Contrast-enhanced Sonography for the Evaluation of Neovascularization in Tendinopathic Tissues

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Objective: The purpose of this study was to evaluate the usefulness of contrast-enhanced ultrasound for detecting neovascularization in tendinopathic tissues using an animal model. Methods: Doses of 100 μL and 50 μL collagenase were injected into the left and right Achilles tendons of six rabbits. Power Doppler ultrasonography was used before and after the administration of a contrast agent to evaluate tendon perfusion on Day 0 and Day 14 after the collagenase injections. The number of color pixels within the region of interest represented the amount of vascularity. Results: The tendon cross-sectional area was similar between Day 0 and Day 14, and comparisons between both tendons also appeared to be insignificantly different between these two times. Noncontrast power Doppler ultrasonography failed to detect more color pixels in the tendons after collagenase injections in comparison with baseline, whereas a higher peak signal intensity was identified using contrast enhancement in the collagenase-treated tendons. In addition, differences in vascularity between the tendons that received different amounts of collagenase were clearly revealed after the administration of the contrast agents. Conclusion: The present study demonstrates that contrast-enhanced ultrasound is superior to the noncontrast ultrasound for measuring hypervascularity in Achilles tendinopathy in a rabbit model.

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Introduction

The Achilles tendon, the largest and strongest tendon in the human body, is derived from the soleus and gastrocnemius muscles and inserts posteriorly to the calcaneus. Achilles tendinopathy commonly occurs in competitive and recreational athletes [1]. Observational data suggest that competitive athletes have a lifetime incidence of Achilles tendinopathy of up to 24% [2]. The etiology of Achilles tendinopathy is multifactorial. Intrinsic factors include impaired tendon perfusion, older age, female sex, heavy weight, taller heel height, and ankle instability [3], whereas extrinsic causes include previous lower limb injury and vigorous physical activities [4]. The Achilles tendon has a hypovascular zone of 4–6 cm caudal to its calcaneal insertion site, where tendon degeneration and tears commonly occur [5, 6]. In the histology of Achilles tendinopathy, interfibrillar glycosaminoglycan proliferation and collagen fiber disarrangement are commonly present, but not with inflammatory cell infiltration [1]. Some previous studies have suggested that neovascularization contributes to painful Achilles tendinopathy. Previous studies have described sclerosing neovessels in order to relieve pain in most patients [7]. One study found that higher levels of glutamate receptors and vascular endothelial growth factors exist in the vicinity of the nerves in patients with painful Achilles tendinopathy, suggesting that the increase in nociceptive nerve fibers and neovascularization occurs in Achilles tendinopathy [8].

Several imaging tools have been used to evaluate Achilles tendinopathy, including plain film, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound. Plain film is inexpensive and can be used to screen for the existence of bony abnormalities. The CT scan demonstrates a higher sensitivity for detecting calcifications and is useful for planning surgeries to treat complicated foot and ankle fractures. However, both imaging modalities fail to delineate tendon morphology and also result in increased radiation exposure. MRI depicts multiplanar anatomy at a high resolution, but it is sensitive to changes in water content in tendon tissues. Ultrasound, in comparison with MRI, is less costly, provides an easy dynamic evaluation, and allows real-time comparisons with painful sites. Therapeutic injection can also be administered under ultrasonographic guidance [5]. Grey-scale sonography has also been used to reveal that normal Achilles tendons are composed of parallel arrays of fibrillar lines in longitudinal planes and appear round-to-ovoid along transverse planes. Focal hypoechoic changes, such as the thickening of the Achilles tendon, are considered pathological findings. Because neovascularization is often associated with painful Achilles tendinopathy, we can use color or power Doppler to detect blood flow in a tendon. A previous study demonstrated neovascularization in all symptomatic tendons [9]. Power Doppler is superior to color Doppler because of its sensitivity to blood flow, and it is relatively independent of the angle of incidence. Nevertheless, power Doppler is still inadequate for recognizing the vascularity of hypovascular structures, such as tendons [7, 10]. Because neovascularization is highly correlated to painful Achilles tendinopathy, this study sought to determine whether contrast-enhanced ultrasound is more effective than traditional power Doppler for investigating hypervascularity in Achilles tendinopathy.

