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Effect of Extracorporeal Shock Wave Therapy on Cultured Tenocytes

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ABSTRACT

Background: Little has been reported about the biologic effect of shock waves on human normal or pathologic tendon tissue. We hypothesized that inflammatory cytokine and MMP production would be down-regulated by shock wave stimulation. **Materials and Methods:** Diseased Achilles tendon tissue and healthy flexor hallucis longus tissue were used. Shock wave treatment was applied to cultured cells at 0.17 mJ/mm² energy 250, 500, 1000, and 2000 times. **Results:** A dose-dependent decrease in cell viability was noted in cells receiving 1000 and 2000 shocks (86.0 ± 5.6%, *p* = 0.01 and 72.4 ± 8.9%, *p* = 0.001) as compared with the normal control. Cell count in the 500-shock group increased by 23.4% as compared with the control (*p* = 0.05). The concentration of MMP 1, 2, and 13 was higher in diseased tenocytes as compared with normal cells (*p* = 0.04, all comparisons). IL-6 levels were higher in the diseased tenocytes as compared with normal tenocytes (44.10 ± 16.72 versus 0.21 ± 0.55 ng/ml, (*p* < 0.05). IL-1 levels in normal cells increased (2.24 ± 5.02 ng/ml to 9.31 ± 6.85 ng/ml) after shock wave treatment (*p* = 0.04). In diseased tenocytes, levels of MMP-1 (1.12 ± 0.23 to 0.75 ± 0.24 ng/ml; *p* = 0.04) and MMP-13 (1.43 ± 0.11 to 0.80 ± 0.15 ng/ml; *p* = 0.04) were significantly decreased after shock wave treatment. The IL-6 level in diseased tenocytes was decreased (44.10 ± 16.72 to 14.66 ±

9.49 ng/ml) after shock wave treatment (*p* = 0.04). **Conclusion:** Higher levels of MMPs and ILs were found in human tendinopathy-affected tenocytes as compared with normal cells. ESWT decreased the expression of several MMPs and ILs. **Clinical Relevance:** This mechanism may play an important role in shock wave treatment of tendinopathy clinically.

Key Words: Extracorporeal Shock Wave; Tendon; Cells; ESWT

INTRODUCTION

Extracorporeal musculoskeletal shock wave treatment (ESWT) is now used worldwide as a nonsurgical treatment method for plantar fasciitis, tennis elbow, Achilles tendinopathy, and other enthesopathies.^{6-10,13} With good clinical success rates, indications of this treatment have expanded to other tendinopathies.⁶ There are several theories for explaining the mechanism of shock wave effect in various cells and tissues.^{4,12} The mechanism of action possibly includes direct stimulation of the healing process, production of microcavitations, neovascularization, destruction of calcium, and neural effects. On the molecular level, these mechanisms may involve alterations in the permeability of cell membranes, which may prevent development of action potentials that transmit painful stimuli. There may be direct suppressive effects on nociceptors, and hyperstimulation of the sensory nerves may block pain perception through the pain gate control mechanism.

Recent research suggests that tendinopathy is mediated by various biochemical pathways in which matrix metalloproteinases (MMPs) and various cytokines play a key role. Studies suggest that MMPs and cytokines such as interleukins (ILs) are excreted from diseased tenocytes and then break down collagen fibers, causing tendon fiber damage.^{1,18} Resolution of tendinopathy with shock wave treatment might be reflected in decreased numbers of these substances. However, little has been reported about the biologic effect of shock waves on human normal or pathologic tendon tissue.

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We hypothesized that inflammatory cytokine and MMP production would be down-regulated by shock wave stimulation. Our objective was to evaluate inflammatory MMP and cytokine production in human diseased Achilles tendon cells and healthy flexor hallucis longus (FHL) tendon cells before and after shock wave stimulation of healthy and diseased human tenocytes in culture.

MATERIALS AND METHODS

Cell sources

Our laboratory procedures were based on a protocol developed in our laboratory to isolate and grow healthy and diseased tendon cells, extract mRNA, and analyze the effects of growth factors and modalities in gene expression and protein production. Informed consent was obtained from all patients, and the protocol was approved by our institutional review board.

