Effect of unfocused extracorporeal shock wave therapy on growth factor gene expression in wounds and intact skin of horses

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Objective—To compare the effect of extracorporeal shock wave therapy (ESWT) on expression of fibroblast growth factor-7 (FGF-7), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor-A (PDGF), and vascular endothelial growth factor-A (VEGF) in skin with surgically created skin wounds and intact skin in horses.

Animals—14 healthy horses.

Procedure—8 horses were treated with ESWT at 6 locations along the neck at 36, 24, 12, 6, 2, or 1 hour prior to collection of full-thickness biopsy specimens from each location; a control specimen was collected from a sham-treated location. In 6 horses, 5 full-thickness wounds were created in each forelimb. Wounds in 1 forelimb/horse received ESWT immediately after creation and subsequently on days 7, 14, and 21; wounds in the contralateral forelimb remained untreated. Biopsy specimens were collected from 1 wound on each forelimb on days 7, 14, 21, 28, and 35. Expression levels of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF were assessed in tissue samples from the horses’ necks and forelimbs.

Results—In surgically created wounds, ESWT treatment was associated with reduced TGF-β1 expression, compared with expression in control wounds, during the entire study period. At 28 days following wound creation, IGF-1 expression was significantly increased for treated and untreated wounds, compared with findings on days 7, 14, 21, and 35. There was no significant effect of treatment on FGF-7, TGF-β1, IGF-1, PDGF, or VEGF expression in intact skin.

Conclusions and Clinical Relevance—Intervention with ESWT to suppress TGF-β1 may decrease granulation tissue production, resulting in improved wound healing on the distal portion of horses’ limbs. (Am J Vet Res 2013;74:324–332)

Extracorporeal shock wave therapy is currently used in equine medicine to treat a wide variety of conditions. Although the exact method of action is unknown, it has been suggested that ESWT may have an effect on cell-to-cell interactions by altering the expression of biochemical signals, such as growth factors and other cytokines.1–3

Wound healing requires that many mediators work together to accomplish successful tissue repair. For example, during the process of fibroplasia, inflammatory cells release chemoattractants and cytokines that stimulate the proliferation and migration of fibroblasts from adjacent tissues.4 In horses, the healing of wounds on the distal portion of limbs can be especially problematic, compared with healing of wounds in other body regions.5 The healing of distal limb wounds is often confounded by the production of excess granulation tissue (thought to be a result of prolonged but weak low-grade inflammation), which prolongs the rehabilitation period.6,7 Excessive fibroplasia, which is regulated by cytokines (eg, TGF-β1), is a feature of exuberant granulation tissue.4 Because ESWT is thought to have an effect on growth factor expression, it is currently being considered as a mode of treatment for distal limb wounds in horses to reduce healing time and improve outcomes. As such, it is important to measure its efficacy, determine its effects on tissue, and develop treatment protocols for its use.

An early study8 to evaluate the effects of ESWT on wound healing was performed on piglets with surgi-
cally induced split-thickness wounds. In that study, a dose-dependent effect of ESWT was identified. Low-energy ESWT treatment (10 shock waves at 14 kV) increased the rate of reepithelialization and the number of microvessels present at the site of treatment and, consequently, enhanced healing in the piglets. However, high-energy ESWT (500 to 1,000 shock waves at 14 kV) delayed reepithelialization. Since that early investigation, the potential use of ESWT as a technique to improve wound repair and tissue regeneration has been further investigated in both human and veterinary medicine. In humans, clinical feasibility studies have been conducted, which revealed that ESWT decreased healing time in various conditions associated with ongoing tissue repair and regeneration, including burns and chronic ulcers of the lower extremities.

Extracorporeal shock wave therapy has been introduced as a treatment for tendon injuries in both humans and horses. The effects of ESWT on ligaments and tendons have been previously investigated to determine the mode of action. Tendon lesions are a frequent cause of lameness and early retirement of performance horses. Extracorporeal shock wave therapy has been reported to reduce healing time and improve quality of tendon repair in horses. In a study of horses with collagenase-induced suspensory desmitis, ESWT treatment resulted in higher fibroblast activity and increased expression of TGF-β1 associated with an increased rate of tissue healing, compared with findings in an untreated control group. In a model of experimentally induced Achilles tendinitis in rats, immunohistochemical examination of ESWT-treated tendons revealed increased expression of TGF-β1 and IGF-1, compared with untreated tendons. Histologically, more fibrous tissue bridges, enhanced neovascularization, and greater extracellular matrix production were detected in the ESWT-treated tendons. In mechanical load-to-failure testing, the ESWT-treated tendons were stronger than affected tendons that were not treated with ESWT. In an in vitro experiment involving a human dermal fibroblast cell line, ESWT activated both fibroblast proliferation and mRNA expression of TGF-β1.

