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Extracorporeal shock waves promote healing of collagenase-induced Achilles tendinitis and increase TGF-β1 and IGF-I expression

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Abstract

Extracorporeal shock waves (ESW) have recently been used in resolving tendinitis. However, mechanisms by which ESW promote tendon repair is not fully understood. In this study, we reported that an optimal ESW treatment promoted healing of Achilles tendintis by inducing TGF-β1 and IGF-I. Rats with the collagenease-induced Achilles tendinitis were given a single ESW treatment (0.16 mJ/mm^2 energy flux density) with 0, 200, 500 and 1000 impulses. Achilles tendons were subjected to biomechanical (load to failure and stiffness), biochemical properties (DNA, glycosaminoglycan and hydroxyproline content) and histological assessment. ESW with 200 impulses restored biomechanical and biochemical characteristics of healing tendons 12 weeks after treatment. However, ESW treatments with 500 and 1000 impulses elicited inhibitory effects on tendinitis repair. Histological observation demonstrated that ESW treatment resolved edema, swelling, and inflammatory cell infiltration in injured tendons. Lesion site underwent intensive tenocyte proliferation, neovascularization and progressive tendon tissue regeneration. Tenocytes at the hypertrophied cellular tissue and newly developed tendon tissue expressed strong proliferating cell nuclear antigen (PCNA) after ESW treatment, suggesting that physical ESW could increase the mitogenic responses of tendons. Moreover, the proliferation of tenocytes adjunct to hypertrophied cell aggregate and newly formed tendon tissue coincided with intensive TGF-β1 and IGF-I expression. Increasing TGF-β1 expression was noted in the early stage of tendon repair, and elevated IGF-I expression was persisted throughout the healing period. Together, low-energy shock wave effectively promoted tendon healing. TGF-β1 and IGF-I played important roles in mediating ESW-stimulated cell proliferation and tissue regeneration of tendon.

Keywords: Achilles tendon; Tendinitis; TGF-β1; IGF-I; Shock waves

Introduction

Insertional tendinitis at the tendon–bone junction is a chronic musculoskeletal disorder that can cause severe pain and impaired function. Repair of a tendon is a complex process requiring inflammatory response, neoangiogenesis, fibrillogenesis, and matrix remodeling [12]. Several methods including growth factor interventions have been used to promote tendon repair [9,11,17].

Extracorporeal shock waves (ESW) are generated by high voltage spark discharge under water, which causes an explosive evaporation of water, producing high-energy acoustic waves. Focusing the acoustic waves with a semi-ellipsoid reflector, we can focus primary shock waves to a specific tissue site [22]. ESW has been found to be an effective non-invasive treatment for resolving calcifying tendinitis of shoulder, painful heel syndrome, lateral epicondylitis of the elbow and bony healing of nonunion [18,31–33]. ESW treatment has been found by scintography and sonography to affect local blood flow and metabolism of rabbit bone and Achilles tendon [20,26]. However, the cellular and biochemical mechanisms by which ESW enhance tendon repair remains to

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be determined. We have recently demonstrated that ESW-promoted osteogenic differentiation of mesenchymal stem cells and healing of segmental femoral defect in rats was achieved by the induction of bone morphogenetic proteins and transforming growth factor-beta 1 (TGF-β1) [35–37]. These findings imply that physical ESW may bring about tissue regeneration by triggering anabolic activities in cells.

Tenocyte growth and neovascularization have been reported to be critical features in the early stage of tendon healing [15]. Tenocytes have been found to convert biophysical stimulation into a biochemical response leading to release of growth factors and cellular adaptation [29]. Of the growth factors regulating tendon repair, TGF-β and insulin-like growth factor-I (IGF-I) have been found to promote tendon regeneration by regulating collagen metabolism and tenocyte proliferation [1,2,5,25]. One of our previous studies has demonstrated that ESW treatment increased the number of neovessels at the normal tendon–bone junction in dogs [34]. One of our more recent of our study has shown that ESW promotes neovascularization through the release of anabolic and bio-active materials [38].

This study aims to elucidate the effect of various impulses of ESW treatment on the healing of collagenase-induced Achilles tendinitis, and to investigate biochemical and biomechanical properties of healing tendons, and to investigate whether physical ESW promotion of tendon repair is linked to increases in tenocyte proliferation and induction of growth factors.