Diseased Achilles tendon tissue from tendon transfer reconstructive surgery for Achilles tendinopathy was used for culture of diseased tenocytes. A remnant of the FHL from the same procedure was used as a source of normal tenocytes. Diseased and healthy tendon cells were obtained from each patient as a control.

Primary culture and tenocyte phenotyping

Tendon tissue was transferred from the operating room and kept in cold Dulbecco's modified Eagle's Medium/F12 (DMEM/F12, GIBCO BRL, Grand Island, NY) that did not contain any fetal bovine serum (FBS). Tissue was washed three times with 10 ml phosphate buffered saline (PBS, GIBCO BRL, Grand Island, NY) in a culture dish. Tissue was then chopped into 1-mm cubes and washed twice with PBS solution. Tissue was put into 5 ml of 1X trypsin solution (Sigma, St. Louis, MO) and incubated at 37 degrees for 5 minutes. Trypsin solution was washed away with 10 ml of PBS solution, and 10 ml of non-FBS DMEM/F12 was added to the culture dish. Tissue was deposited in six-well flasks, incubated for 15 minutes at 37 degrees, and put into 700 μ l of DMEM/F12 containing 10% FBS. After 5 to 7 days of tissue culture 37 degrees and 5% CO₂, tenocytes had migrated and were positioned near the edge of the tissue (Figure 1). The tissue was kept in the flask for 10 to 14 days depending on the rate of cell growth. Medium was changed every 3 days. The tissue was removed after the tenocytes had populated the entire cell culture dish. The cells were fed with 5 ml of 10% FBS DMEM/F12.

Total RNA was prepared from cultured tenocytes using an RNEasy kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized from 1 μ g total RNA by reverse transcription polymerase chain reaction (PCR) at 50 degrees for 30 minutes, and specific DNA fragments were amplified with a one-step RNA PCR kit (Qiagen, Hilden, Germany) using the following oligonucleotide primers: GAPDH: 5'-GGT GAA GGT CGG AGT CAA CG-

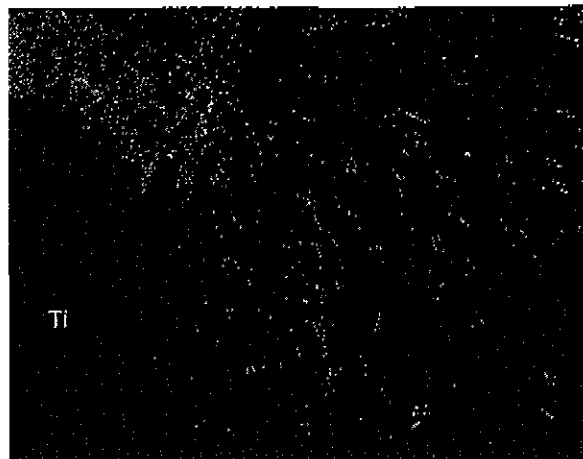


Fig. 1: Microscopic findings of cultured tenocytes (day 7, $\times 200$). Primary tenocytes (Te, black arrow) are migrated from the tendon tissue (Ti) and are well proliferated in the culture flask. The morphology of the culture cells is spindle-like fibroblast.

3' and 5'-CAA AGT TGT CAT GGA TGA CC -3'; scleraxis: 5'-GTGAACACGGCCTTCACGG- 3' and 5'-CTGCGAATCGCTGTCTTTC-3'; and collagen type I: 5'-TCCGACCTCTCTCCTCTGAA- 3' and 5'-GAGTGGGGT-TATGGAGGGAT-3'. PCR amplification was performed at 25 cycles for GAPDH and collagen type I and at 30 cycles for scleraxis for denaturation at 94 degrees for 30 seconds, annealing at 55 degrees for 30 seconds, and extension at 72 degrees for 1 minute. PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide (Sigma, St. Louis, MO) staining.

Shock wave treatment of cultured tenocytes

Tenocytes derived from the Achilles and FHL tendons were treated by shock wave using a modification of a previously described method¹⁹ with a Sonocur[®] system (Siemens Medical Solutions, Iselin, NJ). The shock wave produced by the Sonocur[®] system is pencil shaped with a rounded end, producing an energy focus of 1/4-inch thickness and a length of 2-1/3 inches. Energy is transferred when the sample is placed in the z-axis of the energy wave.

