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Original Article

Sequential inflammation model for Achilles tendinopathy by elastin degradation with treadmill exercise

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ABSTRACT

Background & objective: Tendinopathy is a tendon disease with abnormal mechanical loading to induce chronic repetitive injury. However, lack of a comparable animal model to demonstrate clinical progressions has hindered the understanding of anatomical and pathological changes. The major extracellular matrix (ECM) in the tendon consists of abundant type I collagen (COL) and minimal amount of elastin (ELN).

Methods: To study the ECM breakdown and inflammation, rat Achilles tendon was harvested and ex vivo incubated with specific enzymes of elastase (ELNase) or collagenase (COLase).

Results: The ELNase broke down ELN, loosened the tendon structure, and increased the COL composition. Increases in cyclooxygenase-2 expression levels in tenocytes were revealed to induce inflammation with either ELNase or COLase. However, incubation of COLase for 12 hours severely digested the tendon. To create a proper ELN degradation in rats, the present study used high-frequency ultrasound to guide the injection of ELNase at the paratendon tissue of the Achilles tendon. The effect of mechanically triggered inflammatory responses was investigated by applying treadmill exercise (15 m/min for 20 min per day). After ELNase injection for 14 and 28 days, a significant loss of ELN was observed, and exercise further facilitated the pathological transition of COL. The dynamics of inflammatory cell recruitments was revealed by specific staining of CD-11b (neutrophils) and CD-68 (macrophage) after in vivo injection of ELNase or COLase for 1, 3, 7, 14, and 28 days. The combination of ELNase and exercise caused early recruitment of neutrophil on day 1 and sequential expression of macrophage on day 7 in peritendinous tissue.

Conclusion: These results suggested that ELN degradation with repetitive mechanical loading may present a suitable model for the pathogenesis of tendinopathy.

The Translational potential of this article: This discover the role of elastin degradation in tendinopathy and the interaction of exercise in the histological changes. The established the pathological model mimicking the pathogenesis to the human disease by injecting the elastase using ultrasound guidance and then applying treadmill exercise. The loss of elastin and change of collagen composition in clinical tendinopathy samples were observed in the rats. In addition, the sequential inflammation cascades were observed in the histological outcomes with combination of elastase injection and treadmill exercise. Thus, this model may be used to test the clinical treatment of tendinopathy in different stages.

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Introduction

Tendinopathy causes chronic pain with several pathological processes that may limit the movement during functional activities or increase the risk of tendon rupture, with unclear mechanism [1]. Tendon, a dense connective tissue that contains abundant extracellular matrix (ECM), connects the muscle to bone, thereby assisting the movements of the skeleton and body segments [2]. The collagen fascicle is wrapped by endotenon to separate each fascicle [3]. Epitenon is the outermost layer of the tendon that consists of a smooth surface and vessels for supplying nutrition to the tendon. A loose connective tissue outside the epitenon, called paratenon, lubricates and guides the tendon during movements. In the early phase of tendon healing (3–7 days after injury), inflammations are initiated by neutrophils [4]. The following steps to clean up tissue debris and release inflammatory cytokines are led by monocytes and macrophages. During the middle phase (5–21 days after injury), tenocytes produce ECM to repair the injured tendon. The tendon inflammation during the healing period is characterised as tendinitis. The usage of corticosteroids and nonsteroidal anti-inflammatory drugs provided short-term pain relief but did not demonstrate long-term efficacy. Some other treatment approaches were also suggested, such as prolotherapy, platelet-rich plasma injections, or topical nitric oxide patches.

Repetitive injuries or abnormal loading to prolong the healing process induces chronic tendinopathy [5]. Compared with the surrounding tissue, the blood supply within the tendon is still relatively poor as a result of difficulty in healing after tendon injury. Type I collagen (COL) is the major ECM component of the tendon [6]. The elastin (ELN) is also found in the endotenon and interfascicle space adjacent to the mature tenocytes [7–9]. Tendinopathy is also a major clinical problem in sports medicine and is often difficult to treat [10]. Overuse and overload are believed to be the major factors of failure of the healing processes in the tendon [5]. The impaired healing response might be due to the inappropriate mechanical microenvironment in the injured tendon [10]. The inflammatory mechanism of tendinopathy may involve the increases of cell apoptosis [11], inducible nitric oxide synthase, hypoxia [12], and tumour necrosis factor- α [13]. However, the dynamics of tendon structure and the changes of ECM composition during pathogenesis are still unknown.

Collagenase (COLase) injection is the most popular animal model to induce acute tendinopathy [14], but tendinopathy usually occurs as a chronic pathological process. The elastase (ELNase) is an ECM digestion enzyme that degrades ELN and its cross-link to COL. The amount of ELN affects the mechanical properties of connective tissues. ELN was discovered in tendon [6,8], and ELNase-positive cells were increased in human specimens with grades II and III tendinopathy [15]. ELN was also found in the interfascicular matrix of energy-storing tendons, such as superficial digital flexor tendon, and becomes disorganised with ageing [16]. ELNase can be released by neutrophils and macrophages during inflammatory responses [17]. ELNase was applied in an animal model to induce abdominal aortic aneurysm by degrading the ELN in the arterial wall, which leads to failure in the bearing of blood pressure [18]. The ELNase-treated tendons exhibit significant structural and compositional changes including crimp undulation and release of glycosaminoglycans. ELNase treatments also affect the mechanical properties of tendon, including the ultimate tensile strength and failure strain [6,19]. However, the pathogenesis and involvement of mechanical loading with ELNase are still unknown. We hypothesised that the absence of ELN and the combination of mechanical loading trigger the inflammatory responses in paratenon tissue to induce chronic tendinopathy. In the present study, the *ex vivo* incubation of ELNase in tendon and *in vivo* ELNase injection combined with exercise in rats demonstrated the pathogenesis of tendinopathy via ELN degradation and inflammatory induction in paratenon tissue.