Materials and methods

Animal model

We examined six male New Zealand rabbits (mean weight: 3.2 kg; range: 3.1–3.4 kg) and both Achilles tendons of each rabbit. All of the rabbits were anesthetized with isoflurane, and the hairs of the hind feet were shaved. According to our pilot study, a subcutaneous injection of 100 μL (10 mg/mL) collagenase (Sigma-Aldrich, St. Louis, MO, USA) is adequate for inducing swelling and local erythematous changes over the Achilles tendons, and the gross appearance typically returns to baseline approximately 2 weeks later. We injected 100 μL and 50 μL of collagenase into the left and right leg of each rabbit, respectively, at 1.5 cm proximal to the bony insertions of the Achilles tendons. We performed the ultrasonographic measurement on Day 0 before collagenase injection and on Day 14 after injection. This project was approved by the animal ethics committee of National Taiwan University Hospital.

Ultrasound imaging and protocol

We used an ultrasound machine (Acuson S2000 system, Siemens, Munich, Germany) equipped with a multifrequency transducer (14L5, 6–12 MHz in 2D mode; 5.5–7.5 MHz in Doppler mode) to perform the examinations. Power Doppler was employed to investigate tendon vascularity and was adjusted to the highest sensitivity that did not result in artifacts beneath the bony cortex before administering contrast agents. In order to reduce the blooming effect, the color gain was reduced to 0 dB while contrast agents were being introduced. A video retriever was externally connected to the ultrasound machine for continuous recording. The ultrasound examinations were performed on Day 0 before collagenase injection and on Day 14 after injection. The rabbits were settled in the prone position with the lower limbs immersed in a water tank, and the ultrasound probe was fixed along the long axis of the Achilles tendons (Fig. 1). Grey-color imaging was used to locate the midpoint of the calcaneus bone in the transverse plane, and then the probe was turned parallel to the long axis of the Achilles tendons. The right vertical border of the scan view was adjusted to pass through the caudal edge of the calcaneus bone in order to assure that the same segment was investigated at each time point. We first captured an image in traditional power Doppler mode and then performed contrast-enhanced imaging; this was video recorded for 5 minutes. The total dose of the contrast agent was approximately 0.32 mL (6.4 × 10^8 microbubbles), and this was injected through each rabbit’s large veins in the ears, followed by 1.5 mL of a normal saline flush. The video was analyzed off-line, frame-by-frame, and a freely downloaded software, KM player (KMPlayer_EN_3.0.0.1442) was used to retrieve the appropriate images. We selected...
three mostly enhanced pictures and calculated their averages of pixels within the region of interest for the outcome analysis. We measured color pixels within the entire Achilles tendon to represent the amount of vascularity using Image J software (National Institutes of Health, Bethesda, MD, USA). After adjusting the threshold of the color histogram, the investigator was able to extract the area that was enhanced in power Doppler mode [11]. Then, the “analyze particles” function was used to calculate the target areas of the pixels (Fig. 2).

**Statistics analysis**

We used SPSS Statistics software (version 12; SPSS, IBM Corporation, Somer, NY, USA) to perform the analyses. The statistical significance was set at a \( p \) value \(<0.05\). The difference between Day 0 and Day 14 after the collagenase injection was analyzed using the general linear model with repeated measurements. The Wilcoxon signed-rank test was used to compare data between the tendons that received 100 \( \mu L \) and 50 \( \mu L \) collagenase.

