Ultrastructural and immunocytochemical evaluation of the effects of extracorporeal shock wave treatment in the hind limbs of horses with experimentally induced suspensory ligament desmitis

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**Objective**—To evaluate the effects of extracorporeal shock wave therapy (ESWT) on affected ligaments in the hind limbs of horses with experimentally induced suspensory ligament desmitis by use of ultrasonographic, ultrastructural, and immunocytochemical techniques.

**Animals**—10 horses.

**Procedure**—Suspensory ligament desmitis was induced in both hind limbs of each horse by use of 2 collagenase injections (administered 2 weeks apart) in each suspensory ligament. Two weeks after the second injection, the right hind limb of each horse was treated with ESWT (3 treatments at 3-week intervals); the left hind limb was not treated (control limb). Periodically during the study, the healing process was monitored ultrasonographically and the proportions of ligaments affected with lesions were assessed. Four weeks after the last ESWT treatment, biopsy specimens were collected from all ligaments for ultrastructural evaluation and immunocytochemical analysis of transforming growth factor β-1.

**Results**—The difference in the proportion of the lesion-affected ligament between ESWT-treated and control limbs was significant \( P < 0.05 \) from 3 weeks after the second ESWT treatment to the end of the study. Compared with control ligaments, ESWT-treated ligaments had more small, newly formed collagen fibrils and greater expression of transforming growth factor β-1 4 weeks after the last ESWT treatment was administered.

**Conclusions and Clinical Relevance**—Results have indicated that ESWT appears to facilitate the healing process in horses with experimentally induced hind limb suspensory ligament desmitis. (Am J Vet Res 2005;66:892–896)

In horses, ligaments are frequently affected with lesions that result in degenerative and inflammatory changes. The healing of these lesions is often unsuccessful with regard to restoration of the morphologic and functional characteristics of the ligament, thereby compromising the future athletic performance of or increasing the risk of recurrence of lesions in the affected horses.

Treatment of suspensory ligament desmitis in athletic horses is controversial. Various treatments have been proposed for the treatment of suspensory ligament desmitis, including rest, controlled exercise protocols, intraleisional injections, administration of anti-inflammatory agents, and corrective shoeing; however, these treatments are commonly associated with unsatisfactory results and high recurrence rates, especially when used in horses with hind limb suspensory ligament desmitis.

New treatments such as extracorporeal shock wave therapy (ESWT) may represent an option for the treatment of suspensory ligament desmitis in horses, according to some researchers. In support of this claim, results of 1 study indicated that ESWT-treated lesions in horses with collagenase-induced suspensory ligament desmitis healed faster than non–ESWT-treated lesions, as evaluated ultrasonographically. Results of another recent study in rats also suggest that ESWT is associated with an increase in the production of transforming growth factor β-1 (TGF-β1), with concomitant chemotactic and mitogen effects on osteoblasts.

Few controlled studies have been performed to investigate the experimental use of the extracorporeal shock waves in horses. The purpose of the study reported here was to evaluate the effects of ESWT on affected ligaments in the hind limbs of horses with experimentally induced suspensory ligament desmitis by use of ultrasonographic, ultrastructural, and immunocytochemical techniques.