Methods

Ex vivo ECM degradation

The Sprague–Dawley rats (7-weeks-old) were sacrificed by overdose with CO₂, and the Achilles tendon was dissected from both legs and incubated in normal saline to prevent tissue dry out. To understand the ECM degradation in tendon structures, the fresh, isolated Achilles tendon samples were immediately incubated with ELNase (1 U/mL; Sigma-Aldrich, Inc.) or COLase (COLase type I, Cat# 17018029, 5 mg/mL; Invitrogen, Thermo Fisher Scientific Co.) in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) at 37 °C for 12 h. After incubating with ECM enzymes, the tissues were carefully rinsed twice with phosphate-buffered saline (PBS) and then collected for the following histological assessments.

Animal model of tendinopathy

Sprague–Dawley rats (7-weeks-old, total 148 rats) were provided by the Animal Centre at National Cheng Kung University. The experimental procedures were approved by the Institutional Animal Care and Use Committee. To study the induction of tendinopathy by different ECM enzymes, ELNase (1 U/mL; Sigma) or COLase (10 mg/mL; Invitrogen) was injected in the paratenon area of the Achilles tendon in rats using a 31-G needle, with image guidance using high-frequency ultrasound (HFU) [20,21]. HFU that consisted of a 50-MHz transducer to provide sub-tissue image resolution for identifying the detailed structure in the posterior compartment of rat legs was used. Briefly, the rats were anaesthetised by using isoflurane (Panion & BF Biotech Inc., Taiwan Healthcare Co.), with the hind limbs immersed in a water bath. HFU images were obtained by scanning along the longitudinal axis of the Achilles tendon to acquire the full length (about 14 mm) of the tendon structure. The needle tip was observed under HFU during injection, and ECM enzymes were applied around the paratenon. The injection of equal volume of PBS was used as the sham control group.

To induce repetitive mechanical loading in the rats, treadmill exercise (model T510; Diagnostic & Research Instruments Co., Ltd., Taoyuan, Taiwan) was applied daily at a speed of 15 m/min for 20 min after injection. After ELNase or COLase injection for 1, 3, 7, 14, and 28 days (D1, D3, D7, D14, and D28, respectively), the rats with or without exercise were sacrificed by overdose with carbon dioxide (CO₂) to harvest the whole posterior compartments of the legs. Anatomical changes in the subcutaneous, peritendinous, and tendon structures were observed by applying specific histological staining to reveal the structural changes and inflammatory responses at different healing time points.

Measurement of mRNA

After *ex vivo* incubation of ECM enzymes, the tendon tissue was rinsed twice with PBS and then kept in Trizol (Invitrogen) to prevent mRNA degradation. The tissue was cut into 1 mm × 1 mm pieces and repeatedly freeze-thawed in liquid nitrogen 3 times. Then, the tissue was homogenised using a tissue homogeniser to isolate the mRNA. The gene expression levels of COL and ELN were measured by quantitative polymerase chain reaction (qPCR) after reverse transcribing into cDNA using Super Script III (Invitrogen) [22]. The primers for COL are as follows—forward sequence: TGGTCCTCAAGGTTTCCAAG and reverse sequence: CCTCTGTGTCCCTTCATTCC. Quantification of the gene expressions was assessed by fold changes normalised to the DMEM control and the house-keeping gene GAPDH.

Histological assessments

Several histological stainings were performed to observe the tissue morphology and protein expressions during the tendon pathogenic

process. The clinical tendinopathy tissue was obtained from the tendon of biceps brachii in a patient during surgery with informed consent and with the approval of the Review Board of National Cheng Kung University Hospital. After sample collection, the tissues were rinsed twice with PBS and fixed with 4% paraformaldehyde (Sigma) at 4 °C for 48 h. Haematoxylin–eosin (H&E) staining was performed to reveal the histological structure after the samples were dehydrated, embedded in paraffin, and then sectioned at a 5- μ m thickness [20]. The tissue sections were stained with haematoxylin (Leica Biosystems, Leica Microsystems Inc., USA) for 7 min and then with eosin (Leica) for 5 s. The ELN in the tendon was visualised in black colour using the Verhoeff elastic stain kit (Sigma). Masson's trichrome staining was performed to present the COL composition for original high-tensile COL (red colour) and newly synthesised low-tensile COL (blue colour) [23] in accordance with the staining protocols of the commercial Masson's trichrome kit (Sigma). Specific CD

markers were used in immunohistochemistry (IHC) staining to identify the different inflammatory cells exhibited at different time points. Briefly, the paraffin sections were dewaxed, rehydrated, activated by treating with 3% H₂O₂, and then blocked the nonspecific binding by 5% bovine serum albumin (Sigma) in 0.02% Tris-buffered saline with Tween 20 (Sigma). The sectioned tissues were then incubated with primary antibodies at 4 °C overnight. The CD-11b antibody (1:200; Abcam, Cambridge, UK) was applied to identify the neutrophils, and CD-68 (1:200; Abcam) was used to label the macrophages. The samples labelled with specific primary antibodies were incubated with secondary antibodies (1:500; Abcam) and then visualised by using an AB reagent (Vector, Burlingame, CA, USA) to couple with 3,3'-diaminobenzidin. Tissue sections were counterstained with haematoxylin and then mounted by using a mounting medium (Leica). The expressions of different inflammatory cells and histological images were acquired by