An important effect of ESWT on soft tissue healing is enhanced neovascularization. In a study of collagenase-induced superficial digital flexor tendinitis in horses, increased neovascularization as a result of ESWT was evident histologically. The expression of VEGF is upregulated following ESWT in osteoblast cell cultures and in ischemic skin flaps in rats and mice. In an additional experiment involving an ischemic skin flap model in rats and mice, ESWT increased fibroblast recruitment and leukocyte infiltration, which resulted in better healing. To our knowledge, there are no reports of studies to evaluate the effect of ESWT on growth-factor mediation in skin and wounds in horses.

Fibroblast growth factor-7 is produced by inflammatory cells, fibroblasts, and endothelial cells. It is an important chemotactic and mitogenic factor that signals both fibroblasts and endothelial cells. It is also involved in protein synthesis and angiogenesis. Transforming growth factor-β1 is involved in a range of activities, and a wide variety of cell types respond to it. It is involved in the regulation of its own production by monocytes and activated macrophages, thereby sustaining its expression at wound sites. Transforming growth factor-β1 enhances migration and growth of fibroblasts and endothelial cells. It is also involved in the development of extracellular matrix, promotes synthesis of proteins, inhibits matrix turnover (thereby reducing overall enzymatic activity), and enhances wound contraction (ie, differentiation and tubule formation). Produced by the liver and platelets, IGF-1 is an important chemotactic and mitogenic factor that affects endothelial cells. Insulin-like growth factor-1 is also involved in the migration of epithelial cells, proliferation of fibroblasts, and the synthesis of collagen. Platelet-derived growth factor, as the name implies, is derived from platelets. It is chemotactic to inflammatory cells and fibroblasts and mitogenic to mesenchymal cells. Macrophages, fibroblasts, and endothelial cells produce VEGF, a major contributor to angiogenesis. The purpose of the study reported here was to evaluate the effect of unfocused ESWT on expression of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF in intact (apparently normal) skin of the neck and surgically created wounds in the distal portion of the forelimbs in horses.

Materials and Methods

Animals—Fourteen horses with no abnormal findings on physical examination were used in the study. Eight horses were used to investigate the effect of ESWT on expression of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF in intact (apparently normal) skin of the neck. These 8 horses included 4 Standardbreds, 2 Quarter Horses, 1 Thoroughbred, and 1 Appaloosa (5 mares and 3 geldings). The horses were 4 to 25 years of age (median age, 12.6 years) and weighed 450 to 568 kg (median weight, 496 kg). Six horses were used to investigate the effect of ESWT on expression of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF in tissues of surgically created wounds in the distal portion of the forelimbs. These 8 horses included 3 Standardbreds and 1 Thoroughbred (3 mares and 3 geldings). The horses were 3 to 13 years of age (median age, 8 years) and weighed 404 to 500 kg (median weight, 450 kg). The horses were housed in box stalls and provided hay and water ad libitum for the duration of the study. This study was performed according to guidelines established by the Canada Council on Animal Care and was approved by the University of Guelph’s Animal Care Committee.

ESWT application and biopsy procedures at neck locations—For each of the 8 horses used to investigate the effect of ESWT on expression of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF in intact skin of the neck, a template was placed on the neck to identify 7 circular treatment areas (each 3.39 cm in diameter) from which the hair was clipped. Of the 7 treatment areas, 4 were on the right and the remaining 3 were on the left side of the horse’s neck. Randomly, 6 of the 7 areas were assigned to be treated with ESWT at 1 of 6 time points: 36, 24, 12, 6, 2, or 1 hour prior to biopsy specimen collection from that site. The seventh area was assigned as an untreated control site. Treatments were administered so that the biopsy specimens could be collected during 1 period of sedation.
An electrohydraulic shock wave generator with a wide-focused applicator was used for all of the treatments. Extracorporeal shock wave treatment was performed at an energy flux density of 0.11 mJ/mm² with 100 pulses/cm² (total, 900 pulses). The device’s probe head was 6 cm in diameter, and it was moved in a circular and uniform fashion over the entire selected treatment area. The area identified as the control site received a sham treatment in which the probe was applied without emitting shock waves 1 hour before the biopsy procedure.

