Effects of unfocused extracorporeal shock wave therapy on healing of wounds of the distal portion of the forelimb in horses

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Objective—To determine effects of extracorporeal shock wave therapy (ESWT) on healing of wounds in the distal portion of the forelimb in horses.

Animals—6 horses.

Procedures—Five 6.25-cm² superficial wounds were created over both third metacarpi of 6 horses. Forelimbs were randomly assigned to treatment (ESWT and bandage) or control (bandage only) groups. In treated limbs, each wound was treated with 625 shock wave pulses from an unfocused electrohydraulic shock wave generator. In control limbs, each wound received sham treatment. Wound appearance was recorded weekly as inflamed or healthy and scored for the amount of protruding granulation tissue. Standardized digital photographs were used to determine the area of neoeipithelization and absolute wound area. Biopsy was performed on 1 wound on each limb every week for 6 weeks to evaluate epithelialization, fibroplasia, neovascularization, and inflammation. Immunohistochemical staining for α smooth muscle actin was used to label myofibroblasts.

Results—Control wounds were 1.9 times as likely to appear inflamed, compared with treated wounds. Control wounds had significantly higher scores for exuberant granulation tissue. Treatment did not affect wound size or area of neoeipithelization. No significant difference was found for any of the histologic or immunohistochemical variables between groups.

Conclusions and Clinical Relevance—Treatment with ESWT did not accelerate healing of equine distal limb wounds, but treated wounds had less exuberant granulation tissue and appeared healthier than controls. Therefore, ESWT may be useful to prevent exuberant granulation tissue formation and chronic inflammation of such wounds, but further studies are necessary before recommending ESWT for clinical application. (Am J Vet Res 2010;71:229–234)

Treatment of distal limb wounds in horses can be frustrating and is often complicated by delayed closure, formation of exuberant granulation tissue, and hypertrophic scars.1,2 Equine limb wounds have some particular characteristics, such as weak and persistent inflammation, excessive fibroplasia, and decreased rates of contraction and epithelialization, leading to prolonged healing time.3–6 Several treatment modalities have been proposed to stimulate healing of distal wounds in horses, but so far, no treatment has been completely successful. Therefore, development of a new, effective, and noninvasive treatment of distal limb wounds in horses would be extremely valuable.

Extracorporeal shock wave therapy accelerates healing of chronic wounds in humans.7 Shock waves are pulsed high-energy pressure waves that, when applied to tissues, deflect at zones of different acoustic impedance, resulting in the release of kinetic energy and consequent formation of pressure and shear forces that mechanically react with the tissues.8 Although the exact mechanism of action of ESWT on tissue healing is still unknown, it is believed that the waves perturb cell membranes, inducing cell-signaling effects and consequently altering the expression of cytokines and inflammatory mediators that are responsible for the mechanisms of repair.6 The use of an unfocused shock wave applicator4 to treat wounds has been described.7 The
unfocused waves are generated by a parabolic reflector that allows delivery of nearly parallel waves without a focus point. These waves have low-energy density and a broad acoustic field that stimulates large superficial areas. Unfocused ESWT has been safely and successfully used for the clinical treatment of human wounds and experimentally created cutaneous burns in mice. Extracorporeal shock waves enhance porcine wound healing by stimulating epithelialization and neovascularization. Recently, it was reported that a single application of low-energy unfocused ESWT on experimental full-thickness burn wounds in mice results in downregulation of chemokines, proinflammatory cytokines, and metalloproteinases and reduction of inflammatory cell infiltrate. Interestingly, in that study, ESWT did not affect wound closure, epithelialization, or fibroplasia, but modified the initial inflammatory reaction, which is known to be an important complication of burns.

Moreover, ESWT has a positive effect on myofibroblast differentiation in canine tendons. Myofibroblasts are phenotypically transformed fibroblasts that play a major role in healing tissues by means that include wound contraction and collagen synthesis. Myofibroblasts express α-SMA along the contraction lines, and α-SMA is presently considered to be the most reliable myofibroblast marker. It has been suggested that equine distal limb wounds have lower contraction rates than body wounds because of inferior myofibroblast differentiation and organization. To our knowledge, the effect of ESWT on wound myofibroblasts has not been evaluated. We therefore speculate that ESWT could improve wound contraction by affecting myofibroblast differentiation.

The purpose of the study reported here was to determine whether unfocused ESWT has an effect on the healing of distal limb wounds, compared with bandaged-only control wounds, in horses. Our first hypothesis was that distal limb wounds treated with unfocused ESWT would heal faster and have higher contraction rates than controls. Our second hypothesis was that ESWT-treated wounds would have reduced inflammation, which would result in healthier appearance and decreased exuberant granulation tissue formation. Our third hypothesis was that cellular proliferation (fibroplasia, angiogenesis, and epithelialization) and myofibroblast differentiation and organization would be superior for ESWT-treated wounds.