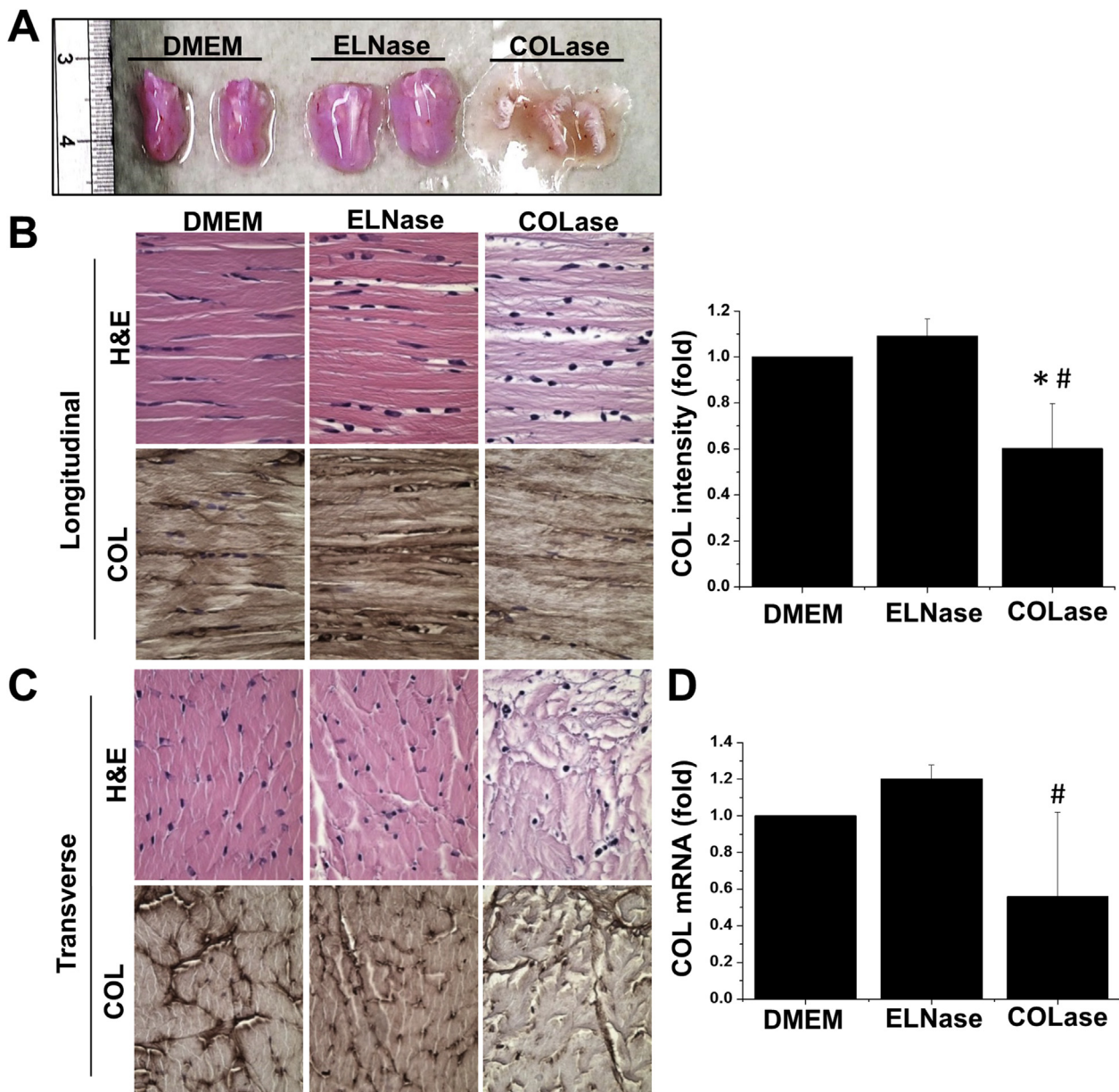


Figure 1. (A) Gross images of *ex vivo* incubation of the Achilles tendon in extracellular matrix (ECM) of elastase (ELNase) or collagenase (COLase) for 12 h. (B) Both the haematoxylin–eosin (H&E) and immunohistochemistry (IHC) stainings of collagen (COL) show severe loss of tendon integrity in the longitudinal tissue sections after COLase incubation (*significant difference from DMEM, $p < 0.05$; #significant difference from ELNase, $p < 0.05$). By contrast, ELNase mildly loosens the tendon and increases the COL expression. (C) Disarrangement of tendon fascicle when treated with COLase is further confirmed in the cross-sectioned tissues. (D) The qPCR showed the tendency of decreased COL expression after COLase incubation (#significant difference from ELNase, $p < 0.05$). DMEM = Dulbecco's Modified Eagle's Medium; qPCR = quantitative polymerase chain reaction.

tissue scanning microscopy (BX51, Olympus, Olympus Co., Japan). In Masson's trichrome images, the composition of high- and low (blue colour)-tensile COL is derived by separating the red and blue colours from the RGB (Red-Blue-Green) image and quantifying the mean intensity in the same visual field. The whole tissue of the posterior leg was scanned to demonstrate the gross changes of histological structures in longitudinal tissue sections. All histological images were randomly acquired for at least 3 repetitions and enlarged to represent a specified anatomical region in each sample.

Frozen section samples were prepared for immunofluorescence (IF) staining to confirm the ECM degradation and tenocyte responses. After dehydration by 30% (*w/v*) sucrose in 0.1 M PBS and then embedding in the frozen tissue matrix (Tissue Freezing Medium, Leica), the *ex vivo* tissue samples were sectioned into 20- μ m-thick slices. Specific primary antibodies against ELN (1:250; Abcam), COL (1:250; Abcam), cyclooxygenase-2 (COX-2, 1:200; Cayman), and tenascin-C (TN-C, 1:100; Abcam) were used to observe the loss of specific ECM within tendon tissue and the induction of inflammation in tenocytes. The primary antibodies were labelled by fluorescence-labelled secondary antibodies (1:200; Invitrogen) and 4',6-diamidino-2-phenylindole, (DAPI, 1:1000;

Invitrogen) for the cell nucleus. Fluorescent signals were observed at excitation–emission wavelengths of 470–505 nm and 596–615 nm by using a $\times 20$ objective lens with a tissue scanning fluorescence microscope (BX51; Olympus).