Materials and methods

Collagenase-induced tendinitis model

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. Three-month-old male Sprague–Dawley rats (National Experimental Animals Production Center, Taipei, Taiwan) were caged in pairs and maintained on rodent chow and water ad libitum. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal® sodium, Abbott Laboratories, IL, USA). Hindlimbs of each rat were shaved and washed in Betadine. A total 250 unit (30 μl) of collagenase or 30 μl saline was injected percutaneously near the osteotendinous junction of the left Achilles tendon using a 30 G needle, respectively. Collagenase (bacterial collagenase type I; Sigma Chemical Inc. St. Louis, MO, USA) was dissolved in normal saline and sterile filtered through a 0.22 μm filter. Chloromycinetin (10 mg/kg, intraperitoneal injection) and analgesia (0.02 mg/kg bupenorphine, subcutaneous injection; Reckitt and Colman Pharmaceutical Inc., Richmond VA, USA) were administered for 2 days. The injected area and ankle did not display infection, edema and redness after injecting 30 μl sterile normal saline. The collagenase injection did not appear to have adverse effects on the animals, because all the rats treated with collagenase maintained their weights or gained weight during the study period.

ESW treatment

Each rat with an Achilles tendinitis was re-anesthetized and placed in prone position with the left heel up. The shock wave tube was focused on the Achilles tendon near the insertion site to the heel bone using a C-arm image intensifier and the treatment device's control guide. The ESW treatment (0.16 mJ/mm^2 energy flux density, 1 Hz; HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was used 3 days after collagenase injection. Ultrasound transmission gel (Pharmaceutical Innovations Inc, Trenton, NJ, USA) was used as contact medium between the ESW apparatus and skin.

Experimental design

Experiment I

The total acoustic energy of ESW treatment applied to tissue is presented as the number of pulses multiplied by the energy per pulse [22]. In a pilot study, 12 weeks after ESW treatment (0.16 mJ/mm^2 energy flux density, 1 Hz, 200 impulses), rats with the Achilles tendinitis were found to restore mechanical properties of tendons. In the present study, we examined the effects of ESW energy on biochemical and biomechanical characteristics of tendons. Seventy-five rats were employed for studies. Sixty rats with an Achilles tendinitis were randomly divided into four groups. Rats in each group received ESW treatment (0.16 mJ/mm^2 energy flux density, 1 Hz) for control ($n = 15$), 200 impulses ($n = 15$), 500 impulses ($n = 15$) and 1000 impulses ($n = 15$), respectively. The remaining rats ($n = 15$) received 30 μl sterile saline and received no ESW treatment were used as vehicle group. Rats in each group were killed with an overdose of pentobarbital sodium 12 weeks after ESW treatment. Twelve Achilles tendons were harvested for biochemical and biomechanical assessment and three Achilles tendons were subjected to histomorphometry.

Experiment II

Forty-eight rats with the tendinitis were randomly divided into ESW and control groups. Twenty-four rats were given ESW at 0.16 mJ/mm^2 for 200 impulses. These rats were killed in 3 days ($n = 4$) after collagenase injection, 1 week ($n = 4$), 2 weeks ($n = 4$), 4 weeks ($n = 4$), 6 weeks ($n = 4$) and 12 weeks ($n = 4$) after ESW treatment. The remaining 24 rats received no ESW treatment. Four rats were killed at the same time point as mentioned for the ESW group and were dissected for histological assessment.

Biomechanical properties determination

Tendons were excised through two incisions, one distal to the musculotendinous junction and the other one proximal to the calcaneal bone. The biomechanical properties of the specimens were assessed using a Material Testing System QT-10 (MTS Crop. Minneapolis, MN, USA) with custom designed cryoclamps. Tendons were loaded at a rate of 20 mm/min. During this test, specimens were kept moistened with normal saline. For each sample, load–displacement curves were plotted, and load-to-failure and stiffness were calculated for each sample.

Glycosaminoglycan, DNA and hydroxyproline content measurement

After biomechanical assessment, each specimen containing both rupture tendons was ground with a mortar and pestle under liquid nitrogen. Each ground specimen was divided into two portions. Specimens were digested in 0.5% papain at 65 °C for 4 h. Glycosaminoglycan contents in tendons were spectrophotometrically determined using dimethylmethylene blue as previously described [13]. Papain digests were incubated at 65 °C for a further 20 h. DNA contents in mixtures were determined using bisbenzimide-flourometric assay [24]. Specimens were acid hydrolyzed with 6 N HCl at 110 °C for 24 h and derivatized with phenylisothiocyanate. Hydroxyproline content was determined using a high performance liquid chromatography (LC-10AD, Shimadze, Tokyo, Japan) equipped with a reverse phase column (4.6 mm internal diameter × 250 mm length; TSK-gel, ODS-80TM). The column was eluted with 0.4% ammonium acetate (pH 7.4) and 75% acetonitrile at a flow rate of 2 ml/min through an isocratic pump. The hydroxyproline concentration in each sample was detected using a fluorescence detector (RF-10AXL, Shimadze, Tokyo, Japan) and integrated from the retention time and area under the eluting peak. Results were normalized with protein concentration in each sample.