Materials and Methods

Horses—Six healthy mature horses with no abnormal findings via physical examination, CBC, and serum biochemical analyses were used in this study: Horses included 3 geldings and 3 mares (5 Standardbreds and 1 Thoroughbred), were 3 to 13 years of age, and weighed 450 to 500 kg. One forelimb of each horse was randomly assigned to receive treatment (ESWT and bandage); the contralateral limb was used as the control (bandage only). The study was approved by the University of Guelph Animal Care Committee.

Wounds—Horses were sedated with xylazine hydrochloride (0.2 to 0.5 mg/kg, IV), and general anesthesia was induced with guaifenesin (100 mg/kg, IV) and ketamine hydrochloride (2.2 mg/kg, IV). Horses were positioned in dorsal recumbency, and general anesthesia was maintained by use of isoflurane in oxygen and intermittent positive-pressure ventilation. Mean blood pressure (measured by use of direct arterial catheterization) was maintained at ≥ 70 mm Hg. Lactated Ringer’s solution was administered IV at a rate of 5 to 10 mL/kg/h to maintain circulating volume and blood pressure. The distal portions of the both forelimbs were aseptically prepared for the surgical procedure. As described, 5 full-thickness wounds (2.5 × 2.5 cm) were created in each limb by use of a sterile template and a scalpel blade. Three wounds were made over the dorsolateral aspect of the metacarpus and 2 over the dorso- medial aspect of the metacarpus.

Postoperative care—All horses received anti-inflammatory treatment (phenylbutazone, 2.2 mg/kg, PO, q 12 h for 3 days and q 24 h for 4 days) and antimicrobial treatment (trimethoprim-sulfamethoxazole, 24 mg/kg, PO, q 12 h for 7 days) after surgery. Light bandages were applied every other day for 8 weeks. Nonocclusive dressings were used to cover the wounds.

Treated and control limbs—The wounds on the treated limbs received ESWT immediately after wound creation during general anesthesia and subsequently on days 7, 14, and 21 via standing sedation (romifidine [0.05 mg/kg, IV] and butorphanol [0.25 mg/kg, IV]). An ultrasound transmission gel was applied to the wounds, and an electrohydraulic shock wave generator with an unfocused applicator was used to apply 0.11 ml/mm² to each wound (625 pulses at level 5/10 of energy and 5/10 of frequency). During application, the probe was gently moved to cover the entire wound area. All wounds were divided into 4 treatment zones (left, right, dorsal, and ventral). A treatment zone consisted of an area of granulation tissue, neoepithelialized border, and adjacent healthy skin. One hundred fifty-six shock wave pulses were applied to each treatment zone.

The wounds from the control limbs were submitted to the same treatment protocol as the treated wounds except that the probe was applied to the wound without emitting shock waves. After treatment application, the remaining ultrasound gel was cleaned from the wound by use of gauze and saline (0.9% NaCl) solution, the limbs were dried, and a bandage was applied.

Clinical evaluation of the wounds—Clinical assessment of all wounds was made by a single investigator (JK), who was unaware of assignment as treatment and control limbs, on days 1, 7, 14, 21, 28, 35, 42, and 56. Overall wound appearance was classified as either inflamed or healthy on the basis of a predetermined scoring scale of 3 variables: presence of inflammatory exudate within the wound (1 = none, 2 = thin film, and 3 = thick crust), appearance of the skin adjacent to the wound (1 = no swelling or hyperemia, 2 = mild swelling and hyperemia, and 3 = substantial swelling and hyperemia), and appearance of the granulation tissue (1 = pink and regular, 2 = red and regular, and 3 = dark and irregular). The wound was classified as healthy if the

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variables of absolute wound area, area of neoeptithelialization, protruding granulation tissue score, histologic inflammation and epithelialization scores, and immunohistochemical scores were analyzed with a general linear mixed model accounting for repeated measures made over time. Appropriate correlation structure was chosen on the basis of the lowest Akaike information criterion. The assumptions of the ANOVA were assessed by use of comprehensive residual analyses. A Shapiro-Wilk test was conducted to assess overall normality. Residuals were plotted against predicted values and explanatory variables to look for patterns in the data that would suggest outliers, unequal variance, or other problems. A square-root transformation was applied to absolute wound area data.

A general linear model for mixed distributions to account for the random effect of horse was used to evaluate the overall appearance of the wounds. This procedure allowed evaluation of a binomial outcome while accounting for independent repeated evaluations within 1 animal. Histologic fibroplasia and angiogenesis were compared at each week with a Wilcoxon signed rank test.