Statistical analysis

The experiments were repeated at least three times, and data were expressed as mean \pm standard deviation. Statistical analysis was performed by using one-way analysis of variance and the Scheffe post hoc test. A *p* value < 0.05 was considered statistically significant.

Results

Ex vivo ECM degradation–induced tenocyte inflammation

To further understand the influence of ECM degradation by enzyme digestion and the induction of inflammatory responses in tenocytes, Achilles tendons were harvested from the age-matched rats. After *ex vivo* incubation of the tendon in ELNase or COLase for 12 h, the gross image

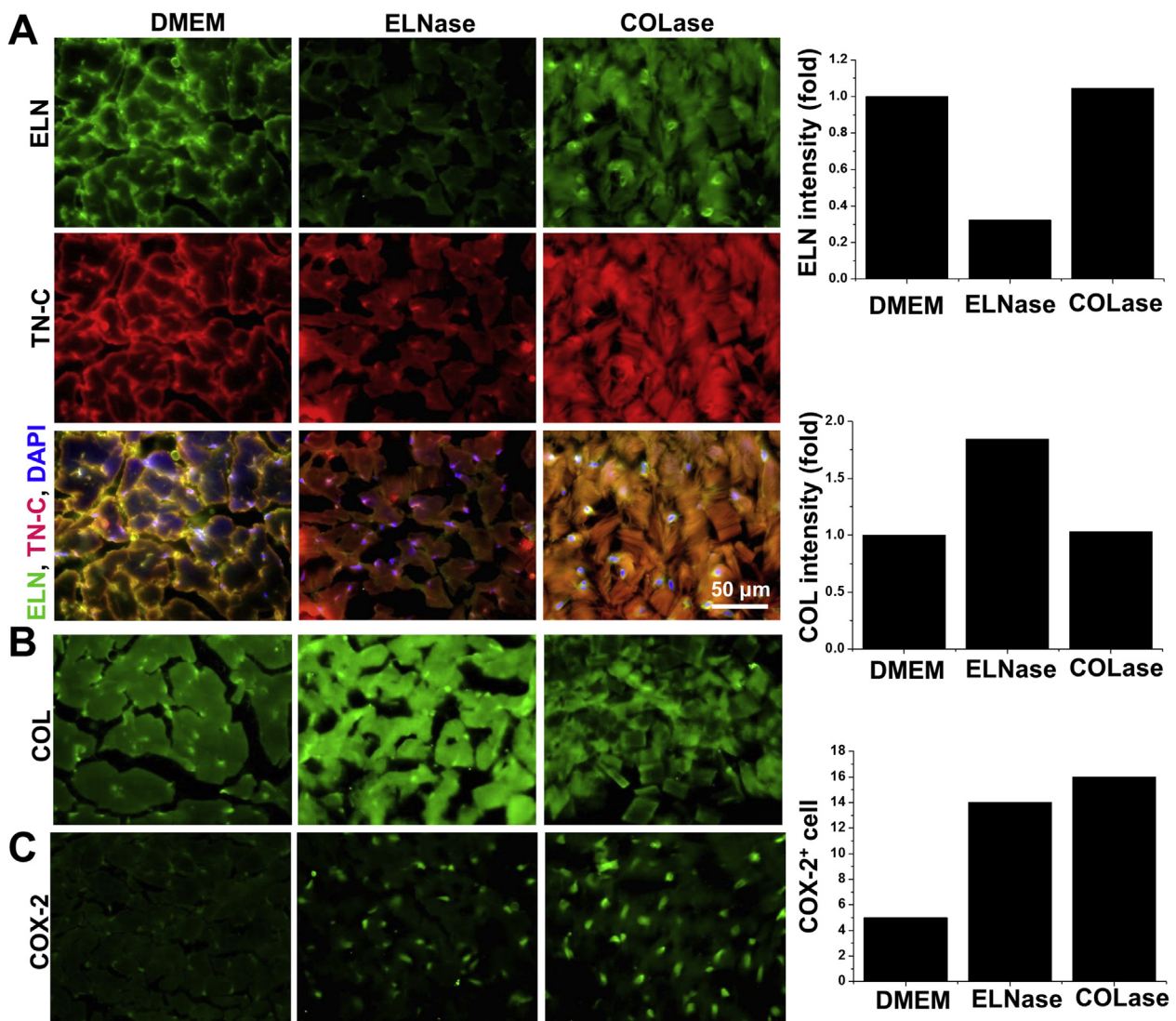


Figure 2. (A) The expression of elastin (ELN, green) colocalized with the tenocytes as labelled by tenascin-C (TN-C, red) in normal tendon (incubated in DMEM). ELNase specifically degrades ELN but does not disrupt the fascicle, as illustrated in the TN-C staining. However, COLase caused severe diffusion of the ELN and TN-C expressions. (B) The increase in COL production during ELNase incubation is confirmed by specific immunofluorescence staining of COL. (C) Both ELNase and COLase increased the cyclooxygenase-2 (COX-2) expressions to induce the inflammation in tenocytes. Scale bar = 50 μ m. DMEM = Dulbecco's Modified Eagle's Medium.

was obtained, which showed obvious changes in tendon morphologies, especially when incubated with COLase (Fig. 1A). The detailed histological structure of the tendon was observed by H&E staining in longitudinal (Fig. 1B) or transverse sections (Fig. 1C). ELNase slightly loosened the tendon structure, but COLase resulted in severe degradation of tissue integrity. The IHC staining of COL further confirmed the tendon disruption with COLase treatment, especially observed in the transverse sections (Fig. 1C, COL staining). We also measured the mRNA expression levels of COL after ELNase and COLase incubation (Fig. 1D). The incubation of COLase showed the tendency to inhibit the COL expression but did not achieve significant difference owing to high variation and difficulty in isolating tenocytes from tendon tissue.