Immunohistochemistry

Specimens were fixed in 4% buffered paraformaldehyde for 48 h and then decalcified in PBS-buffered 10% EDTA. Decalcified tissues were embedded in paraffin. Tendon specimens were cut longitudinally into 5-μm sections and transferred to poly-lysine-coated slides for conventional hematoxylin–eosin staining (Sigma Chemicals Inc, St. Louis, MO, USA). Immunoreactivity in specimens was demonstrated using a horseradish peroxidase (HRP)-3′-, 3′-diaminobenzidine (DAB) cell and tissue staining kit (R&D Systems Inc. Minneapolis, MN, USA) according to manufacturer's instructions. Sections were immunostained for proliferating cell nuclear antigen (PCNA), TGF-β1 and IGF-I (Upstate Biotechnology, Lake Placid, NY, USA). Sections were further incubated with biotinylated secondary antibodies with streptavidin conjugated to HRP, followed by chromogen solution and counterstaining with hematoxylin. Sections were dehydrated and mounted. Sections without primary antibodies were enrolled as negative controls for the immunostaining.

Histomorphologic assessment

Five areas of tendon were randomly chosen for observation from three sections of four rats. To quantify immunostaining, the sections were analyzed using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Gottingen, Germany). An area (3 mm^2) with positive cells was selected for analysis. Three random images of 0.75 mm^2 from each selected area (3 mm^2) were then taken under 400× magnifications. All the images of each specimen were captured using a Cool CCD camera (SNAP-Pro c.f. Digital kit; Media Cybernetics, Sliver Spring, MD, USA). Images were analyzed using an Image-Pro® Plus image-analysis software (Media Cybernetics, Sliver Spring, MD, USA). The number of the hematoxylin-stained nuclei in each image was counted using an Image-Pro® Plus image-analysis software (Media Cybernetics, Sliver Spring, MD, USA). A professional pathologist, blinded to the treatment regimen performed measurement on all sections.

Statistical analysis

All values were expressed as mean + standard error. Wilcoxson test was used to evaluate the difference between the sample of interest and its respective control. For analysis time course, a multiple range of ANOVA was used. A P-value of <0.05 was considered significant.

Results

Animal activity

The injected area and ankle displayed edema and redness 1 day after injection. Rats walked with lameness within 3 days after collagenase injection. ESW with 200 and 500 impulses did not induce skin hemorrhage or ecchymosis, but ESW with 1000 impulses immediately caused skin ecchymosis after treatment. Improvement of lameness and edema of ankle varied with ESW dose. ESW with 200 impulses completely improved lameness and edema of ankle ($n = 15$) 4 weeks after treatment. ESW with 500 impulses was found to resolve lameness to some extent ($n = 5$) 8 weeks after treatment. No improvement in lameness was observed in the control and 1000-impulse groups of animals during the study.

Biomechanical and biochemical properties

All specimens displayed a typical load–displacement curve, with an upward linear slop and a failure response

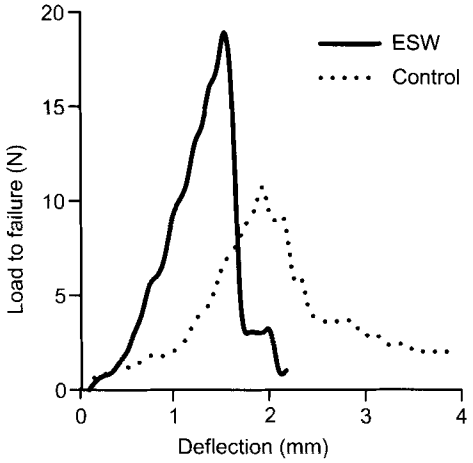

Fig. 1. Representative load failure curves of control and ESW-treated tendons. Achilles tendinitis without or with ESW treatment (0.16 mJ/mm^2, energy flux density, 1 Hz, 200 impulses were subjected to biomechanical determination 12 weeks after ESW).