All biopsy specimens were collected during one 15-minute time period, and each horse was sedated with detomidine hydrochloride (0.01 mg/kg, IV) and romifidine hydrochloride (0.05 mg/kg, IV) and butorphanol tartrate (0.03 mg/kg, IV). An anesthetic line block with 2% lidocaine hydrochloride was performed proximal to the biopsy site. Each treatment area was aseptically prepared, and a sterile template was used to ensure that each of 3 full-thickness biopsy specimens was collected at a point equidistant from the center of the treatment zone. Each of the 3 biopsy specimens from each treatment area was collected by use of a 6-mm-diameter biopsy punch. The 3 biopsy specimens collected at each area were used as biological replicates for the applied treatment. Following the collection of the biopsy specimens from all 7 neck areas, each horse was administered phenylbutazone (4.4 mg/kg, PO).

Each 6-mm-diameter biopsy specimen was bisected. One half of each biopsy specimen was placed in an RNA storage reagent, incubated at 4°C for 24 hours, and then stored at −70°C. The other half of each biopsy specimen was immediately placed in neutral-buffered 10% formalin for 24 hours. Samples were subsequently paraffin embedded via standard histologic methods; sections were cut at 5 μm and stained with H&E stain for histologic evaluation.

ESWT application and biopsy procedures at forelimb wound locations—For each of the 6 horses used to investigate the effect of ESWT on expression of FGF-7, TGF-β1, IGF-1, PDGF, and VEGF in surgically created wounds in the distal portion of the forelimbs, 1 forelimb was randomly assigned to receive treatment (ESWT and bandage); the contralateral limb was used as the control limb (bandage only).

Under anesthesia, 5 square (2.5 × 2.5-cm) full-thickness wounds were created in each forelimb of each horse by use of a sterile template and a scalpel blade. Three wounds were made over the dorsomedial aspect of the metacarpus, and 2 wounds were made over the dorsolateral aspect of the metacarpus (avoiding the extensor tendons). All horses received phenylbutazone (2.2 mg/kg, PO, q 12 h for 3 days and then q 12 h for 4 days) and trimethoprim-sulfamethoxazole (24 mg/kg, PO, q 12 h) for 7 days after surgery. Nonocclusive dressings were used to cover the wounds. Light bandages were applied on both forelimbs every other day for 8 weeks.

For the forelimb that was assigned to receive treatment, all 5 wounds received ESW treatment immediately after wound creation, when each horse remained anesthetized. Subsequently, treatment was applied on days 7, 14, and 21 when each horse had been sedated with romifidine hydrochloride (0.05 mg/kg, IV) and butorphanol tartrate (0.25 mg/kg, IV). With the horse standing, an ultrasound transmission gel was applied to the wounds, and an electrohydraulic shock wave generator with a wide-focused applicator was used to apply 100 pulses/cm² at an energy flux density of 0.11 mJ/mm² to each wound (625 pulses). The device’s probe head was 6 cm in diameter, and it was moved in a circular and uniform fashion over the entire area of each wound. The wounds on the control limb of each horse underwent the same treatment protocol except that the probe was applied to each wound without emitting shock waves.

One wound on both the treated and control forelimb of each horse was randomly selected to undergo a biopsy procedure at each predetermined time point by use of a randomization table. While the horse remained under general anesthesia, 1 baseline biopsy specimen was collected from a randomly assigned wound on both the ESW-treated and sham-treated (control) limb directly following wounding and the first treatment or sham procedure. Following the collection of the first baseline sample, only 1 biopsy specimen was collected from each wound to avoid further compromise of overall wound healing and to avoid detection of changes that were attributable to the biopsy procedure. One biopsy specimen from a treated wound and 1 biopsy specimen from a control wound were collected from each horse on days 7, 14, 21, 28, and 35, after the horse was sedated. An anesthetic line block with 2% lidocaine hydrochloride was performed proximal to the selected wound. Full-thickness biopsy specimens were obtained from the healing border (margin) of the wounds with a 4-mm-diameter punch. A 4-mm-diameter punch was selected for use to reduce the interference with the wound-healing process. The wounds were divided into 4 zones (proximal, distal, left, and right) for randomized biopsy specimen collection, and the sites were uniformly distributed for both the treatment and control forelimbs. Tissue specimens were placed in aluminum foil and snap frozen in liquid nitrogen. The samples were stored at −70°C.

