

Nanoscale Structure and Property Changes in Collagen Fibrils on the Extracorporeal Shockwave Therapy Applications of the Rat Achilles Tendon Injury

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ABSTRACT

Extracorporeal shockwave therapy (ESWT) is popular in the treatment of musculo-skeletal injuries. However, the mechanism by which such therapy might have an effect on tendon injury is unknown. The Achilles tendon consists mainly of collagen Type I fibers that contain collagen fibrils. When the Achilles tendon is injured, it is inflamed. The collagenase-induced Achilles tendinitis (CIAT) model has been widely used to study tendinitis. The major advantages of atomic force microscopy (AFM) over conventional optical and electron microscopy for bioimaging include its non-requirement of a special coating and vacuum, and its capability to perform imaging in all environments. Therefore, the changes in the ultrastructure and adhesion force of the collagen fibrils on the Achilles tendons of rats with CIAT model were observed using AFM. The changes in the structure of the Achilles tendons were evaluated based on the diameter and D-banding of the collagen fibrils. CIAT was induced with the injection of 30 μ l of crude collagenase into male Sprague-Dawley rats (7-week old). The animals were each sacrificed on the each at the 2th, 3th, 7th, 12th, 19th, 26th, 33th, 40th, 47th, and 63th day after the collagenase injection. The normal and injured Achilles tendons were fixed in 4% buffered formalin and dehydrated with increasing concentrations of ethanol. AFM was performed using the non-contact mode at the resolution of 512×512 pixels with a scan speed of 0.8 line per second. The adhesion force was measured via the force-distance curve, resulted from the interactions between the AFM tip and the collagen fibril sample using the contact mode. In intact Achilles tendon, the mean diameter, D-periodicity and adhesion force of collagen fibrils were 130.38 ± 17.40 nm, 67.81 ± 2.37 nm, and 6.48 ± 0.81 nN. CIAT rats reached 128.68 ± 15.75 nm, 72.55 ± 12.60 nm, and 5.03 ± 0.88 nN at the end of the experiment. Then, fibril diameter (138.17%) and adhesion force (187.35%) showed significant maximum increases ($p < 0.0001$ vs. normal) at 40 days after injection and might return to the level of the normal.

Keywords: Rat Achilles tendon, atomic force microscopy (AFM), collagenase-induced Achilles tendinitis (CIAT), collagen fibril, extracorporeal shockwave therapy (ESWT).

1 INTRODUCTION

The Achilles tendon is the largest and strongest tendon in the body. It is located at the back of the lower leg, is attached to the heel bone and connects the leg muscles to the foot. The tendon is composed of a dense connective tissue that consists mainly of Type I collagen fibers that contain collagen fibrils. Collagen is most abundant in tissues as very long fibrils with an axial periodic structure. This is distinct from other proteins in that the molecules consist of three polypeptide chains (α chains) that form a unique triple-helical structure, so-called tropocollagen [1-5]. Currently, there are approximately twenty different types of collagen known [4,5]. The collagen fibrils provide the major biomechanical scaffold for cell attachment and the anchorage of macromolecules, and allow the shape and form of tissues to be maintained [4]. These fibrils are stabilized by the cross-linking of specific lysines and hydroxylsines of the collagen molecules, ordered parallel in a D-period pattern [4,6]. This stagger leads to the characteristic banding pattern of light and dark areas, when negatively stained and observed by electron microscopy (EM) [7]. This D-periodic pattern generally shows a 67 nm axial repeat of the collagen fibrils.

In the Achilles tendons, the collagen fibrils are thick, stiff, and usually form laterally assembled collagen fibers that follow a planar zig-zag pattern, with bending regions that act as flexible hinges [8]. The degree of intramolecular and intermolecular cross-linking between collagen molecules gives the tendon mechanical strength [9].

Since Binning et al. [10] invented atomic force microscopy (AFM) in 1986, it has become a useful technique for characterizing the surface properties of various cells and biological materials [11]. The major advantage of AFM over conventional optical microscopy and scanning electron microscopy (SEM) is its ultra-high resolution without special treatment or vacuum conditions of the specimen. Various literature point out that AFM has been successfully used for the structural analysis of normal collagen. Paige et al. [12] reported the ultrastructure of fibrous long-spacing collagen fibrils that were formed in vitro using AFM. Raspanti et al. [13] investigated the extracellular matrix of rats' tail tendons using AFM and SEM. They showed the three-dimensional structure of the crimps of the rats' Achilles tendons using AFM in air and a

fluid. Although AFM was originally used to obtain the surface topography of a sample, it can precisely measure the interactions between its probe tip and the sample surface from force-distance measurements. It has recently become a fundamental tool in the fields of surface chemistry, biochemistry, and materials science [14,15]. Strasser et al. [16] used AFM as a microdissection tool for probing local elasticity. They measured the force-distance curves on the shell and in the core of native single collagen fibrils to investigate their mechanical property.