at the point of failure (Fig. 1). Compared with the vehicle group, ESW treatment with 200 impulses significantly restored mechanical load-to-failure and stiffness of healing tendons. ESW treatment with 500 impulses and 1000 impulses significantly decreased biomechanical properties of tendons (Table 1). We further determined the influences of ESW on biochemical properties of tendons. ESW treatment with 200 impulses reversed DNA, glycosaminoglycan and hydroxyproline contents of healing tendons, bringing them back to levels comparable to those of normal tendons. ESW treatment with 500 impulses and 1000 impulses elicited inhibitory effects on biochemical characteristics of tendons (Table 1). Histological observation also showed that injured tendons subjected to ESW treatment with 200 impulses displayed well-aligned tendon fiber bundle arrangement 12 weeks after ESW (Fig. 2A). In the 500-impulse group (Fig. 2B) and 1000-impulse group (Fig. 2C), injured tendons were filled with loose fibrous tissues. In control group, the lesion site was degenerative and surrounded by fibrous and connective tissue (Fig. 2D). Results indicated that ESW treatment with 200 impulses promoted tendinitis healing, whereas ESW treatment with greater than 200 impulses may suppress tendon repair. ESW treatment with 200 impulses was then used for the proceeding experiments.

Kinetic histological changes in ESW-promoted healing of Achilles tendinitis

Three days after collagenase injection, areas of surrounding the tendon displayed obvious edema, erythrocyte extravasation and massive inflammation cell infiltration. The tendon integrity was disrupted and the lesion site displayed intensive tendon fiber swelling and

Table 1
Biochemical and biomechanical properties of collagenase-induced Achilles tendinitis with and without ESW treatment

	Vehicle[a]	Control[b]		ESW at 0.16 mJ/mm^2 energy flux density					
		Control	*P*-value[c]	200 impulses	*P*-value[c]	500 impulses	*P*-value[c]	1000 impulses	*P*-value[c]
Failure load (N)	20.6 ± 3.2	11.8 ± 1.4	0.027	18.6 ± 2.3	0.682	12.5 ± 1.8	0.016	8.1 ± 1.5	0.0032
Stiffness (N/mm)	14.8 ± 1.9	9.3 ± 1.3	0.013	13.4 ± 2.1	0.824	6.3 ± 1.2	0.012	5.6 ± 1.4	0.0074
DNA content (μg/mg)	4.1 ± 0.4	2.7 ± 0.2	0.023	3.9 ± 0.3	0.518	2.0 ± 0.2	0.036	1.7 ± 0.3	0.016
Hydroxyproline (pg/μg)	1.45 ± 0.2	0.95 ± 0.16	0.028	1.33 ± 0.18	0.364	0.85 ± 0.18	0.028	0.78 ± 0.15	0.021
Glycosaminoglycan (μg/mg)	25.7 ± 2.9	17.8 ± 1.7	0.019	23.4 ± 2.7	0.617	15.4 ± 1.4	0.011	13.2 ± 0.9	0.015

Results are presented with mean ± standard errors calculated from 12 rats.
[a] Achilles tendons were given 30 μl saline and treated without ESW.
[b] Achilles tendons were given collagenase and treated without ESW.
[c] Comparisons between vehicle group, control group and ESW groups, respectively.

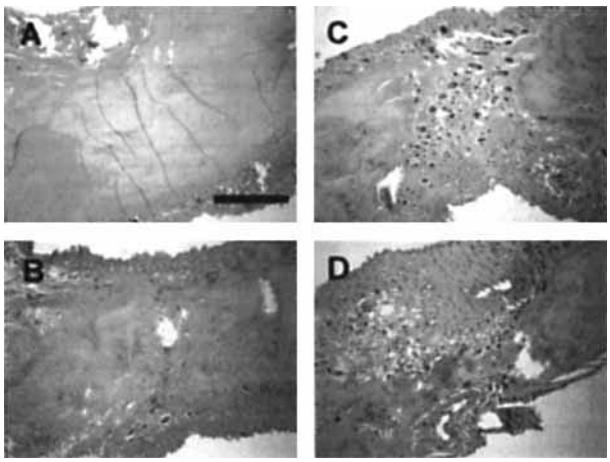

Fig. 2. Histological photographs of collagenase-induced Achilles tendinitis 12 weeks after with or without ESW treatment. (A) Lesion site displayed well-aligned tendon fiber bundle arrangement after 200 impulses of ESW. (B) In the 500-impulse group and (C) 1000-impulse group, injured tendons were filled with loose fibrous tissues. (D) In the control group, the injured tendon was degenerative and surrounded by fibrous and connective tissue. Specimens were stained with conventional hematoxylin–eosin and observed in magnification = 5× and bar scale = 2 mm.

