Histomorphologic evaluation of extracorporeal shock wave therapy of the fourth metatarsal bone and the origin of the suspensory ligament in horses without lameness

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Objective—To determine via histologic examination and scintigraphy the effect of focused extracorporeal shock wave therapy (ESWT) on normal bone and the bone-ligament interface in horses.

Animals—6 horses without lameness.

Procedure—Origins of the suspensory ligament at the metacarpus (35-mm probe depth) and fourth metatarsal bone (5-mm probe depth) were treated twice (days 0 and 16) with 2,000 shocks (energy flux density, 0.15 mJ/mm2). One forelimb and 1 hind limb were randomly treated, and the contralateral limbs served as nontreated controls. Bone scans were performed on days –1 (before ESWT), 3, 16, and 19. Histomorphologic studies of control and treated tissues were performed on day 30.

Results—ESWT significantly increased the number of osteoblasts but caused no damage to associated soft tissue structures and did not induce cortical microfractures. A significant correlation between osteoblast numbers and radiopharmaceutical uptake was noticed on lateral views of the hind limb on days 3 and 16 and on caudal views of the forelimb on day 3.

Conclusions and Clinical Relevance—Results suggested that ESWT has the potential to increase osteoblast numbers in horses. The correlation between increased osteoblast numbers and radiopharmaceutical uptake 3 days and 16 days after the first ESWT suggested that stimulation of osteogenesis occurred soon after ESWT. No damage to bone or the bone-ligament interface should occur at the settings used in this study, and ESWT can therefore be administered safely in horses. (Am J Vet Res 2006;67:577–582)

A common use for ESWT is lithotripsy in human medicine.1–3 Interest in shock wave effects on bone was stimulated by the observation that shock waves interacting with the pelvis during lithotripsy cause an increase in pelvic bone density.4 Subsequent investigations focusing on the effect of shock waves on bone were performed.5–10 An early study11 on the effect of shock waves on bone formation was investigated in a rat fracture model. The potential to stimulate bone formation increased interest in investigation of the effect of ESWT on fracture healing12,13 and healing of hypertrophic nonunions.14 The main orthopedic indications for shock wave therapy in humans and other animals are treatment for insertional desmopathies and stimulation of osteogenesis. In a recent study15 on rabbits with ligament reconstruction of the knee, shock wave treatment resulted in improved healing rate of the tendon-bone interface, leading to significantly higher tensile strength.

To date, there are only few experimental studies of the effects of shock waves on bone that explain the molecular mechanisms underlying the action of shock waves. In vitro studies on human bone cells16 and osteoblastlike cells17 reveal cell stimulation and enhanced osteoblast differentiation. Studies in experimental animals reveal increased transforming growth factor-β1 and vascular endothelial growth factor expression in shock wave–promoted bone regeneration18 as well as promotion of growth and differentiation of bone-marrow stromal cells toward osteoprogenitors.19

The beneficial effect of ESWT in horses with recurrent or CPSD has been reported.20–22 Studies20,21 indicate that ESWT facilitates the healing process of experimentally induced SL desmitis in horses. However, there are still few studies focusing on the morphologic changes in equine bone after shock wave administration. No evidence of microfractures or changes in material properties was observed after ESWT of ex vivo equine bone.23–25 Another study25 found significant effects on bone microcracking after application of 9,000 shock waves in 1 session to distal portions of equine cadaver limbs.

Thirty days after application of ESWT to the equine metacarpus-metatarsus, osteogenic stimulation was detected in an in vivo study.25 All treated specimens had activated osteons throughout the width of the cortex, and there was a notable endosteal response. In another in vivo experiment with 6 nonlame (sound)
horses, no significant effect of ESWT at the origin of the SL and the MTIV, compared to the control limb, could be detected by use of repeated skeletal scintigraphy or thermography after 2 sessions of focused ESWT.26

The objective of the study reported here was to determine via histology and scintigraphy the effect of focused ESWT on normal bone and the bone-ligament interface in horses at possible clinical application sites in horses, such as the MTIV and the OSL-MC. We hypothesized that ESWT may induce bone remodeling and may increase the number of osteoblasts in normal equine bone and the bone-ligament interface. Another objective was to examine the correlation between osteoblast numbers in the present study and those of an earlier study26 of repeated bone scintigraphy at the ROI.

