to bend the forelimb and had hot, painful edema proximal to the bandage. He was sound at the walk. The horse was treated systemically with trimethoprim sulfa (22mg/kg per os q12h) for 10 days and phenylbutazone (1g per os q12h) for 10 days. The heat and edema resolved by day 7 of the study and this horse had no further adverse events.
Gross Appearance of Wounds: The wounds on the treatment limbs had a visible difference in gross appearance throughout the study period, most marked in the early stages of wound healing. In general, the wounds on the control limbs were flat, red in color, with minimal serous discharge. In contrast, wounds on the treatment limbs had mild to severe local edema with a roughened or irregular, cobblestone surface, were tan in color, with varying degrees of increased serous or fibrinous exudate. The difference in edema of treatment vs. control limbs was subjectively graded as mild in 2 horses, moderate in 1 horse, and severe in 1 horse (Figure 3).

Time to Epithelialization: Wounds on the treatment limbs had a longer time to epithelialization than wounds on control limbs (Figure 4). The mean time to epithelialization in treatment wounds was 51.8 days (range 39-63), versus a mean of 46.8 days (range 39-60) for control wounds, but the difference was not statistically significant ($p = 0.23$). The epithelialized area was not significantly different in treatment versus control wounds (Figure 5A). Mean wound size (junction of neoepithelium and wound margin) was smaller in control wounds than treatment wounds at all time points, with a significant difference on days 16-25 and 35 (Figure 5B).

Histopathology: Two samples (one treatment and one control on day 21) were excluded from analysis due to sectioning error. All other wounds were evaluated using H&E at all time points (Figure 6) and Masson’s Trichrome on days 0-35. Masson’s Trichrome was not available for day 84 samples due to poor staining.

Overall mean histopathology scores were lower (inferior) for treatment wounds than controls at all biopsy time points (days 7, 21, 35 and 84). The difference was significant only on day 84 (Table 2).
Additionally, post-mortem tissue was available on day 240 for one horse euthanized for reasons unrelated to the study. There was no difference in histologic appearance of the treatment and control wounds in the post-mortem samples (histologic score of 6 for all wounds). All post-mortem wounds were completely epithelialized with evidence of persistent small nodules of lymphoplasmacytic inflammation in the superficial dermis and free hair shafts surrounded by granulomatous inflammation (furunculosis). Wounds had low numbers of hair follicles and sebaceous glands and very rare apocrine glands (Figure 5). The adnexae that were present were haphazardly oriented with abnormal shape (dysplastic).

**Follow-Up:** Wounds were grossly evaluated for each horse in the study at a range of 7-16 months. Two horses were euthanized for reasons unrelated to this study at 7 months and 16 months. One horse was lost to follow-up at 9 months due to adoption, and one horse remains in the institution’s herd and photographic examination was performed 16 months after wounding. During the 99-day study period, both treatment (n=3 wounds, 2 horses) and control (n=5 wounds, 4 horses) wounds were subject to reinjury, with the neoepithelium becoming traumatized and re-wounded. No additional treatment intervention was required. The long-term cosmetic outcome at the time of follow up was similar between treatment and control wounds (Figure 7).

**Discussion**

The xenogeneic amnion material investigated in this study resulted in an excessive inflammatory response in all treated wounds, leading to delayed time to wound closure in this equine distal wound model. This was an unexpected result based on studies using this material in other
animal species. While this material may be appropriate for non-equine species pending further investigation, it cannot be recommended for use in equine wounds.

Previous literature indicates that 2.5cm² wounds in the horse’s distal limb wound be expected to heal at a mean of 42-60 days, but one study reports healing times of 83 days or longer.²,³,³⁵,³⁶,⁵⁸ Amnion-treated wounds in equids are expected to heal at a similar³⁶ or more rapid²,³ time point compared to controls, and wounds in this population healed at a similar time point to those previously reported. Bandaging has been documented to increase the incidence of exuberant granulation tissue formation, but in this study, exuberant granulation tissue was not encountered.³⁴ This could be attributed to the small sample size, and/or the use of triple antibiotic ointment in the inflammatory phase, as triple antibiotic ointment has been shown to decrease the development of exuberant granulation tissue when equine distal limb wounds are bandaged.³⁵ Altering the timing of treatment application, comparing to unbandaged wounds, or repeating treatment application at multiple bandage changes may alter results.

The tested material caused subjectively substantial edema in the treatment wounds, indicated by focal swelling surrounding the wounds and edema within the granulation tissue on gross evaluation. This was supported by histologic analysis, revealing increased inflammation scores in the treatment wounds. A potential cause for increased inflammation in treatment wounds could be graft rejection of the xenogeneic material. Th1 lymphocytes, for example, promote cytotoxic killing and are prominent in graft rejection reactions.⁵⁹ Further investigation and analysis of leukocyte infiltration of amnion-treated wounds may have determined the source of the increased inflammation, but this was outside the scope of this study. The wounds were not submitted for bacterial culture or bacterial quantification, so bacterial contamination cannot be ruled out as the cause of increased inflammation in treated wounds compared with control.
One horse had a complication (cellulitis) in the treatment limb after wound creation and treatment application. It cannot be definitively determined that the cellulitis in this horse was due to the amnion-derived material, but this must be considered. The horse also required additional local anesthesia to complete the wounding procedure, which may increase risk of cellulitis due to repeated needle penetrations of the skin.\textsuperscript{22}

It is not known if the investigated material would produce a similar inflammatory reaction in humans. Previous research on this material in a porcine model showed no inflammatory reaction and resulted in accelerated healing times.\textsuperscript{56} An equine model was chosen for this study due to the poor healing of wounds on the equine distal limb and the propensity for horses to produce unwanted exuberant granulation tissue, which has similarities to keloid formation in humans.\textsuperscript{11,33-35}

The safety of this human amnion material for use in the horse is challenged by the findings of this study. Increased inflammation of the wounds during healing, slower closure of the wounds and development of cellulitis on the treated limb in one horse all suggest that this amnion preparation is not an appropriate treatment for equine wounds. Due to these adverse effects, and the potential that horses are more sensitive to xenogeneic materials on wounds, the authors suggest conducting similar studies to this for any xenogeneic material being marketed for use in horses, even as a medical device. There remains ample evidence in the literature of the positive effects of equine (allogeneic) amniotic membrane for use in equine wound healing. Further investigation of allogeneic, equine-derived amniotic membrane processed in a similar manner would be ideal.