Trypsinized cells in culture were placed into a 2.5-ml microtube. The center of a foam block was identified and a hole was made that would allow the microtube to lie horizontally in the block. To determine the location of the shock wave, super low-speed film/70-350 PSI (Foot piece, Sensor Production, Inc., Madison, NJ) cut to the size of the foam block was placed on the block. The block was put on a positioning grid, and its position was marked. The shock wave probe was placed on the center of the block. After 20 shocks were delivered, a red dot indicating the location of the shock wave was observed on the photo film. The box was moved on the grid without moving the shock wave probe, and the shock wave was applied again to confirm the dot appeared at the center. The centered position of the block

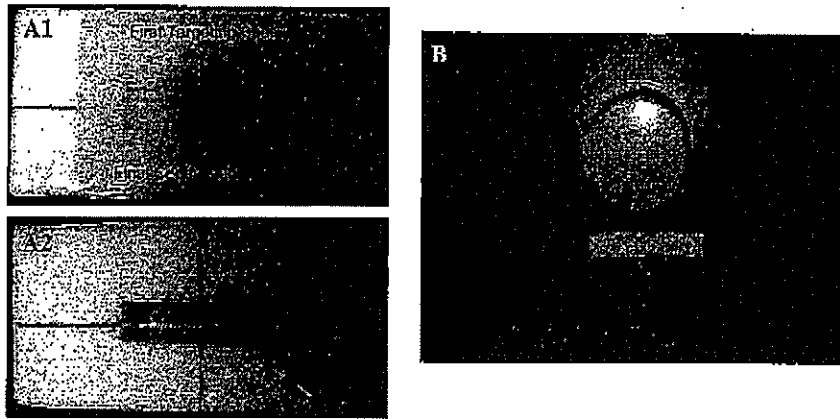


Fig. 2: The shock wave application on test tube and targeting procedure. A1, Using a dot focusing method, the first targeting spot is recognized and moved to the central area on the table. A2, Then the test tube containing tenocytes is placed on the center of the test box. Right upper arrow is a landmark of the testing box. B, Shock wave is applied on the right center area of the test tube.

was marked on the grid. Then the microtube with cells and medium was placed in the foam block, and shock wave was administered (Figure 2).

Cell toxicity assay

Shock wave treatment of 0.17 mJ/mm² energy was applied 250, 500, 1000, and 2000 times on the microtube containing 1 × 10⁶ cells/ml. After shock wave treatment, cell viability in each microtube was measured using 0.4% Trypan blue exclusion assay method.²² Cells with an intact membrane exclude the dye, whereas cells without an intact membrane take up the coloring agents. A 100-μl cell suspension from each test tube was diluted with 100 μl 0.4% Trypan blue solution. A 10-μl dilution was loaded into the counting chamber of a hemocytometer, and the numbers of unstained cells were counted under the microscope. In the following study, equal amounts of viable cells were used in each test.

Cell proliferation assay for tenocyte proliferation

Tenocytes with each shock wave treatment were seeded in a 96-well culture flask at 2 × 10³ cells/well density with 200 μl medium. Viabilities were determined by methylthiazolotetrazolium (MTT, Sigma, St. Louis, MO) assay. The original culture medium was removed with a micropipette, and 150 μl of fresh medium was added per well. Then 100 μl of fresh MTT solution (0.5mg/ml) was added per well, and the culture plates were incubated at 37 degrees for 4 hours. The upper medium was then carefully removed, and the intracellular formazan was solubilized by adding 200 μl of dimethyl sulphoxide (Sigma, St. Louis, MO) into each well. The absorbance of produced formazan was measured at 570 nm three times using a microplate reader (SPECTRAMax 190, Molecular Devices, Sunnyvale, CA).

Enzyme-Linked Immunosorbent Assay (ELISA) for MMP and IL

After shock wave treatment, tenocytes were cultured in a 6-well culture flask for 72 hours. Then medium was

put in 1% volume protease inhibitor solution (Sigma, St. Louis, MO), and stored in a deep freezer at -80 degrees until the experiment. MMP-1, 2, 9, and 13 and IL-1, 6, and 13 in conditioned medium were analyzed using ELISA (Amersham Bioscience, Buckinghamshire, UK) as described by the manufacturer.

