Should we expect similar effects of extracorporeal shockwave therapy on wounds of different species?

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Equine distal limb wounds are common and often heal slowly by second intention. Primary closure of wounds of the distal portion of the limb is often prevented by the lack of soft tissue and immobility of the surrounding skin. Wounds of the distal portion of the limb often heal by second intention and healing is often inhibited by the formation of exuberant granulation tissue. Compared to wounds of the trunk, lacerations of the distal portion of the limb retract more, epithelialize more slowly, and cease to contract sooner. Even within the equine species, there are differences in wound healing. Second-intention healing of wounds occurs faster in ponies than horses. This is the result of a greater and faster contraction of the wound in the ponies. Wounds in horses fill with granulation tissue faster, however in ponies, the granulation tissue is more regular with a smooth surface. Horses often develop exuberant granulation tissue, however this is less common in ponies. This may be explained by differences in the inflammatory response between horses and ponies. Ponies have a greater initial inflammatory response that decreases rapidly after 3 weeks. Horses have less inflammation and fewer neutrophils initially, but the response remains for a longer period of time. During the longer inflammatory period, myofibroblasts are less organized in the horse than the pony. Overall, ponies have a more controlled inflammatory stage and greater organization of myofibroblasts resulting in more rapid and greater wound contraction than the horse. In-vitro studies have shown that there are no inherent differences in fibroblasts and myofibroblasts of horses and ponies therefore, environmental factors such as cytokines and the inflammatory response likely account for the differences.

This is where shock wave therapy may be important to help direct the healing response. Numerous studies have shown an upregulation of multiple cytokines following ESWT. The consistent findings in multiple tissues are an increase in growth factors including VEGF, TGF-B1, and IGF. TGF-B1 has been documented as important in stimulating wound contraction. The horse has lower production of TGF-B2 than ponies which may be one of the reasons for the differences in wound contraction rates between horses and ponies. Another possibility could be oxygen derived free radicals including superoxide and nitric oxide which have been identified in other tissues following ESWT. Increased endothelial nitric oxide synthase has been demonstrated by immunohistochemistry in tendon and bone following shockwave therapy. A nitric oxide releasing gel was shown to increase the rate of epithelialization of burn wounds in rats therefore, nitric oxide could be another potential mechanism for stimulation of wound healing. Associated with the increased growth factors is a resultant increase in neovascularization which should result in faster wound healing.

Undoubtedly, in all species it would be desirable for wounds to heal quickly with a return to normal function. How this is accomplished in each species may be by different mechanisms. In the horse, a mechanism to stimulate the rate and amount of contraction would be beneficial. Contraction is usually beneficial for return to function of limb wounds in horses. It usually occurs faster than epithelialization, and results in a better cosmetic outcome. Therefore, maximal contraction is usually desirable in the horse. Disfiguring and disabling contracture does not occur on the lower limbs of the horse. Excessive contracture can occur with injuries to the lips, muzzle or eyelids, however, even this is not common. Consequently, there is a large difference between horses and other species including humans and dogs where excessive wound contracture is more problematic.

Re-epithelialization is an important step in wound healing in the horse, but not important as contraction. Until a wound on the distal limb of a horse is completely re-epithelialized there is a risk of exuberant granulation tissue formation. Thin, nonpigmented epithelialized scars can result in repetitive injuries to the re-epithelialized tissue. Epithelialization is slow and frequently the most prolonged phase of the process with a maximum rate of 1mm/10 days. In horses, epithelialization is limited until contraction has subsided, therefore, wounds with greater contraction have less epithelialization.

Many drugs and devices to stimulate wound healing have been evaluated on distal limb wounds of horses, but few controlled studies document the benefits of these products. Schumacher, et al. found no benefit of island grafting on the rates of epithelialization and contraction of surgically created wounds on the distal portion of limbs of horses. Equine-derived amnion applied as a dressing to full-thickness wounds on the distal limb of horses significantly sped epithelialization in one study, but this finding could not be repeated in another. Topical medications, including antimicrobial drugs, corticosteroids, and various dressings, have shown little benefit to wound healing. One study did demonstrate that application of a 1% silver sulfadiazine cream resulted in a faster rate of epithelialization.