\textbf{Conclusion}
The xenogeneic amnion material investigated in this study is not recommended for use on equine wounds. The material appeared to induce an inflammatory response, resulting in edema in the wounds and reduced wound closure. In addition, one horse had an adverse response that may be attributed to the material resulting in cellulitis of the treated limb. The effects of topical application of this material in other species cannot be determined with this study. Careful study of any other xenogeneic materials for wound healing in horses is recommended.
FIGURES

Figure 1. Schematic of distribution of wounds on the distal limb. Four wounds (represented by squares) were created at the proximomedial, proximolateral, distomedial and distolateral aspect of the metacarpus. Circles represent location of 6mm biopsy punches at each labeled time point.
Figure 2. Wound Measurement Technique in a wound on day 39. Digital software was used to measure the interface of normal skin with neoepidermis (“Outer epidermal margin,” line A) as well as the junction of neoepidermis with non-epithelialized tissue (“Wound margin,” line B).
Figure 3. Horse with severe focal edema and fibrinous exudate from each of the treatment wounds on Day 14. The control wounds have minimal edema and no discharge.
Figure 4. Images of wounds at selected time points taken at a standard distance. Ruler grid = 5mm.
Figure 5. Wound Measurements. A) Area of wound composed of neoepithelium in mm$^2$ over time. B) Area of wound not epithelialized. Asterisks (*) denote a significant difference ($p < 0.05$) between treatment and control wounds.

A.

![Epithelialized Wound Area](image)

B.

![Wound Area](image)
Figure 6. Day 35 Treatment limb. Re-epithelialization of the wound is incomplete and there is a focally extensive area of ulceration with granulation tissue and hemorrhage (arrow heads). There is fibrosis, vascular proliferation and hemorrhage within the dermis. Hair follicles, adnexal structures, and smooth muscle are absent. H&E stain; bar= 600mm. Inset: Photomicrograph of superficial dermis demonstrating mild neutrophilic and lymphoplasmacytic inflammation (circled), fibrosis, vascular proliferation (arrows) and hemorrhage. The vascular proliferation in this section is more severe than that in the biopsy from the control limb bar= 60mm.
Figure 7. Long-term cosmetic and functional outcome was similar between treatment and control wounds.
Table 1. Histopathology Scoring Rubric. Each wound received a score for each category at Days 0, 7, 21, 35 and 84, as assessed by a board-certified pathologist. Normal skin received a total score of 13.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epidermal Epithelialization</strong></td>
<td>Ulcerated without epithelialization</td>
<td>Ulcerated with partial epithelialization</td>
<td>Complete epithelialization with hyperplasia</td>
<td>Normal/intact skin</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>Moderate to severe</td>
<td>Mild</td>
<td>Absent</td>
<td></td>
</tr>
<tr>
<td><strong>Hair follicles</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glands</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smooth Muscles</strong></td>
<td>Absent</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Collagen Orientation/density</strong></td>
<td>Abnormal deep dermis</td>
<td>Abnormal superficial dermis</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td><strong>Fibroplasia</strong></td>
<td>Present</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vascular Proliferation</strong></td>
<td>Present</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hemorrhage</strong></td>
<td>Present</td>
<td>Absent</td>
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</table>
Table 2. Mean histopathology scores for treatment and control wounds at each biopsy time point,
on a scale of 0 (acutely wounded skin) to 13 (normal, intact skin).

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 35</th>
<th>Day 84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.25</td>
<td>0.33</td>
<td>2.75</td>
<td>2.5</td>
</tr>
<tr>
<td>Control</td>
<td>0.75</td>
<td>1.00</td>
<td>3.75</td>
<td>4.5</td>
</tr>
<tr>
<td>p</td>
<td>0.157</td>
<td>0.083</td>
<td>0.435</td>
<td>0.039*</td>
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</tbody>
</table>

*Difference was significant on day 84
<table>
<thead>
<tr>
<th>Day 0</th>
<th>Wounding</th>
<th>Limbs blocked with local anesthetic Wounds aseptically created Treatment applied Limbs bandaged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>Biopsy</td>
<td>6mm punch biopsy distolateral wound</td>
</tr>
<tr>
<td>Day 9</td>
<td>Discontinue triple antibiotic ointment on all wounds</td>
<td>Continue bandaging with non-adherent dressing on inner layer</td>
</tr>
<tr>
<td>Day 21</td>
<td>Biopsy</td>
<td>6mm punch biopsy distolateral wound</td>
</tr>
<tr>
<td>Day 35</td>
<td>Biopsy</td>
<td>6mm biopsy punch distomedial wound</td>
</tr>
<tr>
<td>Day 63</td>
<td>All wounds epithelialized</td>
<td></td>
</tr>
<tr>
<td>Day 84</td>
<td>Biopsy</td>
<td>6mm punch biopsy proximolateral wound</td>
</tr>
</tbody>
</table>
REFERENCES


