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Effect of shock-wave therapy on patellar tendinopathy in a rabbit model

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Abstract

This study investigated the effect of shock-wave therapy (SWT) on collagenase induced tendinopathy in the rabbit patellar tendon. Eighteen rabbits were treated by ultrasonography-guided injection of 0.025 ml collagenase into the patellar tendon in both knees. After tendinopathy was confirmed at 3 weeks post-treatment by the histological examination, SWT was initiated to the right patellar tendon involving 1500 cycles at 0.29 mJ/mm^2 in two separated weekly courses from 4 weeks post-treatment. The rabbits were randomly divided into two groups, which were sacrificed at the 4th and 16th week after SWT, respectively. The histological examination, the mechanical and biochemical tests then were performed. The ultimate tensile load in the SWT tendon increased 7.03% at 4 week and 10.34% at 16 week after treatment as compared to the sham group. Hydroxyproline concentrations increased in the SWT tendons over both the 4 and 16 weeks after treatment. Moreover, the pyridinoline concentration increased at the 4th week but decreased at 16th week as compared to the sham group. The histological examination demonstrated increased blast-like tenocyte at the 4th week, while more mature tenocyte with neovasculization at the 16th week. The result obtained here validates the effectiveness of the SWT in the established tendinopathy. SWT may increase collagen synthesis and collagen crosslink formation during early healing process.

Introduction

Since early 1980s in Europe, shock-wave therapy (SWT) has received enthusiastic attention regarding its clinical application for various soft-tissue pathologies, including lateral humeral epicondylitis, Achilles tendinitis, rotator cuff calcifying tendinopathy, and plantar fasciitis [11–13,25,26]. Outcomes have been favorable, but the exact mechanism inducing accelerated soft-tissue healing remains unclear. Tendinopathy is a condition of tendon degeneration, and has been attributed to various causes, including local injury, hormone, aging, chemicals, vascular insufficiency and overuse [2,4,5,16,19,23]. Immediately following tendon injury, firstly neutrophil aggregates, then macrophage ensues to phagocyte the necrotic debris and release collagenase, thus causing significant inflammation [18]. Injection of collagenase into the tendon mimics this situation and has been widely employed as a animal tendinopathy model since being reported by Silver et al. in the late 1970 [2,5, 6,29].

In a tendon regeneration process, the reparative cell is tenocyte, which when activated behaves like a modified form of fibroblast or fibrocyte, and can produce collagen, protein mediator of repair and matrix proteoglycan [16]. Initially Type III collagen is deposited in a woven pattern, while type I collagen laid down as it matures along with crosslink formation [3]. In the newly formed collagen, hydroxyproline represents a reliable index. Meanwhile pyridinoline, a trivalent crosslink residue of collagen, reflects a major crosslink in mature collagen in various tissues [8]. However, the biomechanical properties of the healed tissue can reduce by as much as 30% despite the good remodeling status, and biochemical differences in collagen type and arrangement, water content, glycosaminoglycan content persisted indefinitely.

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Notably, the mechanical properties of the healed tissues never equal those of the intact tendon [16].

In this study, the model of ultrasonography-guided injection of collagenase into the patellar tendon in rabbits was adapted to validate the effectiveness of the SWT on the established tendinopathy.

Materials and methods

The experiment complied with the Taiwan law on animal experiments and was approved by the Ministry of Justice. Eighteen male rabbits weighing about 3 kg each were injected with 0.025 ml collagenase (CLS Type I collagenase; Worthington Biochemical Corporation, NJ, USA.) into the patellar tendon over both knees. Before injection, all rabbits were sedated with ketamine. The injection was guided by ultrasonography to confirm intratendinous placement. Following injection, the rabbits were given free reign to move within their cages until sacrifice. One rabbit was sacrificed for the confirmation of the tendinopathy at the 3rd week by the histological examination with appearance of many lymphocyte and macrophages infiltration associated with collagen disruption. SWT (Orthospec™, MEDISPEC, Israel) was initiated over the right patellar tendon with 1500 cycles at 0.29 mJ/mm^2 in two separated weekly courses since the 4th week. This medium energy delivery was selected because no detrimental effect was observed in the literature [28]. The rabbits then were divided into two groups. Group I comprised 8 rabbits, which were sacrificed at the 4th week after SWT. Meanwhile, Group II comprised 9 rabbits, which were sacrificed at the 16th week after SWT. One rabbit from each group was sacrificed for histological examination, while the remainders were employed for the mechanical and biochemical tests.