Recent studies, in which EWS1T was applied to skin grafts and epidermal burns in people, demonstrated a decreased time to healing. When the effects of EWS1T on the healing of partial-thickness wounds of pigs were evaluated, researchers found that the effect of EWS1T on the speed of epithelialization was dose related and that the maximum effect on epithelialization occurred at 10 pulses at 14Kv. Recently, the survival of epigastric skin flaps of rats was shown to be enhanced by the application of EWS1T. Similarly, in a skin flap model, EWS1T stimulated healing as much as did gene therapy using transforming growth factor-β1 (TGF-β1) or vascular endothelial growth factor (VEGF). EWS1T-treated flaps developed an area of necrosis of only 2.5%, whereas 17% of the area of flaps that did not receive EWS1T was necrotic. In another study,
ESWT was shown to induce production of VEGF and perhaps to modulate expression of other growth factors. ESWT was also shown to decrease time to re-epithelialization of deep, partial-thickness burns of human beings.

Frequently equine distal limb wounds are expected to heal by second intention. It was unknown how ESWT would affect the wound healing in the horse.

**Study of second intention healing of distal limb wounds in horses**

In a recent study by the authors of this paper, the effect of ESWT on second intention healing of distal limb wounds of the horse was evaluated. For this study, a 5-cm diameter circle was tattooed on the dorsomedial aspect of the mid-metacarpal region of each fore limb of each of 6 horses 4 weeks before the study began. This allowed for the measurement of wound expansion and contraction. On day 0, a 4-cm diameter, circular defect that included skin, subcutis, and periosteum was created in the center of each tattoo. At the same time, two similar 3-cm diameter wounds were made on the dorsomedial aspect of each metatarsus, one 4 cm proximal and one 4 cm distal to the middle of the metatarsus. These wounds were created to obtain biopsies for immunohistochemical evaluation.

(Figure 3)

On day 1, the wound on one randomly selected MC was covered with ultrasound coupling gel and treated with ESWT using 500 pulses administered at 0.11 mJ/mm². Both MT wounds on one randomly selected MT were treated with 280 pulses which provided an equal number of pulses per square centimeter of wound as the larger forelimb wounds. Treatments were repeated weekly until the wounds were healed. During treatments, the untreated control wounds on the contralateral MC and MT were also covered with ultrasound coupling gel. At each bandage change, the wounds were digitally photographed with a ruler positioned vertically and horizontally close to the wound as reference for the photographs. The wounds were maintained under a non-adherent dressing and bandage until healed. At day 14, full-thickness, full-width of the wound, rectangular excisional biopsies were taken from the distal wound on each metatarsus, and at day 28, full-thickness biopsies were taken from each proximal wound on each metatarsus. Each biopsy was approximately 6 mm wide, oriented in a transverse plane across the center of the wound, and encompassed adjacent unwounded tissue on both the medial and lateral aspects of the wound. Each biopsy sample was placed into a 10% solution of neutral-buffered formalin and then into 70% isopropyl alcohol. The biopsy was embedded in paraffin using an automated system. Samples were cut into 5-µm thick sections.

**Epithelialization and Contraction**

- The digital photographs were analyzed using computer software to determine the area within the tattoo, the area of epithelialization, and the area of the non-epithelialized portion of the wound. The percentage of contraction was calculated using the following formula:

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\% \text{ contraction} = \frac{\text{wdD} - \text{tatD}}{\text{tatD}} \times 100\%
\]

where \( \text{wdD} \) denoted the maximum area of the wound, and \( \text{tatD} \) denoted the maximum area of the tattoo, each of which was determined after the wound enlarged to its maximum extent after its creation, before contraction and epithelialization began. Wound contraction was expressed as a percentage of the wound’s area on day D, D, represented any specific day after D.

**Granulation tissue**

- The quantity of granulation tissue was scored as: 0 = none; 1 = ≤ 5 mm depth and ≤ 1 cm² in area; 2 = ≤ 5 mm depth, entire area of the wound; 3 = ≥ 5 mm depth.