Materials and Methods

Horses, ESWT, and bone scintigraphy—Data regarding horses, ESWT, and bone scintigraphy used in this experiment have been published.26

Histologic examination—At 30 days after ESWT, the horses were euthanatized with a barbiturate overdose and all limbs were collected for histologic evaluation. Immediately after dissection, each limb was perfused with 0.9% NaCl solution that contained heparin (250 U of heparin natrium/mL) followed by 1 L of 4% paraformaldehyde through the median and cranial tibial arteries, respectively. After allowing the 4% paraformaldehyde to penetrate thoroughly into the tissue at 21°C for 24 hours, the limbs were frozen at –20°C. Tissue specimens of the OSL-MC and the treated segment of the MTIV were harvested from the treated limb and the corresponding control limb and preserved in 4% paraformaldehyde for 5 days. Subsequently, the samples were dehydrated in ethanol for 1 week, infiltrated with methylmethacrylate until polymerized. The blocks were sectioned at 150 to 200 µm. The resulting 180 sections were conventionally stained with H&E, Giemsa, or toluidine blue.

From the forelimbs (treated and control), 2 longitudinal sections of the OSL-MC and a transverse and a longitudinal section of the SL were prepared. The sections were from a location equidistant from the second and fourth metacarpal bones and distal to the proximal border of the third metacarpal bone. From the hind limbs, a transverse and a longitudinal section of the SL were prepared. The sections were from a 2-cm segment of the treated MTIV were prepared.

Histologic examination was performed independently and without knowledge of treatment by 2 of the authors (HG and ASB), who was unaware of treatment versus control assignments. Data analysis was performed by 1 author (ASB), who was unaware of treatment versus control assignments.

Results were similar at the MTIV, but the difference was not significant (P = 0.073; Figure 1). In areas with low cell numbers, the osteoblast density was similar in treated and control limbs (OSL-MC, P = 0.739; MTIV, P = 0.188).

Statistical analysis—Data analysis was performed with software.1 An ANOVA was used to assess interactions between treatment and cell count results. Data were preprocessed using the software.1

Results

ESWT had a small but significant effect on osteoblast numbers of the OSL-MC. In areas with high cell numbers, osteoblast density was higher in the treated limbs than in the control limbs (P = 0.001). Results were similar at the MTIV, but the difference was not significant (P = 0.073; Figure 1). In areas with low cell numbers, the osteoblast density was similar in treated and control limbs (OSL-MC, P = 0.739; MTIV, P = 0.188).

Figure 1—Photomicrographs of longitudinal sections of the MTIV of a horse that received ESWT. A—Area with high cell numbers (delineated by green line). B—Area with low cell numbers (delineated by green line). Toluidine blue stain.
Significant positive correlations between peak values of RU in the ROI and osteoblast density in areas with high cell numbers at the OSL-MC were evident on the caudal views 3 days after the first ESWT ($P = 0.047$; Table 1). At the MTIV, a significant correlation was evident on lateral views 3 days ($P = 0.004$) and 16 days ($P = 0.005$) after the first ESWT.

The RU of the ROI as previously reported and osteoblast numbers from areas with high cell numbers were significantly correlated 3 days (correlation coefficient, 0.609 [$P = 0.002$]) and 16 days (correlation coefficient, 0.419 [$P = 0.042$]) after the first ESWT on lateral views of the MTIV but not at the OSL-MC. No significant correlation was found between osteoblast counts of areas with low numbers and scintigraphic data (peak and mean values) at any time point or on any views at the OSL-MC or the MTIV of any limb.

