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Allogeneic Stem Cells Alter Gene Expression and Improve Healing of Distal Limb Wounds in Horses

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Key Words. Mesenchymal stem cells • Tissue regeneration • Animal models • Cord blood • Hypoxia

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Received March 29, 2017; accepted for publication August 25, 2017; first published October 24, 2017.

http://dx.doi.org/10.1002/sctm.17-0071

ABSTRACT

Distal extremity wounds are a significant clinical problem in horses and humans and may benefit from mesenchymal stem cell (MSC) therapy. This study evaluated the effects of direct wound treatment with allogeneic stem cells, in terms of gross, histologic, and transcriptional features of healing. Three full-thickness cutaneous wounds were created on each distal forelimb in six healthy horses, for a total of six wounds per horse. Umbilical cord-blood derived equine MSCs were applied to each wound 1 day after wound creation, in one of four forms: (a) normoxic- or (b) hypoxic-preconditioned cells injected into wound margins, or (c) normoxic- or (d) hypoxic-preconditioned cells embedded in an autologous fibrin gel and applied topically to the wound bed. Controls were one blank (saline) injected wound and one blank fibrin gel-treated wound per horse. Data were collected weekly for 6 weeks and included wound surface area, thermography, gene expression, and histologic scoring. Results indicated that MSC treatment by either delivery method was safe and improved histologic outcomes and wound area. Hypoxic-preconditioning did not offer an advantage. MSC treatment by injection resulted in statistically significant increases in transforming growth factor beta and cyclooxygenase-2 expression at week 1. Histologically, significantly more MSC-treated wounds were categorized as pro-healing than pro-inflammatory. Wound area was significantly affected by treatment: MSC-injected wounds were consistently smaller than gel-treated or control wounds. In conclusion, MSC therapy shows promise for distal extremity wounds in horses, particularly when applied by direct injection into the wound margin. STEM CELLS TRANSLATIONAL MEDICINE *2018;7:98–108*

SIGNIFICANCE STATEMENT

Distal extremity wounds are a significant clinical problem in horses and humans and may benefit from mesenchymal stem cell (MSC) therapy. This study evaluated the effects of direct wound treatment with allogeneic stem cells. This study provides evidence that MSC therapy shows promise for distal extremity wounds in horses, particularly when applied by direct injection into the wound margin. Interestingly, hypoxic preconditioning did not offer an advantage in this study. These findings in a horse model may be directly applicable to chronic wound studies in human patients, and provide insights to cellular based approaches of treatment.

INTRODUCTION

Chronic wound management is a growing problem for human health care around the world [1, 2]. Full thickness wounds and extensive burns are extremely detrimental to patients even with early intervention [3]. Traumatic wounds also commonly occur in the distal limbs of horses and healing is often delayed, due to high skin tension, and minimal subcutaneous stroma for vascular and structural support, and an aberrant inflammatory response. The pathobiology occurring in equine distal limb wounds is characterized by prolonged but somewhat ineffectual inflammation [4, 5], persistent transforming growth factor beta 1 (TGFβ1) signaling leading to a fibroproliferative response [6], and regional hypoxia with microvascular occlusion [7–10]. Epithelialization is slow and there is minimal capacity for wound contraction in this location. Taken together, these factors result in a delayed and often dysplastic healing process [11], as is the case for many human patients as well. Both species demonstrate hypoxia and delayed or absent epithelialization in distal extremity wounds, and horses have been proposed as a translational model for human wound research [12]. The development of new methods to augment healing in distal extremity wounds could have significant clinical impact for both horses and people.

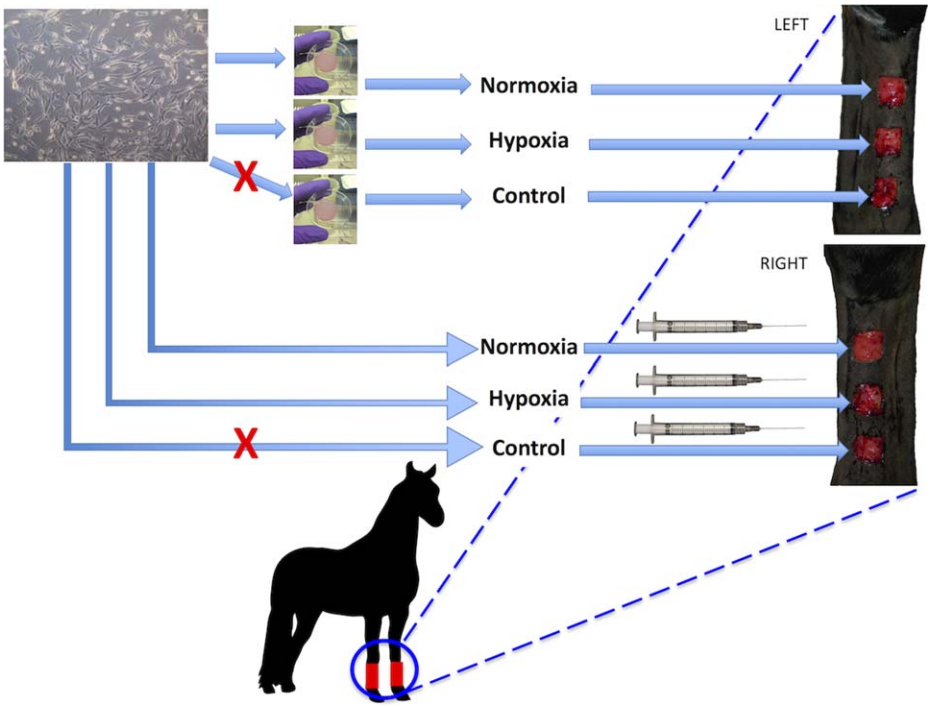

Figure 1. Experimental design. Banked cord blood mesenchymal stem cells from one donor were cultured and either embedded in fibrin gels or suspended in phosphate-buffered saline for treatment of experimental wounds on the distal forelimbs of horses. Prior to wound application, one gel and one culture flask were exposed to hypoxia (2% O_2) for 24 hours. Injected treatments were randomized in terms of right or left limb and proximodistal wound assignment, and the corresponding gel was applied to the corresponding wound on the opposite limb. Control wounds were treated with the corresponding blank vehicle (gel or saline). Treatment was applied 24 hours after wound creation.