Many researches of collagen fibrils using AFM are limited to collagen fibrils that are formed via *in vitro* self-assembly. Although some studies observed the ultrastructure of the crimps in normal Achilles tendons via AFM, there has been no investigation using AFM of the ultrastructural changes in collagen fibrils *in vivo* when the Achilles tendon was injured. Achilles tendons are vulnerable to various types of acute or chronic injuries. Once the Achilles tendon is injured, it is inflamed, which is the first stage in the healing of collagenous tissue wounds [17]. Alaseirlis et al. [18] suggested that an early inflammatory response affects the quality of the healing tissue. The collagenase-induced model has been widely used in the study of tendinitis. It is known that collagenase results in rapid dissolution of fibers, cell necrosis, and vascular damage and inflammation, which mimic many aspects of naturally occurring traumatic injuries.

Extracorporeal shockwave therapy (ESWT) has been found to be an effective non-invasive treatment for musculoskeletal diseases, such as calcifying tendonitis of shoulder, painful heel syndrome, lateral epicondylitis of the elbow and bony healing of nonunion. Several studies reported that ESWT helps Achilles tendon recover from inflammation. However it is not clear if ESWT is effective for Achilles tendonitis. The aim of this study was to observe the effects of ESWT in healing process of the rat CIAT model.

2 MATERLANS AND METHODS

2.1 Animal Preparation

The collagenase-induced Achilles tendinitis (CIAT) was induced with the injection of 30 μ l of crude collagenase into five 7-week old male Sprague-Dawley rats. The CIAT-induced animals were each sacrificed each at the 2th, 3th, 7th, 12th, 19th, 26th, 33th, 40th, 47th, and 63th days after the injection. One normal and five CIAT Achilles tendons were quickly dissected and transferred into a 4% buffered formaldehyde solution. After their fixation, they were dehydrated with increasing concentrations of ethanol. The specimens were immobilized on bio-adhesive tape and placed in the AFM stage.

Each rat of ESWT group (Fig. 1) was anesthetized with xylazine (80 mg/kg, Rompun, Bayer, Monheim, Germany) and placed in prone position. Four sessions of ESWT (Dornier MedTech, 0.085 mJ/mm², 4 Hz, 1000 impulses,

twice a week) were performed. The ESWT tube was placed on the Achilles tendon near the osteotendinous junction.



Figure 1: Experimental ESWT application in rats.

2.2 AFM measurement

Non-contact mode AFM images were obtained using an NANOSTATION II (Surface Imaging Systems, Herzogenrath, Germany) equipped with a 92.5 μ m \times 92.5 μ m \times 6 μ m XYZ scanner and two Zeiss optical microscopes (Epiplan 100 \times and 500 \times). External noise was eliminated by placing the AFM on an active vibration isolation table (TS-150, S.I.S., Herzogenrath, Germany) inside a passive vibration isolation table (Pucotech, Seoul, Republic of Korea). The surfaces of each human scleral sample were scanned in air at a resolution of 512 \times 512 pixels and a scan speed of 0.8 line/sec. AFM imaging was performed at ten min intervals. All the images were scanned with a size of 2 μ m \times 2 μ m. The reflex-coated silicon cantilevers for the contact mode (Surface Imaging Systems, Herzogenrath, Germany) had a frequency range of 6-21 kHz, a spring constant of 0.02-0.07 N/m, a length of 450 μ m, and a resistance of 0.01-0.02 Ohm-cm. The dimensions of the collagen fibrils, including their diameter and D-banding, were measured (double-blind) from the topography images using the Scanning Probe Imaging Processor (SPIP, Image Metrology, Denmark). One hundred and fifty collagen fibrils were selected to measure their diameters in each specimen, and 50 collagen fibrils were selected to measure their D-banding. The force-distance curve measurements were performed to measure the adhesion force of the Achilles tendon. The Achilles tendon tissues were identified in contact imaging mode (nominal spring constant 0.64 N/m) and the force data was obtained at locations with a similar height to avoid edge effects. After the force-distance curve measurements (75 sites), a second image was obtained to ensure that the tissue had not shifted.

