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Original Research

Evaluation of a Hyaluronic Acid-Based Biomaterial to Enhance Wound Healing in the Equine Distal Limb

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ABSTRACT

The objective of this study was to evaluate the ability of a cross-linked hyaluronic acid-based biomaterial (CMHA) to promote healing in an equine distal limb wound model. Three 6.25 cm^2 full thickness skin wounds were surgically created on the dorsomedial metacarpus and metatarsus of each of four limbs of eight normal adult horses under general anesthesia. Three days after surgery, wounds were photographed and one of four treatments was applied to all three wounds on a single limb: control (no CMHA), single application of CMHA gel, multiple applications of CMHA gel, and multiple applications of CMHA film. Wounds were assessed for quality of repair tissue and photographed for image analysis at bandage changes every 4 days throughout the 47-day study. Exuberant granulation tissue was excised as needed and weighed. Size of granulating wounds was analyzed by repeated measures analysis of covariance and rate of wound healing assessed using a logistic decay model fitted to the data by nonlinear least-squares regression. Significance was set at $P < .05$. No adverse effects of CMHA were noted. There was a significant day by treatment interaction; however, overall, there were no significant differences among treatment groups ($P = .09$). Wounds treated with CMHA films decreased to half their original size significantly faster, were significantly smaller on day 31, and healed with higher quality, less fragile epithelium than wounds in other treatment groups. Treatment of distal limb wounds in horses may benefit from use of CMHA films to improve healing.

1. Introduction

Traumatic wounds are common in horses, and those on the distal limb can be especially challenging due to tissue loss, relatively poor blood supply, and/or damage to the blood supply [1]. They are under relatively high tension and are prone to the formation of exuberant granulation tissue [2,3]. Delayed healing in the distal limb of horses can be the result of prolonged inflammation, slow epithelialization, high motion, presence of a foreign body or necrotic tissue, and cessation of wound contraction early in the healing process [4–6]. These and other factors result in the need for protracted wound care. Even with optimal care, suboptimal results frequently occur due to formation of exuberant granulation tissue and/or excessive scar tissue that may cause chronic lameness, unacceptable cosmesis, or fragile scar prone to reinjury. Many of the problems encountered in equine wound healing present similar challenges in

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human medicine, including delayed wound healing and scar formation [7,8].

Over the past 20 years, authors have reported on a variety of novel dressings and topical medications designed to speed or improve the quality of healing; however, the results have been mixed [9–13]. Wound dressings perform a variety of functions including protection from bacterial infection, maintenance of a moist environment, absorption of exudates, and stimulation of keratinocyte proliferation and differentiation [14]. Biologic agents are receiving increased attention as a means of stimulating optimal wound healing [11,12,15,16]. Hyaluronic acid (HA) is an attractive material for tissue regeneration applications such as wound healing because of its natural role in tissue repair, its biocompatibility, and the ability to optimize its physical properties [17–22].

Hyaluronic acid is a nonsulfated glycosaminoglycan ubiquitous in biological fluids and tissues and is a critical component in the structure and organization of extracellular matrix (ECM) [23]. Hyaluronic acid is a linear poly saccharide comprised of alternating units of β-1,4-linked D-glucuronic acid and (β-1,3) *N*-acetyl-D-glucosamine. Despite its simple structure, HA has diverse biological functions [24]. However, the mechanisms by which it actively participates in tissue repair remain unclear. In addition to its structural role in the ECM, HA influences cell behavior such as cell proliferation, differentiation, adhesion, and migration by its unique hygroscopic, rheologic, and viscoelastic properties [17,25–27]. Finally, HA is known to directly affect cell function by binding to cell surface receptors and mediating a variety of downstream effects important in wound healing, including increased expression of proinflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-1β, interleukin-8), cell migration, cell proliferation, and organization of granulation tissue matrix [17,25,28,29].

Although the exact role of HA in fetal wound healing is not known, the findings of numerous studies suggest that HA plays an important role in fetal scarless wound healing [26,30–32]. Hyaluronic acid and its receptors are found in greater abundance in fetal tissues compared to adult tissues [29,33,34]. The density of the HA cell surface receptor, CD44, was approximately four times higher on fetal rabbit dermal fibroblasts compared to adult rabbit fibroblasts [29]. Hyaluronic acid increased more rapidly and remained elevated longer in fluid from experimental wounds in lambs [35,36]. The amount of HA-stimulating activity was greater in fetal wounds compared to adult wounds in a rabbit model of wound healing [33]. Fetal rabbit wounds treated with the enzyme hyaluronidase to reduce the HA content showed markedly altered healing compared to control wounds, resulting in increased fibroblast infiltration, collagen deposition, angiogenesis, and infiltration of inflammatory cells [37]. Considered together, these data strongly support the role of HA and its regulation in wound healing.

Hyaluronic acid, in its unmodified form, has been used for drug delivery and viscosurgery; however, its poor biomechanical properties and rapid degradation limit its clinical applications [38]. Hyaluronic acid is, however, amenable to a variety of chemical modifications that can make it more mechanically and chemically robust for in vivo implantation. Carboxymethylation of HA creates more available sites for thiol modification [39,40]. Thiol modification creates active thiol groups available to form chemical cross-links, which decrease the degradation rate of the modified HA [38–40]. Cross-links formed by this method can be created in a biocompatible manner rather than having to use cross-linking agents that produce cytotoxic products. The result is a thiolated carboxymethyl HA (CMHA)–based biomaterial that is biocompatible and supports cell proliferation [17–22,38,41].

Numerous studies document the ability of both topical and systemic HA to improve wound healing in laboratory animals [21,41–46]. Topical application accelerates reepithelialization and decreases fibrosis and scar tissue formation of dermal wounds in mice and rats [14,18,21,41,44] and improves corneal wound healing in rabbits [21]. Mechanical properties of wounds were increased in diabetic mice following systemic administration of HA [46]. In vivo studies evaluating the effects of chemically modified HA hydrogels and films are limited; however, a preliminary study comparing healing in rats, dogs, and horses found that cross-linked HA-based biomaterials enhance wound healing [41]. Wound areas in rats 7 days after CMHA hydrogel application and in horses 17 and 26 days following CMHA film application were significantly smaller than control wounds, and the wound beds of all species were grossly healthier in appearance in CMHA-treated wounds [41]. Although smaller than control wounds, the change in wound size in dogs treated with CMHA films failed to reach statistical significance at 14 and 21 days [41]. The results of this preliminary study support further investigation of CMHA hydrogels and films for the treatment of distal limb wounds in horses.