Recent studies have demonstrated beneficial effects of mesenchymal stem cells (MSC) on wound repair in laboratory animals [13–15] and in one case series of horses [16]. It has also been reported that umbilical cord derived MSCs have improved burn repair in rodent models [15, 17]. Preconditioning MSCs in a hypoxic environment prior to tissue delivery appears to increase prosurvival and proangiogenic gene expression [18, 19]. Since these cells may improve neovascularization, reduce inflammatory cytokines, and promote wound contraction [19], they may be of particular benefit in the inherently hypoxic and persistently inflamed distal limb wounds of horses. Several MSC delivery methods have been reported, including intradermal injection [13, 16, 20] and topical application of cells within a synthetic [21] or natural polymer construct [22], such as fibrin [3, 23, 24].

Autologous and allogeneic MSCs have been widely used in both research and clinical applications in the horse, particularly in the treatment of musculoskeletal tissues [25, 26]. Recent studies have also reported stem cell therapy to improve equine wound healing in vitro and in vivo [16, 27–29]. Multipotent cells have been sourced from equine peripheral blood [16], bone marrow, adipose tissue, tendon, umbilical cord blood and tissue [30], amnion [31] and skin [32], and pluripotent cells have been obtained from embryos or induced from fibroblasts and keratinocytes [33–37]. Induced pluripotent cells of fetal origin have also been directed into keratinocyte lineage with a view toward cultured equine skin constructs [33]. Umbilical cord blood MSCs have been well-characterized in our laboratory [38–40] and were selected for this study based on their stability within fibrin gels during pilot studies and their nitric oxide production [39], which could prove advantageous in the hypoxic wound environment of the distal extremity.

In this study, we evaluated the application of allogeneic cord-blood derived MSCs to experimentally created distal limb wounds in horses. We compared hypoxic to normoxic-preconditioning of the cells, and also compared their delivery by direct injection versus topical application embedded within a fibrin gel. We hypothesized that MSC-treated wounds would have improved outcomes in wound area, thermography, gene expression, and histology when compared to untreated controls. We further hypothesized that hypoxic-preconditioned MSCs would be of greater benefit for wound healing than normoxic MSCs.

Methods

Animals

Six adult horses (three females, three castrated males) of varying breeds (four Quarter Horses, two Warmbloods) and ages (range: 5–19 years; mean: 14 years) were selected for the study. Horses were housed in outdoor individual stalls at the Center for Equine Health, University of California, Davis and were examined prior to enrollment to ensure they were healthy and free of any scars or skin disease on the distal limbs. The study was conducted according to an approved institutional animal care and use protocol.

Experimental Overview. Equine cord blood MSCs from one allogeneic donor were expanded and cultured exactly as previously described; [40] cells were trypsinized and prepared for wound treatment at passage 4–5. Cells were continued in culture or transferred into fibrin gels, and were then either exposed to hypoxic conditions or maintained in normoxia, before application to wounds (Fig. 1). In vitro preparation began approximately 2

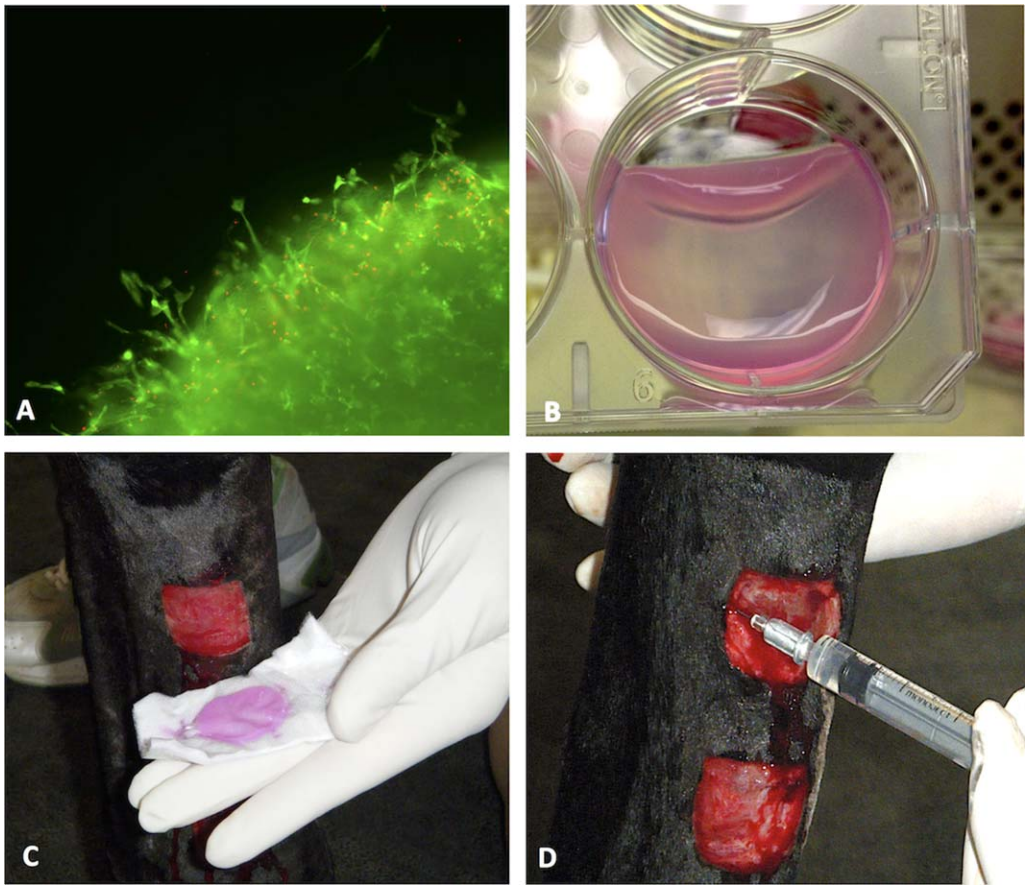

Figure 2. Experimental execution. Cord blood mesenchymal stem cells (MSCs) from one donor were suspended in autologous fibrinogen from each experimental subject to create fibrin gels. Pilot studies confirmed cell viability within the gels (calcein-ethidium staining) and migration from the gel margins at 48 hours after gels were released from wells **(A)**. On the morning of wound treatment, gels were released from the well just prior to wound application **(B)**. (Pink staining is a result of phenol red in cell culture medium.) Wounds were treated by topical application of MSCs in fibrin gels **(C)** or by direct subcutaneous injection of MSCs in saline **(D)**.

weeks before wound treatment and data was collected weekly for 6 weeks thereafter (Supporting Information Fig. S1).