RNA extraction and cDNA synthesis—Each tissue specimen was homogenized, and total RNA extraction was performed with a phenol reagent following methods outlined by the manufacturer. A spectrophotometer was used to quantify the RNA. The quality of the RNA was assessed by calculation of the ratio of absorbance at 260 and 280 nm; all values were within acceptable limits (ratio values of 1.8 to 2.2). A cDNA synthesis kit was used to remove genomic DNA contamination and to reverse transcribe 1 μg of total RNA following the manufacturer’s protocol.

Cytokine gene expression analysis—Primers were designed with publically available software and sequences available in the National Center for Biotechnology Information’s nucleotide sequence database (Appendix). Identities of PCR products were confirmed via DNA sequencing. ß2-microglobulin and GAPDH were selected as reference genes. Quantitative real-time PCR procedures were performed with a multwell plate detection system and SYBR a mix according to the manufacturer’s recom
mended protocol, with final primer concentrations of 0.5 μM for all reactions. The PCR cycling regimen was as follows: denaturation and preincubation (at 95°C for 5 minutes), followed by 45 cycles of denaturation (at 95°C for 10 seconds), annealing (at 56°C for 10 seconds for all reactions), elongation (at 72°C for 30 seconds), and detection. Standard curves for each gene were generated by use of serially diluted cDNA samples analyzed in quintuplicate; all reactions had an efficiency between 1.8 and 2.2 (ie, within recommended limits of 2 ± 10%), and all samples were analyzed in duplicate. Results were determined by quantification software and were expressed as ratios of target gene expression to the geometric mean of the gene expression of the 2 reference genes.

Histologic evaluation of intact skin biopsy specimens from neck locations—Two observers (BNL and BLP), who were blinded to the origin of the biopsy specimens, evaluated the amount of inflammation and angiogenesis and the degree of fibroblast organization. The grading scale developed for use in the study was modified from that used by Lepault et al. Inflammation was graded on a 4-point scale from 0 to 3 on the basis of the degree of infiltration by polymorphonuclear and mononuclear cells, fibrin, and platelets as follows: 0 = normal, 1 = mild, 2 = moderate, or 3 = severe. Vascular reaction was graded on a 4-point scale from 0 to 3 on the basis of the extent of new or reactive capillary clusters (ie, ≥ 5 reactive capillaries grouped together) as follows: 0 = none apparent, 1 = rare clusters, 2 = occasional clusters, or 3 = many clusters. Fibroplasia was graded on a 3-point scale from 0 to 2 on the basis of the presence of reactive fibroblasts or the degree of dermal fibrous connective tissue deposition as follows: 0 = none, 1 = local or minor fibroplasia, or 2 = widespread or major fibroplasia.

### Results

In the present study, there was a significant (P = 0.033) effect of treatment on the level of expression of TGF-β1 in the surgically created wounds on the forelimbs of horses (Table 1). At all time points, 

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<tr>
<td>Time (day of study)</td>
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<td>Treatment (ESWT vs control)</td>
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<td>Time and treatment interaction</td>
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Gene expressions were determined in tissue specimens from 5 surgically created wounds on the distal portion of 1 forelimb of each of 6 horses and from 6 areas of intact (apparently normal) skin on the necks of 8 horses after skin areas did or did not receive ESWT.

Statistical analysis—Expression of the target genes FGF-7, TGF-β1, IGF-1, PDGF, and VEGF relative to gene expression of both β2-microglobulin and GAPDH was determined. The ratios of the target gene expression to geometric mean expression of the β2-microglobulin and GAPDH reference genes were calculated to generate the data set used for statistical analysis. Computer software was used to perform the statistical analysis for the study. All data underwent logarithmic transformation, and a Shapiro-Wilk test was performed to ensure the data were normally distributed. Data were analyzed with a general linear mixed-model ANOVA, accounting for the random effects of horse and the fixed effects of time, treatment, and their interaction. Significance was set at a value of P < 0.05.