At the OSL-MC, the subjectively graded scoring of bone remodeling revealed no significant ($P = 0.99$) difference between treated ($2.25 \pm 0.52$) and control ($2.25 \pm 0.27$) limbs. No other lesions of the SL were identified. There was no evidence of microfractures or microfissures in the third metacarpal bone.

Bone remodeling scores of the MTIV revealed a slight but not significant ($P = 0.152$) increase in bone remodeling in the treated limbs ($2.06 \pm 0.73$), compared with the control limbs ($1.77 \pm 0.66$ [Figures 2 and 3]). The areas with the highest bone turnover were situated beneath the periosteum and were dominated by osteons in early stages of formation. In these peripheral regions, osteoblast density was increased and intercellular substance was calcified (3, arrows). Toluidine blue stain.

Discussion

The osteogenic effect of ESWT has been detected in rabbits, dogs, and recently in horses. Thirty days after ESWT, equine metacarpal and metatarsal bones had a larger number of active osteons labeled by oxytetracycline than after simple periosteal elevation.

In the study reported here, the treatment locations in the forelimbs were chosen because of good results reported after ESWT in horses with proximal suspensory desmitis. The treatment locations in the hind limbs were chosen because of reports of osteogenesis in an intact bone model and a healing bone model.

Table 1—Results of correlation analyses (r) of osteoblast numbers in areas of bone with high cell numbers and maximum RU before ESWT (day 0) and on days 3, 16, and 19, measured via various scintigraphic views of the origin of the OSL-MC and the MTIV in horses.

<table>
<thead>
<tr>
<th>Location</th>
<th>Day 0 ($P = 0.765$)</th>
<th>Day 3 ($P = 0.885$)</th>
<th>Day 16 ($P = 0.987$)</th>
<th>Day 19 ($P = 0.145$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSL-MC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cranial</td>
<td>0.065</td>
<td>0.037</td>
<td>0.003</td>
<td>0.307</td>
</tr>
<tr>
<td>Lateral</td>
<td>0.031</td>
<td>0.117</td>
<td>0.253</td>
<td>0.141</td>
</tr>
<tr>
<td>Caudal</td>
<td>0.092</td>
<td>0.410</td>
<td>0.178</td>
<td>0.263</td>
</tr>
<tr>
<td>MTIV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral</td>
<td>0.357</td>
<td>0.561</td>
<td>0.553</td>
<td>0.061</td>
</tr>
<tr>
<td>Caudal</td>
<td>0.037</td>
<td>0.234</td>
<td>0.246</td>
<td>0.073</td>
</tr>
</tbody>
</table>

Figures 2—Photomicrograph of a transverse section of the MTIV in a horse that received ESWT. Features of bone remodeling are evident. Osteons in the early stages of formation and numerous osteoblasts (1, arrows) are located in peripheral regions of the MTIV. Osteoid is not yet fully calcified, and the calcification front (2, arrow) can be detected. Osteons located deeper in the bone are fully developed, and intercellular substance is calcified (3, arrows). Toluidine blue stain.

Figure 3—Photomicrograph of a transverse section of a control MTIV of the same horse as in Figure 2. Notice fully developed osteons in the periphery of the bone, signifying normal physiologic bone remodeling. Toluidine blue stain.
In the hind limb, the MTIV was favored over the third metacarpal bone because many horses have nonunion fractures and exostoses of the MTIV as well as intermetatarsal ligament problems. The MTIV is also more accessible for ESWT and scintigraphy.

In our study, a significant difference in number of osteoblasts between treatment and control limbs was found at the OSL-MC, although not at the MTIV. This was presumably attributable to the osteogenesis properties of ESWT, although the exact mechanism of stimulation of osteoblasts is not yet fully elucidated and may not be detectable via histologic examination. However, in vitro studies have revealed several biochemical explanations for the increased rate of metabolism, proliferation, and differentiation of osteoblasts. Low-dose shock wave treatment on human osteoblast-like cells results in stimulation of osteoblasts attributable to an increase in nitric oxide, osteocalcin, and tumor growth factor-β1.