MSCs in Fibrin Gels

Autologous fibrinogen was isolated from each study horse for fibrin gel preparation as described previously [41]. Centrifugation was applied with minimal acceleration and deceleration and prostaglandin E1 (10 μg/ml) (Sigma, St. Louis, MO) was added to plasma to prevent platelet activation. Precipitated fibrinogen was dissolved in DPBS and quantified by a STA Compact Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ). Fibrin gels were prepared in six-well cell culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Each gel was 2.375 ml total volume (approximately 2.5 mm thick), 9.5 cm^2 in area, and contained 20 mg/ml fibrinogen and 1.66 $\times$ 10^6 MSCs. Blank control gels were prepared without MSCs. On day 7, half of the gels were transferred to a 5% CO_2, 2% O_2 hypoxic incubator, and grown for 24 hours before application to wounds. Normoxic and control gels remained in standard culture conditions.

Based on in vitro pilot studies and reported doubling times for cord blood cells [42], we estimated the cell population to be 10–20 $\times$ 10^6 cells per gel after 8 days of incubation (i.e., at the time of application to wounds). Cell viability in the gels was confirmed by visual assessment on a light microscope, based on the observation of elongated cell morphology and refractility; migration from the gel margin was also confirmed during pilot studies (Fig. 2A). On the day of wound treatment, gels were lifted from wells in the laboratory hood (Fig. 2B) and transferred onto a Telfa pad (Telfa pad, Covidien, Minneapolis, MN) for transport to the horse housing facility.

MSCs for Injection

Twenty-four hours before wound treatment, half of the flasks were transferred to a 5% CO_2, 2% O_2 hypoxic incubator. Cells were trypsinized, washed, and resuspended to a final concentration of 17.5 $\times$ 10^6 cells per milliliter in saline. One milliliter of cells was injected for each treatment; 1 ml injectable saline was used as a vehicle control. The target cell dose was 15–20 $\times$ 10^6 cells; the cell concentration for the first horse's injection was 17.5 $\times$ 10^6 cells per milliliter and we therefore matched all subsequent injections to that same concentration.

Wound Creation

Surgical wounding was performed under field anesthesia: after placing a 14 g IV catheter in the left jugular vein, horses were treated with phenylbutazone (2 g IV) and then premedicated with xylazine (1 mg/kg IV). Anesthesia was induced with ketamine

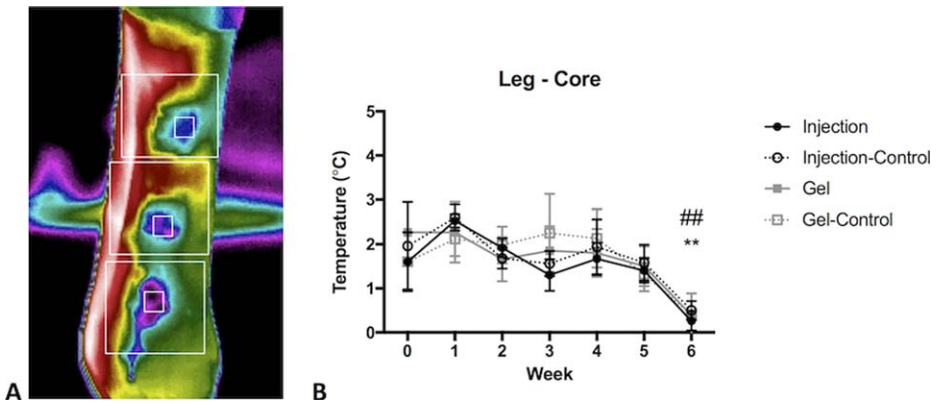

Figure 3. Thermography. **(A):** Thermographic data were analyzed according to core wound temperature (small square gates centered on wounds) and overall Leg temperature (larger square gates). **(B):** A significant effect of time was detected for all groups ($p < .0001$). By 6 weeks, the difference between core wound and surrounding leg temperature is nearly zero, that is, temperature of the wound bed has normalized. There was a significant decrease of temperature variation in wounds treated with mesenchymal stem cell (MSC) embedded gels and injected MSCs at Week 6. Data are displayed as mean (SE). Week 0 data was collected on day 1 after wounding, prior to treatment application. Symbols: * denotes statistically significant difference for Injection (*, $p < .05$; **, $p < .01$; ***, $p < .001$); # denotes statistically significant difference for Gel (#, $p < .05$; ##, $p < .01$).

(3 mg/kg IV) and diazepam (0.1 mg/kg IV) and was maintained with repeat doses of xylazine (0.5 mg/kg IV) and ketamine (1.5 mg/kg IV) every 10 minutes thereafter. Horses were positioned in lateral recumbency and the dorsomedial aspect of the down limb was aseptically prepared. Three square full-thickness skin wounds (2.5 cm × 2.5 cm, spaced 2.5 cm apart and vertically aligned; Fig. 1) were created using a sterile adherent template and a #10 scalpel blade. The limb was bandaged in standard fashion (Telfa pad, roll gauze (Kling, Johnson & Johnson, New Brunswick, NJ), cotton, brown gauze, VetWrap (3M, St. Paul, MN) and Elastikon (Johnson & Johnson, New Brunswick, NJ)). The procedure was repeated on the opposite forelimb.

Wound Treatment

Treatments were assigned as follows: MSC injection was randomly assigned to the right or left limb, and then control, normoxic MSCs, or hypoxic MSCs were each randomly assigned to one of the three wounds on that leg. On the opposite forelimb, corresponding MSC-fibrin gel treatments (control, normoxic, hypoxic) were assigned to the corresponding wound (i.e., proximal, middle, distal, respectively) (Fig. 1). Treatments were applied on day 1 after wounding; horses were sedated with 0.01 mg detomidine IV. Gels were lifted from wells in the laboratory hood by gently rimming the margin with a P200 pipette tip and then passing the tip back and forth under one released edge. The pipette tip was moved across the well under the gel in an advancing manner as the gel was allowed to fold down on itself (Fig. 2B). Spatula-tipped forceps were used to transfer the gel onto a Telfa pad (Telfa pad, Covidien, Minneapolis, MN) set within a sterile individual Petri dish for transport to the horse housing facility. Gels were applied directly to the wound surface by handling the Telfa pad (Fig. 2C), and were secured to the limb with a single circumferential wrap of Elastikon. The gels were placed to ensure full contact with the wound bed and no overlap with the wound margin. Injections were applied subcutaneously at the mid-point of each border of the square wound: 0.25 ml was injected at each site using a 25 g needle (Fig. 2D). Control wounds received either saline injection or a blank fibrin gel with no MSCs.