The purpose of our study was to evaluate the ability of CMHA [41,47,48] to enhance wound healing using a model of equine distal limb injury. We hypothesized that application of CMHA to surgically created full thickness wounds on the distal limbs of horses would result in an increased rate and quality of wound healing compared to wounds managed with bandaging alone, regardless of product formulation (hydrogel or film) or application frequency.

2. Materials and Methods

2.1. Experimental Animals

Adult mixed breed horses (n = 8; mean, 3.4 years; range, 2–8) were examined clinically to ensure that no preexisting skin defects were present on the distal limbs. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC). Horses were housed in individual 10 × 10 foot stalls and allowed to acclimate for 2 to 4 days before surgery. A normal complete blood count was confirmed for each horse before study initiation.

2.2. Surgical Procedure

Each horse was treated perioperatively with trimethoprim sulfa (30 mg/kg orally twice daily for 3 days) and

phenylbutazone (4.4 mg/kg orally once daily for 3 days). Feed was withheld for 12 hours before surgery. An intravenous catheter was placed in the left jugular vein, and horses were sedated with xylazine (1.1 mg/kg intravenously [IV]). Anesthesia was induced with diazepam (0.02 mg/kg IV) and ketamine hydrochloride (2.2 mg/kg IV), orotracheal intubation with a cuffed endotracheal tube was performed, and horses were administered oxygen at 10 to 15 L/min via the tube. Anesthesia was maintained with a combination of xylazine, ketamine, and guaifenesin (500 mg, 2 g, and 50 g, respectively) in 1 L of sterile 0.9% NaCl solution administered IV to effect (approximately 1 mL/kg/hr). The third metacarpi and metatarsi were clipped and aseptically prepared using povidone-iodine and 70% isopropyl alcohol. Three, 6.25 cm^2, full thickness skin wounds (2.5 cm × 2.5 cm) were created on the dorsomedial aspect of each metacarpus and metatarsus using an established model of wound healing (Fig. 1) [9,10,49,50]. Wounds were centered and aligned vertically on the long axis of the bone and were spaced 2 cm apart. Wounds were standardized using a sterile template. The skin was incised using a #10 scalpel blade, separated from the underlying subcutaneous tissues and the skin excised using mayo scissors. Care was taken to leave the subcutaneous tissues intact. Wounds were bandaged routinely (nonadherent dressing, sterile gauze, elastic adhesive tape, and cotton standing bandage). All horses recovered uneventfully without assistance.

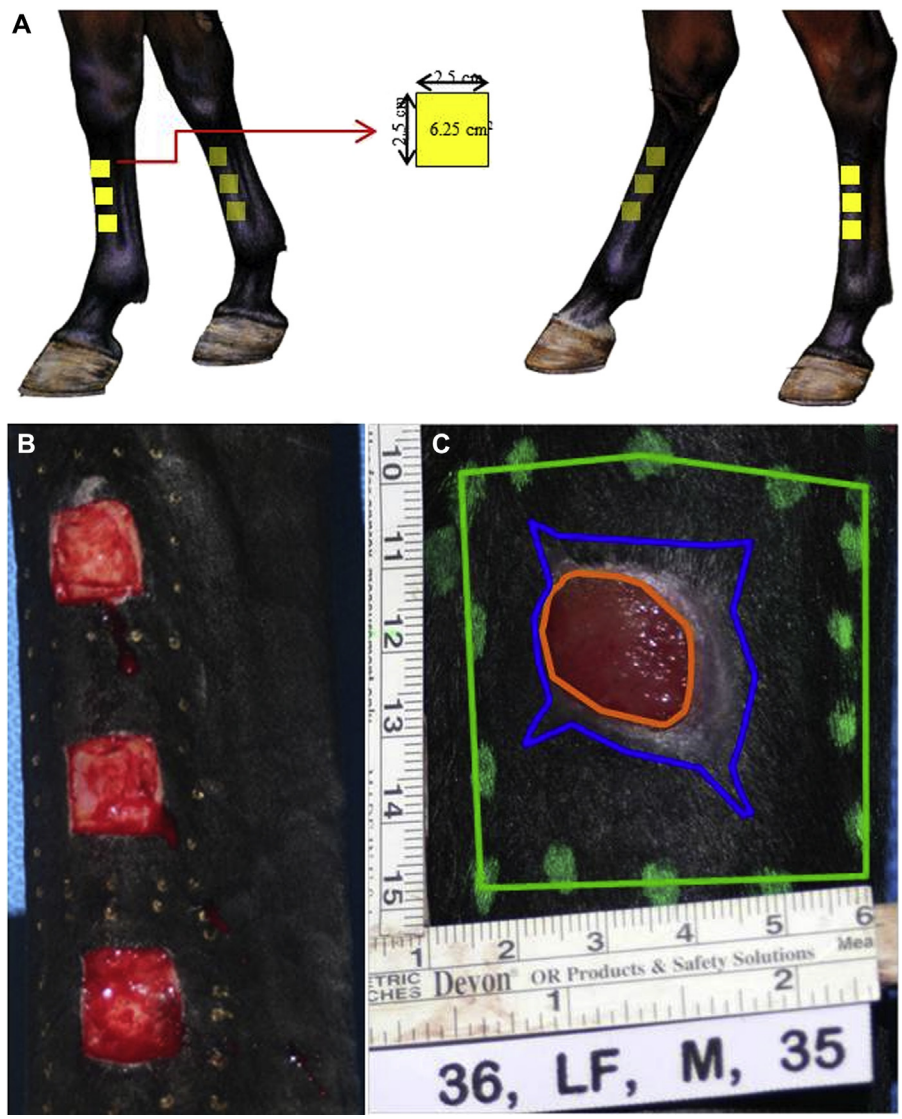


Fig. 1. Study design. (A) Schematic showing size, location, and arrangement of limb wounds; (B) representative wounds on a single limb 3 days following surgery before the start of treatments; (C) Example of a healing wound following image preparation for planimetry analysis with the healing wound (blue) and granulating wound bed (orange) outlined. The difference of the area surrounded by the blue and that surrounded by the orange is the area that has healed by epithelialization. A ruler is included for internal calibration, and the label identifies the individual horse, the wound location on the limb, and the day after surgery.

