EXPERIMENTAL STUDY

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The effects of extracorporeal shock waves on carrageenan-induced Achilles tendinitis in rats: a biomechanical and histological analysis

Bekir Murat ÇINAR[1], Esra ÇİRCİ[2], Cenk BALÇIK[3], Gülnur GÜVEN[4], Sercan AKPINAR[1], Alihan DERİNCEK[1]

[1]*Department of Orthopaedics and Traumatology, Faculty of Medicine, Başkent University, Ankara, Turkey;*
[2]*Department of Orthopaedics and Traumatology, İstanbul Training and Research Hospital, İstanbul, Turkey;*
[3]*Department of Mechanical Engineering, Faculty of Engineering, Başkent University, Ankara, Turkey;*
[4]*Department of Pathology, Faculty of Medicine, Başkent University, Ankara, Turkey*

Objective: The aim of this study was to investigate the influence of low-dose extracorporeal shock waves (ESW) on the healing potential of Achilles tendinitis in the rat.

Methods: The 36 adult Sprague-Dawley rats used in this study were randomly divided into four groups. Group A (n=10) were injected with carrageenan, Group B (n=10) were injected with carrageenan and received ESW, Group C (n=10) received ESW only, and Group D (n=6) was a sham group. Rats were injected with 10 microliters of 3% carrageenan or a saline solution eight times during a one-week period with a subcutaneous needle. One week following the final injection, ESW was applied at a rate of 500 impulses in 5 minutes at 2 bars (comparative to 0.09 mJ/mm^2) to rats in Groups B and C. Rats were sacrificed three weeks later. Tensile strength, inflammation, and vascularity and collagen density were measured.

Results: Failure of the tendon ultimate loads was significantly lower in the study groups than in the control group ($p<0.05$). Collagen fiber density was higher in the control group than in the other groups ($p=0.59$). No other histological differences were found.

Conclusion: Low-dose ESW has a negative effect on tendon tensile strength in this animal model.

Key words: Achilles tendinitis; biomechanical analysis; extracorporeal shock wave; histological analysis; rat.

Tendinopathies are common soft tissue problems associated with significant morbidity. They are linked to overuse, local trauma, aging, chemical agents, and vascular inadequacy; all of which have been implicated in their development.[1-3] However, their etiology and natural history are not exactly understood[4,5] and therapies can often be of long duration, resulting in continuing morbidity.

The pathology and pathophysiology of tendinopathy in humans is currently poorly understood, making validation using animal models difficult. However, histopathological changes and mechanical weakening of the tendon are associated with tendinopathy in humans.[6] In the literature, chemical agents, such as collagenase, cytokine and prostaglandin E1 have been applied to tendon tissue and the resulting histological

Correspondence: Esra Çirci, MD. İstanbul Eğitim ve Araştırma Hastanesi, Ortopedi ve Travmatoloji Kliniği, Org. Abdurrahman Nafiz Gürman Cad., 34098, Fatih, İstanbul, Turkey.
Tel: +90 212 - 459 60 00 e-mail: esracirci@hotmail.com

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changes have been observed. These include an increase in the number of inflammatory cells, increased capillary growth and changes in the collagen organization[6-9] Carrageenan has been used for many years in order to study the effects of inflammation on tendons.[10,11] Tillander et al. revealed that repeated injection of carrageenan resulted in a degenerative matrix, macrophage infiltration and bone and fibrocartilaginous metaplasia in the rat tendon.[10]

More recently, extracorporeal shock wave treatment (ESWT) has also been used to treat bone and soft tissue disorders, such as nonunion, calcific tendinitis of the shoulder, epicondylitis, trochanteric bursitis, patella tendinitis, Achilles tendinitis, and plantar fasciitis.[12,13] Despite its success in clinical application, the exact mechanism of shock wave therapy remains unknown. It is assumed that the application of the shock waves induces long-lasting analgesia and stimulates healing potential in tendons through microtraumatic lesions of avascular or hypovascular tissues. This is thought to encourage revascularization and release local growth factors, contributing to normal tissue healing.[7,14] However, there have been some reports on the dose-dependent alteration of tissue or damage to the tendon after shock wave therapy.[15,16]

Pathophysiological effects of ESW have been explored in the normal tendon tissue in various experimental studies.[14,17-19] However, only a few studies have examined its effect on tendinitis. Despite excellent clinical and basic science observations related to tendinitis, very little is known about the healing response.[20] We used histological and biomechanical parameters to assess the contribution of shock waves to tendon healing.[1,17,21]

The aim of this study was to investigate the effect of ESW on the healing potential of carrageenan-induced Achilles tendinitis using histological and mechanical tendon properties in a rat model.

