In vivo photoacoustic micro-imaging of microvascular changes for Achilles tendon injury on a mouse model

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Abstract: Since neovascularization has been reported that it is associated with tendinopathy, assessments of vascularity are important for both diagnosis and treatment estimation. Photoacoustic imaging, taking the advantages of good ultrasonic resolution and high optical absorption contrast, has been shown a promising tool for vascular imaging. In this study, we explore the feasibility of photoacoustic micro-imaging in noninvasive monitoring of microvascular changes in Achilles tendon injuries on a mouse model in vivo. During collagenase-induced tendinitis, a 25-MHz photoacoustic microscope was used to image microvascular changes in Achilles tendons of mice longitudinally up to 23 days. In addition, complementary tissue structural information was revealed by collateral 25-MHz ultrasound microscopy. Morphological changes and proliferation of new blood vessels in Achilles tendons were observed during and after the acute inflammation. Observed microvascular changes during tendinitis were similar to the findings in the literatures. This study demonstrates that photoacoustic imaging can potentially be a complementary tool for high sensitive diagnosis and assessment of treatment performance in tendinopathy.

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1. Introduction

In the past few decades, the incidence of the Achilles tendon injuries has risen due to the popularity of recreational and competitive sporting activities, especially in runners [1]. Efforts on current imaging techniques such as radiography, computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound show their capability of clinical diagnosis. However, radiography and CT are limited to the diagnosis of tendon structural abnormality [2]. In clinical situations, MRI and ultrasound imaging are the most common imaging technologies used to elucidate the fine internal structures of tendons noninvasively for both diagnosis and treatment monitoring [2,3]. Typically, because of its widely available, easy to use, relatively inexpensive, and exhibiting a high lesion detection rate, ultrasound has recently become the first line examination tool [4]. In addition, ultrasound color and power Doppler imaging can offer additional flow information, which is an essential adjunct to B-mode imaging in assessments of vascularity. Several studies have indicated that neovascularization accompanies with tendinitis (i.e., acute inflammation) and painful tendinosis (i.e., chronic tendon degeneration) [5–8], and may be a contributory factor to the pain and chronicity of the disease. In addition, it also has been suggested that the existence of new blood vessels affects the performance of treatment [9]. Therefore, assessments of vascularity are important when assessing inflammation changes in tendon injuries. However, it is still challenging for ultrasound to accurately detect the tiny blood flow of normal vasculature and neovasculature inside the tendon, and it may cause huge variability to the diagnosis results. For example, Snellenberg et al. indicate that the presence of neovascularization is not exclusive to Achilles tendons with chronic pain [10] which is not in correspondence with the result discovered by Lars Öhberg et al. [6].

Photoacoustic imaging (PAI), which is a novel hybrid imaging modality owning high optical absorption contrast and good ultrasonic resolution, has been shown as a promising tool for vascular imaging by means of laser-induced ultrasound [11,12]: In PAI, a short pulsed laser source is used to excite a biological sample (e.g., tendon). Ultrasonic waves are then generated due to thermoelastic expansion when the biological tissues absorb the light energy. A wideband ultrasonic transducer then detects the induced photoacoustic waves to form an image representing the distribution of optical absorption in the imaged sample. With adequate...
excitation laser wavelength, blood vessels can be imaged with high intrinsic optical absorption contrast and high ultrasound resolution [13]. PAI forms a good alternative of power Doppler ultrasound to assess tendon vascularity with higher sensitivity and specificity. The non-ionizing excitation source makes it suitable for long-term monitoring of vascular changes and treatment evaluation.

Typically, photoacoustic microscopy (PAM) [13–16], i.e. high-frequency (> 20 MHz) PAI, offers a unique opportunity to monitor micro-vascular changes in tendon injuries on a mouse model in vivo, which high frequency ultrasound Doppler imaging still has difficulties to detect [17]. In this study, we show the potential of PAM in exploring the morphological and microvascular changes of the injured mouse Achilles tendons in vivo. A mouse model of collagenase-induced Achilles tendinitis was adopted here [18]. A custom-made 25-MHz dark-field confocal PAM was used to image micro-vascular changes in injured Achilles tendons of mice during collagenase-induced tendinitis in vivo. Collateral 25-MHz ultrasound, i.e. ultrasonic microscopy (USM), B-mode images were also incorporated to provide complementary structural information.