**Immunohistochemical staining for IGF-1, VEGF and TGF-81**

For immunohistochemical staining the IGF-1 antibodies were diluted 1:10, and the VEGF and TGF-81 antibodies were diluted 1:50 in Tris/PBS/BSA solution. Sections were bathed in the primary antibody solutions for 2 hours at room temperature. Endogenous peroxidase activity was inhibited by applying 3% H₂O₂ for 10 minutes. For IGF-1 and VEGF, a multilink, goat, anti-immunoglobulin secondary antibody was used at a dilution of 1:80 and were incubated for 15 minutes at room temperature. For TGF-81, a goat anti-rabbit secondary antibody was used at a 1:500 dilution. Samples were incubated for 15 minutes at room temperature. All slides were then exposed to a 1:200 dilution of Horse-radish Peroxidase-Streptavidin for 15 minutes then stained with Nova Red for 5 minutes and counterstained with one-quarter strength Shandon’s hematoxilin for 2 minutes. Negative controls were incubated in PBS instead of with the primary antibodies. Normal equine pancreas was used as the positive control for IGF-1. A section of skin with an extensive focus of granulation tissue was used for both the VEGF and TGF-81 controls.

For each time frame and treatment group, five fields of immature, loose, granulating fibrous connective tissue were randomly chosen for examination at 600X. The connective tissue was subjectively evaluated for intensity of cytoplasmic staining and objectively evaluated for the density of cells with positive staining. A field of view was assigned a score of 1 if the uptake of stain was low in intensity. This corresponded to <15 cells with stain uptake /600X field. The field of view was assigned a score of 2 if the uptake of stain was moderate in intensity. This corresponded to 15-40 cells stained /600X field. The field of view was assigned a score of 3 if the uptake of stain was high in intensity. This score corresponded to >40 cells stained /600X field.

**Results**

**Epithelialization and Contraction**

- The mean length of time for wound healing was 76 days for wounds treated with ESWT, and 90 days for the untreated control wounds (\( P = 0.051 \)). The healed treated and control wounds had similar areas of epithelialization (\( T = 4.5 \) cm²; \( C = 3.9 \) cm²; \( P = 0.48 \)) and percentages of contraction (\( T = 61.3\%; C = 61.0\%; P = 0.96 \)).

(Figures 4 & 5)

**Granulation tissue**

- The mean sum of the granulation tissue scores did not differ significantly throughout the study period (\( P = 0.52 \)). When the wounds were healed, the sum of the granulation tissue scores


throughout the study period was 10 for the treated wounds and 8 for the control wounds.

**Immunohistochemical staining**

In study tissues, IGFL-1 staining was observed in the cytoplasm of macrophages, fibroblasts, neutrophils, and plump, immature endothelial cells. Positive staining for VEGF in control and study tissues was identified in the cytoplasm of fibroblasts, endothelial cells, macrophages, and smooth muscle cells. Staining for TGF-β1 was identified in the fibrinous exudate, and the cytoplasm of macrophages, fibroblasts, and endothelial cells.

The density of staining of growth factors varied between regions of the section examined. Density of staining was greatest in areas of wound repair that were composed of immature, loose, granulating, fibrous connective tissue. There were no statistical differences in the score for cells with positive staining for VEGF (P = 1.0 and P = 0.37), IGFL-1 (P = 0.15 and P = 0.31), and TGF-β1 (P = 1.0 and P = 0.37) between treatment and control wounds at day 14, or day 28, respectively. There was a statistically significant decrease in density score for cells staining positive for VEGF (P = 0.03) in control wounds from day 14 (mean 3) to day 28 (mean 2.17). A similar trend in VEGF score was noted in the treatment wounds (d 14 = 3; d 28 = 2.33; P = 0.06).

There was a significant decrease in the IGFL-1 density score between the initial and second biopsies among the control wounds (d 14 = 2.83; d 28 = 1.67; P = 0.015), but not among the treatment wounds (d 14 = 2.83; d 28 = 2.33; P = 0.25). TGF-β1 scores decreased significantly during the time from the first to second biopsy in both the treatment (d 14 = 3.0; d 28 = 1.5; P = 0.015) and control (d 14 = 3.0; d 28 = 1.67; P = 0.015) wounds.