**Results**

The cross-sectional area (CSA), which is correlated with tendon thickness, was not significantly different between Day 0 and Day 14 after the collagenase injection into the bilateral Achilles tendons. Compared with the tendons that received 50 \( \mu L \) collagenase, those injected with 100 \( \mu L \) collagenase did not demonstrate an enlarged CSA. Similar to the CSA comparisons, noncontrast power Doppler ultrasound failed to detect any changes or discrepancies over time between both sides via measurement of the

**Fig. 1** Platform and settings used for sonographic evaluations. The rabbit is settled in the prone position with both hind-feet immersed in a gasless water tank at a constant temperature. The ultrasound probe is fixed along the long axis of the Achilles tendon using a self-made hinge device.

**Fig. 2** Quantitative analysis of the power Doppler signal intensity. (A) First, pictures of the enhanced areas were viewed with the naked eye, then the KM player was then used to retrieve the appropriate images. (B) Second, we adjusted the hue, saturation, and brightness of each selected image using Image J software. Then we extracted the enhanced area from the region of interest. (C) Third, we altered the color threshold and changed the color area to red. (D) Finally, we used the “analyze particles” function to calculate the enhanced areas of the pixels.
number of color pixels. After administering the ultrasound contrast agents, on Day 14 we identified a peak signal intensity (PSI) in both tendons that was higher than their basal values ($p = 0.025$ and 0.009 for the left and right side, respectively). In addition, PSI was not significantly different between the bilateral tendons on Day 0, whereas the values on Day 14 appeared more elevated in the tendons that were administered 100 μL collagenase than those that were administered 50 μL collagenase ($p = 0.046$; Table 1 and Fig. 3).

### Discussion

In our study, we found that the CSA 14 days after collagenase injection was not significantly different from baseline in either Achilles tendon. Regarding comparisons between the bilateral Achilles tendons, the differences in CSA and vascularity measured using grey-scale or noncontrast power Doppler ultrasound were insignificant 14 days after collagenase injection. However, using contrast-enhanced ultrasound (CEUS) allowed us to detect increased vascularity in the tendons that were treated using a higher dosage of collagenase in comparison with the contralateral side. A higher number of neo-vascular vessels were also detected in both postinjected tendons in comparison with the basal amounts that were measured prior to collagenase injection.

Achilles tendinopathy may be initiated by injuries to the tendons via inflammatory cell infiltration, but Achilles tendinopathy is actually caused by an increase in matrix proliferation and extensive neovascularization with the absence of inflammation [12]. There are two types of animal tendinopathic models: those that are provoked by chemical irritants and those provoked by mechanical overuse. Collagenase, the most common chemical material used to induce tendinopathy, is a protease that is secreted by fibroblasts or inflammatory cells and it can degrade the triple helical structure of collagen, leading to reproducible and consistent damage to tendons and degeneration followed by an inflammatory response [13]. The collagenase we used in this study was specific to type I collagen. Since type I collagen composes about 85% of the tendon, we chose this kind of collagenase. According to previous studies, the number of neutrophils and macrophages might increase after collagenase injections, but these numbers typically return to baseline values within 7–14 days [14]. Therefore, we chose 14 days after the injection of collagenase as the time to measure these values. The advantage of using collagenase to elicit tendinopathy is its similarity to naturally occurring tendinopathy, which represents matrix degradation, cellular proliferation, and hyper-vascularity. Furthermore, the number of neo-vessels is proportional to the injected dose, which allows for comparisons between experimental designs [13].

Contrast-enhanced ultrasound, an emerging imaging modality that can be used to evaluate angiogenesis, uses gas-filled and shell-stabilized microbubbles as contrast agents. The size of the microbubbles ranges between 1–8 μm [15], which can augment backscattered signals through strong reflections from the gas-fluid interfaces [10,16,17]. Adverse reactions of using microbubbles as a contrast agent are rare in humans and are usually transient without being life threatening, if they occur at all [18]. In contrast to iodinated CT or gadolinium-based MRI contrast agents, there have been no reports of nephrotoxicity using microbubbles [19]. CEUS allows the assessment of the macro- and microvasculature in various organs, including the liver, heart, and kidney [17], and has a higher sensitivity than traditional power Doppler ultrasound for revealing the microvasculature [20]. CEUS has been used to detect and characterize focal liver lesions [21], improves the delineation of the endocardial border and assessment of myocardial perfusion, and is beneficial for detecting renal transplant rejection [15]. Nevertheless, based on the relevant studies that have been published in the literature, few studies have applied this technique to evaluate tendinopathy [10].