Bandaging

For the first week post-wounding, wounds were bandaged continuously, as previously described (changed on days 1 and 5). Bandages were also temporarily applied for 24 hours after each biopsy to control bleeding. For the remainder of the experiment, wounds were left unbandaged with the intent of minimizing exuberant granulation tissue (EGT) formation.

Thermography

Thermal images were acquired using a Flir Vet T-420 thermography camera (FLIR Systems, Inc., North Billerica, MA) at a consistent distance of 1 m. A standard emissivity value of 0.98 was chosen based on recently published equine studies [43]. Bandages were removed 15 minutes before acquisition of thermal images, in an environmentally controlled room. One dorsopalmar image of the metacarpus was acquired per limb with the thermography camera before treatment of day 1 wounds (Week 0, baseline), and at 1, 2, 3, 4, 5, and 6 weeks. Using the FLIR Systems' software program (FLIR Systems, Inc., Wilsonville, OR), standard delineated areas were superimposed on saved digital thermal images at specific wound sites (Fig. 3) to determine core and whole leg temperatures. The operator was blinded to the management of the wounds (gel vs. injection, normoxic vs. hypoxic).

Wound Surface Area

Digital photography of each forelimb was performed on seven occasions beginning the day after wound creation and then weekly for 6 weeks thereafter. On each occasion, a clipboard containing horse identification, date, limb identification, and a metric ruler was placed behind each limb and included in the photograph. Each picture was taken parallel to the ground and included all three wounds. Care was taken to ensure that the true margins of each wound were completely visible (i.e., not obscured by bleeding, exudate, or regrown hair); any obscuring exudate was gently removed using a saline-soaked gauze prior to photography. Images were calibrated to scale and were analyzed using Image J [44] (www.imagej.net) to trace wound margins and calculate surface area. Each wound area was then expressed as a percentage of its original area on day 1 (just prior to treatment).

Wound Biopsy

Biopsies were obtained weekly for 6 weeks beginning on Week 1 (day 8), immediately following wound photography. Biopsies were taken beginning from the distal right vertical border on Week 1 and proceeded proximally on Weeks 2 and 3, such that there was no overlap with the previous biopsy site. On Weeks 4–6 the same procedures were repeated on the left vertical margin of the wound. Prior to biopsy, horses were sedated (0.01 mg/kg detomidine IV) and an assistant wearing latex gloves cleaned the wound margins and adjacent skin with saline-soaked gauze. Surgeons wearing non-sterile latex gloves injected local anesthetic (2% lidocaine without epinephrine, 2 ml total) subcutaneously through the intact skin proximal and adjacent to the upper wound corner above the intended biopsy site. Biopsies were then collected using sterile technique: the 8 mm skin biopsy punch was centered on the wound margin in order to obtain $\frac{1}{2}$ wound bed and $\frac{1}{2}$ adjacent intact skin in each biopsy. Samples were then cut in half transversely: $\frac{1}{2}$ was placed in formalin for histology and the other $\frac{1}{2}$ was used for PCR analysis. For the PCR samples, intact skin was removed and the remaining wound bed portion was cut into approximately 4 pieces, placed in cryovials containing RNAlater (Qiagen, Valencia, CA) and frozen at -80°C.

Gene Expression

Biopsy samples were thawed, minced, and washed with DPBS prior to cell lysis in modified (1% β-Mercaptoethanol) RLT buffer (Qiagen). Tissues were then placed in a preheated (55°C) water bath sonicator (Branson, Shelton, CT) for 10 minutes. Lysed tissues were further run through a QIAshredder column (Qiagen) per manufacturer's instructions. Tissue lysate was centrifuged, supernatant was removed and subjected to RNA extraction with RNeasy mini-kit (Qiagen) per manufacturer's instructions. Extracted RNA was then converted to cDNA using a First-strand cDNA Synthesis kit (Origene, Rockville, MD) per manufacturer's instructions. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed using primers that were purchased from a commercial vendor ($TGF\beta_1$, Kingfisher Biotech, Saint Paul, MN) or designed via on-line software (peptidylpropyl isomerase B, cyclooxygenase 2 [COX2], vascular endothelial growth factor [VEGF], and basic fibroblast growth factor 2 [FGF2], www.idtdna.com. See Supporting Information Table 1 for primers' details), and Fast SYBR Green Master MIX (AB Applied Biosystems, Foster City, CA) and run on a 7300 Real Time PCR System (AB Applied Biosystems) thermocycler. All PCR products sizes were confirmed by electrophoresing PCR products on a 2% agarose gel. Gene expression data were reported as fold-change over corresponding controls from each time point.

Histology

Biopsies were processed for routine H&E-stained tissue sections. Tissue sections were randomly assigned a number value and blindly evaluated by a board-certified veterinary pathologist. Sections were scored 0–4 for each of four categories: degree of epithelialization, inflammation, vascularity, and fibrosis. For all scores, 0 was assigned to cases lacking the histologic feature, and 4 was assigned when the feature was prominent.

Statistical Analysis

Significance for all analyses was set at $p < .05$ and data were reported as mean +/– SE.

For wound area, thermography, and gene expression, three dependent variables were considered; treatment of wound by MSC, hypoxic preconditioning, and modality of MSC application. Data were analyzed using R software. Due to the small sample size of the current study, data was log-transformed to achieve normality. A Wilk-Shapiro test of normality revealed all transformed data sets had error distributions within a normal range ($W > 0.95$). An ANOVA using a mixed model procedure was performed to assess the array of treatments fixed effects by MSCs, oxygen and application. No effects of hypoxic preconditions were observed, therefore groups were combined to compare injection versus gel over a 6-week time frame. Statistical outliers were removed, only if alterations were suspect of causation by automutilation. Finalized data were analyzed using GraphPad Prism 6 for Mac OS X. Ordinary two-way ANOVA testing was performed to detect significant differences between treatment groups over time. Repeated measures could not be performed due to removal of experimental wounds from data sets caused by self-mutilation by some study animals. Using the Sidak multiple comparison test, post-tests were performed to first compare treatment groups at each time point and then to compare all time points within each treatment group.

Histologic scores were entered into a matrix and blindly categorized by hierarchical clustering based on scores across all four variables. Hierarchical clustering was performed using package GMD in R software. In addition, the number of horses within each group (MSC-treated vs. control) was tabulated and a Fisher's exact test was performed using R software.