For multiple comparisons, the Tukey post hoc test was applied when the F test result was significant. Computer software was used for the statistical analysis. A value of $P < 0.05$ was considered significant.
Results

Thirty wounds of 6 horses were included in each treatment group. Clinical assessment of the wounds revealed that control wounds were classified as inflamed 1.9 times (95% confidence interval, 1.389 to 3.171) as frequently as were the ESWT wounds.

All wounds developed exuberant granulation tissue at some time; however, control wounds had significantly ($P = 0.015$) higher scores for exuberant granulation tissue than did ESWT-treated wounds. Mean score of the protruding granulation tissue score at all observations was 1.06 in the treatment wounds and 1.47 in the control wounds (Figure 1). Measurements obtained from digital pictures revealed that ESWT affected neither the absolute wound area nor the neoepithelialization area (Figures 2 and 3).

The absolute wound area and area of neoepithelialization were not significantly different between the groups at any time. At the eighth week after wound creation, 89% of all wounds had healed such that >80% of the original wound area had healed. There was no significant difference in the percentage of healed area between the treatment and control groups. Biopsy did not have a significant ($P = 0.727$) effect on the absolute wound area, compared with measurements made after biopsy. Data from the clinical evaluation and absolute area were discarded after the sixth and eighth week after wound creation, respectively. At that time, a dry scab had formed over the wounds, resulting in inaccurate wound scoring and area measurement. No significant difference between the study groups was found for any of the histologic variables, including $\alpha$-SMA as evaluated by use of immunohistochemical staining.

Discussion

Findings of this study did not support the first hypothesis that ESWT-treated wounds would heal faster and with higher contraction rates than controls; there was no significant difference in the absolute wound area between the groups at any time. At the end of the trial, 8 weeks after wound creation, 89% of the all wounds had the absolute wound area reduced by 80%. Interestingly, the healing time of the wounds in the present study was different than that in 3 other studies\textsuperscript{15–17} where healing times of approximately 32, 42, and 72 days were obtained with the same wound creation protocol. Interestingly, the healing time in all studies seems to be proportional to the number of wounds that developed exuberant granulation tissue. Thus, studies that had an increased number of wounds with exuberant granulation tissue also had a longer healing time. In contrast to those studies, all wounds reported here developed at least 1 mm of exuberant granulation tissue, which was not excised when protruding above the skin level. We decided to avoid interfering with the formation of exuberant granulation tissue to consistently evaluate the effects of ESWT on fibroplasia, but it is possible that the exuberant granulation tissue mechanically limited cellular migration and contraction, resulting in the prolonged healing time of the wounds. The large number of wounds that developed exuberant granulation tissue in the present study most likely resulted from the fact that the limbs were kept bandaged throughout the repair. Bandages increase the oxygen gradient between tissue and the wound surface, which stimulates angiogenesis and fibroblast proliferation.\textsuperscript{18,19}

Although the ESWT protocol was associated with faster healing time of human wounds,\textsuperscript{7} the same results were not seen in the present study or in cutaneous burn
injuries in mice, in which wound closure and epithelialization were not stimulated.11 It is possible that a different shock wave dose is required to achieve better results in each species. Furthermore, the effect of ESWT is highly dose dependent. In a study10 of use of ESWT for the healing of wounds in piglets, substantial stimulation of healing was seen with low-dose treatment, whereas high-dose application of shock waves resulted in inhibition of wound epithelialization. In the same study, intermediate doses of ESWT had no effect.

In agreement with our second hypothesis, ESWT-treated wounds appeared less inflamed and had reduced formation of exuberant granulation tissue. Overall wound appearance was classified as inflamed 1.9 times as frequently in control wounds as in the ESWT-treated wounds, which suggested that ESWT had an anti-inflammatory effect on distal limb wounds. It has recently been reported that unfocused ESWT at 0.11 mJ/mm² results in reduced inflammatory cell infiltration, which was assumed to be associated with global suppression of the expression of proinflammatory cytokines, chemokines, and matrix metalloproteinases, as detected by use of real-time quantitative PCR gene profiling for proinflammatory transcripts. This finding suggests that cellular signaling changes follow ESWT, causing a reduction in the inflammatory response. The reduced exuberant granulation tissue seen in ESWT-treated wounds in the present study could have also been the result of a suppression of the inflammatory response.

Although a macroscopic effect of ESWT on equine wounds was detected, none of the histologic variables were significantly affected by treatment. This could be associated with the large variation in healing among the horses, which resulted in large SDs of the data, especially for the histologic variables. For instance, the power to detect a significant difference of 1 scoring point for histologic inflammation was 36.76% and to detect a difference of 2 scoring points was 61.42%. The number of horses required to achieve a power of 90% would be 18 for 1 point and 12 for 2 points. Additionally, it is possible that the low number of samples available for histologic evaluation limited the power of the analysis. We decided to perform only 1 biopsy/wound to avoid further changes in wound size; however, a higher number of specimens would have allowed higher statistical power.