Materials and methods

This study was approved by the University Ethical Committee for Experimental Research on Animals and supported by the University Research Fund.

Thirty-six rats with a mean age of 12 months and mean weight of 294±38 grams were included in the study. All rats were divided randomly into four groups. Group A (n=10) were injected with carrageenan, Group B (n=10) were injected with carrageenan and received ESW, Group C (n=10) received ESW only, and Group D (n=6) was a sham group.

Rats were acclimatized to caged laboratory conditions and were allowed to feed with a standard diet and water ad libitum. The room temperature and humidity were maintained at 20 to 24°C and at 50 to 60% respectively. The light cycle was fixed at 12 hours.

Rats in Groups A and B were injected with 10 microliter carrageenan (3%) with a 26 gauge needle eight times for one week. Rats in Groups C and D received sterile saline injections using the same protocol. Before injection, all rats were sedated with ketamine. Rats were injected using the aseptic technique percutaneously near the osteotendinous junction in both Achilles tendons. Following injection, the rats were free to move within their cages.

Extracorporeal shock waves were applied by a Swiss Dolorclast® (Electro Medical Systems, Nyon, Switzerland) one week following the final injection to rats in Groups B and C. Rats received 500 impulses at 2 bars (comparative to 0.09 mJ/mm^2) for 5 minutes. Rats were re-anesthetized and placed in the prone position. The shock wave tube was focused on the Achilles tendon near the insertion site to the heel bone and a single shock wave application was given. Three weeks later, all rats were sacrificed with an intraperitoneal injection of 200 mg/kg thiopental. The sham group (Group D) did not receive any shock waves or carrageenan injection. Each rat was analyzed either biomechanically or histologically, with randomized selection into one of these groups.

Samples were dissected and the muscle detached at the musculotendinous junction, leaving the tendinous part and calcaneus intact. Specimens were stored at -20°C until testing. During biomechanical assessments, the tensile strength of the Achilles tendon was measured using electromagnetic testing equipment (Instron Tensometer, Model 8874, Automated Materials Testing System; Instron Corp., Canton, MA, USA). Room temperature and humidity were stabilized at 20°C and at 40%, respectively. The fresh-frozen specimens were thawed at room temperature on the day of biomechanical analysis and kept moist with normal saline throughout the test. The calcaneus was placed on a model stone (Amberok, dental model stone) with its long axis in the horizontal plane. The proximal end of the Achilles tendon was fixed between two pieces of sandpaper and clamped vertically in a custom made cryoclamp. The system was loaded to 250 N with a displacement rate of 5 mm/min. Failure loads were determined for each specimen.

Tissue samples were fixed in 10% neutral buffered formalin overnight then dehydrated with alcohol. The fixed tissue was processed, embedded in paraffin, and sectioned at 3 micrometers. Finally, tissue sections

were stained with hematoxylin and eosin according to standard protocols for evaluation of inflammation and vascularity. Masson's trichrome staining was used to investigate collagen density.

The presence or absence of chondroid or osseous metaplasia was recorded. Inflammation, vascularity, fibroblast density, and adhesion and epitenon thickness were graded on a four-point grading system on the following scale; (0) no tissue response, (1) mild tissue response, (2) moderate tissue response, and (3) severe tissue response. The percentage of collagen density was also determined.

Two slides were prepared for each staining technique. The area of specimen showing the most advanced pathological changes was selected and the worst possible results for each slide were used in this study. Slides were examined under a light microscope (Olympus B×50) by the same pathologist who was blinded to the samples.

Data were evaluated using the Statistical Package for Social Sciences (SPSS) for Windows 13.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics including frequencies, mean, standard deviation and minimum and maximum values were calculated. The Kruskal-Wallis test was used to compare three or more groups. The Mann-Whitney U test was applied to two group differences in the biomechanical analysis. The chi-squared test was applied to obtain the differences in the histological analysis between the groups. Results of $p<0.05$ were considered statistically significant.

Results

There were no macroscopic changes of the tendons in neither the control nor the study groups.