2.3 Statistics

The results of three parameters including the diameter, D-periodicity, and the adhesion force, were expressed as the mean \pm standard deviation (SD). A statistical analysis was performed to compare the ultrastructural change between the normal and CIAT rats using a two-tailed Student's t-test.

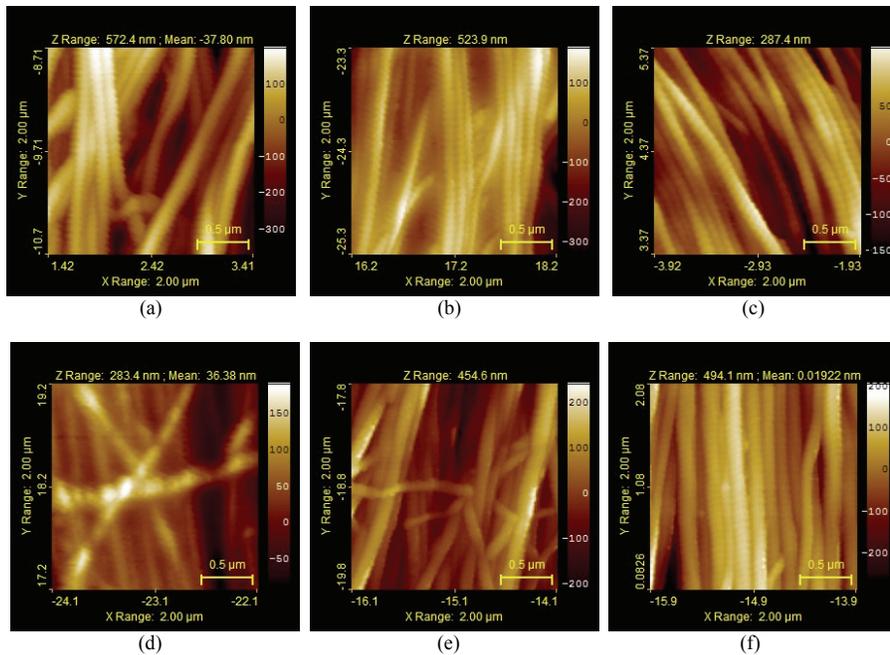


Figure 2: Representative AFM topographical images of the ultrastructural changes in the collagen fibrils of the rats' Achilles tendons during the postoperative period after the collagenase injection. Normal: No injection (a), and control group: 1 day (d), 7 days (b) and 33 days (c) after inducing tendinitis. ESWT group: 7 days (e) and 33 days (f) after inducing tendinitis.

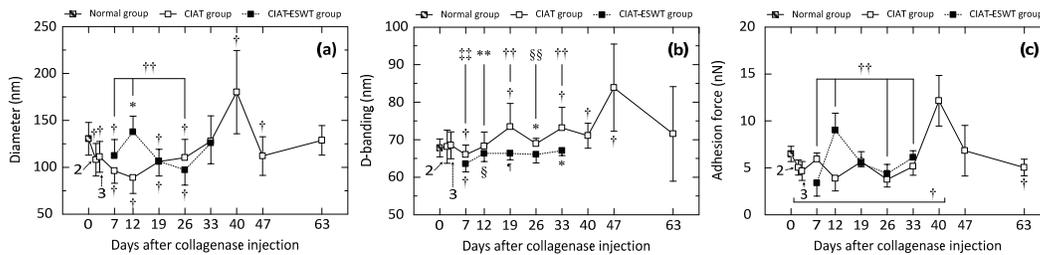


Figure 3: Nanostructure and property changes in collagen fibril diameter (a), D-banding (b), and adhesion force (c) in the rat Achilles tendon during the experiment. In figures, each point represents the mean \pm standard deviation. * $p < 0.05$ vs. normal, § $p < 0.005$ vs. normal, ¶ $p < 0.0005$ vs. normal, † $p < 0.0001$ vs. normal, ** $p < 0.05$ vs. CIAT, §§ $p < 0.005$ vs. CIAT, †† $p = 0.0001$ vs. CIAT, ††† $p < 0.0001$ vs. CIAT.

p -Values less than 0.05 were considered statistically significant.