2.3. Treatments

Three days after surgery, bandages were changed, wounds were photographed, and one of four treatments was applied. A Latin square design was used to control for the two sources of variation (horse and limb location) with one of the limbs on each horse serving as a control [51,52]. Treatments were randomized among the four limbs of the first horse, and a rotating pattern was used on each horse thereafter so that each treatment was applied to each limb location twice in the eight horses. The result of this allocation of treatments was an amalgamation of two Latin squares to form a 4 × 8 rectangular design [51]. Each of the three wounds on a particular limb received the same treatment to avoid potential confounding that may have occurred with different treatments applied to the same limb. Two sterile CMHA formulations, gels and films, were used in our study; their synthesis is described in detail elsewhere [41]. Briefly, the starting product for both was thiolated carboxymethyl HA that was then either cross-linked with poly(ethylene glycol) diacrylate (films) or disulfide cross-linked (gels). Films were then cast onto nonstick gauze pads. The four treatment groups were as follows: control (bandaging only), single application of CMHA gel (single gel) (equitrX Gel, SentrX Animal Care, Salt Lake City, UT), multiple applications of CMHA gel (multiple gel), and multiple applications of CMHA film (multiple film) (equitrX Film-On-Gauze (5 × 5 cm), SentrX Animal Care). Single application occurred only on day 3, whereas multiple applications were performed every fourth day at the time of each bandage change (11 total applications). Carboxymethyl HA gel was applied in a thin layer covering the entire wound using a gloved finger. One 5 × 5 cm film was applied over each of the three wounds per leg. Bandages were changed and wounds photographed on the third postoperative day and every fourth day thereafter until the completion of the 47 days study.

2.4. Wound Management

Horses were sedated with detomidine (5–10 μg/kg IV), butorphanol (0.01–0.02 mg/kg IV), and acepromazine (0.03 mg/kg IV) for each bandage change. Granulation tissue was considered exuberant when the periphery of the granulation bed was above the surrounding skin and covering the advancing epithelial margin; wounds were considered healed when they were completely covered with epithelium. Exuberant granulation tissue was trimmed to the level of the surrounding skin or migrating epithelium by simple excision using a single edge razor blade with horses standing under sedation. The number of excisions was recorded for each wound and the excised tissue frozen and saved for later quantification of total weight of trimmed tissue. Granulation tissue was trimmed only as needed, but not excessively, in a manner similar to that used in clinical practice. At the time of each bandage change, wounds were gently cleaned of debris and exudate using sterile saline from a spray bottle and blotted dry before photography. First appearance of granulation tissue and first appearance of new epithelium were recorded for each wound. The general appearance of wounds and any details associated with wounds were also recorded.

2.5. Assessment of Wound Healing

Wounds were photographed at the time of each bandage change using a digital camera (EOS 20D, Canon USA, Inc, Lake Success, NY) placed on a tripod [9]. As needed, the hair surrounding the wounds was clipped to ensure accurate identification of wound margins. Photographs were taken before trimming exuberant granulation tissue. Horizontal and vertical metric scales forming a right angle were included in each photograph for calibration, and a label was placed below the wound for identification of horse, treatment, day, and wound location. Images were first prepared using commercial software (PowerPoint 2007, Microsoft Corporation, Seattle, WA) by tracing the hair margin of the wound periphery (wound area) and the margin between the granulation tissue and the epithelium (granulation tissue area). Images were then saved for determination of total wound area and granulation tissue area using planimetry software (ImageJ 1.42, National Institutes of Health, Bethesda, MD) (Fig. 1). Quality of healed tissue in day 47 photographs was graded based on the following criteria: color of advancing epithelium (4 = healthy pink, 3 = mild discoloration, 2 = moderate discoloration, and 1 = gross discoloration); flatness of advancing epithelium (4 = flat and regular, 3 = small raised areas, 2 = multiple raised areas, and 1 = grossly raised); quality of adherence to underlying granulation tissue (4 = fully adhered, 3 = small areas lacking adherence, 2 = multiple areas lacking adherence, and 1 = marked lack of adherence); and the degree to which epithelium tore during wound preparation (4 = no tearing, 3 = minor tearing, 2 = moderate tearing, and 1 = severe tearing). Scores for each criterion and for all three wounds on a single limb were combined to achieve a single score per limb with a higher score indicating a higher quality of healed tissue.

2.6. Statistical Analysis

Data for wound size and granulation area were averaged for the three wounds on each limb for all analyses. Repeated measures analysis of covariance (ANCOVA) was used to assess differences over time and differences between groups based at specific time points (SAS version 9.2, SAS Institute, Inc, Cary, NC). Size of the wound on day 3 at the time of initial treatment application was used as the covariate. In addition, nonlinear least squares (LS) regression was performed (TableCurve 2D 5.0, SYSTAT Software Inc, Richmond, CA) to fit data on the relationship between mean size of granulation area and time using the logistic decay function [53,54]:

$$Y(t) = \frac{A}{1 + \exp(b^*(t - c))}$$

where for each treatment, $Y(t)$ is mean size of granulating wound on a particular day (t); the constant A represents the mean size of granulating wound at the start of observations

Table 1
Number of days to first need to trim granulation tissue, number of times granulation tissue required trimming, total weight of granulation tissue trimmed, and number of days to first appearance of new epithelium.

Treatment	Days to First Tissue Trim	Number of Times Tissue Trimmed	Total Grams of Tissue Trimmed	Days to First New Epithelium
Control	19.5 ± 4.50	2.38 ± 0.92[ab]	5.47 ± 2.82	18.0 ± 8.21
Single gel	22.0 ± 10.6	2.25 ± 1.28[a]	5.03 ± 3.89	19.5 ± 8.93
Multiple gel	15.0 ± 0.00	3.25 ± 1.16[b]	6.88 ± 4.25	20.0 ± 5.13
Multiple film	16.5 ± 3.00	2.75 ± 0.71[ab]	5.93 ± 3.58	18.5 ± 6.21

Abbreviation: SD, standard deviation.
Mean ± SD. Superscript letters indicate significant differences between groups.

in each treatment; *b* is a measure of the initial rate of change (decrease) in the mean size of granulating wound; and *c* is the time (number of days) it takes a granulating wound to decrease to half its original mean size (A). Significant differences in the mean values for each of the parameters, A, *b*, and *c*, among the treatments were determined by nonoverlapping 84% confidence intervals, which equates to a significance level of $P < .05$ [55,56].

Differences in time to first trimming of granulation tissue, number of times granulation tissue was trimmed, weight of trimmed tissue, and quality of healed tissue were analyzed by mixed model analysis of variance (ANOVA) (SAS version 9.2, SAS Institute Inc, Cary, NC). Time to first appearance of new epithelium was analyzed by Friedman's chi-square analysis (SAS version 9.2). Significance was set at $P < .05$.