Statistical analysis

All data were expressed as mean ± standard deviation. Comparison between the shock wave groups and the control group were performed using a Wilcoxon signed rank test and a paired two-tailed t-test with statistical significance set at p ≤ 0.05.

RESULTS

Primary cell culture

All cells proved positive by PCR gel for GADPH, scleraxis, and collagen 1, markers specific to human fibroblasts, confirming the cells originated from tendon tissue (Figure 3).

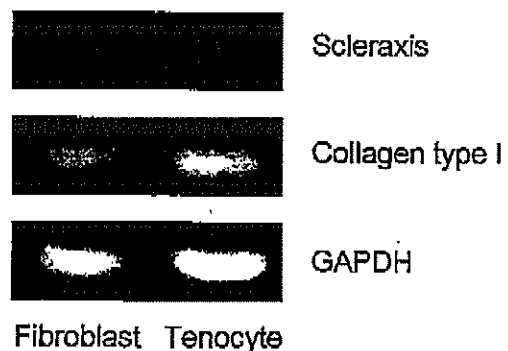


Fig. 3: Results of RT-PCR of cultured tenocytes. Scleraxis and collagen type I, the phenotype marker genes of tenocytes, are well expressed by RT-PCR as compared with skin fibroblasts. These results confirm that the cells cultured by our methods are tenocytes, not other kinds of fibroblasts.

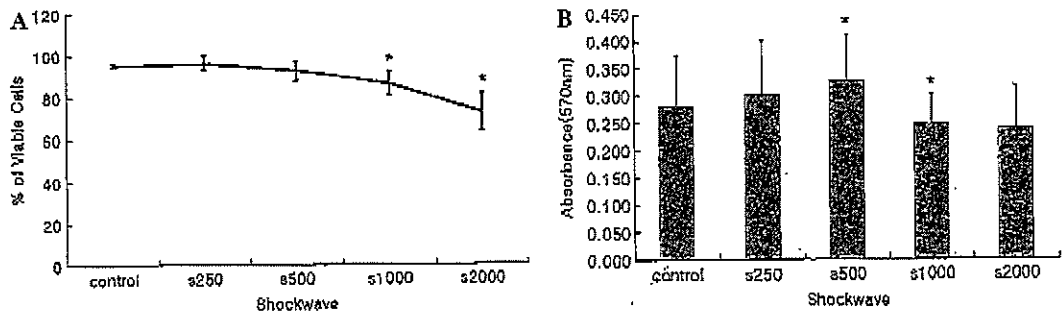


Fig. 4: Cell toxicity and cell proliferation graphs for each shock wave treatment. A, Cell toxicity is significantly increased above 1000 shocks of 0.17 mmJ/mm². B, Cell proliferation increased until 500 shocks of 0.17 mmJ/mm² and significantly decreased above the 1000 shocks of 0.17 mmJ/mm². (*, *p* < 0.05).

Cell toxicity assay

Cell death was not prominent in cells treated with 250 and 500 shocks (95.1% ± 1.0%, *p* = 0.64, and 96.0 ± 3.5%, *p* = 0.20) compared with the normal control. A significant dose-dependent decrease in cell viability was noted in cells receiving 1000 and 2000 shocks (86.0 ± 5.6%, *p* = 0.01 and 72.4 ± 8.9%, *p* = 0.001) as compared with the normal control. The cell viability of the 2000 shock group decreased by 23% compared to the 500 shock group (*p* = 0.0016) (Figure 4A).

Cell proliferation assay

Compared to the control group, cell count in the 250-shock cells increased by 16.5%, which was not significant (*p* = 0.35). Cell count in the 500-shock group increased by 23.4% as compared with the control (*p* = 0.05). The 1000-shock and 2000-shock group showed a decrease in cell count by 7.3% and 5.1% each (*p* = 0.05, *p* = 0.04, respectively) compared with the normal control (Figure 4B).

MMP and IL excretion concentration from normal and diseased tenocytes

The concentration of MMP 1, 2, and 13 were increased in the diseased tenocytes as compared with normal cells (*p* = 0.04, *p* = 0.04, *p* = 0.04, respectively) (Figure 5A). IL-6 levels were significantly increased in the diseased tenocytes

as compared with normal tenocytes (44.10 ± 16.72 versus 0.21 ± 0.55 ng/ml, respectively; (*p* < 0.05) (Figure 5B).