2. System setup

A 25-MHz dark-field confocal PAM (Fig. 1(A)) was conducted for in vivo imaging of mouse Achilles tendons. A frequency-doubled Nd:YAG Q-switched laser (Surlite II-10, Continuum) with pulse width of 6.5 ns, pulse repetition frequency (PRF) of 10 Hz, and wavelength of 532 nm was employed to provide laser pulses. Passing through the optical path shown as Fig. 1(A), laser light was then uniformly delivered to the Achilles tendon in the dark-field form. The incident energy density on the sample surface was <1 mJ/cm², which is well within the ANSI safety limit of 20 mJ/cm². Since blood owns strong optical absorption at 532 nm, it guarantees that the excited photoacoustic signals mainly come from the vessels. To receive the photoacoustic signals, we used a 25-MHz focused ultrasonic transducer (v324, Olympus) with −6dB fractional bandwidth of 55%, focal length of 12.7 mm, and 6-mm active element size. Spatial resolution of this system was measured by imaging the cross-section of a 6-μm carbon fiber in a turbid solution. This PAM system owned an −6 dB axial resolution of 68 μm and a lateral resolution of 185 μm.

To perform a two-dimensional photoacoustic scanning (i.e., three-dimensional imaging), the optical elements and the transducer was loaded on and driven by a two-dimensional motorized stage. The B-mode images would section the Achilles tendon along the longitudinal direction.
direction of it (see Fig. 1(B)) in a 6 mm by 2 mm scanning region which covered the tendon as shown in Fig. 1(C). The step sizes in x and y directions of Fig. 1 were both 60 μm. During the scanning, the de-ionized water in the water tank and the Achilles tendon were kept in constant temperature by a thermostat. The photoacoustic signals received by the transducer were pre-amplified by 59 dB with a low-noise amplifier (AU-3A-0110, MITEQ), filtered by an ultrasonic pulser/receiver (5073 PR, Olympus) and a low-pass filter (BLP-50+, Mini-Circuits), digitized by a digital oscilloscope (TDS 5034B, Tektronix), and then stored in the PC. The whole mechanical scanning procedure and further data processing were based on customized MATLAB programs (MATLAB®, The MathWorks).

Note that the photoacoustic images shown in the following section were taken without signal averaging to reduce the scanning time. By changing the excitation source from the laser to the ultrasonic pulser, we could switch the imaging mode from PAM to USM, for providing complementary structural information with 56-μm axial and 125-μm lateral resolution, respectively, and also for initially rapid positioning of the imaged tendon. Each acquired ultrasound signal was averaged for 64 times to increase the signal-to-noise ratio (SNR). It took about 16 minutes for both PAM and USM to complete a two-dimensional scanning. Note that synthetic aperture focusing technique and coherence factor weighting were applied to all the photoacoustic images to improve the signal-to-noise (SNR) ratio and the spatial resolution in the non-focused area [19,20].

3. Animal handling

Injection of collagenase into the tendon has been widely employed as a tendinopathy model [18], [21,22]. Here we adopted a collagenase-induced tendinitis model [18] of mice to demonstrate the feasibility of PAM in exploring the vascular changes before and after the acute inflammation of Achilles tendinitis. Balb/c mice with body weight of about 20 grams (National Laboratory Animal Center, Taiwan) were used for in vivo experiments (n = 6). The handling of mice was in accordance with the regulations of the Laboratory Animal Center of National Tsing Hua University, Taiwan.

Mice were anesthetized by gas anesthesia of isoflurane with a dose of 1% of pure oxygen at 1-L/min flow rate in all the following procedures. Hair on hind limbs of mice was shaved and then further removed with a hair remover lotion before collagenase administration. Collagenase (bacterial collagenase type I, Sigma) was dissolved in normal saline and sterile filtered through a 0.22-μm filter. 20-μL collagenase with concentration of 125 unit/30μL was percutaneously injected near the osteotendinous junction of the left Achilles tendon using a 30-G needle. As a control group, the right Achilles tendon was injected with the same amount of sterile normal saline at the same site. The injected area and ankle at the right Achilles tendon did not display infection, edema, and redness after injecting 20-μL normal saline while acute inflammation of the left Achilles tendon occurred immediately after collagenase injection. The in vivo experiments were performed before saline and collagenase administration and on the 12th and 23rd days post injection to trace the microvascular changes in the Achilles tendons during collagenase-induced tendinitis. Prior to imaging, the mouse was restrained using a home-made mount and the hind limbs were positioned as shown in Fig. 1(C). To facilitate the identification of the Achilles tendon in the acquired images, partial calcaneum and crus muscle were encompassed into the images as imaging markers and the angle between the Achilles tendon and the sole was adjusted to close to 90 degrees to extend the tendon as possible. During imaging, the body temperature of mice was maintained with a warming lamp and the thermostat in the water tank. The mice were sacrificed after the experiment on the 23rd day, and parts of Achilles tendons were then used for histological verification.