3. Results

All wounds in all treatment groups progressed well during the study period and were at least 85% covered with epithelium at study completion; most wounds were >90% epithelialized. No detrimental effects of CMHA were detected for any wounds or treatment groups. Two wounds (both from the CMHA film-treated group) were completely epithelialized at the end of the study period. One wound on one limb of a single horse in the single gel group did not require trimming of granulation tissue at all. There was a significant difference between groups in the number of times granulation tissue required trimming ($P = .04$; Table 1). Wounds treated with multiple applications of CMHA gel required a significantly greater number of trimmings compared to the single application of gel. There were no significant differences between groups in the amount of granulation tissue removed (by weight) over the course of the study ($P = .44$; Table 1). The mean time to first trim of granulation tissue ($P = .27$) and the first appearance of new epithelium ($P = .78$) were not significantly different between treatment groups (Table 1). Wound quality at day 47 was significantly different between treatment groups ($P = .002$; Fig. 2). Wounds treated with CMHA film had a significantly higher score for the quality of the healed tissue compared to control ($P = .020$), single gel ($P = .005$), and multiple gel ($P = .005$) groups (Fig. 3).

There were no significant differences overall in the LS means for the size of the granulation tissue area ($P = .09$) or wound ($P = .35$) among treatment groups; however, day by treatment interaction was significant for both ($P = .002$ and .013, respectively) indicating that the treatments resulted in different patterns in the size of the granulation tissue and wound area across time (Fig. 4). Because the graphs appear similar, the more detailed results are presented for granulation tissue area only. Mean size of the granulation tissue area of wounds treated with the multiple film application was smaller than control wounds on day 19 ($P = .07$) and significantly smaller on day 31 ($P = .02$) (Fig. 5). Mean granulation tissue area for the single gel group was significantly smaller than means for the multiple gel or multiple film groups ($P = .04$ and $= .05$, respectively) on day 11. Mean granulation tissue area for the multiple film group was significantly smaller than that of the multiple gel group on day 15 ($P = .04$).

Data were fitted by nonlinear LS regression to the logistic decay model. The ANOVA of the regression for the curve fit of the data under each of the treatments was significant ($P < .001$) with $R^2 > .90$, confirming that the curves (and parameters) were a good fit to the respective data sets. No significant differences were observed among treatments with respect to original size of granulating wounds indicating that all treatments started with equivalently sized wounds (Table 2). The initial mean rates of decrease in size of the granulating wounds were greater in the single gel and multiple film groups; however, these rates were not significantly different from the control and multiple gel groups. The time it took for the granulating wounds to decrease to 50% of their original size was significantly shorter for the multiple film treatment compared with the other three treatments ($P < .05$),

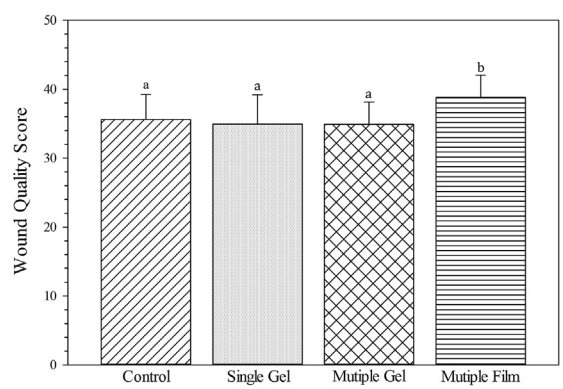
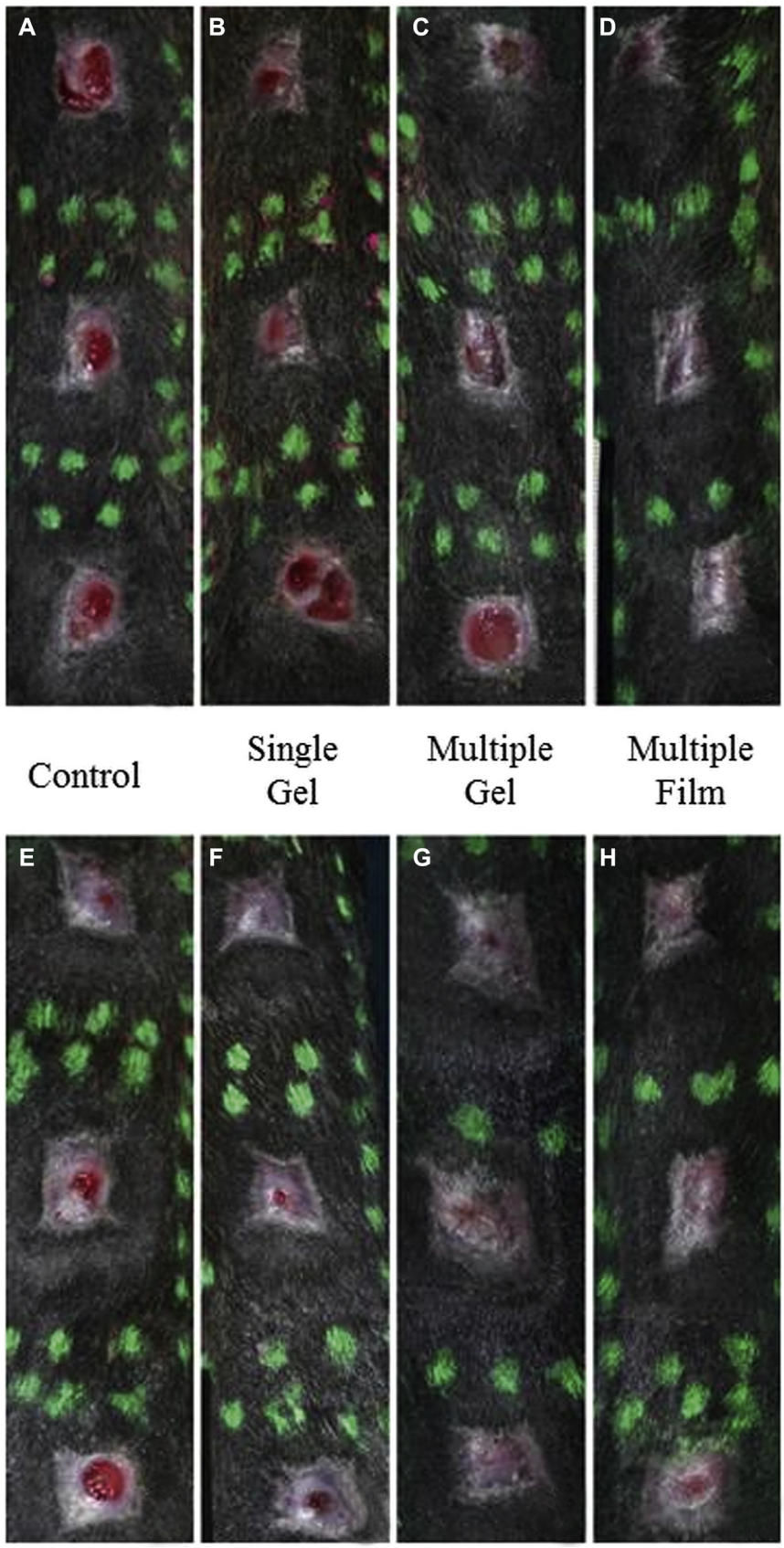