necrosis (Fig. 3A). One week after ESW treatment, inflammation was gradually resolved and intensive blood capillaries and extracellular matrix production were observed in lesion site (Fig. 3B). Four weeks after ESW treatment, lesion site appeared to be fused by a fibrous bridge. The granulation tissue and inflammation cell infiltration was completely improved. Well-aligned tendon fiber bundles gradually formed parallel to the long axis of the tendon (Fig. 3C). Six weeks following ESW, fibrous tissue in lesion site was replaced by newly developed tendon tissue. Tendon fiber density was increased and matrix remodeling was in process (Fig. 3D). We further assessed histological changes in sections using high power microscopy. Three days after collagenase injection, lesion site was largely filled with loose fibrous tissue, granulation tissue and connective tissue and intensive inflammatory cells accumulation (Fig. 3E). One week after treatment, a large number of plum-shaped and spindle-shaped fibroblasts from peritendon recruited into the lesion site and turned into hypertrophied cellular tissue 1 week after ESW treatment (Fig. 3F). Four weeks after ESW, spindle-shaped tenocytes gradually oriented into tendon bundles. Intensive tendon fibril continued to be produced. Fibril increased in size to become histologically visible thin wavy forms (Fig. 3G). Six weeks following ESW, newly formed tendon tissue displayed increasing vascularity within the new tendon and the longitudinal orientation of the collagen bundles (Fig. 3H).

Immunohistochemisty

We examined the effect of ESW treatment on the tenocyte proliferation. Proliferating cells were immunostained for PCNA, a protein associated with the S-phase of a dividing cell. The nuclei of cells with positive PCNA expression appeared as brown color. Proliferating cells were concentrated at the lesion site. Within 1 week and 6 weeks after ESW, cubodial-, plum-shaped (Fig. 4A) and spindle-shaped tenocytes (Fig. 4B) adjacent to hypertrophied cellular tissue and newly formed tendon tissue matrix were found to have undergone intensive PCNA expression. Cells at the fibrous tissue in the control group displayed slight PCNA expression throughout the study period (Fig. 4C and D). We compared the number of proliferating cells in injured tendon of both groups and found ESW treatment able to stimulate a significant increase in cell proliferation within 6 weeks of treatment (Table 2).

We further investigated immunohistochemically whether ESW promotion of tenocyte proliferation and tendon healing was linked to TGF-β1 and IGF-I induction. One week after ESW, plum-shaped tenocytes at hypertrophied cellular tissue were found to express intensive TGF-β1 (Fig. 5A). Four weeks after treatment, spindle-shaped tenocytes at the newly developed tendon tissue