Results

Gross Wound Evaluation

All wounds healed and no adverse effects were noted specific to MSC therapy. Pilot studies using continuous bandaging resulted in significant EGT formation. This problem was largely eliminated using the short-term and intermittent (i.e., after biopsy) bandaging protocol as described in "Methods" section. However, in the absence of protective bandages, fly irritation and self-mutilation were confounding factors in some horses; four affected wounds (two Injection, one Gel, and one Injection-Control) were excluded from data analysis. Notably, gels were not grossly visible in the wound bed at the first bandage change (day 5), suggesting proteolytic digestion.

No Effect of Hypoxic Preconditioning on Wound Healing Outcomes

Based on the existing literature and our pilot studies, which showed altered gene expression after hypoxic preconditioning, we expected to find a positive effect of hypoxic-MSC treatment. Surprisingly, we did not detect significant differences in any outcome on the basis of hypoxic versus normoxic MSC culture conditions. Data were therefore combined and results are reported as four groups (Injection, Injection-Control, Gel, Gel-Control).

Thermographic Imaging Tracked Wound Healing In Situ

In order to noninvasively evaluate wound physiology in vivo, we used thermographic imaging to detect changes in blood flow post-wounding. This complementary modality correlated well with histologic assessment of vascularization. Core wound temperature and leg temperature changed throughout the study as

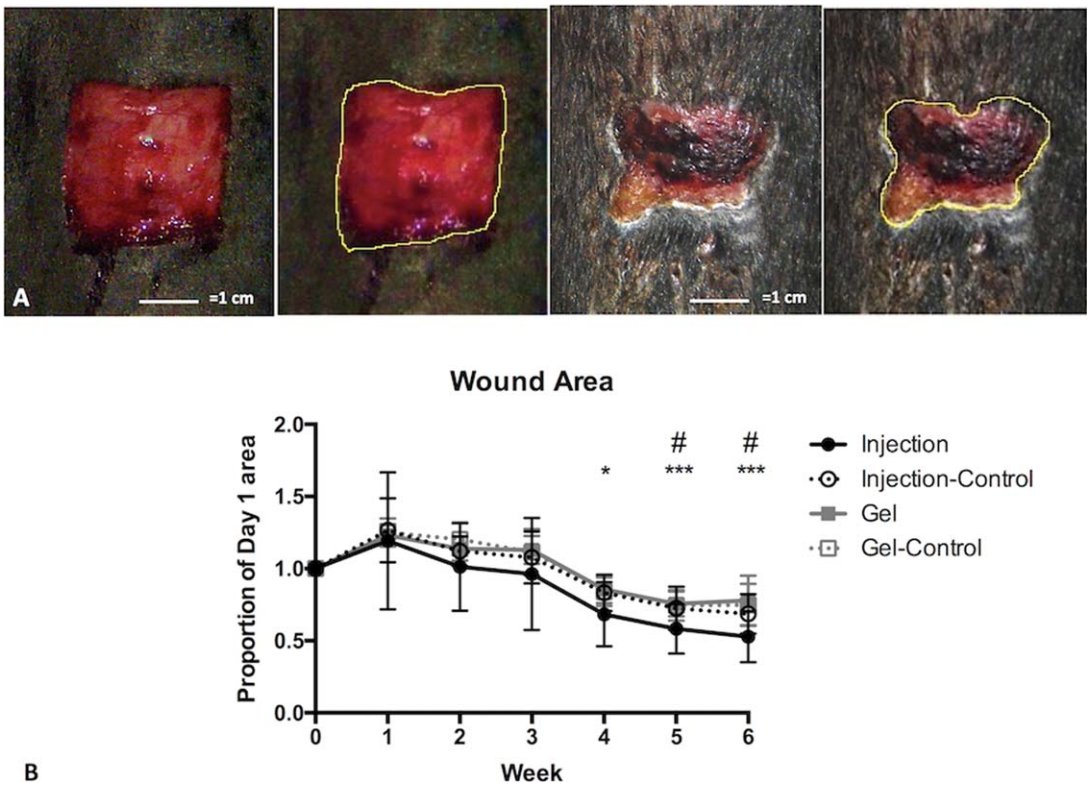

Figure 4. Wound area. Wound surface area was calculated from wound margins traced on calibrated digital images. **(A):** Example images of the same wound on day 1 (left) and day 36 (right), with corresponding outlines of the same images. **(B):** Wound area decreased significantly in Weeks 4–6 for mesenchymal stem cell (MSC) Injected wounds, compared to Week 1 values (largest wound area); wounds treated with MSC Gel followed suit at Weeks 5–6 but to a lesser extent. Data is displayed as mean (SE). Symbols: * denotes statistically significant difference for Injection (*, $p < .05$; **, $p < .01$; ***, $p < .001$); # denotes statistically significant difference for Gel (#, $p < .05$; ##, $p < .01$).

we expected: core wound temperature increased overall, as vascularization of the wound bed occurred. Surrounding leg temperature initially increased, during the acute inflammatory phase after wounding, and then decreased as healing progressed and inflammation subsided (Fig. 3). The difference between these two values ("Leg-Core") was calculated as an index of healing, that is, in the absence of a wound, there would be no difference in temperature at these two sites, and the Leg-Core value would therefore equal zero. As healing progresses, therefore, the Leg-Core value should trend toward zero. There was an overall significant effect of time ($p < .0001$), that is, as wounds healed, they returned to the temperature of the surrounding intact skin. MSC treated wounds began to normalize temperature (delta leg-core approaching zero) at Week 6 in both gel and injection groups ($p < .01$).

MSC Treatment Accelerates Reduction in Wound Area

Wounds initially enlarged in size, as expected for equine limb wounds, and then reduced to 72% (controls), 78% (MSC-gel), and 53% (MSC-injected) of original wound area by Week 6 (Fig. 4). A significant treatment effect was detected ($p = .039$) but post hoc multiple comparisons failed to further clarify this effect. Nonetheless, mean wound area was smallest in the Injected group for all time points. A significant effect of time was also detected for treated wounds but not for control wounds: wound area was significantly less at Weeks 4, 5, and 6 for Injection and at Weeks 5 and 6 for Gel, in comparison to Week 1 values (largest wound areas).