Fig. 2. Mean (±SD) wound quality scores for day 47 control wounds and those treated with single gel, multiple gel, or multiple film applications of CMHA. Letters indicate significant differences between groups. SD, standard deviation. CMHA, carboxymethyl HA-based biomaterial.


A
B
C
D
Control
Single Gel
Multiple Gel
Multiple Film
E
F
G
H

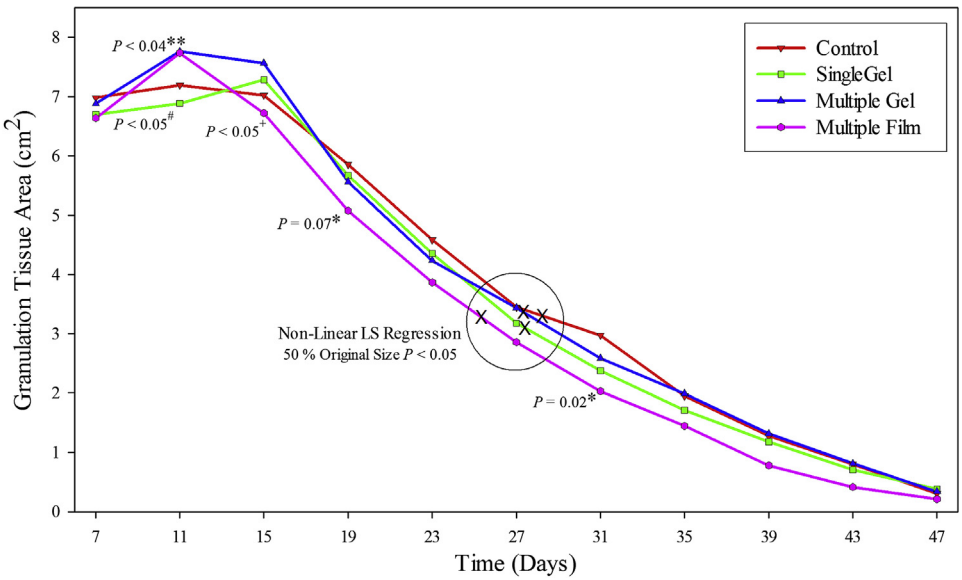


Fig. 4. Least squares means and nonlinear LS regression for the size of the granulating wounds over time for each of four treatments. There were no significant differences among treatment groups overall; however, there was a significant day by treatment interaction, indicating that the treatments resulted in different patterns in the size of the granulation tissue area across time. X marks the mean time point at which wounds in each treatment group reached 50% of their original size. Differences of interest between individual groups are indicated: **single gel versus multiple gel; #single gel versus multiple film; +multiple film versus multiple gel; *multiple film versus control.

indicating that, overall, wound healing occurred faster with this treatment.

4. Discussion

The majority of wounds heal with minimal medical attention; however, in both people and horses, a subset fails to heal normally and complications associated with their healing commonly necessitate prolonged treatment and/or frequent medical attention. There is a strong need for the development of novel wound dressings capable of actively participating in and therefore helping direct the healing process. Hyaluronic acid-based dressings show great potential to be successfully used in such a manner [14,17,19]. The results of our study support the existing literature establishing HA as a logical selection for a biological dressing. Wounds treated with CMHA films healed with superior tissue quality and the granulation tissue area reached 50% its original size significantly earlier than control wounds.

Wound healing over time was analyzed in two different ways to evaluate both size of the granulation tissue area (ANCOVA) and rate at which size changed (LS regression). In both analyses, wounds treated with multiple applications of the film preparation showed significant improvement compared to the other treatments. The results of the wound size analyses, along with the higher wound quality scores, support the potential benefits of the film-on-gauze preparation for treatment of full thickness wounds on the distal limb of horses. Our results and those obtained in other animal species lend further support to the usefulness of HA-based biologic dressings in the management of wounds across species, including people.

The gel-based preparations failed to demonstrate a clear benefit over the control wounds. After the initial 15 days, during which all wounds retracted slightly, the HA-treated wounds were at no time larger than controls. Wound quality scores were not significantly different between controls and gel-based preparations. Based on the results presented, a single application of gel appears to be superior for full thickness wounds such as those created in this study than multiple applications of the gel, which resulted in increased granulation tissue formation. Large degloving wounds with significant tissue deficits, where stimulation of granulation tissue is desirable to fill the void, may be an excellent application for repeated application of CMHA gel.

Formation of granulation tissue is essential for successful second intention wound healing. Granulation tissue physically fills a wound deficit created by significant tissue loss, forms a protective barrier against environmental contaminants, and eventually creates a scaffold across which the epithelium can migrate [2,57]. Myofibroblasts contained within granulation tissue are responsible for wound contraction, an essential part of second intention healing [58]. Degloving wounds with significant tissue loss

Fig. 3. Representative images of wounds from two horses on day 47 (A and E) control; (B and F) single gel; (C and G) multiple gel; and (D and H) multiple film. Wounds treated with CMHA films healed with significantly greater mean scores for quality of tissue demonstrating superior color, uniformity, adherence to the underlying granulation tissue, and resistance to tearing at the periphery. CMHA, carboxymethyl HA-based biomaterial.