All specimens were analyzed successfully during biomechanical testing. The expected load deformation curves were determined in the tendons. The tendon ruptured in the mid-zone in both the study and control groups (Fig. 1). Biomechanical testing results of Groups A, B and C were inferior to the control group. Failure of tendon ultimate load was significantly lower in the shock wave applied group (Group C) than the control group (Group D) ($p=0.03$). Ultimate failure load was significantly lower in the carrageenan injected group (Group A) and carrageenan injected and ESW applied group (Group B) than the control group (Group D) ($p=0.03$ and $p=0.01$, respectively). However, significant differences were not found between the study groups themselves. Biomechanical testing results are summarized in Table 1.

Fig. 1. Biomechanic analysis of the Achilles tendon. The tendon ruptured at mid-substance. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Histological evaluations are shown in Table 2. When examined under microscope, carrageenan solution covering the whole tendon tissue was observed (Fig. 2). Characteristic mononuclear cell infiltration was found in all specimens of the carrageenan injected groups (Group A and B), indicating an inflammatory response. Figure 3 shows the inflammatory response of the Achilles tendon in Group B. No signs of inflammation were seen in Groups C and D. No significant differences were found between Groups A and B ($p=0.06$).

While collagen fiber density was higher in the control than in other groups, the differences were not significant ($p=0.59$). Figure 4 shows the greater organization of collagen (90% in the tendon) in the control group.

Fibroblast intensity and epitenon thickness were significantly different between the groups ($p<0.05$ and

Table 1. Ultimate load±standard deviations (in Newtons) to failure in biomechanical analysis.

	Study groups			Control group
	Carrageenan-received group (Group A; n=10)	Carrageenan and ESWL-received group (Group B; n=10)	ESWL-received group (Group C; n=10)	Sham group (Group D; n=6)
Load to failure	57.8±12.6	52.4±9.6	54.6±11.3	69.4±8.9

Table 2. Histological evaluation of the groups.

Histological parameter	Carrageenan-received group (Group A; n=10)	Carrageenan and ESWL-received group (Group B; n=10)	ESWL-received group (Group C; n=10)	Sham group (Group D; n=6)
Chronic inflammation grade*				
0	-	-	10	6
1	3	-	-	-
2	6	5	-	-
3	1	5	-	-
Vascularity grade*				
0	2	1	3	5
1	2	3	2	1
2	3	2	2	-
3	3	4	3	-
Fibroblast intensity grade*				
0	1	-	10	4
1	4	4	-	2
2	3	5	-	-
3	2	1	-	-
Adhesion grade*				
0	7	6	10	5
1	2	2	-	1
2	0	1	-	-
3	1	1	-	-
Epitenon thickness grade*				
0	2	4	7	6
1	4	6	3	-
2	4	-	-	-
Collagen fiber orientation†	50±20	62±28	57±28	62±27
Chondroid metaplasia‡	5 (50%)	3 (33%)	-	1 (16%)
Osseous metaplasia‡	4 (25%)	3 (33%)	-	-

*Values are given in numbers. †Values are given in mean percentage±standard deviation. ‡Values are given in numbers (percentage).

p<0.02, respectively). These differences were found between the carrageenan-received and the other groups (p<0.05 and p<0.05, respectively). However, there was no significant difference between Groups A and B (p=0.8 and p=0.18, respectively).

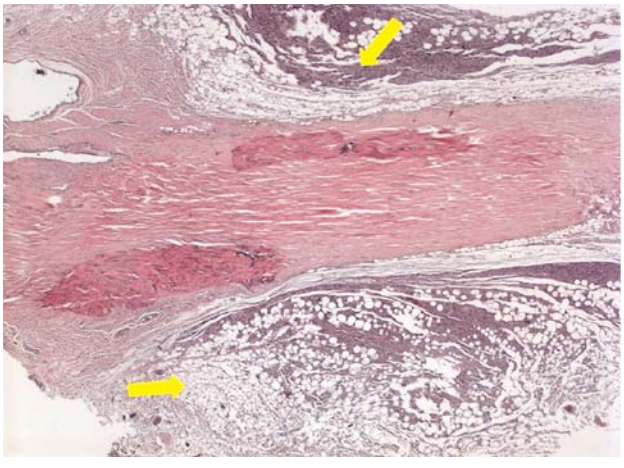

Fig. 2. Photomicrographs showing a longitudinal section of a rat tendon from the carrageenan-induced group with carrageenan solutions covered the whole tendon (arrows) (H&E ×40). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

There was no significant difference between groups in terms of the number of capillaries and adhesion (p=0.5 and p=0.75, respectively). Eight of the 20 specimens (40%) had chondroid metaplasia and seven (35%) osseous metaplasia in Groups A and B. There was no significant difference between the groups (p=0.16 and p=0.17).