Contrary to our third hypothesis, ESWT did not enhance any of the histologic variables of cellular proliferation and myofibroblast differentiation. Absence of treatment effect for epithelialization histologically and clinically indicated that the ESWT protocol did not affect epithelial cell proliferation and migration. As discussed, ESWT has a dose-dependent effect on epithelialization, and we postulate that higher shock wave intensity would have resulted in greater epithelial cell proliferation. Increased neovascularization seen in ischemic skin flaps treated with the same ESWT protocol in mice was not detected in the present study. Similar to epithelialization, the shock wave intensity used in the present study could be inadequate to induce increased neovascularization in horses. However, the lack of improvement in neovascularization could also be attributable to the fact that maximal angiogenic stimulation is already present in equine distal limb wounds, in response to the hypoxia and microvascular occlusion that occur in this region.10 Moreover, in the present study, ESWT did not alter fibroblast proliferation, organization, or collagen production. Indeed, ESWT has a positive effect on fibroplasia and collagen organization in equine suspensory ligaments, but this effect has never been detected in any experimental wound model.11 Perhaps it can be speculated that ESWT does not affect fibroblast activity in cutaneous wounds.

There was no treatment effect of ESWT as evaluated via immunohistochemical analysis for localization of α-SMA, suggesting that there was no direct effect of ESWT on myofibroblast concentration and organization. These results combined with the similar healing time for both groups indicated that ESWT at this dosage did not affect wound contraction.

Administration of unfocused ESWT was considered painless for most of the treated human patients in a feasibility study.7 In the present study, all horses tolerated treatment application well, allowing uniform distribution of the shocks and gentle movement of the probe along the wound. It is possible that the application of the shock waves overlapped in some regions; however, dividing the wound into treatment zones reduced this problem. The direct mechanical effects of the shock wave applicator over the wounds have not been reported; however, it is possible that the vibration caused during shock wave generation could alter the mechanism of repair. Movement of the probe over the wounds could cause irritation and inflammation, but we believe that application of ultrasonographic gel can reduce the friction effect and improve transmission of shock waves.

Application of ESWT immediately after wound creation could have negatively affected the wound repair in this study. The anti-inflammatory effect of ESWT could have been detrimental for early stages of wound healing, when a strong inflammatory response would have been desired.

The present study revealed that ESWT-treated wounds had decreased exuberant granulation tissue formation and were less clinically inflamed than were the control wounds; however, ESWT did not accelerate overall wound healing. Therefore, ESWT may be useful to prevent exuberant granulation tissue formation and chronic inflammation of distal limb wounds in horses, but further studies are necessary before recommending ESWT for clinical application. Future studies might include evaluation of the effects of different shock wave protocols on wound healing in horses as well as evaluation of the application of ESWT 2 weeks after wound creation, as an attempt to prevent the chronic inflammatory response.

b. Rompun, Bayer Animal Health, Etobicoke, ON, Canada.
c. Gualenisin (3%) solution, Baxter, Mississauga, ON, Canada.
d. Ketalar, Bayer Animal Health, Etobicoke, ON, Canada.
e. Lactate Ringer's solution, Baxter, Mississauga, ON, Canada.
f. Phenylbutazone tablets, Dominion Veterinary Laboratories Ltd, Toronto, ON, Canada.
g. APO Sulfatrim DS, Apotex Inc, Toronto, ON, Canada.

h. Pansement Telfa dressing, Kendall Canada Inc, Peterborough, ON, Canada.
i. Sedivet, Boehringer Ingelheim, Burlington, ON, Canada.
j. Torbogesic, Wyeth Canada, Saint Laurent, QC, Canada.
k. Eco gel 200, multipurpose ultrasound gel, Eco-Med Pharmaceutical Inc, Mississauga, ON, Canada.
m. ImageJ, Windows version of the NIH image program, Scion Corp, Frederick, Md.

n. Ultra V block, Lab vision, Medcor Inc, Montreal, QC, Canada.
o. Anti-human rabbit polyclonal IgG, Abcam ab5694, 0.20 mg/mL, Medcor Inc, Montreal, QC, Canada.
p. HRP Polymer, Medcor Inc, Montreal, QC, Canada.
q. DAB chromagen, DAKO Corp, Carpinteria, Calif.
r. Cytoseal Mounting Media, Cole-Parmer Canada Inc, Montreal, QC, Canada.
s. SAS OnlineDoc, version 9.1.3, SAS Institute Inc, Cary, NC.

References