Histological analysis [16] was conducted around the injection site to determine the different responses of the matrix and tendon cells to the collagenase injection and the effects of SWT. After the animals were sacrificed with lethal doses of anesthesia, the bilateral patellar tendons were dissected and embedded in formalin solution. Next, 10 μm thickness sections were cut and stained with haematoxyline and eosin (H&E). [14,34]. The changes in cellularity, presence of angiogenesis, evidence of matrix degeneration, and accompanying changes to the peritendon tissue, such as fibrosis, then were assessed under a light microscope.

Mechanical testing

For biomechanical analysis [13,14,37], the hind limb was dissected into a patella–patellar tendon–tibia complex. The patella and tibia were transfixed with Kirschner wire and potted in polymethylmethacrylate in an inline fashion, and the complex was then placed in specially designed clamp. The clamped specimen was mounted on a material testing machine (Instron, Canton, MA, USA). Next, 10 cycles of reconditioning at 2–4% strain rate were performed to minimize viscoelastic effects. Load to failure was performed at a rate of 1 mm/min. Finally, ultimate tensile failure load was determined and recorded on a computer.

Biochemical test

Biochemical analysis [2,5,24,30–32,37] was performed after completing mechanical testing, and 1 cm of the central tendon specimen was dissected free of surrounding connective tissues. The specimen was washed in cold distilled water and dried by lyophilization for 18 h. Next, tissue was extracted in 1 M Guadinium hydroxychloride for 24 h, then lyophilized again. Subsequently, the dried sample was hydrolyzed in 6 M HCl at 108 °C for 18 h. The hydrolysate was then neutralized by dilution in water and dried on a savat Speed Vac concentrator. The dried samples were reconstituted in 1% *N*-heptafluorobutyric acid (HFBA). Finally, small aliquots of these samples were taken to react with chloramine-T and *p*-dimethylamino-benzaldehyde (DAB) and spectrometrically assessed at 537 nm (DU 650, Beckman-Coulter, California, USA) to determine collagen content using hydroxyproline [35]. A known hydroxyproline standard (Sigma, Japan) ranging form 0–10 μg/ml was plotted for comparison.

Pyridinoline concentration was assessed by reverse phase high pressure liquid chromatograph (HPLC) as described by Eyre et al. [8]. The HPLC (Angilent 1100, Helwett Packard, USA) was equipped with a 0.46 cm × 25 cm reverse C-18 column. Notably, HFBA was used as an ion-pairing agent. The mobile phase included solvent A: 0.01 M HFBA in 5% acetonitrile, and solvent B: 0.01 M HFBA in acetonitrile. A flow rate of 1 ml/min was used with a linear gradient of acetonitrile (21–25%). The column effluent was monitored for fluorescence by excitation at a wavelength of 297 nm using an emission filter that cut off below a wavelength of 380 nm. Pyridinoline was eluted at 7.47 min. The pyridinoline standard was kindly provided by Dr. Simon P Robbins (Matrix biochemistry group. Rowett Research Institute, Bucksburn, Scotland, UK).

Statistical analysis

The bilateral knees of the same rabbit were analyzed with the student's paired *t*-test using Microsoft Excel (Microsoft Corp, Seattle, WA, USA), with statistical significance $p < 0.05$.

Results

Histological examination

In Group I, immature fibroblast-like soft tissue forming cell was embedded in a pale eosinophilic matrix over the SWT tendon, while denser and plump fibroblast-like cell in disarrayed collagen was demonstrated in the control tendon. New capillaries in the peritentious tissue were more obvious in the SWT tendon. In Group II, increased cellularity with mature clustered fibroblast was observed in the SWT tendon, while immature fibroblast was still noted in the non-SWT tendon (Fig. 1). Larger blood vessels, including differentiated venules and arterioles, as well as capillaries, were found in the SWT group, while only scattered capillaries were observed on the control tendon. Notably, the collagen array was more oriented in the SWT group (Fig. 2).