MMP and IL change after shock wave treatment

IL-1 levels in normal cells were increased (2.24 ± 5.02 ng/ml to 9.31 ± 6.85 ng/ml) after shock wave treatment (*p* = 0.04). MMPs and IL-6 and 13, however, showed no change (*p* = 1.0 and *p* = 0.07, respectively) (Figure 6).

In diseased tenocytes, levels of MMP-1 (1.12 ± 0.23 ng/ml to 0.75 ± 0.24 ng/ml; *p* = 0.04) and MMP-13 (1.43 ± 0.11 ng/ml to 0.80 ± 0.15; *p* = 0.04) were significantly decreased after shock wave treatment. The IL-6 level in diseased tenocytes was decreased (44.10 ± 16.72 ng/ml to 14.66 ± 9.49 ng/ml) after shock wave treatment (*p* = 0.04) (Figure 7).

DISCUSSION

Shock wave stimulation has been shown to be an effective treatment for tendinopathy clinically,^{6,13} but the mechanism of the effect has not been established. In this study of matched healthy and diseased human tendon cells, we found that shock wave treatment is safe for tendon tissue. In healthy tissue, there was change only in IL-1 expression after shock wave treatment. This finding may be an early indication of

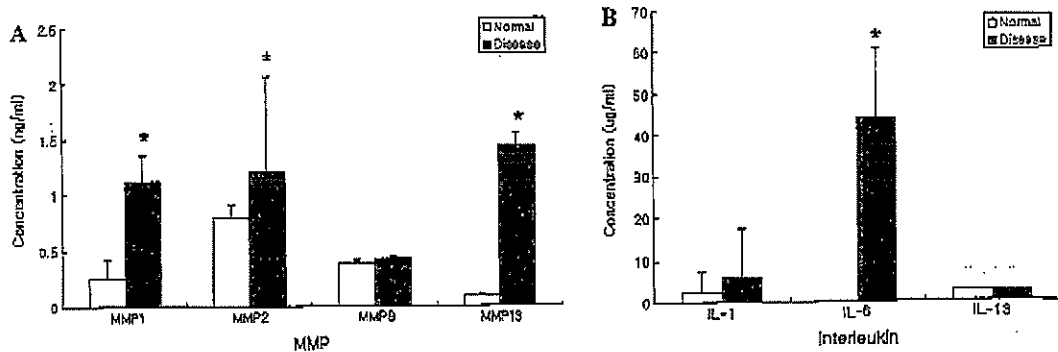


Fig. 5: Excretion concentration of MMPs and interleukins from normal and disease tenocytes. A, The concentration of MMP 1, 2, and 13 were significantly increased in the diseased tenocytes as compared with normal cells. B, The concentration of IL-6 was significantly increased in the diseased tenocytes. (*, *p* < 0.05).

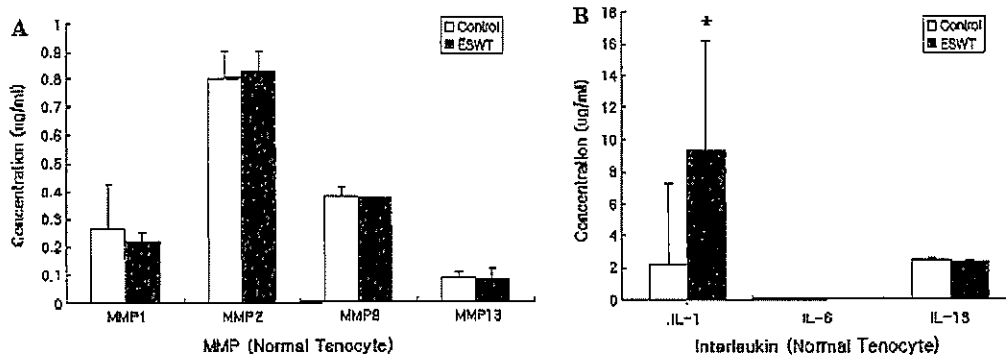


Fig. 6: Excretion concentration change of MMPs and interleukins in normal tenocytes after shock wave treatment. A, The concentration of MMPs did not change after shockwave treatment. B, The concentration of IL-1 significantly increased after treatment (*, $p < 0.05$).