Gene Expression Varies After MSC Injection but Not Gel Application

Injected MSCs clearly impacted gene expression within the wound, suggesting that direct application into the tissue allowed immediate interaction and functionality within the wound environment. Alternatively, it is possible that the physical process of injection alone resulted in activation of the MSCs and altered their gene expression profile.

Transcriptional changes did not occur when cells were embedded within a three-dimensional fibrin matrix and applied to the wounds.

Cyclooxygenase 2 (COX-2) expression changed significantly based on time and treatment. Specifically, a 20-fold increase in COX-2 expression was observed at Week 1 after MSC injection (Fig. 5). This value was significantly greater than after MSC gel application at the same time point ($p < .0001$) and was also significantly greater than all subsequent time points for MSC injection ($p < .0001$). In contrast, there were no significant differences in COX-2 expression with MSC gels.

$TGF\beta_1$ expression was also significantly altered after MSC injection but not after gel application: MSC injection significantly increased expression of $TGF\beta_1$ at Week 1 only, as compared to gel application ($p < .01$). Expression of $TGF\beta_1$ at Week 1 was also significantly higher than Weeks 3–6 after MSC injection ($p < .05$). No alterations in $TGF\beta_1$ expression occurred after MSC-gel application.

For VEGF and FGF, there were no significant differences based on treatment or time. There was a notable inversion of VEGF expression: MSCs embedded in gel were associated with a twofold

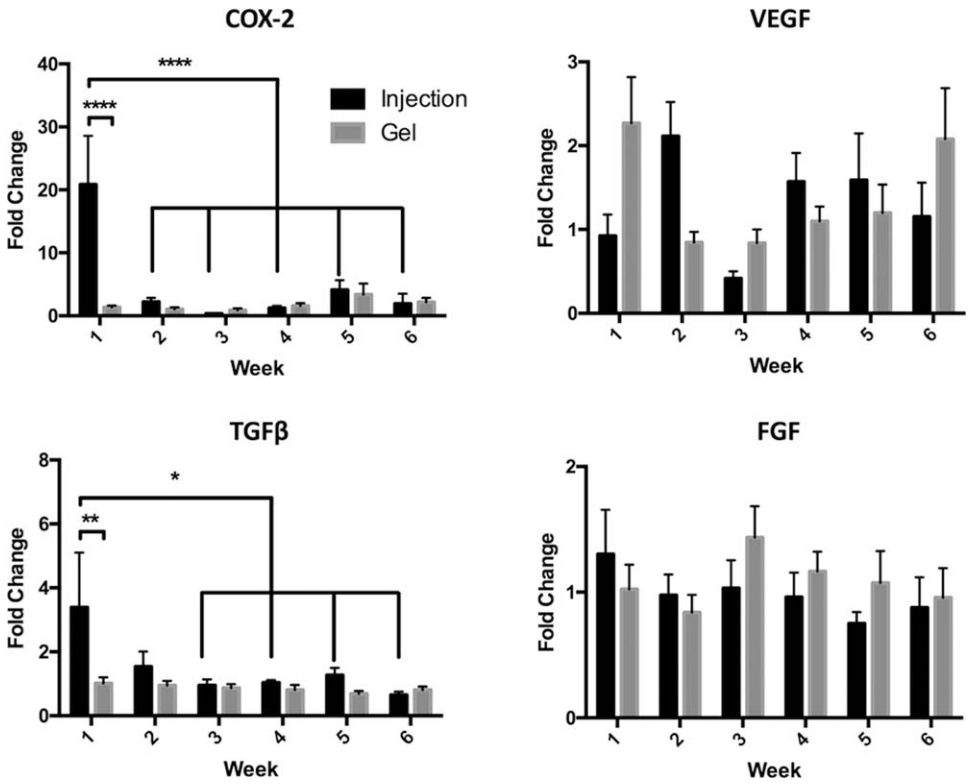

Figure 5. Gene expression. PCR Data (mean [SE]), expressed as fold-change over corresponding controls. One week after treatment, COX-2 and TGFβ gene expression were significantly greater after mesenchymal stem cell (MSC)-Injection in comparison to MSC-Gels. Week 1 expression after Injection was also significantly greater than that measured in all subsequent weeks (COX-2) and Weeks 3–6 (TGFβ). No significant differences were detected for VEGF or FGF. Symbols: *, $p < .05$; **, $p < .01$; ****, $p < .0001$. Abbreviations: COX-2, cyclooxygenase-2; FGF, fibroblast growth factor; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor.

increase in expression at Week 1 and returned to control levels at Week 2; MSC injection results were just the opposite. We were surprised not to see a greater impact on VEGF expression, particularly in light of improved histologic scores for vascularity with MSC treatment and the known nitric oxide production of cord blood MSCs [39].

MSC Treatment Improves Histologic Outcomes

Histologic appearances varied widely and ranged from early vascularization, fibrosis, and epithelialization to chronic inflammation and ulceration (Fig. 6). In order to identify trends within this large data set, histologic scores were distributed in a dendrogram with a heatmap matrix (Supporting Information Fig. 2) superimposed, using assigned colors to illustrate clusters of data. The following clusters of wound characteristics were identified: (1A) increased vascularity and epithelialization (1B) increased fibrosis ("Pro-Healing"), (2A) lack of epithelialization, or ulceration, and (2B) increased inflammation ("Ulcerative/Pro-Inflammatory"). Although significant differences could not be detected between Injected versus Gel treatment groups, there was a significantly greater number of MSC-treated wounds (Injected and Gel combined) in the Pro-Healing Group than in the Ulcerative/Pro-Inflammatory Group; (Fig. 7; $p = .0136$).

Discussion

Chronic wounds represent an enormous burden for the individual patient and society as a whole. The impact of this "silent epidemic" is felt on many levels, from lost work days to permanent disability (amputation) to death of the patient, and the economic implications of chronic wound care are alarming ($25 billion annually in the U.S.) [1]. Impaired healing can occur in severely injured normal tissue, such as a burn, or as a result of comorbidities which create compromised tissue integrity, such as decubital sores or diabetic foot ulcers (DFU). Distal limb wounds in horses share many of the complexities of chronic wounds in people, in that they heal mainly by epithelialization, rather than contraction, and are persistently hypoxic and pro-inflammatory during the healing period. This study adds to the accumulating evidence [16, 29, 45–52] that allogeneic MSC therapy can facilitate wound healing: MSC-treated wounds showed significant differences in wound area, gene expression and histologic scores. MSCs are believed to impact wound healing by paracrine immunomodulatory, antifibrotic [50, 51] and anti-inflammatory effects [15, 39, 45, 46], enhanced neovascularization [15, 21], reversal of collagen dysregulation [52], and in some reports, by transdifferentiation and engraftment [13, 53].