With regard to the histologic evaluation scores for intact skin biopsy specimens collected at neck locations, a mean score for inflammation, angiogenesis, and fibroplasia was each calculated for the control site and treatment sites in each horse at each time point. An overall mean score for each of those characteristics in treated intact skin specimens at each time point and for the control site was calculated. A total reaction score was also generated for treated intact skin specimens at each time point and for the control site by calculation of the mean sum of the 3 scores. The variation between the mean sums of the scores for the treated intact tissue was compared with that of the control tissue for each characteristic as well as compared with the total reaction score. Intraobserver agreement was calculated.

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*For this gene, there was a significant (P ≤ 0.05) effect of time. †For this gene, there was a significant (P ≤ 0.05) effect of ESWT.
the expression of TGF-β1 was suppressed in wounds that received ESWT, compared with the expression in wounds that did not receive ESWT (Figure 1). However, there was no effect of treatment on the expression of FGF-7, IGF-1, PDGF, or VEGF in surgically created wounds. Insulin-like growth factor-1 was the only gene for which there was a detectable effect of time on expression in the forelimb wounds; IGF-1 expression was increased (P = 0.006) on day 28 in both treated and control wound biopsy specimens, compared with findings on days 7, 14, 21, and 35. Extracorporeal shock wave therapy had no significant effect on cytokine expression in intact (apparently normal) skin (Figure 2).

Histologic evaluation of the H&E-stained specimens of intact skin tissue collected at locations on horses’ necks revealed no significant differences in the mean sum of the scores for inflammation, angiogenesis, or fibroplasia between the untreated control intact skin specimens and the specimens of intact skin that had
received ESWT (Table 2). On the basis of those findings, we concluded that ESWT did not have a significant effect on normal equine skin. Intraobserver agreement was strong; 91.2% of the values were identical for both observers. The remaining 8.8% of scores for both observers varied by only 1 score point. Results of the histologic evaluation of the surgically created wounds have been reported previously.20

Discussion

The results of the present study indicated that ESWT has no significant effect on the expression of the evaluated growth factors in intact (apparently normal) skin of horses. Histologic examination of the intact skin biopsy specimens revealed no effect of treatment, and the treated tissue specimens had characteristics of normal skin. These results were similar to those of another study20 investigating the effect of ESWT on healing of wounds in the distal portion of the forelimb in horses; in that study, shock wave treatment did not change the histologic appearance (in terms of neovascularization, cell proliferation, and myofibroblast differentiation) of the biopsy specimens obtained from wound margins.6 The design of the present study was based on an assumption that ESWT applied to healthy intact skin of horses would induce an angiogenic response, as observed in other studies.16,18,26 A vascular response occurs during the acute inflammatory phase of healing as injured endothelial cell membranes release phospholipids that control vessel tone and permeability.4 Changes in vessel permeability enable transport of cytokines and other mediators of neovascularization to the site of inflammation.4 Changes in endothelial permeability as well as increased expression of VEGF resulting in improved neo-

Figure 2—Mean ± SE LRR* of expression of FGF-7 (A), IGF-1 (B), PDGF (C), TGF-β1 (D), and VEGF (E) in tissue specimens collected from areas of intact (apparently normal) skin on the necks of 8 horses at intervals after localized application of ESWT or sham treatment (control). In each horse, 7 areas were assessed; randomly, 6 of the 7 areas were assigned to receive ESWT at 1 of 6 time points: at 36, 24, 12, 6, 2, or 1 hour prior to biopsy specimen collection from that site. The seventh area was assigned as a sham treated control site. By use of an electrohydraulic shock wave generator with a wide-focused applicator, ESWT was applied at an energy flux density of 0.11 mJ/mm2 with 100 pulses/cm2 (total, 900 pulses). The device’s probe head was 6 cm in diameter and it was moved in a circular and uniform fashion over the entire selected treatment area. The area identified as the control site received a sham treatment in which the probe was applied without emitting shock waves 1 hour before the biopsy procedure. See Figure 1 for key.
In an investigation of the efficacy of ESWT on ischemic tissue, we aimed to evaluate a dose-dependent effect of ESWT. We used different energy flux density, number of treatment pulses in each treatment, and number of treatments in the present study. The present study was not designed to evaluate a dose-dependent effect of ESWT. We also compared the present study with the aforementioned studies in that species. It is possible that the delicate epigastric tissue of a rat is more susceptible to shock waves than is the forelimb skin of a horse; therefore, higher energy or a greater number of pulses may be required to elicit a response in horses. It is interesting, however, that treatment of metacarpal wounds in horses at a dose of 100 pulses/cm² at 0.11 mJ/mm² resulted in a significant reduction in granulation tissue and inflammation scores (assigned by a blinded observer), which would suggest that this dose might be appropriate to elicit a response, at least in wounded skin.