**Discussion**

It is very apparent that we must look at each study individually. Not only do we need to account for EFD, pulse numbers, waveform, and device, we need to be aware of species differences. The extrapolation between species is only viable as starting points for research. The wound type must also be considered. Most burn wounds and skin flaps have some degree of dermis present. In our study we evaluated the rate of healing of full-thickness, cutaneous defects. These wounds included all skin, subcutaneous tissue and periosteum. To our knowledge, similar wounds have not been evaluated in other species.

While the results of this study indicate that ESWT may speed the rate of healing of wounds on the distal portion of the fore limbs of horses, it only resulted in a 14 day improvement. When healed, the treated and control limbs had similar percentages of contraction and epithelialization. Our results show that the increased rate of healing was primarily due to an accelerated rate of epithelialization and to a lesser degree contraction during the early wound healing period. Clearly there are some benefits for ESWT in wounds that must heal by second intention in the distal limb of the horse. However, from this study and previous studies, there may be additional clinical indications.

Chronic non-healing wounds with exuberant granulation tissue are common in the horse. In these wounds, any mechanism to stimulate healing after debriding the exuberant granulation tissue is needed. In the study presented here, the effect of ESWT was seen predominantly in the first 3-5 weeks after wounding. If ESWT could "restart" the early phases of wound healing it could be beneficial to these chronic wounds.

Limb wounds in the horse frequently have flaps of tissue, which are often lost due to avascular necrosis. The benefit of ESWT on the epigastric flaps in rats resulted in an 14.5% decrease in flap loss. This could be important in these wounds where there is limited soft tissue covering. (Figure 6)

The increase in epithelialization was the primary contributor to the differences seen in this study. Distal limb wounds in the horse frequently require skin grafting to achieve healing. The stimulation of epithelialization from skin grafts would greatly speed the healing of these grafted wounds. (Figure 7)

Additionally, ESWT in conjunction with other wound therapies may provide a way to enhance the response and further maximize the rate of wound healing. The topical application of platelet-rich plasma has been shown to accelerate epithelial differentiation, which potentially could be synergistic with the ESWT. In equine lower limb injuries where contraction is limited and skin grafts are often required, the value of ESWT on graft take and epithelialization should be investigated. There were no complications seen with the treatment in this study and no contraindications were found involving 208 human patients. The effects appear to be most predominant early in the healing process, so treatment may best be concentrated early after injury.

*Equitron, Sanuwave Inc., Marietta, GA.*

*Image J 1.37v, National Institutes of Health.*

*Antibody source: IGFL-1 (H-70)-sc-9013, TGFβ1 (V)-sc-146, VEGF (A-20)-sc-152: Santa Cruz Biotechnology, Inc., 2145 Delaware Avenue, Santa Cruz, CA 95060

**References**

12. Wang CJ, Huang HY, Pai CH. Shock wave-enhanced neovascularization at the...
Figure 4. From top to bottom pictures taken on day 0, 30, 62 and 90 days after the creation of the wounds. For this horse the left limb (on the left) was treated and the right was the untreated control. The dark skin in this horse made it difficult to see the tattoo so the tattoo was marked over with a white marker for pictures.

Figure 5. The total area of the control (C) and treated (T) wounds increased in size from day 0 to day 14 when they began to decrease in size from contraction and epithelialization. There was a greater area of contraction in the control wounds because they took longer to heal. The majority of the difference between treated and control wounds was the difference in the area of epithelialization from 17 through 34 days.

Figure 6. Lower limb injuries in the horse commonly result in the formation of tissue flaps with poor perfusion that become avascular. Decreasing the area of tissue lost from avascular necrosis would notably improve healing of these wounds.

Figure 7. This mesh graft was applied 24 hours previously to a laceration over the pastern region. Shock wave therapy could potentially improving the rate of epithelialization from grafts and shorten healing times.