**Materials and Methods**

This study was approved by the Ethics in Animal Experimentation Committee of UNESP-Botucatu. Ten adult horses (4 geldings and 6 mares) were included in the study. Prior to induction of suspensory desmitis, each horse underwent an initial clinical lameness and ultrasonographic examination of the hindlimbs. Suspensory desmitis was induced in both hind limbs of each horse by use of 2 collagenase injections/limb administered 2 weeks apart.
each collagenase treatment, the horses were each sedated with romifidine (0.1 mg/kg, IV) and 2% lidocaine was injected perineurally in each hind limb to provide local anesthesia of the planter metatarsal and plantar nerves; the skin of the lateral metatarsal region was prepared aseptically. With ultrasound guidance, collagenase injections were administered in the body of the suspensory ligament (approx 20 cm from the point of the hock) of both hind limbs. The first injection comprised 0.5 mL (2.5 mg/mL) of collagenase and the second injection (administered 2 weeks later) comprised 0.5 mL (5.0 mg/mL) of collagenase. Immediately before and 1 week after each collagenase treatment, ultrasonographic evaluation of the collagenase-induced lesions in each hind limb was performed by use of a 7.5-MHz linear transducer, and the extent of each lesion was calculated from the measurement of the area of the ligament and the area of the lesion. Two weeks after the second collagenase treatment, the horses were each sedated with romifidine (0.1 mg/kg, IV) and were treated in the right hind limb with focused ESWT. The ESWT procedure involved application of 500 shocks (5-mm focus) over the lateral aspect, 350 shocks (3.5-mm focus) over the medial aspect, and 500 shocks (3.5-mm focus) over the plantar aspect of the mapped region of suspensory ligament. The energy density was 0.15 mJ/mm². On 3 occasions at 3-week intervals, the horses were sedated and their right hind limbs were treated with ESWT. The left hind limb of each horse was not treated with ESWT (control ligaments). The progression of the healing process in the ESWT-treated and control hind limbs was monitored ultrasonographically at intervals throughout the study. The area of the ligament, area of the lesion within the ligament, and proportion of the ligament affected in each hind limb were assessed ultrasonographically before and 1 week after the first collagenase treatment (weeks 0 and 1, respectively) and before 1 week after the second collagenase treatment (weeks 2 and 3, respectively). Ultrasonographic evaluations were also performed before each of the 3 treatments with ESWT (weeks 4, 7, and 10) and 2 and 4 weeks after the last ESWT (weeks 12 and 14). At the end of the study (14 weeks after the first collagenase treatment), a small (1.0 X 0.5 cm) biopsy specimen of the suspensory ligament was obtained from the right and left hind limbs of each horse for immunocytochemical evaluation of the expression of TGFβ-1 and ultrastructural evaluation via transmission electron microscopy. For collection of biopsy specimens, each horse was premedicated with xylazine (1.1 mg/kg, IV) and anesthesia was induced with ketamine (2 mg/kg, IV) and diazepam (0.05 mg/kg, IV) and maintained with continuous IV infusion of guaifenesin (100 mg/kg), ketamine (2 mg/kg), and xylazine (1.1 mg/kg). Subsequently, horses were allowed to recover from anesthesia, were kept grouped in paddocks (200 m²), and received postoperative care, including analgesics, antimicrobials, and rest until recovery.

For ultrastructural evaluation, specimens of suspensory ligament were fixed in 4% glutaraldehyde at 4°C, postfixed in osmium tetroxide, stained with uranyl acetate, embedded in resin, and mounted in copper grids. Transmission electron microscopy, collagen fibers were measured and counted, counting the mean number of fibers per photomicrograph of the chosen fields of view from the affected area of the specimens, and morphologic characteristics of the cellular structures in the suspensory ligament were determined.

For immunocytochemical evaluation of the expression of TGFβ-1, samples of suspensory ligament were fixed in 10% buffered formalin and paraffin embedded. After deparaffinization, samples were processed with the monoclonal antibody against TGFβ-1. Antigen unmasking was done in a hot bath at 96.5°C in buffered citrate solution; endogenous peroxidase blockade was performed with hydrogen peroxide and water (1:1), and background staining was reduced with 5% bovine serum albumin. Samples were incubated with primary antibody for approximately 12 hours at 4°C, and dianamobenzidine was used as the chromogen. The TGFβ-1 immunoreactivity of samples was assessed subjectively by use of a scale from 0 to 3, where 0 was the absence of immunoreactivity. 1 was low immunoreactivity, 2 was moderate immunoreactivity, and 3 was high immunoreactivity, with 3 examinations by different investigators for each specimen.

Statistical analyses were performed by use of a Student t test for paired samples and Wilcoxon t test for nonparametric paired samples. Values of P < 0.05 were considered significant, when comparing treated and control limbs at each time point. Data are reported as mean values ± SD. All data representing scores were analyzed as nonparametric paired samples.

Results

Results of the ultrastructural evaluation indicated that greater amounts of small collagen fibrils (diameter, 20 to 70 nm) that represented newly formed collagen fibrils and mitochondria were present in the samples of suspensory ligament obtained from the ESWT-treated limbs, compared with findings in the specimens from untreated control limbs (Figure 1). Overall, the mean number of fibrils per field of the affected area in the evaluated photomicrographs was 759 ± 42 in ESWT-treated specimens, compared with a value of 69 ± 14 in the control specimens.

The immunocytochemical evaluation of the presence of TGFβ-1 revealed intense cytoplasmic staining of the activated fibroblasts in the specimens of suspensory ligament obtained from ESWT-treated limbs; in comparison, less expression of TGFβ-1 was detected in specimens from control limbs (Figure 2). The median scores for TGFβ-1 immunoreactivity of the specimens of suspensory ligament from the ESWT-treated (2.8 ± 0.16) and control (1.2 ± 0.16) limbs were significantly (P < 0.05) different.