We evaluated several variables in the preparation and application of MSCs to wounds, including hypoxic-preconditioning and topical versus injectable delivery methods. In contrast to several other reports [18, 19], hypoxia during MSC culture was not advantageous in our study. Our pilot studies indicated an obvious in vitro effect of hypoxia on MSC gene expression (induction of VEGF expression), but this did not translate into a detectable effect in vivo. With regard to delivery method, we investigated direct injection of MSCs as the simplest means for applying these cells to

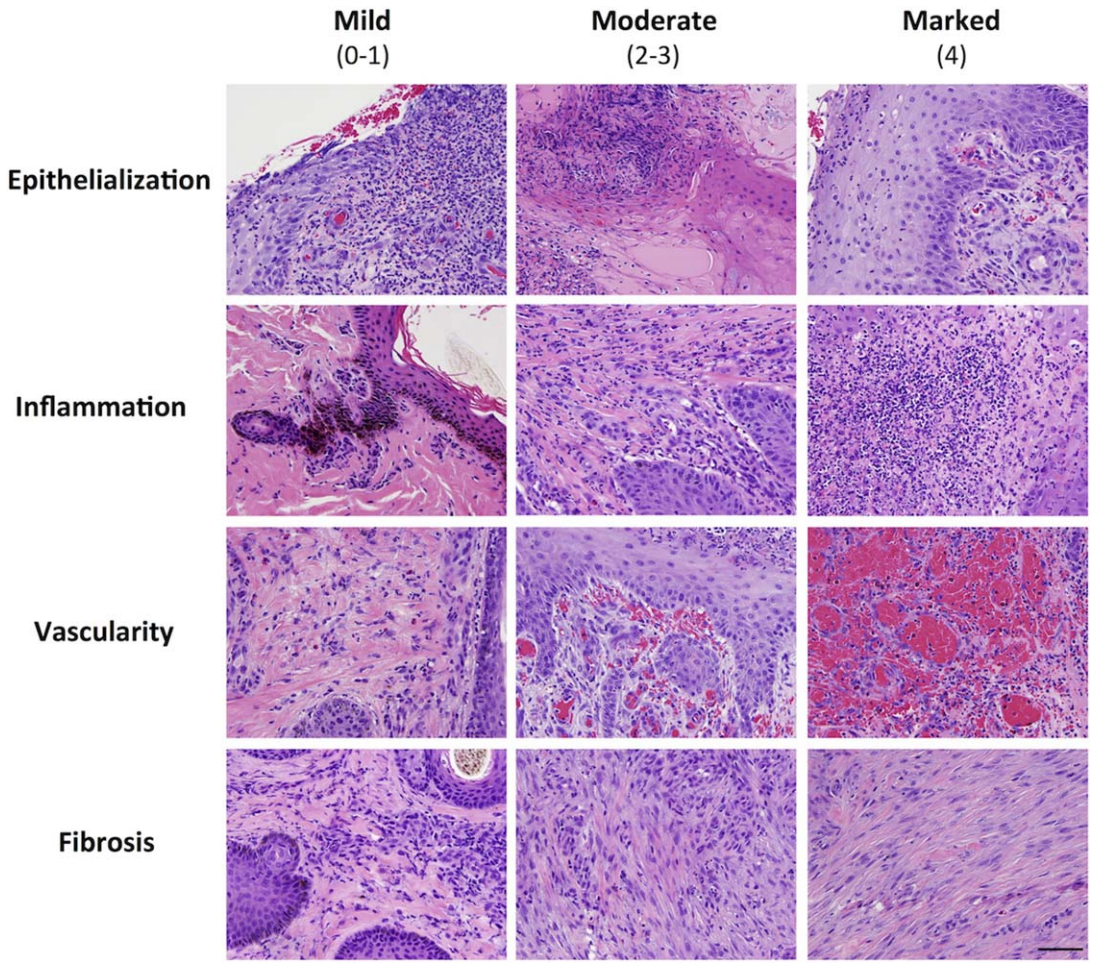

Figure 6. Histology. Representative photomicrographs demonstrating the histologic scoring criteria for epithelialization, inflammation, vascularity, and fibrosis. H&E staining, bar = 50 μm.

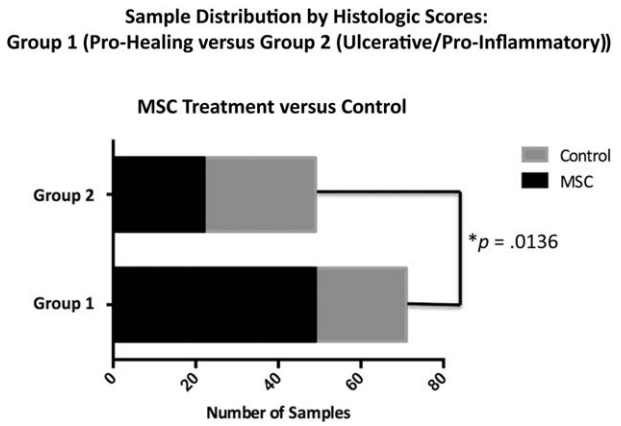

Figure 7. Histology results. Fisher's exact results comparing the proportion of mesenchymal stem cell (MSC)-Treated versus control wounds classified as either Histologic Score Group 1 (Pro-Healing) or Group 2 (Ulcerative/Pro-Inflammatory). MSC-treated wounds comprised a significantly greater proportion of the Pro-Healing Group ($p = .0136$).

tissue, but were also interested in the impact of MSCs in a hydrogel. Burns and DFUs are preferentially treated with some type of biological matrix to provide a physical barrier as well as to "instruct" or contribute to the wound bed directly with molecules such as hyaluronic acid [54], or conceivably, resident cells [49]. We chose autologous fibrin to provide the most native provisional matrix possible for each wound, as well as naturally occurring adhesion motifs to allow MSC anchorage and interaction within the gel. The MSCs in gels were applied to wounds in what we believed to be a "primed" state for biological activity: they were elongated, attached to a matrix, migrating, and presumably interacting with other cells in the gel through signal transduction, in contrast to the dissociated, recently trypsinized cells used for injection. In a recent study, protein synthesis by MSC spheroids improved dramatically when embedded in an RGD-modified alginate gels, presumably based on the ability of the cells to interact with the matrix [55]. Another study on MSCs in a PEGylated fibrin system showed significant increases in vascularization in a burn model, and MSCs also transmigrated from the gel into the wound bed [49]. However, in our study MSC-injection appeared superior to topical application of MSC-laden gels. Wounds treated by MSC-injection showed an earlier decrease in wound area, and significant differences in early $TGF\beta_1$ and COX-2 gene expression were detected in the injected but not the gel-treated wounds. Injection was logistically easier when compared to gel preparation: significantly less time and materials are required for injection and the entire cell dose is delivered directly into the tissue. Our gels

appeared to be digested rapidly within the wound, such that the MSCs may have been liberated faster than they could migrate into tissue; gel modification to reduce proteolytic destruction (such as PEGylation) may have changed our results.