Another study to evaluate the effect of ESWT on distal limb wounds in horses used an experimental design and treatment protocol very similar to the portion of the present study in which surgically created wounds were evaluated. In that study by Morgan et al., shock wave treatments were administered at 0.11 mJ/mm² (500 pulses/12.6-cm² wound) directly after surgery and weekly thereafter. To assess histologic and immunohistochemical effects, full-thickness biopsy specimens were collected on days 14 and 28 after surgery. Immunohistochemical analysis of those specimens collected following ESWT revealed increased expression of TGF-β1. In those studies and in other investigations, an induction of cytokine expression by ESWT was evident. However, those studies and the present study differed in experimental design and in the species and tissue type evaluated. In addition, compared with the present study, the aforementioned studies used different energy flux density, number of pulses in each treatment, and number of treatments in the same location. The present study was not designed to evaluate a dose-dependent effect of ESWT. In an investigation of the efficacy of ESWT on ischemic tissue in rats, the effect of the number of shock wave pulses was assessed. In those rats, surgically created 8 × 8-cm epigastric skin flaps were treated with an energy flux density of 0.11 mJ/mm² directly after surgery; the number of pulses used ranged from 200 to 5,000. Treatment and control areas were compared after 7 days for area of necrotic tissue. The smallest mean area of necrosis was associated with a treatment level of 500 pulses. Similar improvements were found for treatment levels of 1,500 and 2,000 pulses, whereas a treatment level of 5,000 pulses (approx 78 pulses/cm²) was found to increase the extent of necrotic tissue. The lack of histologic tissue changes, including necrosis, despite application of a higher number of pulses per unit area in the present study may reflect a different healing mechanism (related to the severity of vascular compromise) in the skin flap model. However, the differences in species and tissue type make it difficult to draw a parallel. A skin flap model study in horses would help to determine the most effective dose of ESWT for different applications in that species. It is possible that the delicate epigastric tissue of a rat is more susceptible to shock waves than is the forelimb skin of a horse; therefore, higher energy or a greater number of pulses may be required to elicit a response in horses. It is interesting, however, that treatment of metacarpal wounds in horses at a dose of 100 pulses/cm² at 0.11 mJ/mm² resulted in a significant reduction in granulation tissue and inflammation scores (assigned by a blinded observer), which would suggest that this dose might be appropriate to elicit a response, at least in wounded skin.

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to 28. However, TGF-β1 expression on day 28 in the treated tissues was significantly lower than the level of expression on day 14. These results support the present study’s finding that ESWT suppressed TGF-β1 expression in surgically created wounds on horses’ forelimbs.

Reduction in TGF-β1 expression during the chronic inflammatory phase of wound healing decreases the induction of fibrosis. Transforming growth factor-β1 has an important role in the differentiation of fibroblasts to the myofibroblast phenotype. This process regulates the production of the extracellular matrix and determines the degree of extracellular matrix turnover. Prolonged expression of TGF-β1 results in extracellular matrix synthesis and favors delayed wound contraction, reduced epithelialization, and tissue hypoxia, which lead to the production of granulation tissue formation. Alterations in the expression of TGF-β1 may explain why significantly less granulation tissue was observed in distal forelimb wounds that received ESWT, compared with findings in wounds that did not receive ESWT. There is little information in the veterinary medical literature that describes and quantifies temporal or spatial variation in growth factor expression during healing in horses. However, an ELISA has been used to measure the concentration of TGF-β1 in wounds in horses, allowing comparison of expression among anatomic wound locations. Data from that study indicated that TGF-β1 expression peaked at 24 hours in both limb and thoracic wounds. In the thoracic wounds, TGF-β1 expression returned to prewounding baseline level after 14 days; however, in the limb wounds, expression remained elevated for the duration of the study. The differences between healing of thoracic wounds, compared with healing of distal limb wounds, in horses can be explained by the persistence of profibrotic factors, such as TGF-β1. It would therefore be expected that intervention with ESWT to suppress TGF-β1 expression would result in improved healing of distal limb wounds.