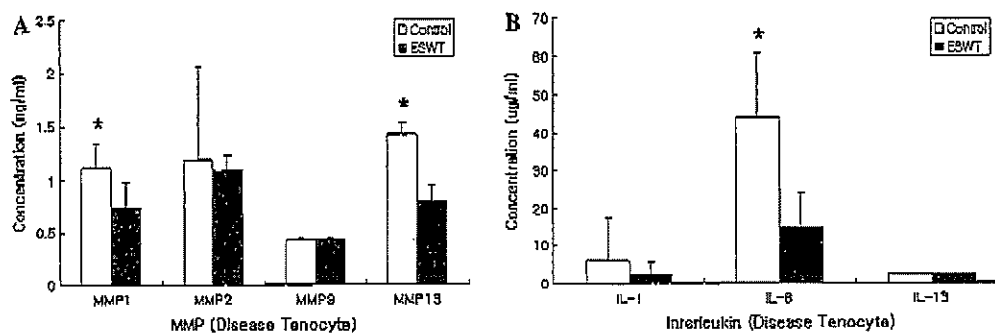


Fig. 7: Excretion concentration change of MMPs and interleukins in diseased tenocytes after shock wave treatment. The concentration of MMP-1 and MMP-13 significantly decreased after treatment. A, The concentration of MMP-2 and MMP-9 did not change significantly. B, The concentration of IL-6 significantly decreased after treatment (*, $p < 0.05$).

inflammation, but we did not find general increases in MMPs or ILs in normal cells after shock wave treatment. Treatment with 500 shocks proved to be within the safe range in the energy intensity of $0.17\text{mJ}/\text{mm}^2$ (Figure 4A).^{19,20} Further, we found that pathologic expression of MMPs 1 and 13 and IL-6 was decreased in diseased cells with shock wave treatment.

Most studies on biological effects of ESWT have focused on growth factors and cell growth, without investigating the etiologic proteins of tendinopathy. Wang et al. found Ras activation and subsequent osteogenesis in bone marrow stromal cells after shock wave therapy. Similar studies using mesenchymal cells from the umbilical cord and found oxygen radical-mediated osteogenesis.²¹ ESWT is also known to increase the production of TGF- β 1. Chen et al. reported tendon healing after shock wave treatment in rat tendinopathy tissue and found an increase in TGF- β 1 and IGF-I expression. They have proposed that these kinds of growth factors may play a role in tendinopathy healing.⁴ Chao et al. reported collagen increase and accelerated cell growth after shock wave treatment in normal tenocytes, especially noting the increase in TGF- β 1 and proliferating cell nuclear antigen.³

In the current study, a significant decrease in certain ILs and MMPs were noted after shock wave treatment, possibly

shedding light on another biological effect of ESWT. MMPs and ILs are markers of tendinopathy and may have a role in the pathomechanics of tendinopathy.^{2,15,17,18} These preliminary findings suggest a mechanism of action of shock wave treatment and may partially indicate the reason shock wave treatment works clinically. The findings call into question other theories of shock wave mechanism of action based on adverse effect on cells.

MMPs play a crucial role in degrading collagen matrix,^{5,11,16} a major constituent of tendon. While important in regulating the regeneration of tendon, its level is increased in tendinopathy. MMP production is thought to be mediated by cytokines such as interleukins.^{14,17,18} Such cell signal transduction, as well as the effects of mechanical stimulation such as ESWT, warrant further investigation.

The study is limited in that we used cells and not whole tendon tissue. The optimal setting for testing would be to use live tissue, treat with shock wave stimulation, culture cells, and analyze the sample while administering stretch and stimulation, changing the environment and stresses, and adjusting the shock wave dose. That study would be valuable in the future. The cells were not in a matrix as in whole tissue and may not behave exactly the same as cells in whole tissue. Although we tested human tendon cells in culture rather than

whole tissue, our model has the advantage of using human matched tendon samples with direct applicability to clinical injury.

CONCLUSION

The current study showed higher levels of MMPs and ILs in human tendinopathy-affected tenocytes. ESWT decreased the expression of several MMPs and ILs. This mechanism may play a role in shock wave treatment of tendinopathy clinically.

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