In the present study, noncontrast power Doppler ultrasound failed to recognize neovascularization in tendinopathic models, whereas CEUS could detect higher vascularity in Achilles tendons 2 weeks after collagenase injection. In addition, differences in hypervascularity following the administration of various amounts of collagenase injection could be identified using CEUS. One explanation is that the newly formed vessels are extremely small, preventing traditional power Doppler

<table>
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<tr>
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<th>Day 0</th>
<th>Day 14</th>
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<tr>
<td>Tendon cross-sectional area (no. of pixels)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Achilles tendon</td>
<td>38,772.5 ± 3906.6</td>
<td>41,081.7 ± 10,089.4</td>
</tr>
<tr>
<td>Right Achilles tendon</td>
<td>38,927.2 ± 6497.6</td>
<td>40,573.7 ± 5170.0</td>
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<tr>
<td>Color pixels determined using noncontrast power Doppler (no. of pixels)</td>
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<tr>
<td>Left Achilles tendon</td>
<td>3258.8 ± 803.3</td>
<td>2979.2 ± 1477.0</td>
</tr>
<tr>
<td>Right Achilles tendon</td>
<td>2391.0 ± 1059.5</td>
<td>3985.6 ± 2152.0</td>
</tr>
<tr>
<td>Color pixels determined using contrast-enhanced ultrasound (no. of pixels)</td>
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<tr>
<td>Left Achilles tendon</td>
<td>13,475.0 ± 4256.9*</td>
<td>22,667.8 ± 5402.5**</td>
</tr>
<tr>
<td>Right Achilles tendon</td>
<td>12,271.5 ± 1991.1*</td>
<td>18,211.0 ± 3921.0**</td>
</tr>
</tbody>
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* Indicates significant difference between the parameters of tendons at either side on Day 0 and Day 14.
** Indicates significant difference between the parameters in the left and right Achilles tendons.
ultrasound from observing their existence. In contrast, contrast agents augment the Doppler signals that are derived from the intravascular microbubbles and facilitate the depiction of focal perfusion [20]. Another confounding factor resulted from the accompanying arteries within the normal Achilles tendons of rabbits. Because we did not synchronize measurements with their heart beats, the pulsatile properties of the arteries lead to inaccurate estimates of the power Doppler signals when using their color area as a gauge of intensity. Nevertheless, microbubbles could have oversaturated the adjacent midsized vessels and significantly reduced the influence of arterial pulsation. Therefore, when using contrast-enhanced techniques, comparisons of the status before and after collagenase injection and both sides of the Achilles tendons mainly depend on the newly developed small caliber vessels [22].

Two limitations to this study must be acknowledged. First, we chose 14 days after collagenase injection as the time to evaluate vascularity; therefore, there might be greater differences in vascularity or cross-sectional area during the acute stage. Serial follow-up with shortened intervals may be helpful. Second, in the present study, we selected three maximally enhanced pictures using the naked eye; therefore, vascularity could have been inaccurately estimated because of the pulsatile properties of the accompanied arteries. Future work may use imaging processing software to automatically analyze the enhanced area and plot the intensity changes according to time. Further analysis of the time-intensity curves may contribute information that is more useful to the perfusion dynamics of tissues with tendinopathy.

Conclusions

In the present study, we demonstrated that using CEUS is more effective than using noncontrast-enhanced power Doppler ultrasound for detecting hypervascularity in a rabbit model of Achilles tendinopathy. Our future work will be dedicated to determining whether this technique can monitor the therapeutic responses to neovascularization and promoting its application in humans.

References