Immunohistochemical assay and subtractive hybridization have been used to generate profiles of cytokine expression during the proliferative phase of wound healing in horses, but there is a lack of data regarding quantification of the expression of growth factors in wounds, which could be used to draw direct comparison to the results of the present study. More research in this area is required to quantify the expected level of cytokine expression during all phases of wound healing in horses. A profile of cytokine expression based on wound location would be of particular interest because there are considerable differences in the healing characteristics at different sites in horses. Results of the present study have indicated that the effect of ESWT on distal limb wounds in horses is a reduction in the expression of TGF-β1, which may be responsible for the decreased granulation tissue production observed in a previous study by investigating the effect of ESWT on healing of wounds in the distal portion of the forelimb in horses.

References


b. Dormosedan, 10 mg/mL; Pfizer Canada, Kirkland, QC, Canada.
c. Torzogesic, 10 mg/mL; Wyeth Canada, Saint Laurent, QC, Canada.
d. Lidocaine hydrochloride, 20 mg/mL; Alveda Pharmaceuticals, Toronto, ON, Canada.
e. 2-Propanol, 70% USP vol/vol, Commercial Alcohols Inc, Brampton, ON, Canada.
f. Baxedin Pre-Op (chlorhexidine gluconate solution, 0.5% wt/vol in 70% isopropl alcohol), Omega Standard Laboratories, Montreal, QC, Canada.
g. Germi-Stat (chlorhexidine gluconate, 4% wt/vol), Gremiphene Corp, Bramford, ON, Canada.
h. Picu-Punch, Acuderm Inc, Fort Lauderdale, Fl.
i. Phenylbutazone tablets, Dominion Veterinary Laboratories Ltd, Toronto, ON, Canada.
j. RNAalater stabilization solution, Applied Biosystems Life Technologies Corp, Ambion, Carlsbad, Calif.
k. APO Sulfatrim DS, Apexon Inc, Toronto, ON, Canada.
l. Sedivet, 10 mg/mL; Boehringer Ingelheim, Burlington, ON, Canada.
m. Eco gel 200, multipurpose ultrasound gel, Eco-Med Pharmaceuticals Inc, Mississauga, ON, Canada.
n. TRizol reagent, Invitrogen Life Technologies, Carlsbad, Calif.
p. QuantiTect cDNA synthesis kit, Qiagen Inc, Toronto, ON, Canada.
r. Sigma-Aldrich Canada Ltd, Oakville, ON, Canada.
s. Laboratory Services, University of Guelph, Guelph ON, Canada.
t. LightCycler 480, Roche Applied Science, Mannheim, Germany.
u. SYBR Green I Master, Roche Applied Science, Mannheim, Germany.
w. Roche LightCycler 480 relative quantification software, Roche Applied Science, Mannheim, Germany.


Appendix

Target and reference gene amplicon length and oligonucleotides for gene expression PCR analysis performed on tissue specimens from distal limb wounds or intact skin neck that had or had not undergone ESWT in horses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon length (bp)</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF-7</td>
<td>246</td>
<td>5′-agcccagggacagtac-3′</td>
<td>5′-tccttgcttcctttctgtc-3′</td>
<td>NM_001633896 XM_001503945</td>
</tr>
<tr>
<td>IGF-1</td>
<td>240</td>
<td>5′-tgctgttcttgattgtc-3′</td>
<td>5′-gtccacgatgcctgtctga-3′</td>
<td>NM_00182498</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>236</td>
<td>5′-ccatctccctgctctgg-3′</td>
<td>5′-gtccacgatgcctgtctga-3′</td>
<td>XM_001915189 X03795</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>214</td>
<td>5′-atggtgaaagaaagaa-3′</td>
<td>5′-tccttgcttcctttctgtc-3′</td>
<td>XM_001915189 X03795</td>
</tr>
<tr>
<td>b2M</td>
<td>228</td>
<td>5′-gagacctcgggagacacttc-3′</td>
<td>5′-gttccttgcttcctttctgtc-3′</td>
<td>NM_001081849</td>
</tr>
<tr>
<td>GAPDH</td>
<td>230</td>
<td>5′-gggacctcgggagacacttc-3′</td>
<td>5′-tccttgcttcctttctgtc-3′</td>
<td>NM_001081849</td>
</tr>
</tbody>
</table>

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