Mechanical testing

The typical diagram of the load–elongation curve derived from tensile ligament testing. Low stiffness (toe region) and high stiffness (linear region) were well demonstrated before the ultimate tensile load was reached (Fig. 3). In Group I, the SWT tendon displayed higher ultimate tensile load (379.91 ± 58.91 N: 354.93 ± 60.97 N, $p < 0.05$). Moreover, an identical result was obtained in Group II (441.62 ± 78.83 N: 400.25 ± 82.74 N, $p < 0.05$). Group II displayed higher ultimate tensile load than Group I ($p < 0.05$), both in the SWT and control tendons (Fig. 4). The ultimate tensile load in the SWT tendon increased 7.03% at 4th week and 10.34% at 16th week after treatment as compared to the sham group.

Biochemical assessment

The hydroxyproline concentrations in the tendon tissue increased at 16th week for both the SWT and

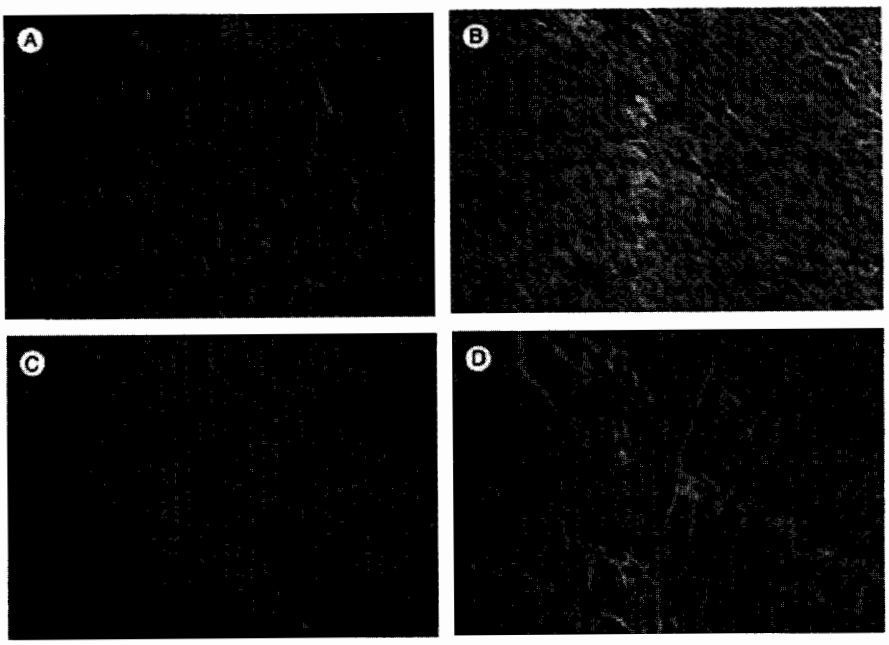


Fig. 1. A: Histological section at 4 weeks after SWT, blast-like tenocyte containing vacuoles was revealed. B: Histological section at 4 weeks in the control group, plump immature tenocyte in disarrayed collagen matrix was demonstrated. C: Histological section of the SWT tendon at 16 weeks, mature tenocyte in parallel array of collagen was noted. D: Histologic section of control group at 16 weeks, coarse mature tenocyte was noted.

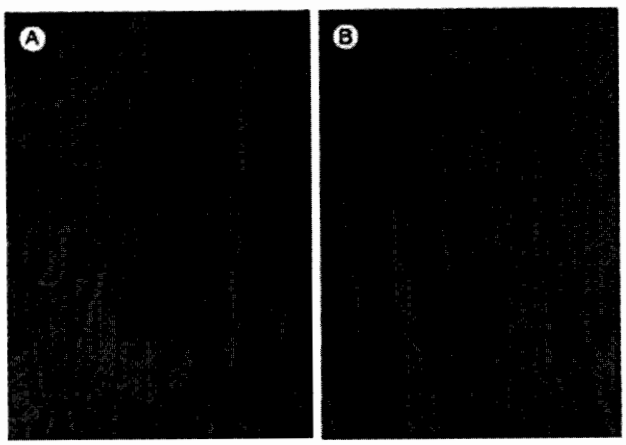


Fig. 2. The histological section of the paratendinous tissue of Group II. A: In the SWT tendon, more mature vessel formation. B: In the control group, capillaries was noted.