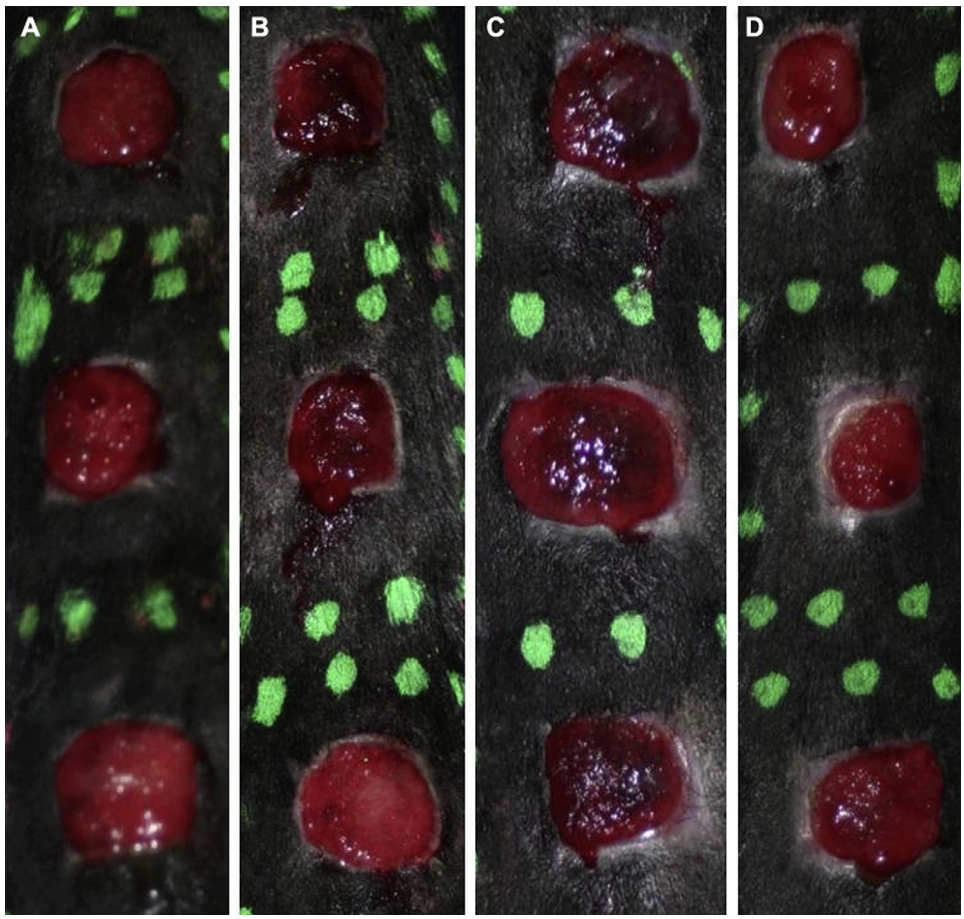


Fig. 5. Representative images of wounds from a single horse on day 31. (A) control; (B) single gel; (C) multiple gel; and (D) multiple film. At this time point, the mean granulation tissue area of those wounds treated with multiple films was significantly smaller than the controls.

are especially common on the distal limb of horses and failure of a wound to fill with healthy granulation tissue due to exposure of bone can result in significantly delayed wound healing [59,60]. Multiple applications of the gel formulation early in the healing process to stimulate the formation of granulation tissue and to fill a large defect, if followed by the application of the film-on-gauze formulation of CMHA, may provide an ideal combination to speed and optimize the healing process.

A specific reason for the differences in healing noted between the gel and film preparations is unknown and is worthy of further investigation. Although the CHMA starting material is identical between the gel and film products, there are several minor structural differences as a result of the two cross-linking methods that could impact wound healing [41]. The lower degree of cross-linking present in the gel makes the HA more readily accessible for aiding wound healing. On the other hand, the film rapidly hydrates by absorbing blood and wound exudate. Growth factors and cytokines present in absorbed fluids could then be rereleased as the film degrades. In addition, the higher degree of cross-linking in the films compared to the gels results in slower degradation, prolonging the release of HA. Finally, the presence of the film's gauze backing, similar to that found in routinely used semi-occlusive nonstick dressings, may have resulted in a more favorable microenvironment for wound healing. The non-adherent dressing used was not specifically semiocclusive

Table 2
Nonlinear least-squares curve fit of the relationship between mean size of granulation tissue area (cm^2) and time (days) after treatment.

Treatment	Granulation Tissue Area—Day 3 (cm^2)	Initial Rate of Decrease of Granulation Tissue Area	Days to 50% Original Size
Control	6.99 ± 0.26	0.145 ± 0.014	28.17 ± 0.86[a]
Single gel	6.64 ± 0.28	0.153 ± 0.018	27.64 ± 0.97[a]
Multiple gel	6.96 ± 0.27	0.135 ± 0.013	27.39 ± 0.94[a]
Multiple film	6.84 ± 0.16	0.151 ± 0.009	25.26 ± 0.53[b]

Abbreviation: SEM, standard error of the mean.
Mean ± SEM. Letters indicate significant differences between groups.

and was selected for ease of covering the three wounds under one bandage and to avoid slippage. In retrospect, it may have been useful to have substituted a gauze bandage more similar to that used as the CMHA film.

Our study was designed to compare several factors of interest: healing resulting from any of the three forms of CMHA to controls, gel versus film formulations, and single application of gel versus repeated applications. In a rodent wound healing model, single gel application resulted in significantly improved wound healing compared to controls [18]. However, because wounds are commonly cleaned at the time of bandage changes in the horse, multiple applications of the gel were included in our study design to mimic how it might be used in clinical practice.

There are several factors that could account for the lack of a difference seen in our study and that performed in rodents [18,41]. First, there are tremendous variations in wound healing between species and horses are notorious for delayed wound healing. Rodent wounds were created on the dorsum of mice and rats, and the majority of the healing would have been expected to occur as a result of wound contraction rather than epithelialization compared to the distal limb. Creation of three wounds per limb in our model would have reduced the amount of healing that could occur via contraction, and our model was therefore even more dependent on epithelialization. No clear conclusions can be drawn from our study regarding the frequency of application of CMHA products. However, because it is common to clean equine wounds at the time of each bandage change to remove accumulated exudates and because of the potential for multiple applications of gel to stimulate granulation tissue formation, reapplication of either form of the product at the time of bandage change is a logical treatment protocol.