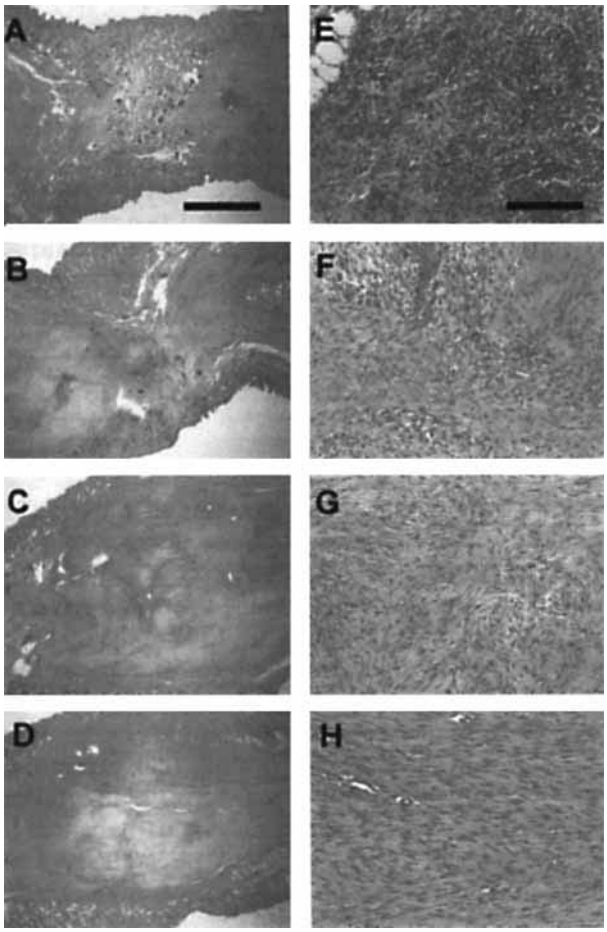

Fig. 3. Histological changes in ESW promotion of Achilles tendinitis repair. (A) Tendon displayed evident swelling, edema, and partially ruptured 3 days after percutaneous injection of collagenase. (B) Inflammation was clearly resolved and extracellular matrix was produced 1 week after ESW treatment. (C) The ruptured end filled with well-aligned tendon fibrils within 4 weeks after treatment. (D) The lesion site was replaced by newly formed tendon fiber 6 weeks after ESW treatment. (E) Lesion site displayed intensive inflammatory cell infiltration 3 days after percutaneous injection of collagenase. (F) Plum-shaped tendocyted aggregated as hypertrophied cellular tissue was adjunct to the injured tendon 1 week after ESW treatment. (G) Intensive tendon fibril production and formed into wave forms 4 weeks after ESW treatment (H) Newly developed tendon tissue appeared to increase vascularity within the repair neotendon 6 weeks after ESW treatment. Rats with the Achilles tendinitis were given a single ESW treatment (0.16 mJ/mm^2 energy flux density, 1 Hz, 200 impulses) 3 days after injection of collagenase. Specimens were stained with conventional hematoxylin–eosin and observed in magnification = 5×, bar scale = 2 mm (A, B, C, and D) and in magnification = 20×, bar scale = 100 μm (E, F, G, and H).

expressed strong TGF-β1 (Fig. 5B). Mature spindle-shaped tendocytes in the dense tendon fiber showed weak TGF-β1 expression in the later stage of healing process (Fig. 5B). In the control sections, cuboidal- and spindle-shape fibroblastic cells located at the lesion site exhibited slight TGF-β1 expression during experiments (Fig. 5C and D). One week after treatment, tenocytes adjacent to the hypertrophied showed intensive IGF-I expression

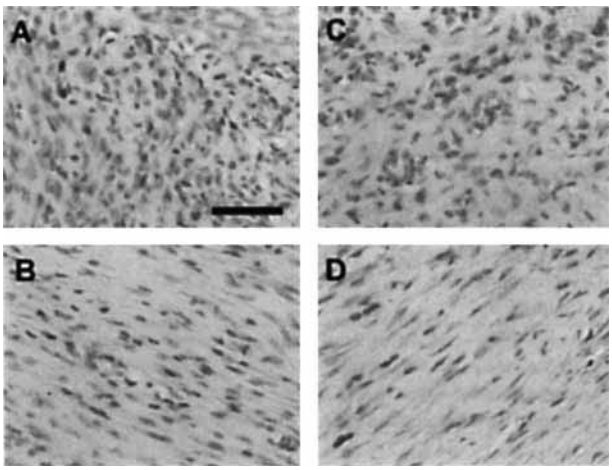

Fig. 4. PCNA expression in the repair of collagenase-induced tendinitis with and without ESW treatment. Within 1 week and 6 weeks after ESW, a large number of plum-shaped and spindle-shaped tenocytes at (A) hypertrophied cellular tissue and (B) newly developed tendon tissue displayed intensive PCNA expression after ESW treatment. Plum- and spindle-shaped tenocytes at (C) lesion site and (D) fibrous tissue of the controls without ESW treatment displayed weak PCNA immunoreactivity. The positive PCNA immunostained cells showed brown color in the nuclei of cells. Specimens were observed in magnification = 40× and bar scale = 50 μm.

(Fig. 6A). Four weeks after ESW, cells at newly formed tendon bundles showed intensive IGF-I expression (Fig. 6B). In control sections, cells at the loose fibrous tissue expressed weak IGF-I expression (Fig. 6C and D). Intensities of TGF-β1 and IGF-I expression varied with the stages of tendon regeneration. Table 2 summarizes the number of tendocytes that exhibited positive TGF-β1 and IGF-I expression during ESW-promoted tendon repair. There were significant increases in TGF-β1 expression within 4 weeks of ESW treatment. Elevating IGF-I expression was noted throughout the study period.

Discussion

In this study, we demonstrate that physical ESW treatment promotes repair of collagenease-induced Achilles tendinitis. Injured tendons receiving ESW treatment resolved inflammation, increased tenocyte proliferation and restored tendon integrity, leading to restoration of biomechanical properties. While a number of reports implicating ESW treatment have been used for tendinitis, little has been done to define the role of growth factor expression in the repair processes. The findings in this study provide the first evidence that ESW promotion of Achilles tendinitis repair coincides with increases in TGF-β1 and IGF-I. We propose that it is the increased mitogenic and anabolic responses of tendon tissue that bring about the clinical success of ESW treatment in resolving tendinitis.