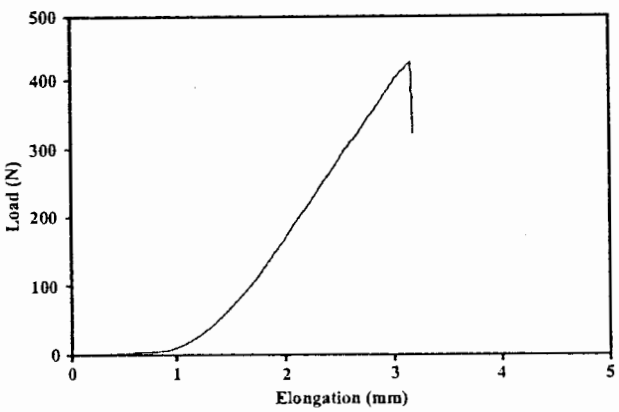


Fig. 3. The load–elongation curve from tensile testing of the ligament. Low stiffness (toe region) and high stiffness (linear region) was well demonstrated before the ultimate tensile load was reached.

control groups. Meanwhile, Groups I and II both displayed a higher concentration of hydroxyproline in the SWT tendon (Group I: 25.32 ± 2.49 mg/ml: 17.83 ± 2.16 mg/ml, $p < 0.05$; Group II: 29.82 ± 3.35 mg/ml: 22.05 ± 2.98 mg/ml, $p < 0.05$) (Fig. 5).

The synthetic pyridinoline standard was eluted at 7.4 min in the HPLC machine, and different concentrations were tested to establish a standard curve of pyridinoline concentration ($R = 0.99$). The comparable peak at the same time (7.41 min) in the sample group was calculated for the sample concentration (Fig. 6). Group I (4 weeks) displayed a concentration of pyridinoline in the SWT tendon that was 10 times that of the control group (0.460 ± 0.082 ng/ml: 0.031 ± 0.010 ng/ml, $p < 0.05$). Meanwhile, the concentration of pyridinoline was higher in the control tendon (SWT: control $= 4.70 \pm 1.19$ ng/ml: 6.70 ± 1.53 ng/ml, $p > 0.05$) (Fig. 7). Comparing Groups I and II reveals a steady increase in pyridinoline concentration over time.

Discussion

Since the introduction of SWT in the musculoskeletal system, successful treatments of tendinopathy have been

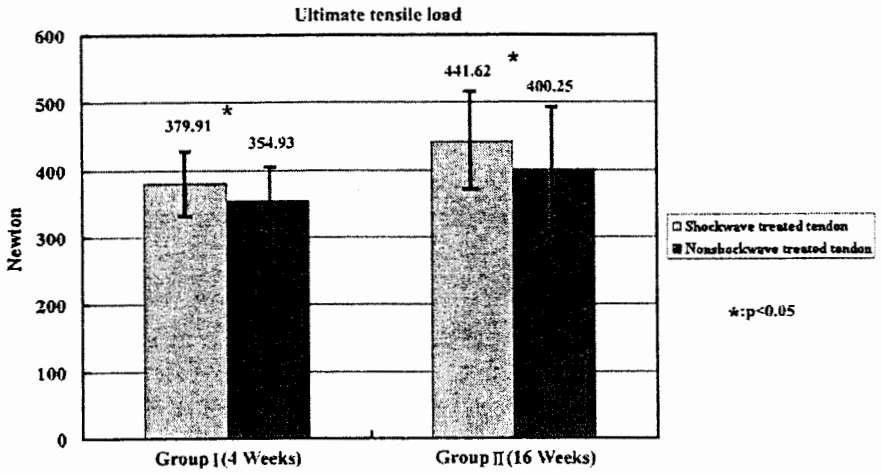


Fig. 4. The ultimate tensile load of the patella-tendon-tibia complex. Both in Group I and Group II, higher ultimate load was revealed in the SWT tendon.