The detailed arrangement of ELN was detected through IF staining of the frozen transverse-sectioned tendon (Fig. 2A). In the normal tendon incubated with DMEM, ELN was observed at the outer layer of tendon fascicles (green). The tenocyte was identified by tenascin-C staining (TN-C, red) and found adjacent to the ELN fibres (yellow colour in the merged image). ELN in the tendon was also digested by *ex vivo* incubation of ELNase, as indicated by decreasing ELN (green). The degradation of ELN by ELNase did not affect the arrangement of TN-C (intact red colour in ELNase treatment). Instead, the application of COLase caused the unconfined tendon fascicles and diffused staining in both ELN and TN-C. It is interesting that both the IHC and IF stainings showed increases in COL when treated with ELNase, suggesting that ELNase may induce COL synthesis after ELN degradation (Fig. 2B). The increase of inflammation in tenocytes was demonstrated by COX-2 staining with ELNase and COLase treatments (Fig. 2C). This *ex vivo* evidence discloses the interaction of ECM structure when applying different ECM enzymes to tendon tissue. The inflammation of tenocytes was induced when disrupting either ELN or COL by ELNase or COLase, respectively.

Exhibition of tendinopathy pattern in rats with combination of ELNase injection and exercise

To understand the loss of ECM in the pathogenesis of tendinopathy, ECM enzymes were precisely injected in the paratendon tissue of rat Achilles tendon under living-animal image guidance by using a 50-MHz HFU system in the rats with age matching to the *ex vivo* ECM digestion model (Fig. 3A). The needle tip was clearly observed under HFU and can be guided to the paratendon tissue for ELNase or COLase injection,

without touching or injuring the tendon. To demonstrate the high resolution of the HFU system, the rat was immediately sacrificed to perform the histological staining after HFU scanning. High agreements of tissue structures on ultrasonic images (HFU) were observed in the longitudinal sections, and the same histological structures for the posterior compartment of rat legs was shown in H&E images (Fig. 3B). The echo images of the same rats were monitored and acquired by HFU before and after ELNase injection on D1, D3, D7, D14, and D28 with or without exercise (Fig. 3C).

An increased number of ELNase-positive cells in pathological lesion sites in human tendon tissue were discovered in our previous study [15, 24]. To illustrate the histological findings for the pathogenesis of the tendon, changes in ELN and COL expressions in clinical tendinopathy tissue were stained with Verhoeff's and the Masson's trichrome stains, respectively (Fig. 4A). Normal human tendon tissue contains a small amount of ELN (green arrows, Verhoeff's stain) and high-tension COL (red colour, Masson's trichrome stain). The ELN was lost, and COL was turned into blue colour to indicate the remodelling of newly synthesised COL in the pathological site. In the rat model, the gross images of Masson's trichrome staining demonstrated the success of creating a pathological rat model of tendinopathy induced by injecting ELNase into the paratendon tissue for 14 and 28 days, especially with the combination of treadmill exercise (Ex) (Fig. 4B). The gross images of Masson's trichrome staining demonstrated significant COL changes in the posterior compartments of legs. Similar to normal human tendon, the Achilles tendon of healthy rats showed high-tensile COL (red colour) in Masson's trichrome staining (Fig. 4B, zoomed-in image). ELNase increases the new synthesis of low-tension COL (blue colour) after injection for 14 and 28 days. The ELN (green arrows in normal tendon) also exhibited a similar degradation pattern as that in the pathological clinical sample of tendinopathy (Fig. 4B, Verhoeff's staining). By specific extraction and quantification of the red and blue colour on Masson's trichrome images, the decreases in high-tension COL and increases in low-tension COL were observed in the rats after ELNase injection (Fig. 4C). The ratio of COL composition was further derived by dividing the low-tension COL by the high-tension COL (low/high ratio) per image field. As compared with the ELNase injection without exercise for 14 and 28 days, the subject of daily treadmill running further facilitated the changes in COL composition and the transition from high-tension to low-tension COL (Fig. 4D). These results highlighted the importance of mechanical loading to promote