Primary sheep osteoblast cultures exposed to low-dose shock wave treatment have increased cell metabolism in terms of enzyme, bone matrix protein, and cytokine activity. In bone marrow cells harvested from the proximal portion of the femur of rats treated with 500 pulses at 0.16 mJ/mm², O₂⁻⁻ production is induced and resulted in osteogenic cell growth and maturation. Increased proliferation of cultured bone cells is found between the third and eighth day after shock wave treatment, and an increase in CFUs is found at 12 days.

It is doubtful whether the results of in vitro studies that used only 1 type of cell can be used to explain the effects of ESWT on complex tissue such as bone or tendon. However, a recent study on rat femoral shafts harvested 4 to 7 days after shock wave administration reveals that gene expression patterns of shock wave–induced osteogenesis are similar to those of periosteal hard callus formation during fracture healing. Thus, shock waves can induce much activation of cells in normal long bones and drive the cells to express genes for osteogenesis. In horses with experimentally induced SL desmitis, greater expression of tumor growth factor-β1 is detected 4 weeks after shock wave administration.

The difference in osteoblast numbers between treated and control limbs was only significant for areas with high cell numbers and not for areas with low cell numbers. When shock waves are focused on bone, 35% of the energy is reflected and the remaining energy undergoes an 80% to 90% reduction within 1 to 2 cm of the surface. Therefore, ESWT does not have an effect throughout the whole bone; the effect is limited to the focal point of treatment and the areas immediately adjacent. High-energy treatment of rabbit bone causes cortical thickening weeks after shock wave therapy but only minor trabecular remodeling in the medullary cavity.

In vivo, equine metacarpal-metatarsal bones treated with 1,000 pulses at 0.89 mJ/mm² have circular areas of reddening (0.3 mm diameter) that extend only 1 to 2 mm into the bone, although this level of energy is relatively high. Therefore, we presumed that areas with high cell numbers represented areas in and close to the focus of the shock waves, although the precise anatomic location of an in vivo treatment is difficult to confirm. This explains the lack of significant differences between control and treated limbs regarding areas with low cell numbers, which received little or no energy.

There was no consistency in the location at which high cell numbers were observed. The MTIV was treated over a 2-cm distance at 2 angles and at 2 time points. Because the treatment was quite local, certain areas may have been more intensively treated than others and may have received more stimulation for cell activation. Conversely, treatment of the MTIV was broader and more uniform, and the entire bone may have been equally treated. This seems probable because the MTIV was 3.5 × 2.8 mm in cross section at the treatment site. Perhaps there are regional differences in bone metabolism and cell dynamics that result in different numbers of osteoblasts at the same time point in the same bone. Thus, this may explain why areas of high cell numbers were found at different locations.

In contrast to osteoblast numbers, the difference in bone remodeling between treated and control limbs was more distinct for the MTIV than for the OSL-MC. The effect of shock waves is thought to vary because of the differences in acoustic impedance of the treated tissue and the surrounding structures. The ensuing differences in energy release may lead to varying levels of tissue activation. Thus, bone remodeling at the OSL-MC may have been at a more advanced stage with less open lacunae and higher numbers of osteoblasts than at the MTIV, where more open lacunae and less osteoblasts were evident.

Bone scintigraphy is a sensitive method for assessment of early changes in bone metabolism. Because a scintigraphic study on rabbit femora treated with ESWT detected an increase in local blood flow and bone metabolism at 28 days after the first shock wave application, a further scintigraphic study at a later date may have revealed an increase in uptake in the treated regions.

A noteworthy finding in this study was the significant positive correlation between scintigraphic uptake and the density of osteoblasts 3 days after the first ESWT at both locations and on day 16 in the MTIV. This effect was seen on the caudal view of the OSL-MC and on the lateral view of the MTIV, and it was more distinct when peak values instead of mean values of uptake in the ROI were used. In these views, scintigraphic evaluation was most sensitive and provided clearer images because the uptake was minimally influenced by overlapping bony structures.