There are a several additional aspects of our study that merit further discussion. A model of wound healing was selected to achieve standardized size and depth of wounds. Translation of the results of our study to naturally occurring wounds in horses must be made with caution. Granulation tissue was trimmed from each wound based on clinical need; however, differences in the number of times granulation tissue was removed between groups could affect the rate and quality of wound healing. Because of differences in healing rates between treatment groups, not all wounds were followed to complete healing. The ability to follow all wounds to complete closure may have revealed additional information on delayed healing or even nonhealing wounds. Finally, we chose to use perioperative antibiotics in our horses because of the multiple wounds on a single limb and in compliance with our IACUC. It is difficult to determine how this may relate to the treatment of clinical cases and whether the use of short-term antibiotics could have resulted in a different outcome compared to no antibiotic therapy. Some horses may not receive immediate antibiotic therapy; however, a portion of wounds are identified early and treated with prophylactic antibiotics. Whether the effects of the CMHA biomaterials used in this study would be altered in the absence of antibiotic therapy is unknown. Our study design ensured that all wounds were treated the same way with respect to antibiotic therapy, avoiding treatment bias within our study.

5. Conclusions

There is a need for novel wound dressings with the potential to participate in and direct the healing process, promoting wound contraction and epithelialization. Based on evidence for an active role of HA in tissue repair and the results of our study, treatment of distal limb wounds in horses may benefit from use of CMHA films to improve the rate and quality of healing. Early stimulation of healthy granulation tissue by multiple applications of CMHA gel to speed the rate of filling of a large defect followed by application of CMHA films should be considered as a useful adjunct to conventional wound management by second intention healing. Further studies documenting histological, gene expression, and mechanical differences in wounds following application of either the film or gel preparation would provide more detail into the mechanisms by which CMHA could aid wound healing. Clinical investigation in naturally occurring wounds might help further define the potential benefits of both CMHA products.

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References

[1] Wilson DA. New and innovative approaches to wound closure. In: Stashak TS, Theoret CL, editors. Equine wound management. 2nd ed. Ames: Wiley-Blackwell; 2008. p. 225–37.
[2] Wilmink JM, Van Weeren PR. Treatment of exuberant granulation tissue. Clin Tech Equine Pract 2004;3:141–7.
[3] Theoret CL. Physiology of wound healing. In: Stashak TS, Theoret CL, editors. Equine wound management. 2nd ed. Ames: Wiley-Blackwell; 2008. p. 5–28.
[4] Jacobs KA, Leach DH, Fretz PB, Townsend HGG. Comparative aspects of the healing of excisional wounds on the leg and body of horses. Vet Surg 1984;13:83–90.
[5] Wilmink JM, Stolk PW, van Weeren PR, Barneveld A. Differences in second-intention wound healing between horses and ponies: macroscopic aspects. Equine Vet J 1999;31:53–60.
[6] Wilmink JM, van Weeren PR, Stolk PW, Van Mil FN, Barneveld A. Differences in second-intention wound healing between horses and ponies: histological aspects. Equine Vet J 1999;31:61–7.
[7] Dechert TA, Ducale AE, Ward SI, Yager DR. Hyaluronan in human acute and chronic dermal wounds. Wound Repair Regen 2006;14:252–8.
[8] Namazi MR, Fallahzadeh MK, Schwartz RA. Strategies for prevention of scars: what can we learn from fetal skin? Int J Dermatol 2011;50:85–93.
[9] Berry 2nd DB, Sullins KE. Effects of topical application of antimicrobials and bandaging on healing and granulation tissue formation in wounds of the distal aspect of the limbs in horses. Am J Vet Res 2003;64:88–92.
[10] Howard RD, Stashak TS, Baxter GM. Evaluation of occlusive dressings for management of full-thickness excisional wounds on the distal portion of the limbs of horses. Am J Vet Res 1993;54:2150–4.
[11] Monteiro SO, Lepage OM, Theoret CL. Effects of platelet-rich plasma on the repair of wounds on the distal aspect of the forelimb in horses. Am J Vet Res 2009;70:277–82.
[12] Goodrich LR, Moll HD, Crisman MV, Lessard P, Bigbie RB. Comparison of equine amnion and a nonadherent wound dressing material for bandaging pinch-grafted wounds in ponies. Am J Vet Res 2000;61:326–9.
[13] Bigbie RB, Schumacher J, Swaim SF, Purohit RC, Wright JC. Effects of amnion and live yeast cell derivative on second-intention healing in horses. Am J Vet Res 1991;52:1376–82.