Discussion

Achilles tendinitis is a common clinical problem characterized by pain on active movement and focal tendon tenderness. Pathological assessment of the Achilles tendon may reveal inflammatory peritendinitis, regressive tendinosis or, more frequently, mixed features of both. Histological appearance of the peritendinitis may include an increase in inflammatory cells, edema, and both thrombosed and dilated capillary veins. Different histopathological features are observed in accordance with the characteristics of the degenerative process: hyaline, mucoid, fibrinoid or fatty degeneration, areas of calcification and areas of cartilage or bone metaplasia.[1,5]

In the literature, extrinsic and intrinsic factors are used to explain the experimental tendinopathy model.[6,8] Mechanical overload is the most common

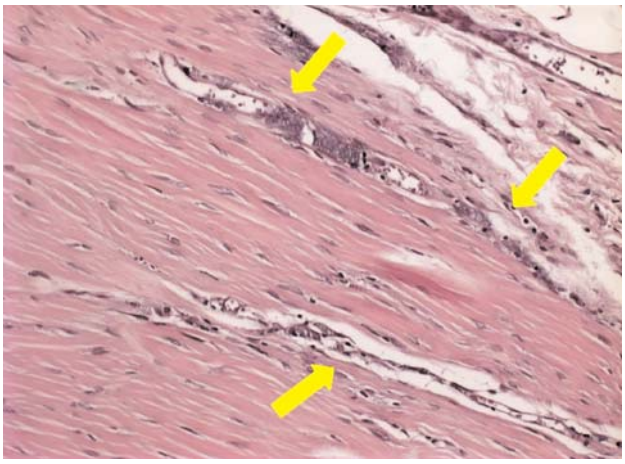

Fig. 3. Photomicrograph of a longitudinal section of a rat tendon from the carrageenan and shock wave-applied group. Arrows indicate streaks of inflammatory cells and an increase in the vascularity between the bundles of the tendinous collagen (H&E ×100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

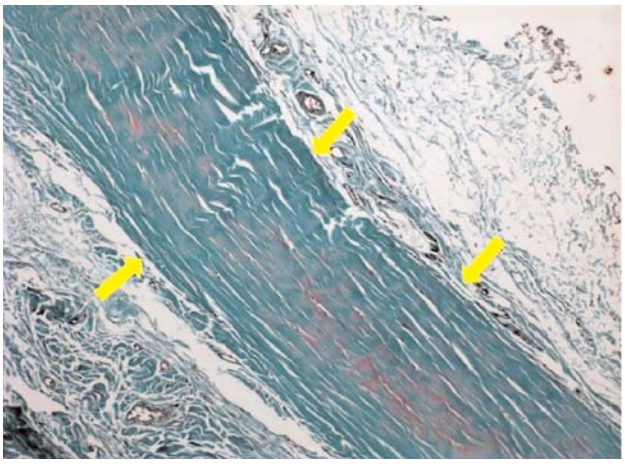

Fig. 4. Photomicrograph of a longitudinal section of a rat tendon from the control group. There is an apparent organization of the collagen colored green in the tendon tissue (arrows) (Masson's trichrome ×100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

extrinsic risk factor for the development of tendinitis in animal models[22] while collagenase, PGE1, PGE2, corticosteroids and cytokines are determined as the intrinsic factors. Carrageenan, a vegetable polysaccharide, is devoid of endogenous proteolytic activity in healthy tendons. Carrageenan has been used for many years in order to study the effects of inflammation on joints and tendons.[23] Carrageenan-induced tendinitis can be confirmed on histological examination by the presence of neutrophilic, lymphocytic and macrophage infiltration associated with collagen disruption, and in some animals, even fibrocartilaginous and bone metaplasia. Thus, while carrageenan-induced inflammation can be considered a suitable model for inflammation, it may not exactly mimic Achilles tendinopathy in humans.