Compared with changes detected in lesions in the suspensory ligaments in control limbs, ultrasonographic evaluations revealed a greater decrease in the proportion of ligament that was affected by the collagenase-induced lesions and in the area of the lesions after onset of treatment with ESWT (Table 1). The difference in the proportion of the lesion-affected ligament between ESWT-treated and control limbs was significant (P < 0.05) from the 10th week to the end of the study. At weeks 10, 12, and 14, the mean percentage of suspensory ligament affected by lesions was 31.85 ± 9.57%, 20.45 ± 6.70%, and 21.47 ± 6.51%, respectively, for ESWT-treated limbs and 43.99 ± 9.96%, 35.32 ± 6.62%, and 31.92 ± 5.22%, respectively, for control limbs.

After the collagenase injection, the area of the suspensory ligament increased in treated and control limbs but started to decrease by the fourth week. After initiation of the ESWT, mean ligament area was 1.60 ± 0.29 cm², 1.52 ± 0.28 cm², 1.54 ± 0.29 cm², and 1.49 ± 0.22 cm² for treated limbs at weeks 7, 10, 12, and 14, respectively; in control limbs, mean ligament area was 1.84 ± 0.37 cm², 1.83 ± 0.31 cm², 1.90 ± 0.35 cm².
The mean ligament areas of ESWT-treated and control limbs were significantly (P < 0.05) different from the seventh week to the end of the study. Measures of the area of lesions in the suspensory ligaments increased after collagenase injections in treated and control limbs. In ESWT-treated limbs, the greatest lesion area was measured at week 7, after which the lesion area began to decrease through to the end of the study. In control limbs, the greatest lesion area was measured at week 10, after which the lesion area began to decrease through to the end of the study. At weeks 10, 12, and 14, mean lesion area was 0.49 ± 0.21 cm², 0.32 ± 0.13 cm², and 0.32 ± 0.11 cm², respectively, in ESWT-treated limbs and 0.82 ± 0.27 cm², 0.69 ± 0.20 cm², and 0.56 ± 0.15 cm², respectively, in control limbs. The values were significantly (P < 0.05) different for the treated limbs at each of these time points.
Discussion

Injection of collagenase into ligaments causes dissolution of collagen and destruction of noncollagenous extracellular matrix, and collagenase-induced desmitis in horses has been considered an efficient model for the study of tissue repair.7-10 The repair process can be monitored by use of noninvasive methods, such as ultrasonography, allowing quantitative identification of the hypoechoic fibers and the measurement of the cross-sectional area of the lesions within the ligament; alternatively, monitoring can be achieved by use of invasive methods, such as collection of biopsy specimens of ligaments for histologic examination, allowing evaluation of the crimped pattern of the collagen fibers and the cellularity and the degree of disorganization of the extracellular matrix.7 The ultrastructural examination allows cellular morphologic characterization and assessment of matrix production.11,12 In the horses with collagenase-induced suspensory ligament desmitis used in the present study, ultrasonographic evaluations revealed that treatment of affected ligaments with ESWT resulted in significantly greater decreases in ligament area, area of the lesion within the ligament, and proportion of the ligament affected by lesion than that achieved during the same time period in control ligaments; our data corroborate findings of a study7 of the use of ESWT in horses with collagenase-induced suspensory ligament desmitis.

In the present study, ultrastructural examination of specimens of suspensory ligaments obtained from the horses with suspensory ligament desmitis revealed an increase in the amount of small-diameter collagen fibrils (20 to 70 nm in diameter) in ESWT-treated limbs, compared with the amount of those fibrils in control limbs. These small-caliber collagen fibrils may be a product of the degeneration of large fibers or represent newly synthesized fibrils that are formed as a result of the stimulation of fibroblasts. The latter process appears more likely because a greater number of mitochondria were detected in ESWT-treated ligaments (compared with the number of control ligaments), which suggests a greater potential for cellular metabolism and greater ability of fibroblasts to synthesize and secrete collagen, extracellular matrix components, and growing factors, as indicated by data collected by other researchers.13-15

The findings of the immunocytochemical evaluation of ligament specimens involving a monoclonal antibody against TGFβ-1 indicated that there was greater immunoreactivity in the ligaments of ESWT-treated limbs, compared with ligaments of control limbs. This finding corroborates data from a study by Chang et al.,16 in which TGFβ-1 increased extracellular matrix deposition and accelerated the tissue repair process in injured flexor tendons of rabbits. Detection of the expression of TGFβ-1 may explain the diminished presence of inflammatory cells in the proliferative phase of healing.17 In the early phase of tissue repair, TGFβ-1 has a proinflammatory action and also modulates the deposition of extracellular matrix components and enhances collagen, fibronectin, and glycosaminoglycan synthesis from fibroblasts.16 Wang et al16 have suggested that one of the possible mechanisms of action of ESWT is mediated through the action of TGFβ-1.