Table 2
Expression of PCNA, TGF-β1 and IGF-I in collagenase-induced Achilles tendinitis with and without ESW treatment

		Week(s) after ESW treatment				
		1	2	4	6	12
PCNA	ESW[a]	932 ± 183	1024 ± 198	814 ± 206	498 ± 102	376 ± 92
	Control	306 ± 86	324 ± 89	287 ± 64	275 ± 53	283 ± 57
	P-value[b]	<0.001	<0.001	<0.001	0.016	0.37
TGF-β1	ESW	614 ± 102	532 ± 93	417 ± 79	318 ± 53	312 ± 48
	Control	226 ± 43	257 ± 51	235 ± 46	231 ± 51	242 ± 47
	P-value	0.0074	0.011	0.018	0.023	0.46
IGF-I	ESW	562 ± 67	654 ± 98	736 ± 105	514 ± 82	467 ± 58
	Control	265 ± 58	249 ± 63	247 ± 72	238 ± 54	256 ± 52
	P-value	0.013	0.0034	0.0078	0.021	0.016

[a] Results are presented with mean ± standard errors calculated from five tendon of areas from three sections of four rats after ESW treatment at 0.16 mJ/mm^2 energy flux density, 1 Hz, 200 impulses.
[b] Comparison between ESW and control groups.

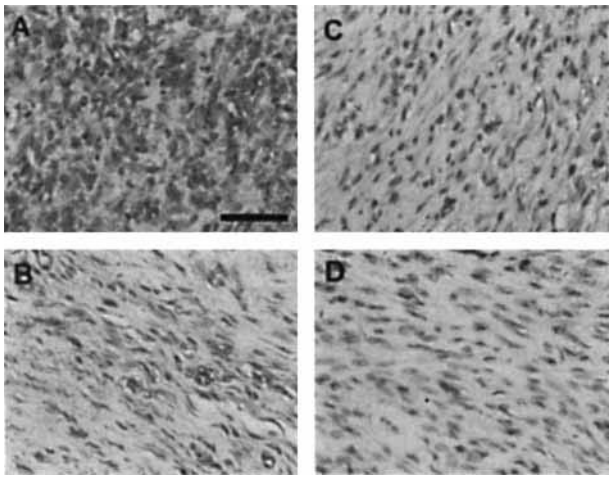

Fig. 5. Expression of TGF-β1 in collagenase-induced tendinitis with and without ESW treatment. Cells adjunct to (A) hypertrophied cellular tissue 1 week after treatment and (B) newly developed tendinous tissue were found to have intensive TGF-β1 expression after 4 weeks after ESW treatment. In control sections, plum- and spindle-shaped tenocytes at (C) lesion site and (D) fibrous tissue expressed weak TGF-β1. Specimens were observed in magnification = 40× and bar scale = 50 μm.

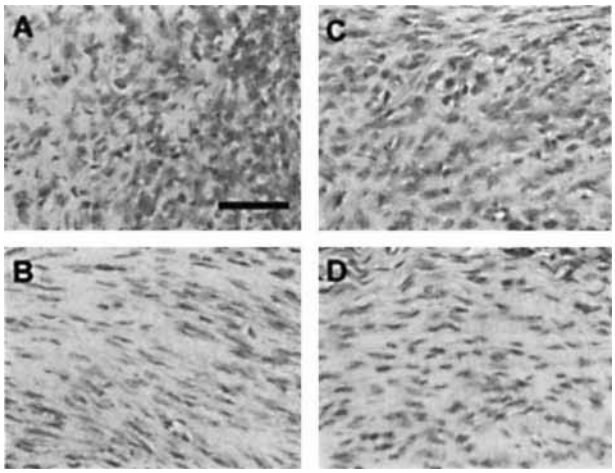

Fig. 6. Expression of IGF-I in collagenase-induced tendinitis with and without ESW treatment. Cells adjunct to (A) hypertrophied cellular tissue 1 week after treatment and (B) newly developed tendinous tissue were found to have intensive IGF-I expression after 4 weeks after ESW treatment. In control sections, plum- and spindle-shaped tenocytes at (C) lesion site and (D) fibrous tissue of the controls displayed weak TGF-β1 immunoreactivity. Specimens were observed in magnification = 40× and bar scale = 50 μm.

The etiologies of tendinitis are multifactorial including avascular changes, degenerative change and metabolic disturbance, neural factors and neovascularization [3,4,9]. Previous reports have demonstrated the effect of ESW treatment on pathophysiology of tendon [19,26]. However, such studies were performed on normal tendons, and their observation should not be extrapolated to explain ESW-promoted tendinitis repair. Collagenase-induced model is a well-established model for the studies of tendinitis. The acute swelling, inflammation and matrix destruction in tendons are similar to those seen in naturally occurring tendon injuries [1,9,10,21]. Histological observation in the present study revealed that lesion sites in injured tendons displayed massive inflammation cell infiltration and tendon degeneration. This model also clearly showed that the physical ESW treatment stimulated the regeneration of tendon. Our study finds the use of collagenase-induced Achilles tendinitis to an excellent model for clarifying the cellular and histological mechanism that biophysical strategies such as ESW treatment uses to stimulate tendon repair in vivo. In contrast to previous studies demonstrating injured tendons healed eventually over time after tendotomy [7,23], our current study showed that collagenase-induced Achilles tendinitis displayed incomplete healing or degenerative tendinitis. We speculate this discrepancy that tendinitis healing may depend on the model system and experimental animals used. There may be a chronic

tendinopathy that does not respond to normal healing time.