We expected that MSC treatment would mitigate the prolonged inflammatory response that contributes to delayed healing of equine limb wounds. Indeed, MSC-treated wounds had lower histologic scores for inflammation and appeared to progress through the stages of wound healing earlier than control wounds. It was therefore initially surprising to observe a dramatic increase in expression of COX-2 in MSC-injected wounds at Week 1. This increased expression was early and transient in nature. Although COX-2 and its eicosanoid products are hallmarks of inflammation, they (specifically COX-2 and Prostaglandin E2) are also recognized as critical components of early wound healing in multiple tissues including skin [56–59]. The increased COX-2 gene expression in our study appears to represent an amplification of this key signal in the healing process, as stimulated by MSC injection. Further research into COX-2 protein expression by MSCs or their paracrine targets during wound repair, including the timing and spatial localization of this event, may clarify the mechanism for our observed MSC treatment effect.

$TGF\beta_1$ dysregulation has been implicated in excessive scar formation in people and EGT in the horse. This pro-fibrotic marker has been extensively studied in the equine wound literature [60–63], and is known to peak early in normal healing and then subside. In contrast, $TGF\beta_1$ persists in wounds healing with EGT [6] and inhibits epithelialization [62]. We found that $TGF\beta_1$ expression was significantly increased (threefold) at Week 1 after MSC injection, and then returned to control values. Interestingly, a more intense burst of $TGF\beta_1$ has been reported in wounds in ponies than in horses, and has been proposed as an explanation for the more rapid and uncomplicated healing that ponies exhibit [63]. In that sense, an early but transient increase in $TGF\beta_1$, as observed here, may be a favorable event mediated by MSC treatment of wounds. A recent study by Fang et al. [50] documented paracrine suppression of the TGFβ/SMAD pathway by microsomal RNA released from umbilical cord tissue-derived MSC exosomes. This fascinating phenomenon resulted in reduced myofibroblast function and ultimately reduced scar production in vivo. Future studies to investigate expression of the antifibrotic isoform, $TGF\beta_3$ [6, 64] may reveal additional favorable matrix effects of MSCs or MSC-derived treatment in wounds, as reported in other species [23, 51]. Clinically, this could translate into less EGT production and scarring, as suggested by other authors [27].

Extrapolation from acute, experimentally created wounds to naturally occurring, traumatic, chronic, and dysplastic wound encountered in the clinical patient is obviously an imperfect science, and is an inherent limitation of most wound studies. Nonetheless, the study of acute wounds can hopefully identify targets and timing for early therapeutic intervention. Any favorable modulation of acute wound events may prevent complications from developing in "at-risk" wounds, such as complex traumatic wounds with extensive soft tissue deficit or wounds with reduced regenerative capacity (i.e., burns or DFUs). The horse may be a more critical translational model for wound research than the traditional murine model, based on the hypoxic nature and minimal contraction of wounds on their distal limbs. Environmental challenges to wound healing were also present in our study, as horses were housed outside during the summer months and wounds were left largely unbandaged to avoid the confounding effect of EGT. Fly irritation and self-mutilation occurred in several wounds, which were eliminated from the data set and thereby reduced study power. A positive treatment effect was still detected despite these limitations, and this model perhaps better represented the complicated wounds for which we aim to improve treatment.

Our study employed allogeneic cord blood MSCs from one donor. We selected cord blood MSCs based on favorable culture characteristics, the perceived greater pluripotency of cord blood cells, nitric oxide production [39], greater gene expression changes with cord blood cells than with bone-marrow-derived MSCs during our pilot studies, and better physical characteristics and gel stability with cord-blood cells than with bone-marrow-derived MSCs. The clinical safety of allogeneic MSCs has been confirmed in the horse [26, 38, 65] and comparable efficacy to autologous cells for wound healing has been demonstrated in other species [66]. However, some limitations to allogeneic cell use may exist: a recent study reported a slight advantage of autologous MSCs over allogeneic cells in terms of treatment effect on equine wounds [27], and allogeneic cells do elicit some immune response [67–69]. Nonetheless, allogeneic cells obviously provide logistical advantages, such as a readily available supply of banked cells (thereby preventing delays in treatment) and selection of cell lines with favorable and consistent growth characteristics. Future work in this field will no doubt be directed at optimizing donor-recipient suitability and thereby, presumably, treatment response.

Summary

Allogeneic cord blood cells enhanced early healing in this critical wound model in horses. This effect was greater for cells delivered by injection than for cells applied topically in fibrin gel. Hypoxic preconditioning did not offer an advantage in this study.

Acknowledgments

This study was supported by the Center of Equine Health and a gift from Dick and Carolyn Randall. Statistical analysis and consultation was provided by Dr. Neil Willits of the UC Davis Statistics Laboratory. We would like to thank the following student volunteers for their time and contributions to this study: Monica Plank, Lyndsey Marsh and Bailey McBride.

Author Contributions

J.A.T.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; K.C.C.: collection and assembly of data, data analysis and interpretation, administrative support, other: animal work; N.J.W.: other: technical support, collection and assembly of data; F.A.A.: collection and assembly of data, other: animal work; A.K.: collection and assembly of data, data analysis and interpretation; K.D.W.: collection and assembly of data, data analysis and interpretation; S.S.L.: collection and assembly of data; A.B.: collection and assembly of data; A.D.: collection and assembly of data; S.N.G.: other: animal work; L.K.B.-W.: other: animal work; D.L.B.: conception and design, financial support, data analysis and interpretation, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Note Added in Proof

This article was published online on 24 October 2017. Minor edits have been made that do not affect data. This notice is included in the online and print versions to indicate that both have been corrected 28 December 2017.

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