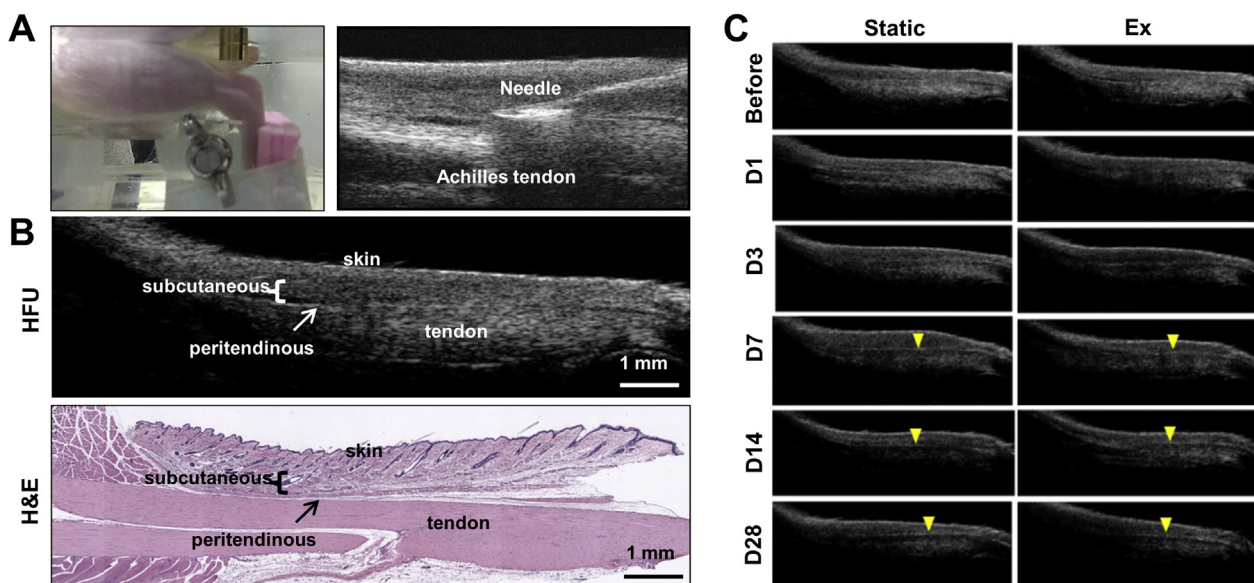


Figure 3. (A) High-frequency ultrasound (HFU) guiding the injection of ECM enzymes to the Achilles tendon of rats. (B) HFU provides subtissue resolution to detect detailed histological structures, as illustrated by the H&E staining in the same rat. (C) The pathological progresses in echo image dynamics after ELNase injection with or without treadmill exercise (Ex) were also monitored by using HFU. H&E = haematoxylin-eosin.

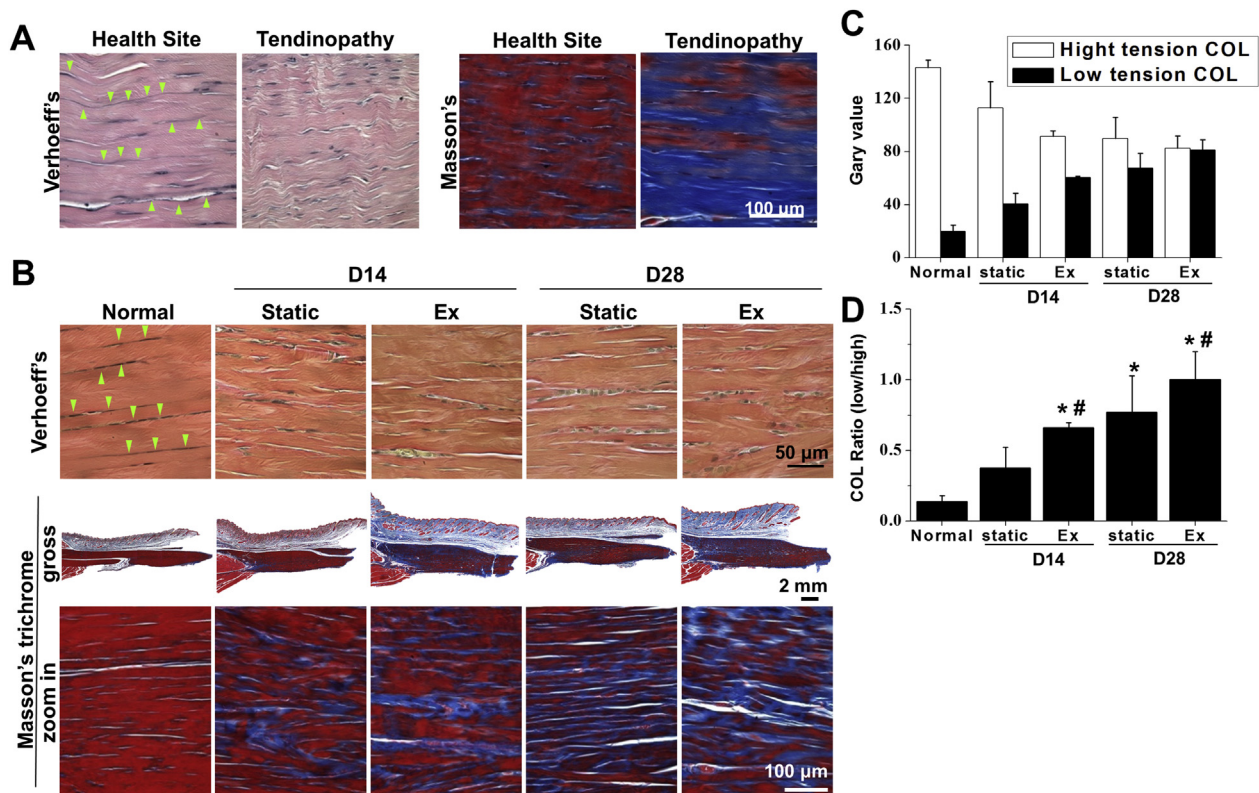


Figure 4. (A) Pathological changes in ELN and COL composition in human tendon tissue with tendinopathy as shown in Masson's trichrome and Verhoeff's stainings. (B) After the ELNase injection for 14 (D14) and 28 (D28) days, significant loss of ELN was observed by Verhoeff's staining (green arrows). Masson's trichrome staining demonstrated the compositional changes of high (red) and low (blue) tensile COL in the Achilles tendon with or without Ex. (C) The red and blue colours are separated to indicate the high- and low-tensile COL. (D) The ratio of COL (low/high COL) was significantly increased in rats after ELNase injections for 28 days or the combination of ELNase and Ex for 14 and 28 days (*significant difference from normal tendon, $p < 0.05$). The exercise facilitated the pathological transition of COL after ELNase digestion (#significant difference from normal tendon, $p < 0.05$).

tendon pathogenesis after ELN degradation.