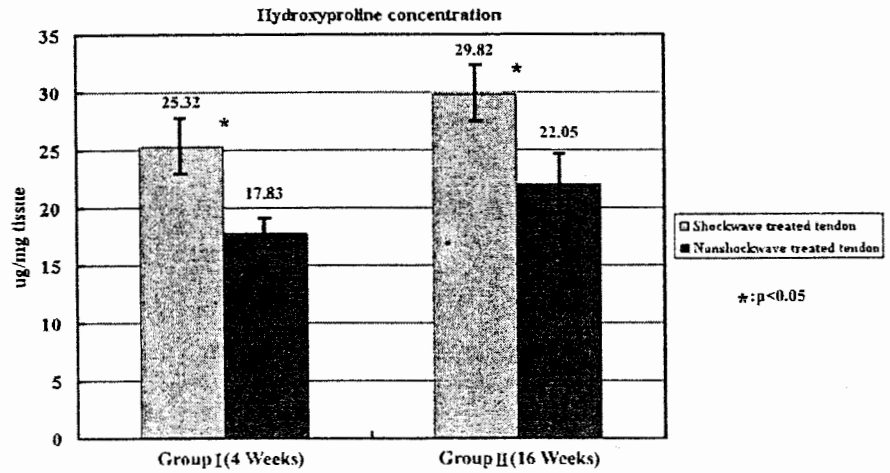
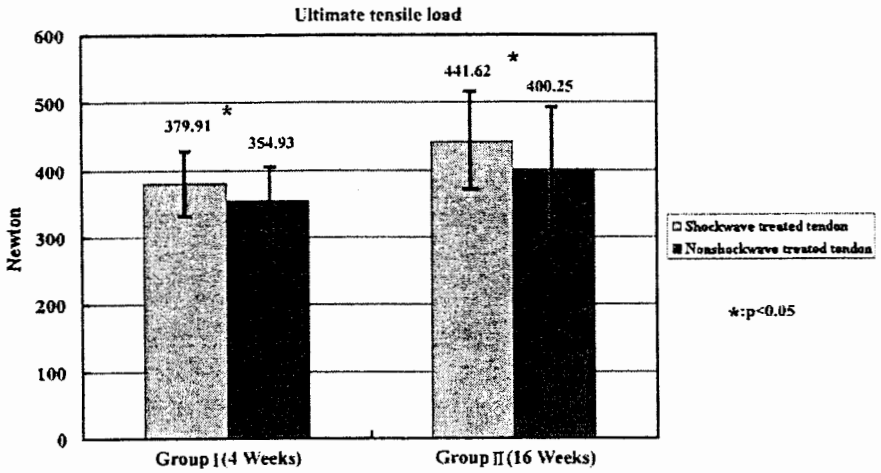


Fig. 5. The hydroxyproline concentration: Higher concentration of the shock-wave treated group was demonstrated both in the Group I and Group II.

reported, including lateral epicondylitis, patellar tendinitis, and plantar fascitis [11,12,25,26]. Most reports have postulated the effect of SWT to be provocation of a painful level of stimulation that achieves pain relief or analgesia through hyperstimulation and increased vascularity [11,15,21,25,27]. However, due to the widespread use of conservative treatment, the effect of SWT on tendon remains poorly understood. This work assessed the effect of SWT on tendinopathy in a collagenase induced tendinopathy model. Collagenase was injected to both patellar tendons of the rabbits to induce tendinopathy, minimizing serum-transmitted interference and the eliminating individual variation among rabbits [8].

Previous studies have reported high energy shock-waves (0.60 mJ/mm^2) to have a detrimental effect on normal tendon tissue [28]. This study used medium-energy SWT(0.29 mJ/mm^2) which was reported with no long term detrimental changes to normal tendon. Since shock-wave therapy was applied to the injured tendon, the effect of median-energy shock-wave on tendinopathy should be assessed. In tendon repair and regeneration, fibroblast-like tenocyte was essential in the synthesis of new collagen. That is, tenocyte became hyperplasia, yet cell vacuolation and blast-like change in morphology might behave like the characteristic of the stress-responsive tenocyte [14]. The histological examination displayed more stress-responsive tenocyte in the SWT tendon at 4 weeks, and more new capillary formation, both of which phenomenon correlated closely with the increased concentration of collagen. The histological examination of Group II revealed mature tenocyte in more parallel array and more new vessel formation, implying the end of repair processes in the SWT tendons, while in the control group, coarse mature tenocyte was demonstrated in the disarray collagen. Since mature tenocyte was classified as a stable cell, only low collagen