Overall, our data have indicated that the use of ESWT in horses with suspensory ligament desmitis resulted in increased amounts of collagen fibrils and extracellular matrix components and greater immunoreactivity for TGFβ-1 (which may possibly represent increased activity of fibroblasts) in affected ligaments, compared with findings in affected ligaments in control limbs that did not receive ESWT. On the basis of these data, we suggest that in horses with collagenase-induced hind limb suspensory ligament desmitis, the rate of tissue repair in ligaments after treatment with ESWT is greater than that achieved in ligaments that receive no treatment with ESWT.


b. Collagenase type I. Sigma-Aldrich Corp, St Louis, Mo.
c. Sedivet, Boehringer Ingelheim, São Paulo, SP, Brazil.
d. Xylestesin 2%, Cristália, Itapira, SP, Brazil.
e. SSD 900, Aloka Inc, Tokyo, Japan.
f. Versatron, HMT High Medical Technologies AG, Lengwil, Switzerland.

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Table 1—Mean values ± SD of suspensory ligament area, area of suspensory ligament lesions, and percentage of suspensory ligament affected by lesions assessed ultrasonographically in 10 horses during induction of collagenase-induced suspensory desmitis in both hind limbs and at intervals after initiation of treatment of the right hind limb with extracorporeal shock wave therapy (ESWT). The left hind limb of each horse was not treated with ESWT (control limb).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Limb group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>10</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligament area (cm²)</td>
<td>Control</td>
<td>1.31 ± 0.26</td>
<td>1.65 ± 0.43</td>
<td>1.63 ± 0.40</td>
<td>1.94 ± 0.40</td>
<td>1.91 ± 0.43</td>
<td>1.84 ± 0.37</td>
<td>1.83 ± 0.31</td>
<td>1.90 ± 0.35</td>
<td>1.72 ± 0.23</td>
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<tr>
<td>ESWT-treated</td>
<td>1.28 ± 0.29</td>
<td>1.57 ± 0.38</td>
<td>1.56 ± 0.38</td>
<td>1.90 ± 0.37</td>
<td>1.87 ± 0.39</td>
<td>1.60 ± 0.20*</td>
<td>1.52 ± 0.28*</td>
<td>1.54 ± 0.28*</td>
<td>1.49 ± 0.28*</td>
<td></td>
</tr>
<tr>
<td>Area of lesion (cm²)</td>
<td>Control</td>
<td>0.00 ± 0.00</td>
<td>0.29 ± 0.11</td>
<td>0.22 ± 0.14</td>
<td>0.53 ± 0.23</td>
<td>0.83 ± 0.23</td>
<td>0.74 ± 0.24</td>
<td>0.82 ± 0.27</td>
<td>0.69 ± 0.20</td>
<td>0.56 ± 0.15</td>
</tr>
<tr>
<td>ESWT-treated</td>
<td>0.00 ± 0.00</td>
<td>0.29 ± 0.11</td>
<td>0.21 ± 0.12</td>
<td>0.47 ± 0.20</td>
<td>0.62 ± 0.24</td>
<td>0.63 ± 0.23</td>
<td>0.49 ± 0.21</td>
<td>0.32 ± 0.13</td>
<td>0.32 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Percentage of ligament affected (%)</td>
<td>Control</td>
<td>0.00 ± 0.00</td>
<td>18.63 ± 3.71</td>
<td>13.45 ± 6.17</td>
<td>26.73 ± 9.24</td>
<td>32.39 ± 7.62</td>
<td>39.55 ± 7.72</td>
<td>43.99 ± 9.96</td>
<td>35.82 ± 6.62</td>
<td>31.99 ± 5.22</td>
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<tr>
<td>ESWT-treated</td>
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<td>18.18 ± 3.50</td>
<td>13.04 ± 6.24</td>
<td>24.89 ± 8.66</td>
<td>33.31 ± 10.70</td>
<td>31.85 ± 9.57</td>
<td>31.85 ± 9.57</td>
<td>20.45 ± 6.70*</td>
<td>21.47 ± 6.51‡</td>
<td></td>
</tr>
</tbody>
</table>

*Value significantly (P < 0.05) different from value for control limbs. †Value significantly (P < 0.05) different from value for control limbs.
References