Extracorporeal shock wave directs a shock wave directly to the affected area and uses accurate, high-energy beams of ultrasound waves. The energy flux density is a measure of the energy per square released by the sonic pulse at a specific point. It is expressed in milliJoules (mJ) per square millimeter. Rompe et al. proposed the categorization of ESW into low-energy (<0.08 mJ/mm^2), medium-energy (0.08 to 0.28 mJ/mm^2) and high-energy (>0.6 mJ/mm^2) treatment.[15] Studies have shown that ESWs may provide analgesic, resorptive and osteoinductive reactions with almost no side effects.[18] However, potential complications have been revealed. There have been some reports of dose-dependent alteration of tissue or damage to the tendon after shock wave therapy. Orhan et al. observed that no histological changes were evident when impulses of 1000 and 1500 with a shock wave of 0.15 mJ/mm^2 were used in the rat tendon, whereas 2000 impulses of 0.20 mJ/mm^2 was associated with detrimental tissue effects such as collagen fiber disorganization.[16] In another experimental study using primary cultures of rat tenocytes, low- to medium-energy level shock waves with low impulses (0.36 mJ/mm^2 with 50 and 100 impulses) had positive stimulatory effects, but high-energy level shock waves with high impulses (0.68 mJ/mm^2 with 250 and 500 impulses) had significant inhibitory effects.[19] There is still debate over the appropriate shock wave dosage and number of sessions required for therapeutic effect. To avoid potential side effects, in this study we used low-energy shock waves.

Tendon healing is a complex process involving an inflammatory response, angiogenesis, and matrix remodeling. Extracorporeal shock wave therapy has been popularized as a therapy for tendinitis, but little is known about its effects on tendon healing. Chao et al.[19] revealed that low-energy shock waves can stimulate tenocyte proliferation and collagen synthesis. The associated tenocyte proliferation is mediated by the upregulation of PCNA. The mechanisms by which collagen synthesis is stimulated both at protein and mRNA level is likely mediated by endogenous NO release and upregulation of TGF-β1. Chen et al.[24] showed that low-dose shock waves at 200 impulses resolved inflammatory cell infiltration and promoted tendon regeneration. They also concluded that 500 impulses and 1000 impulses are not beneficial for tendon repair. In our study, although low-energy was

applied, a greater number of impulses were used. However, a recent clinical study demonstrated that low-dose shock waves did not improve tendon healing.[25] Our findings suggested that 0.09 mJ/mm^2 and 500 impulses of shock wave application did not change the tendon inflammatory response and vascularity; while we demonstrated increased epitenon thickness and fibroblast density in the carrageenan-induced rats, 0.09 mJ/mm^2 and 500 impulses of shock wave application did not promote tendon repair. The number of impulses could therefore be as important as intensity.

We identified a considerable inflammatory cell reaction in the carrageenan-injected group. Inflammation may damage healthy tissue,[20] often resulting in scarring, fibrosis and a decreased functional capacity. However, the formation of fibrosis is essential for tendon healing following injury and raises the question of whether or not inflammation is beneficial or detrimental for tendon healing and tendon tensile strength. Marsolais et al. showed that accumulation of inflammatory cells does not lead to tendon degradation and biomechanical compromise.[9] Recent studies of tendon biomechanics showed that ESW increases tensile forces in the tendon tissue via induced collagen synthesis and regulated collagen modeling.[16,19,26] In this study, however, we obtained decreased tendon mechanical strength in the presence of tendinitis. This study also identified a detrimental collagen fiber organization present in the tendinitis. However, although a trend towards improved collagen fiber organization was seen in Group B as compared to the other groups, no statistically significant differences were found between the groups. Regarding the tensile strength of the tendons, all study groups showed significantly decreased tensile strength compared to the control group, despite inflammatory changes seen on histologic examination. This suggests that the amount of energy and number of impulses generated by ESWT has an effect on the ultimate outcome of tendon healing.

Limitations of this study include the fact that no cross-sectional area was measured using laser micrometry; therefore, the ultimate load could reflect the structural properties of the tissue rather than the mechanical properties.

In conclusion, collagen organization decreased and any tendon healing potential in rat tendons was not evident as detected under light microscopy. Low-dose ESW application and decreased collagen fiber orientation may affect tendon tensile strength in carrageenan-induced Achilles tendinitis. It is therefore unlikely that the results of animal studies are sufficient to determine the appropriate shock wave dose and impulses for clinical application. Given its current popularity as a treatment modality, further studies should aim to clarify the duration and intensity of ESW application.

Conflicts of Interest: No conflicts declared.

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