Dynamics of inflammatory cell infiltration during tendon pathogenesis

To understand the inflammatory cell recruitment during ECM degradation, the rats were injected with ELNase or COLase by using the HFU guidance system as aforementioned. The influence of mechanical loading during tendon healing processes was studied by assigning daily treadmill exercise. The H&E staining demonstrated the increases of inflammatory cells in paratendon tissue after ECM enzyme injection (Fig. 5A). The COLase injections more significantly increased the inflammatory cells than ELNase did at all different time points of D1, D3, D7, D14, and D28 (Supplementary Fig. 1A). The application of exercise in both ELNase and COLase also has the tendency to increase the number of inflammatory cells at different time points. The specific stainings of neutrophils and macrophages by CD-11b and CD-68 validated the dynamics of inflammatory cell infiltration. The neutrophils played a major role in the accumulation around the paratendon tissue to induce inflammation at the early stage (D1 and D3). The CD-11b staining showed the application of exercise further boosted the number of neutrophils (red arrows) in both ELNase and COLase groups (Fig. 5B and quantification in Supplementary Fig. 1B). Unlike the prolonged inflammation in COLase-injected rats, the combination of ELNase and exercise transiently increased the CD-11b-positive cells at D1 and then decreased at D3. The quantification results showed significant difference between static and exercise of ELNase injection at D1, but not at D3 (Supplementary Fig. 1B). We further used the CD-68 staining to observe the prolonged expression profiles of macrophage at D1, D3, D7, and D14 (Fig. 5C). Macrophages were also expressed at the early phase (D1) in the ELNase and exercise combination (ELNase + Ex) (Fig. 5C and

quantification in Supplementary Fig. 1C). The addition of exercise in the ELNase-injected rats increased the number of macrophages at D3 and D7 (Fig. 5C, red arrows). Regardless of exercise, the COLase injection showed increases of inflammatory cells. Taken together, this *in vivo* evidence demonstrated a successful animal model by combining ELNase and exercise to present the dynamic infiltrations of neutrophils at the early phase and then switched to macrophages at the middle phase of tendon pathogenesis.

Discussion

The importance of ELN in the tendon was discovered by conducting both *in vivo* and *ex vivo* experiments in the present study. The parallel alignment of COL fascicles and other composition of ECM is required to form sufficient tension-bearing structure for the force to transmit from muscle to bone [8,25]. The distribution of ELN in the intrafascicle tissue was identified in both the present results and previous research studies [15,24]. The location of ELN was first revealed in flexor digitorum longus tendon [8] and recently discovered with higher amount in the anterior cruciate ligament than other knee periarticular tendons and ligaments [7]. The elastic property of ELN helps the tissue to return back to its original length after release from mechanical deformation [26]. ELN may provide the elastic recoil to prevent the damage of nerves and vessels in the tendon [27]. The specific digestion of ECM in *ex vivo* tendon tissue, especially by administrating the ELNase (Figs. 1 and 2), may provide a rapid platform to understand the cellular responses and tissue structural changes in the comprehensive healthy tendon. Although the ELN degradation by ELNase did not severely damage the tendon structure and alignments, the transmission of mechanical stimuli and the mechanical force applied to tenocytes may be changed as implicated from the tissue

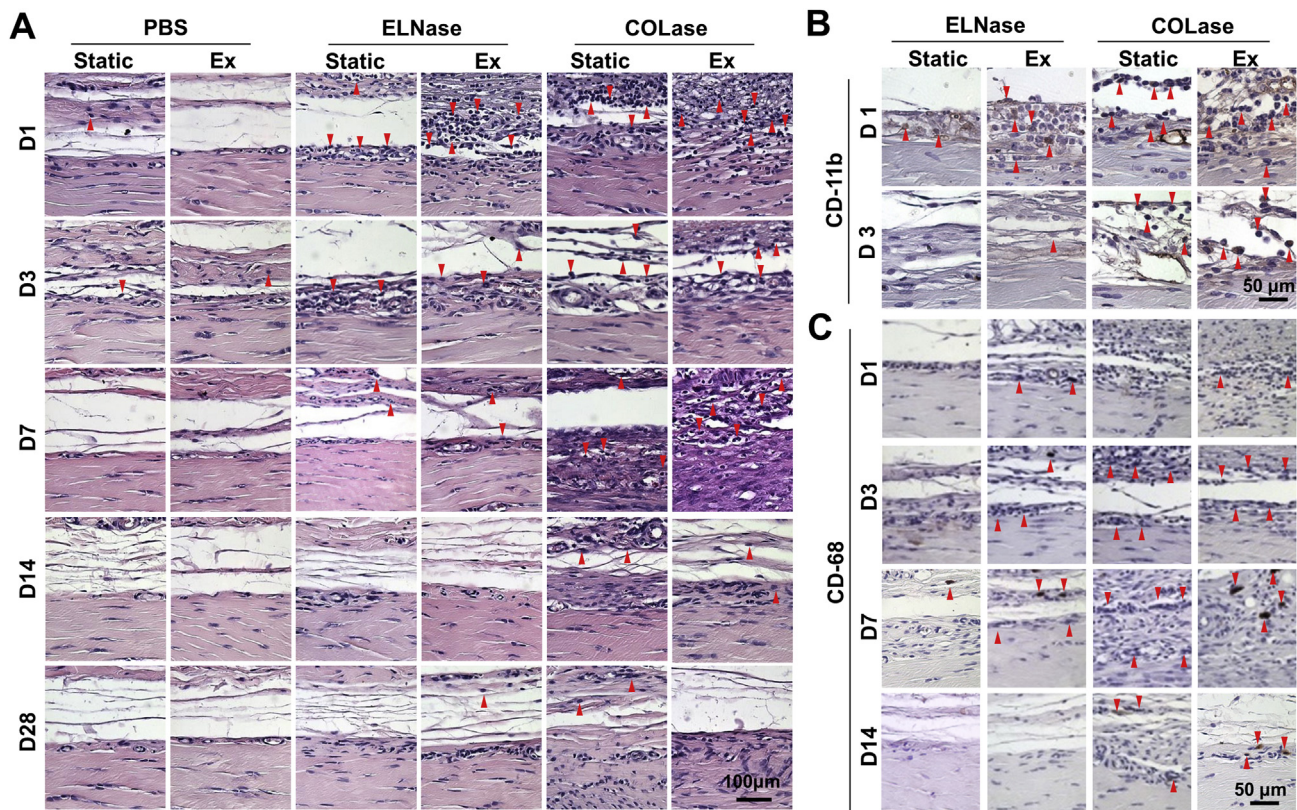


Figure 5. (A) Dynamic histological changes in paratendon tissue can be observed in the H&E staining after different ECM enzyme injections for 1, 3, 7, 14, and 28 days. Exercise promoted the pathological progressions of tendinopathy. (B) The IHC staining of CD-11b shows the increases of neutrophil recruitment at D1 when ELNase was combined with exercise. COLase injections severely damaged the tendon structure and induced large amounts of inflammatory cell accumulation at different time points. (C) The combination of ELNase and exercise shows prolonged inflammation that may have contributed by increasing the number of CD-68–positive macrophages. ECM = extracellular matrix; IHC = immunohistochemistry; H&E = haematoxylin–eosin.