Our present study indicates that high-energy ESW treatment impairs DNA, extracellular matrix synthesis and biomechanical characteristics of tendons. Mechanical or physical impact on tissues exposed to ESW has been found to depend on tissue impedance. ESW-induced damage has been reported as a function of the total amount of acoustic energy absorbed in a finite volume [8]. Previous studies have demonstrated that high-energy ESW treatment can induce fibrinoid necrosis, paratenon fibrosis and inflammatory cell infiltration in normal rabbit Achilles tendons [26] and impaired tensile strength of tendons [19]. Histological findings of our study showed that loose fibrous connective tissue and degenerative tendon formed in the 500-impulse and 1000-impulse groups, indicating high-energy ESW treatment is not beneficial for Achilles tendinitis repair in our model.

ESW with 200 impulses restored biomechanical and biochemical characteristics of tendons. Histological findings showed that 200 impulses improved edema and inflammatory cell infiltration in injured tendon and progressively promoted tendon regeneration. Improvement of lameness also provides an explanation for the trend toward tendon healing promoted by low-energy ESW treatment. Our findings suggest that optimal ESW energy magnitude is beneficial for collagenase-induced Achilles tendinitis healing in rats. In contrast to these findings in rats, human subjects treated for chronic plantar fasciitis and calcifying tendinitis with ESW of more than 200 impulses improved without side effects [27,28]. It has also been our clinical experience that patients treated for painful heel and calcifying tendinitis improved without complications [31,32]. Our findings suggest that ESW stronger than 0.16 mJ/mm^2, 500 impulses should not be used for treating Achilles tendinitis in rats. Soft tissue in rats may be more sensitive to ESW. The impact of high-energy ESW treatments on soft tissue in experimental animals may not be readily extrapolated to humans. Effects of ESW energy magnitude on treating Achilles tendinitis have not been clarified. In one more recent study demonstrated that patients with lateral epicondylitis receiving low-energy ESW (0.07–0.09 mJ/mm^2) did not improve [16]. More research is needed in ESW energy magnitude required for treating Achilles tendinitis in patients.

Increasing fibroblast proliferation and biosynthesis of extracellular matrix and collagen are crucial stage for the return of normal tendon strength [9,10]. In the present study, ESW treatment significantly reversed DNA, glycosaminoglycan and hydroxyproline contents. Histological findings also showed that intensive PCNA expression and new tendon fibril formation were prominent findings in our study of ESW treatment for tendon repair. These findings indicate that ESW treatment raises mitogenic and morphogenic responses, which stimulates tenocyte growth and typical tendon microstructure formation.

Increased tenocyte growth and tissue regeneration indicated that some growth factors were responsible for initiating the ESW-induced mitogenic and morphogenic responses of injured tendon. Intensive PCNA expression of tenocytes and new tendon bundle formation coincided with intensive TGF-β1 and IGF-I expression after ESW treatment. These findings suggest that TGF-β1 and IGF-I, at least in part, are involved in ESW promotion of tendon repair. These growth factors have been found to up-regulate extracellular matrix biosynthesis of tenocytes [1,2,30]. Tenocytes responded to mechanical stimulation by increasing TGF-β1 gene expression [6]. Our findings indicate that tendon tissue can convert ESW stimulation into biochemical signals via release of TGF-β1 and IGF-I for tendinitis repair. Moreover, TGF-β1 has been reported to act as a potent inhibitor of marcophages-induced extracellular matrix degradation and inflammation during the healing of a wound [14,39]. Intensive TGF-β1 expression in the early stage of ESW-augmented tendon repair may explain the improvement in inflammation. Our findings suggest that TGF-β1 and IGF-I are actively involved in tendinitis healing. Each growth factor has a distinct temporal expression pattern and each has a potentially unique role in repair of tendinitis. In summary, our research findings provide evidence that an optimal ESW treatment promotes tendon regeneration, with TGF-β1 and IGF-I playing important mitogenic and anabolic roles in signaling physical ESW stimulation.

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