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# The effect of focused extracorporeal shock wave therapy on collagen matrix and gene expression in normal tendons and ligaments

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## Materials and methods

### *Experimental set-up*

The ponies were sedated with detomidine[1] (Domosedan, 0.01 mg/kg bwt. i.v). and butorphanol tartrate (Torbugesic, 0.1 mg/kg bwt i.v.)[2]. Extracorporeal shock waves were generated electro-hydraulically using an Equitron device[3]. In one weightbearing front limb ESWT was applied to the mid-metacarpal region of the superficial digital flexor tendon (SDFT) over an area of 5 cm and to the origin of the suspensory ligament (SL), which was accessed from the lateral side. Using a probe with the focal point at 5 mm, 600 shocks were applied to both sites, at an energy level of E6, giving an energy flux density (EFD) of 0.14 mJ/mm$^2$. The other front limb was left untreated.

After 6 weeks, ESWT was applied to the same limbs, using an identical protocol, but now at the mid-metacarpal region of the common digital extensor tendon (ET) and at the insertion on the extensor process of the 3rd phalanx of this tendon (EP). To access the EP the probe was placed dorsally just above the coronary band, perpendicular to the skin. Three hours after the last treatment the animals were subjected to euthanasia using an overdose (200 mg/kg bwt i.v.) of Pentobarbital (Euthesate)[4] after sedation with 0.01 mg/kg bwt detomidine.

Samples were obtained from all treated and contralateral untreated control tendons directly after euthanasia. Specimens were taken from the core region of the tendons and the samples were divided for different analyses (histology, biochemistry, gene expression).

### *Collagen content, hydroxylysine and cross links*

After lyophilising for 24 h, approximately 1 mg tendon sample was hydrolysed (110°C, 18–20 h) in 6 mol/l HCl for mass spectrometric (MS) determination of the amino acids hydroxyproline (Hyp) and hydroxylysine (Hyl), and the enzymatic collagen cross links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

The hydrolysed tendon samples were vacuum-dried and dissolved in an internal standard solution (2.4 mmol/l homo-arginine). After centrifugation at 13,000 ***g*** for 20 min, the supernatants were subjected to HPLC/MS.

HPLC/MS analysis was performed using a 4000 Q-TRAP mass-spectrometer[5] equipped with an electrospray ion source in positive-mode. MS was performed at a source temperature of 400ºC, and a spray voltage of 4.5 kV. Amino acids were separated on a Synergi MAX-RP 80A (250 x 3 mm, 4 $\mu$m) column[6] at a flow rate of 200 $\mu$l/min, using a linear gradient from MilliQ water to acetonitrile. Both eluents contained 1.2 mmol/l tridecafluoroheptanoic acid as an ion paring substance. The amino acids were further analysed and identified by mass-spectrometer in MRM mode using the following transitions 429.0/82.0, 413.0/84.0, 189.2/143.7, 131.8/67.8 and 163.2/127.9 (Mz) for HP, LP, homo-arginine, Hyp and Hyl, respectively.

### *Degraded collagen*

The assay for degraded collagen is based on the observation that $\alpha$-chymotrypsin ($\alpha$CT) digests denatured collagen but not the intact triple helix (Bank *et al*. 1997).

The tendon samples were homogenised using a Mikro-Dismembrator[7]. Homogenates were extracted with 1 ml 4 mmol/l guanidine-HCl to remove proteoglycans. After removal of the guanidine-HCl, the denatured collagen was digested overnight at 37ºC using 0.25 mg of $\alpha$CT. After treatment with $\alpha$CT, the digested collagen containing supernatant was separated from the remaining insoluble matrix, containing the intact collagen.

**TABLE o1: Oligonucleotide primer sequences for polymerase chain reaction (PCR) assays**

| Target | Forward primer | Reverse primer |
|---|---|---|
| COL1 | TGCCATCAAAGTCTTCTGCAA | CGCCATACTCGAACTGGAATC |
| COL3 | TACTTCTCGCTCTGCTTCATCC | GAACGGATCCTGAGTCACAGA |
| MMP3 | TTTTGGCCATCTCTTCCTTCA | TGTGGATGCCTCTTGGGTATC |
| MMP13 | CAAGGGATCCAGTCTCTCTATGGT | GGATAAGGAAGGGTCACATTTGTC |
| MMP14 | GGACTGTCCGGAATGAGGATCT | TTGGAATGCTCAAGGCCCA |
| GAPDH | GTCAACGGATTTGGTCGTATTGGG | TGCCATGGGTGGAATCATATTGG |

Collagen contents in both supernatant and pellet were determined by measuring hydroxyproline levels as described before (Creemers *et al*. 1997). The concentration of degraded collagen was calculated as follows: $Hyp_{supernatant}/(Hyp_{supernatant} + Hyp_{pellet})$ x 100% (Bank *et al*. 1997).

*Gene expression*

For gene expression, tissue was snap frozen in liquid nitrogen and stored at -80°C until further analysis. Expression levels of genes COL1 and COL3 and for MMP3, MMP13 and MMP14, as well as for the household gene GAPDH were determined, using real-time RT-PCR.

Homogenised tendon specimens were suspended in 1.8 ml/ 100 mg RNA-Bee[8]. RNA was isolated over RNeasy purification columns[9] and samples were treated with DNAse to degrade any DNA residues. One $\mu$g total RNA was reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit[10], according to the manufacturer's guidelines. Primers were designed to bind to separate exons to avoid co-amplification of genomic DNA and to meet SYBRGreen I[11] requirements, using PrimerExpress 2.0 software[12]. BLASTn searches were conducted to ensure gene specificity of all primers listed in Table o1. Amplification was performed in 20 $\mu$l reactions using qPCR Mastermix Plus for SYBRGreen I according to the manufacturer's guidelines. Real-time RT-PCR (QPCR) was performed using an ABI PRISM 7000 with SDS software version 1.7. Data were normalised to the household gene GAPDH, which was shown to be stably expressed across samples. Relative expression was calculated according to the $2^{-\Delta CT}$ formula (Livak and Schmittgen 2001).

## Manufacturers' addresses

[1]Orion Pharma, Espoo, Finland.
[2]Fort Dodge Animal Health, Ft. Dodge, Iowa, USA.
[3]High Medical Technologies, Lengwil, Switzerland.
[4]Ceva Sante Animale, Naaldwijk, The Netherlands.
[5]Applied Biosystems/MDS Sciex, Concord, Ontario, Canada.
[6]Phenomenex Inc, Torrance, California, USA.
[7]BioTech International Inc, Needville, Texas.
[8]TEL-TEST, Friendswood, Texas, USA.
[9]Qiagen, Hilden, Germany.
[10]MBI Fermentas, St. Leon-Rot, Germany.
[11]Eurogentec Nederland B.V., Maastricht, The Netherlands.
[12]Applied Biosystems, Foster City, California, USA.

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