The osteogenic effect of ESWT is expected to result in a local increase in bone metabolism, measurable during the bone phase of the scintigraphic examination. A microautoradiographic study without shock wave application at 2 hours reveals that radiopharmaceutical localizes at mineralization fronts between existing bone and osteoid and at borders of osteocyte lacunae. In this study, histologic examination of the focus region 30 days after the first ESWT detected a difference in bone remodeling of the MTIV between treated and control limbs. The total time for a remodeling cycle (activation, resorption, and formation) is estimated to be 12 weeks in a healthy human.
days for activation, approx 22 days for resorption, and 57 days for formation).\textsuperscript{37} Under the assumption that shock wave–induced cytokine activation or cavitation-mediated mechanical strain leads to activation of a remodeling cycle, this would indicate that our specimens harvested at 30 days after ESWT would be in the late resorption or early formation phase. A correlation would therefore be expected between uptake and osteoblast numbers on day 19 rather than on days 3 and 16, respectively. This was not observed and might be explained by the dynamics of bone remodeling. Perhaps ESWT represents an extremely strong activation stimulus, leading to a much shorter bone remodeling cycle. Accumulation of radiopharmaceutical is predominantly mediated by osteoblastic activity, although other mechanisms may exist, because positive results of bone scans may also be seen in humans with osteomalacia, in which matrix turnover is high but mineralization fails.\textsuperscript{38} Correlation of uptake and osteoblast numbers as early as on days 3 and 16 may indicate that the primary mechanism for the stimulation of osteogenesis takes place shortly after shock wave therapy. After the second ESWT on day 19, no positive correlation between uptake and osteoblast numbers was detected. Perhaps bone tissue that has already been stimulated is in a refractory state and cannot react to a subsequent stimulus.

After ESWT, no microfissures or microfractures were detected. Results of other in vivo studies of rodents,\textsuperscript{39} sheep,\textsuperscript{40} dogs,\textsuperscript{41} and horses\textsuperscript{23,25} support this finding and indicate that the primary mechanisms for stimulation of osteogenesis do not include induction of microfractures. A study\textsuperscript{42} on equine cadaver limbs revealed microcracks after focal application of a high number of shock waves (9,000 shock waves [0.15 mJ/mm\textsuperscript{2}] in 1 session). In a clinical setting, such a high number of shocks are not applied in 1 session.

Investigations of dose-dependent bone changes indicate that the applied energy level is relevant.\textsuperscript{30,44} The number of shock waves applied in 1 session is also important to consider. Kusnerczak et al.\textsuperscript{45} concluded that the effect of ESWT on bone cells mainly depends on the number of impulses applied. The ability to control the number and intensity of shock waves permits testing the hypothesis that shock waves at low energy levels stimulate cells, whereas use of high-energy shock waves causes destruction, necrosis, and scar formation. In previous studies, this hypothesis was proved to be true for the healing of split-thickness skin wounds in a standardized model in pigs\textsuperscript{2} and enhancement of skin flap survival in plastic surgery.\textsuperscript{5} This hypothesis was also confirmed in our study.

On the basis of our results, it is unlikely that focused ESWT with 2,000 shocks/session and an energy flux density of 0.15 mJ/mm\textsuperscript{2} will damage equine cortical bone or tissue at the bone-ligament interface. Shock wave treatment at these settings can be used safely in horses, and a beneficial osteogenic effect is expected.


\textsuperscript{e} Siedler C. Proximal suspensory desmitis in the horse: extracorporeal shock wave therapy compared to injections according to Dr. Müller-Wolffahrt. Doctoral thesis, Universitätsklinik für Orthopädie bei Haft- und Klauentieren und Institut für Anatomie, Veterinärmedizinische Universität Wien, Vienna, Austria, 2002.

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\textsuperscript{h} Leica Microsystems (Schweiz) AG, Glatthürg, Switzerland.

\textsuperscript{i} SPSS, SPSS Inc, Chicago, Ill.

\textbf{References}