[14] Kirker KR, Luo Y, Nielson JH, Shelby J, Prestwich GD. Glycosaminoglycan hydrogel films as bio-interactive dressings for wound healing. Biomaterials 2002;23:3661–71.
[15] Gomez JH, Schumacher J, Lauten SD, Sartin EA, Hathcock TL, Swaim SF. Effects of 3 biologic dressings on healing of cutaneous wounds on the limbs of horses. Can J Vet Res 2004;68:49–55.
[16] Stashak TS, Farstvedt E, Othic A. Update on wound dressings: indications and best use. Clin Tech Equine Pract 2004;3:148–63.
[17] Chen WYJ, Abatangelo G. Functions of hyaluronan in wound repair. Wound Repair Regen 1999;7:79–89.
[18] Prestwich GD. Engineering a clinically-useful matrix for cell therapy. Organogenesis 2008;4:42–7.
[19] Prestwich GD, Luo Y, Ziebell MR, Vercruysse KP, Kirker KR, McMaster JS. Chemically modified hyaluronan: new biomaterials and probes for cell biology. New frontiers in medical sciences: redefining hyaluronan. New York: Elsevier Science; 2000. p. 181–94.
[20] Vanderhooft JL, Mann BK, Prestwich GD. Synthesis and characterization of novel thiol-reactive poly(ethylene glycol) cross-linkers for extracellular-matrix-mimetic biomaterials. Biomacromolecules 2007;8:2883–9.
[21] Yang G, Espandar L, Mamalis N, Prestwich GD. A cross-linked hyaluronan gel accelerates healing of corneal epithelial abrasion and alkali burn injuries in rabbits. Vet Ophthalmol 2010;13:144–50.
[22] Zheng Shu X, Liu Y, Palumbo FS, Luo Y, Prestwich GD. In situ crosslinkable hyaluronan hydrogels for tissue engineering. Biomaterials 2004;25:1339–48.
[23] Luo Y, Kirker KR, Prestwich GD. Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. J Control Release 2000;69:169–84.
[24] McDonald J, Hascall VC. Hyaluronan minireview series. J Biol Chem 2002;277:4575–9.
[25] Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. J Biol Chem 2002;277:4589–92.
[26] Mack JA, Abramson SR, Ben Y, Coffin JC, Rothrock JK, Maytin EV, et al. Hoxb13 knockout adult skin exhibits high levels of hyaluronan and enhanced wound healing. FASEB J 2003;17:1352–4.
[27] Culp LA, Murray BA, Rollins BJ. Fibronectin and proteoglycans as determinants of cell-substratum adhesion. J Supramol Struct 1979; 11:401–27.
[28] Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435–61.
[29] Alaish SM, Yager D, Diegelmann RF, Cohen IK. Biology of fetal wound healing: hyaluronate receptor expression in fetal fibroblasts. J Pediatr Surg 1994;29:1040–3.
[30] Bullard KM, Longaker MT, Lorenz HP. Fetal wound healing: current biology. World J Surg 2003;27:54–61.
[31] Iocono JA, Krummel TM, Keefer KA, Allison GM, Paul H. Repeated additions of hyaluronan alters granulation tissue deposition in sponge implants in mice. Wound Repair Regen 1998;6:442–8.
[32] Tammi RH, Tammi MI. Hyaluronan accumulation in wounded epidermis: a mediator of keratinocyte activation. J Invest Dermatol 2009;129:1858–60.
[33] Longaker MT, Chiu ES, Harrison MR, Crombleholme TM, Langer JC, Duncan BW, et al. Studies in fetal wound healing. IV. Hyaluronic acid-stimulating activity distinguishes fetal wound fluid from adult wound fluid. Ann Surg 1989;210:667–72.
[34] Krummel TM, Mast BA, Haynes JH, Diegelmann RF, Cohen IK. Characteristics of fetal repair. Prog Clin Biol Res 1991;365: 167–76.
[35] Longaker MT, Chiu ES, Adzick NS, Stern M, Harrison MR, Stern R. Studies in fetal wound healing. V. A prolonged presence of hyaluronic acid characterizes fetal wound fluid. Ann Surg 1991;213: 292–6.
[36] Estes JM, Scott Adzick N, Harrison MR, Longaker MT, Stern R. Hyaluronate metabolism undergoes and ontogenic transition during fetal development: implications for scar-free wound healing. J Pediatr Surg 1993;28:1227–31.
[37] Mast BA, Haynes JH, Krummel TM, Diegelmann RF, Cohen IK. In vivo degradation of fetal wound hyaluronic acid results in increased fibroplasia, collagen deposition, and neovascularization. Plast Reconstr Surg 1992;89:503–9.
[38] Shu XZ, Liu Y, Luo Y, Roberts MC, Prestwich GD. Disulfide cross-linked hyaluronan hydrogels. Biomacromolecules 2002;3: 1304–11.
[39] Condie R, Prestwich GD. Designing clinically useful substitutes for the extracellular matrix. In: Vernon B, editor. Injectable biomaterials: science and applications. Oxford: Woodhead Publishing; 2011. p. 3–23.
[40] Prestwich GD. Simplifying the extracellular matrix for 3-D cell culture and tissue engineering: a pragmatic approach. J Cell Biochem 2007;101:1370–83.
[41] Yang G, Prestwich GD, Mann BK. Thiolated carboxymethyl-hyaluronic-acid-based biomaterials enhance wound healing in rats, dogs, and horses. ISRN Vet Sci 2012;2011:851593.
[42] Tammi MI, Day AJ, Turley EA. Hyaluronan and homeostasis: a balancing act. J Biol Chem 2002;277:4581–4.
[43] Orlandi RR, Shu XZ, McGill L, Petersen E, Prestwich GD. Structural variations in a single hyaluronan derivative significantly alter wound-healing effects in the rabbit maxillary sinus. Laryngoscope 2007;117:1288–95.
[44] Gao F, Liu Y, He Y, Yang C, Wang Y, Shi X, et al. Hyaluronan oligosaccharides promote excisional wound healing through enhanced angiogenesis. Matrix Biol 2010;29:107–16.
[45] McCarty MF. Glucosamine for wound healing. Med Hypotheses 1996;47:273–5.
[46] Galeano M, Polito F, Bitto A, Irrera N, Campo GM, Avenoso A, et al. Systemic administration of high-molecular weight hyaluronan stimulates wound healing in genetically diabetic mice. Biochim Biophys Acta 2011;1812:752–9.
[47] Liu Y, Zheng Shu X, Prestwich GD. Biocompatibility and stability of disulfide-crosslinked hyaluronan films. Biomaterials 2005;26: 4737–46.
[48] Vanderhooft JL, Alcoutlabi M, Magda JJ, Prestwich GD. Rheological properties of cross-linked hyaluronan-gelatin hydrogels for tissue engineering. Macromol Biosci 2009;9:20–8.
[49] Ducharme-Desjarlais M, Celeste CJ, Lepault E, Theoret CL. Effect of a silicone-containing dressing on exuberant granulation tissue formation and wound repair in horses. Am J Vet Res 2005;66:1133–9.
[50] Theoret CL, Barber SM, Moyana TN, Gordon JR. Expression of transforming growth factor beta(1), beta(3), and basic fibroblast growth factor in full-thickness skin wounds of equine limbs and thorax. Vet Surg 2001;30:269–77.
[51] Mead R, Curnow RN. Statistical methods in agriculture and experimental biology. London; New York: Chapman and Hall; 1983.
[52] Ott L, Longnecker M. An introduction to statistical methods and data analysis. Australia; Pacific Grove, CA: Duxbury; 2001.
[53] Moragas A, Castells C, Sans M. Mathematical morphologic analysis of aging-related epidermal changes. Anal Quant Cytol Histol 1993; 15:75–82.
[54] Brown D, Rothery P. Models in biology: mathematics, statistics, and computing. Chichester, West Sussex, England; New York: Wiley; 1993.
[55] Julious SA. Using confidence intervals around individual means to assess statistical significance between two means. Pharm Stat 2004; 3:217–22.
[56] Polanco AM, Miller DM, Brewster CC. Survivorship during starvation for Cimex lectularius L. Insects 2011;2:232–42.
[57] Theoret CL. Treatment of exuberant granulation tissue. In: Stashak TS, Theoret CL, editors. Equine wound management. 2nd ed. Ames: Wiley-Blackwell; 2008. p. 445–62.
[58] Darby IA, Laverdet B, Bonte F, Desmouliere A. Fibroblasts and myofibroblasts in wound healing. Clin Cosmet Investig Dermatol 2014;7:301–11.
[59] Clem MF, DeBowes RM, Yovich JV, Douglass JP, Bennett SM. Osseous sequestration in the horse. A review of 68 cases. Vet Surg 1988;17: 2–5.
[60] Gift LJ, DeBowes RM. Wounds associated with osseous sequestration and penetrating foreign bodies. Vet Clin North Am Equine Pract 1989;5:695–708.