3 RESULTS AND DISCUSSIONS

Figs. 2 and 3 show the ultrastructural changes in the collagen fibrils in the rats' Achilles tendons over the course of one week after the injection of collagenase. The normal rat Achilles tendons showed a regular parallel arrangement of collagen fibrils and D-banding, but the CIAT rat, on the first day after the collagenase injection, clearly showed changes in the collagen network structure at the ultrastructural level. The collagen fibrils seemed to lose their cylindrical shape and exhibit severe D-banding deformation on the first day after the injection.

In Fig. 3, the changes in the collagen fibril diameter and

D-banding in the rats' Achilles tendon during the postoperative days were quantitatively analyzed from the AFM topography image (Fig. 2). The mean diameter of the collagen fibrils in the rats' Achilles tendons significantly decreased throughout the one-week postoperative period, and the correlation coefficient for the linear regression was $R^2 = 0.8717$ ($p < 0.05$). In intact Achilles tendon, the mean diameter, D-banding and adhesion force of collagen fibrils were 130.38 ± 17.40 nm, 67.81 ± 2.37 nm, and 6.48 ± 0.81 nN. CIAT rats reached 128.68 ± 15.75 nm, 72.55 ± 12.60 nm, and 5.03 ± 0.88 nN at the end of the experiment. The sudden decrease of diameter ($p < 0.0001$ vs. normal, $n = 150$) and adhesion force ($p < 0.0001$ vs. normal, $n = 75$) was examined 2 days after collagenase injection. These changes might be maintained till 12 days after injection. Then, fibril diameter (138.17%) and adhesion force

(187.35%) showed significant maximum increases ($p < 0.0001$ vs. normal) at 40 days after injection and might return to the level of the normal. Interestingly, the collagen fibril diameter and adhesion force in CIAT rats with ESWT application showed the maximum increases ($p < 0.0001$ vs. CIAT) at 12 days after injection. Further, the pattern of change in two parameters during period (7d to 26d) with ESWT was similar to that of post-period (33d to 63d) without ESWT.

When tendons are injured, they are inflamed, which is the initial phase of the tissue healing response. Alaseirli et al. [18] showed via TEM that the diameter of the collagen fibril in an injured tendon was smaller than that in a non-injured tendon. They suggested that the tumor necrosis factor (TNF)- α , an inflammatory cytokine, influences tissue wound healing by regulating macrophage differentiation, increasing the synthesis of collagenase, and downregulating the production of collagen. Långsjö et al. [19] reported that collagenase digestion caused a significant decrease in fibril diameter, collagen volume and surface densities after incubation for 24 and 48 hr. And they revealed that collagenase did not cut the fibrils in fragments but digested along the fibrils, which resulted in reduced fibril diameter. From the resulting fibril diameter, it was thought that collagen fibrils within rat Achilles tendons are affected by inflammation from the second day after the collagenase injection.

Fig. 3(c) shows the change in the adhesion force of the collagen fibrils with CIAT during the postoperative days via force-distance measurement. The mean adhesion force in the normal and healthy fibrils was 6.48 ± 0.81 nN. On the first day after the collagenase injection, the mean adhesion force of the fibrils increased, albeit not significantly, to 1.24 nN. The adhesion force decreased until the fifth day after the collagenase injection but increased on the seventh postoperative day ($p < 0.0001$, $n = 75$).

From the resulting structural and property changes, it is concluded that an initial period of inflammation might last for at least five days and overlap with a subsequent period of proliferation on the seventh day in CIAT. Many therapeutic approaches have been used to improve tendon healing, including physical therapy, the use of anti-inflammatory drugs, surgical intervention, and the use of a support bandage.^{22,23} In another study, the effects of various therapies on the CIAT rat model will be observed via AFM. Based on the results of such study, the therapy will be administered before the seventh day after the collagenase injection for effective treatment within the early inflammation phase.

4 CONCLUSIONS

Direct measurement of the dimension of collagen fibrils using AFM images enabled successful observation of the ultrastructural changes in the collagen fibrils of a rat's Achilles tendon. The results were satisfactory and very

promising. We suggest that ESWT might have the effect in reducing the healing period in the Achilles tendonitis. However, further experimental and clinical studies about collagen fibrillogenesis in CIAT rats are required to provide more definitive statements¹.

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