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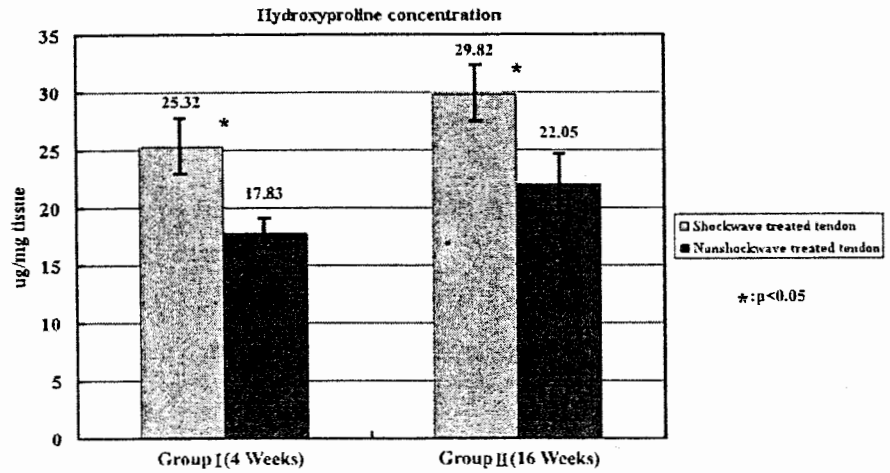

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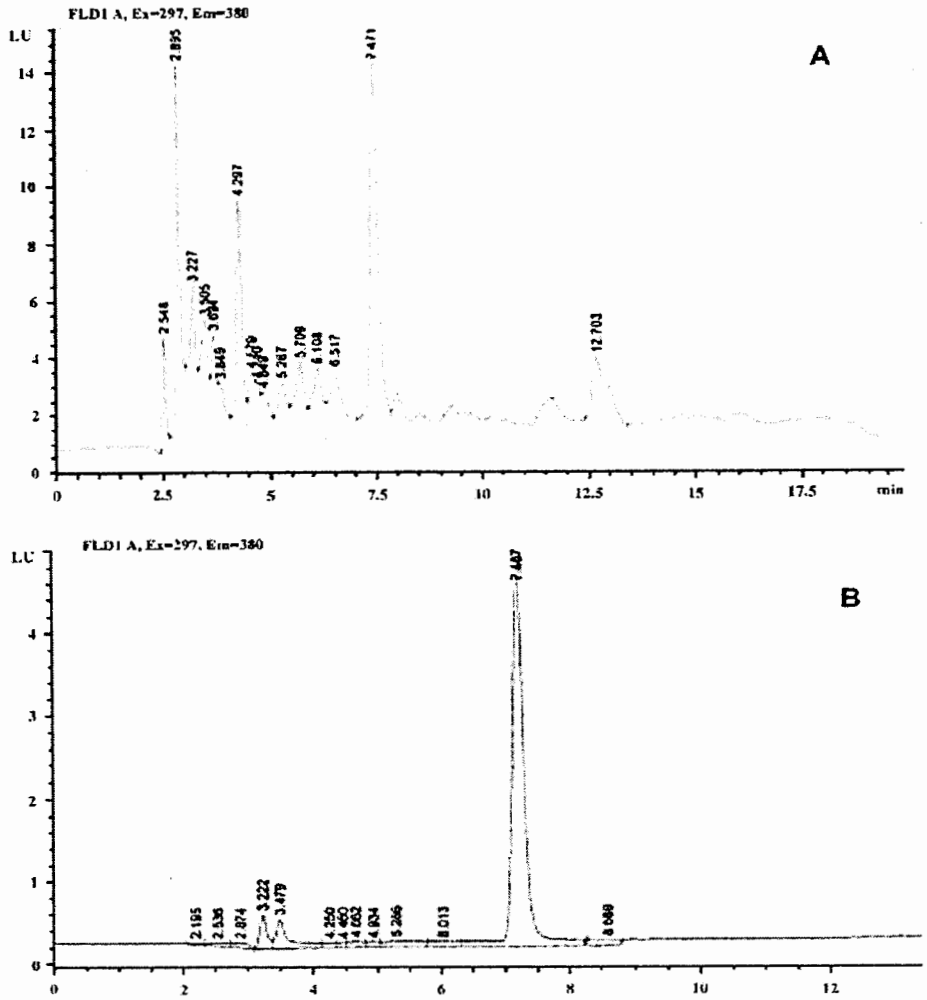

Fig. 6. The reverse-phase chromatography of the pyridinoline. (A) The sample eluted at 7.4 min. (B) The pyridinoline standard eluted at 7.4 min.