morphology and mechanical property by losing ELN in tendon. We also try to isolate the mRNA from *ex vivo* tissue and perform the quantitative polymerase chain reaction to detect the mRNA expression levels of tenocytes during COLase and ELNase incubation. However, the variations are high and difficult to isolate good quality of RNA from dense COL tissue in tendon. The potential alternative approach may use *in situ* hybridisation to observe the gene expression of tenocytes in the future. However, the tenocytes and ELN fibres showed three-dimensional consistent connections with the long axis of tendon [9]. The inflammatory responses in tenocytes after ELN degradation (Fig. 2) may also provide a good *ex vivo* platform to test the potential treatment strategies, such as pharmacological drugs, platelet-rich plasma (PRP), and physical therapies, for tendinopathy prevention [10].

There were many inflammatory molecular targets and cytokines identified in overuse tendinopathy [5,28]. Other inflammatory cells may also participate during the pathogenesis of tendon. Lubrication of paratendon tissue for tendon gliding and sliding was disturbed after injury [29] and gradually results in tendinopathy [30]. The inflammation of tenocytes induced by ECM enzymes was triggered by COX-2 signals (Fig. 2C). The pathological process of the tendon in the clinical samples showed increases in prostaglandin E2 levels [31]. The loss of tension in the tendon also increased the inflammatory genes in the tendon [32]. Prostaglandin E2 is synthesised by prostaglandin-endoperoxide synthase, which can be regulated by COX, particularly COX-2 [33]. We also performed IF staining of other cytokines such as interleukin-1 β and tumour necrosis factor- α , but did not observe significant changes after *ex vivo* incubation under static condition with ELNase or COLase for 12 h (data not shown). The addition of mechanical stimuli by daily exercise caused the malfunction of tissue remodelling and led to pathological progression

to increase the messenger RNA in MMP1, COX-2, and interleukin-1 β genes [34]. The present study demonstrated the neutrophil expression in the rats with only ELNase injection showed a mild immune response, which usually declines to basal level after 48 h (Fig. 5A). On the other hand, the combination of ELNase with exercise (ELNase + Ex) in a rat model triggered the pathogenesis in regards to the anatomical and histological changes in the Achilles tendon. Thus, prevention of mechanical stimuli after ELN degradation may be required for tendon tissue to complete the healing process.

The direct intratendinous injection of COLase is the common animal model for tendinopathy. However, there are several concerns about COLase injection [35]. First, direct injection inside the tendon can damage the tendon structure. Tendon calcification was simply induced at the location of needle penetration. Second, the COLase degraded a large amount of COL in the tendon, triggering a huge and quick neutrophil infiltration between 1 and 3 days and switched to macrophages after COLase injection for 3 days [14]. The need for alternative approaches to study tendinopathy from direct COLase injection has been proposed [36]. Both of our *ex vivo* and *in vivo* models confirmed the rapid tendon digestion as early as 12 h after COLase application (Fig. 1A) and prolonged inflammatory responses (Fig. 5). In clinic settings, tendinopathy is a chronic pathological process with positive staining of tissue inflammation but rare inflammatory cell infiltration [37]. Macrophages were only identified in the early pathogenesis of paratendon tissue in clinical patients [4]. Although we modified the COLase injection at paratendon tissue in rats, COLase still caused severe tendon damage and large amount of inflammatory cell clustering (Fig. 5). Severe neutrophil infiltration may induce excessive tissue inflammation and further damage the injured tissue [38]. Therefore, COLase should be used with care in a

pathological model of tendon injury.

Ultrasonography is usually used in the diagnosis of the pathological changes of soft tissue in clinical practice, but ultrasonography systems usually have a low frequency (5–15 MHz), which can only provide global changes in musculoskeletal structures. HFU uses a transducer with a frequency higher than 15 MHz to provide better image resolution for detecting the remodelling of detailed tissue structures [39]. HFU was used to detect the pathological changes of adenomyomatosis in the gallbladder and showed similar image sensitivity and high accuracy as compared with magnetic resonance cholangiopancreatography [40]. Tissue inflammation in mice after fracture injury was monitored by HFU, and high agreement with ultrasonic signals was shown [20]. In the present study, we used HFU to guide the injection of ECM enzyme without damaging the tendon tissue (Fig. 3A), which showed high accuracy in terms of tissue resolution for rat Achilles tendon (Fig. 3B). Using the HFU to provide a dynamic monitoring system for the inflammation and histological remodelling was very useful in muscle [41] and may correlate the echo image with inflammatory responses for tendon in future studies.

Taken together, this study demonstrated the tissue responses in different ECM enzymes by *ex vivo* incubation of COLase or ELNase. The ELNase broke down the elastin and loosened the tendon structure, whereas COLase severely digested the tendon. Good agreements of the pathological model for mimicking the pathogenesis to human tendinopathy were established by injecting the ELNase with ultrasound guidance and then applying treadmill exercise. The sequential inflammation cascades were discovered in the histological outcomes in rats to demonstrate the mechanical induction of tendinopathy after ELN degradation.

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Conflicts of Interest Statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical approval for animals

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and approved by the IACUC of National Cheng Kung University.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jot.2020.03.004>.

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