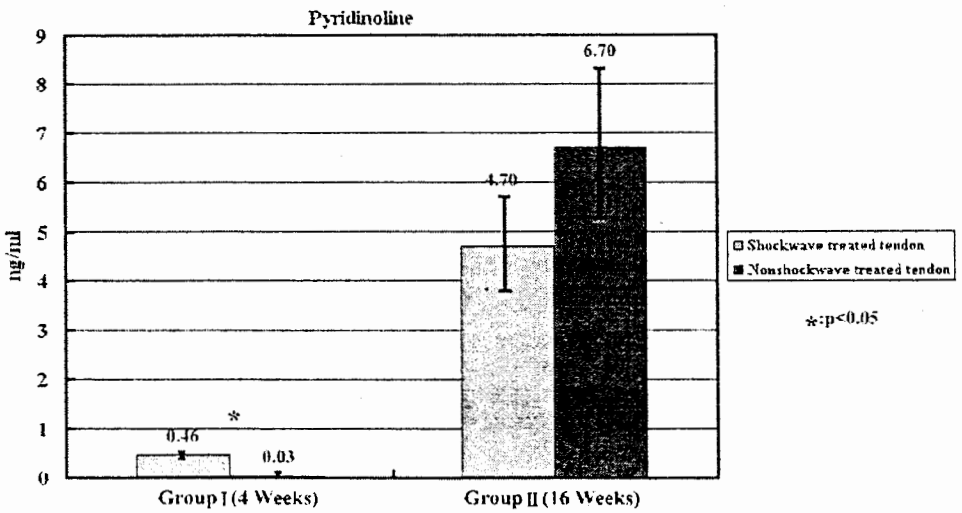

Fig. 7. The pyridinoline crosslink concentration: 10 times greater concentration of the SWT tendon at 4 week was demonstrated. However, higher concentration was revealed at control group at 16 weeks.

turnover can progress after this period. In the present study, the results of hydroxyproline concentration revealed more collagen synthesis was deposited in the SWT group, and the amount of collagen increased with healing time. Therefore, SWT might enhance synthesized collagen formation following the repair process. The pyridinoline crosslink residue in some previous reports suggested the collagen maturity, while other reports declared its correlation with material strength [17]. This study found increasing concentration (10 times) of pyridinoline crosslink residue in the SWT tendon at 4th week, implying that it positively impacted the maturation of the newly synthesized collagen, despite the actual mechanism being unclear.

Based on the assessment of the mechanical testing, mid-substance failure was demonstrated, which correlates with the findings of David stone et al. in a collagenase induced tendinopathy model. The ultimate tensile load of soft tissue was associated with collagen content [1,7,20,22,33], larger cross-section area [36], collagen crosslink formation [9] and architectural arrangement of collagen fiber [10]. This study has shown that, for the rabbit patellar tendon, the collagen concentration and collagen architecture correlate well with ultimate tensile load, which was increased by SWT. However, the single abbert data in the collagen crosslink at 16 weeks that disagrees with the ultimate tensile load may result from its partial contribution to the structural properties. The result of mechanical testing significantly validates the effectiveness of SWT.

This study is indeed a preliminary evaluation of the shock-wave therapy in an animal model. The limitation of this work includes that no cross-section area was measured using laser micrometry; therefore, the ultimate load reflects only the structural properties instead of the mechanical properties. Further, transmission electron microscopy was not performed to assess the diameter of the newly synthesized collagen. Meanwhile, individual collagen typing as Type I, III, V, was not performed to assess the content of newly synthesized collagen. Further studies to determine the cross-section area, the diameter of the newly formed collagen and the individual collagen typing, the dose–response relationship as well as the possible optimal dosage will be undertaken to further substantiate this therapeutic effect. And the exploration of the molecular mechanism responsible for the positive change in the SWT healing tendon will also be planned in the further investigation.

Conclusion

SWT may enhance the healing of collagenase induced tendinopathy, helping achieve superior ultimate tensile load and higher collagen concentration. Consequently, the long term effects of SWT on tendon healing deserve further investigation.

Acknowledgements

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