4. Results

The first scanning after the administration of saline and collagenase was conducted on the 12th day because the acute inflammation was reduced on the 12th day. Note that because of the spatial resolution of our PAM and the small size of the imaged Achilles tendons of the
mouse, the neovasculature in the injured tendons cannot be well resolved in the projected C-scan images; thus, we do not present projected C-scan images of the tendons here. Instead, only photoacoustic B-mode images are provided in this study which also facilitates the head to head comparison with ultrasonic B-mode counterparts. Figure 2 shows the photoacoustic envelope images of the Achilles tendon and their ultrasonic B-mode counterparts in the

![Figure 2](image_url)

**Fig. 2.** Images of the Achilles tendons acquired on the 12th day after the injection of saline and collagenase. Left panels are images of the controlled Achilles tendon and right panels are images of the injured Achilles tendon. (A) and (B): ultrasound images; (C) and (D): photoacoustic images; (E) and (F): combined photoacoustic and ultrasound images where pseudo-color represents photoacoustic signals. The ultrasound images are displayed in a 40-dB dynamic range, and the photoacoustic images are shown in a linear scale. The terms C, D, M, and AT in (A) represent calcaneus, dermis, muscle, and Achilles tendon, respectively. The terms SV and TV in (E) represent subcutaneous vessels and tendon vessels respectively.
longitudinal view on the 12th day. The back-scattered ultrasound signals from the Achilles tendon are lower than those from its surrounding tissues, and thus the tendon appears as a dark band in the longitudinal view, as shown in Fig. 2(A). Via this dark-band appearance of the tendon, USM clearly identifies the contour of the Achilles tendon and other structures such as the calcaneus, the dermis, and the muscles of the right hind limb (Fig. 2(A)), while PAM reveals the vasculature information (Fig. 2(C)). When overlapping the photoacoustic image on the ultrasound one which is similar to power Doppler ultrasound mode, Fig. 2(E) shows the necessity and advantage of the dual-modal – photoacoustic and ultrasound – imaging for tendon vascular imaging – it is useful for distinguishing the vessels inside Achilles tendon from the other vasculatures around it. Comparing to our previous high frequency power Doppler ultrasound results where only blood flow in the peritendinous space can be detected [17], PAM shows a better sensitivity to the microvasculature of the hind limb injected with normal saline. As proven in the literature [23], PAM is more effective than high frequency power Doppler ultrasound for detecting small vessels with low flow speeds which is in line with the case of Achilles tendon. Although without any signal averaging, the subcutaneous vessels, tendon vessels, and even the ones supporting the muscles and under the calcaneus are clearly identified by PAM. The SNR of the labeled tendon vessel in Fig. 2(E) is about 24.5 dB. In the injured Achilles tendon suffering tendinitis shown in Figs. 2(B), (D), and (F), there are some significant changes in both tissue structure and vasculature. The dermis is thicken and filled with blood vessels because of inflammation and swelling. Compressed by the swollen dermis, the boundary of the Achilles tendon is distorted and blurred; moreover, there is an explosive growth of blood content inside and around the tendon possibly resulting from neovascularization and hemorrhage. Focal hypoechoic regions are also observable in Fig. 2(B), which is similar to the reported results in the literature [7].

On the 23rd day post injection, the scanning was conducted again to trace the vascular changes of the Achilles tendon. The results are shown in Fig. 3. There are no observable changes in both tissue structure and vasculature between Figs. 2(E) and 3(E) as we expect while some critical changes occur in the injured one. Figure 3(B) shows that the Achilles tendon is reshaped and homogenized again like the controlled one. Blood content inside the tendon diminishes (Fig. 3(F)) which is in correspondence to the findings in the literature [9]. In the histological verification, Fig. 3(G) shows that the healthy Achilles tendon tissues are with good fibrillar homogeneity and there are no observable vessels while as revealed in Fig. 3(F), Fig. 3(H) indicates that there are more blood vessels in the peritendinous space of the injured Achilles tendon tissues which are with poor homogeneity.

In addition to qualitative observation from Figs. 2 and 3, quantitative analysis of the vascular changes was performed. Since the spatial resolution provided by our 25-MHz PAM is not good enough to resolve single microvessels smaller than 68 μm; tendon blood signals detected by our PAM may represent the overall contribution of microvessels within the sample volume. Considering this point, vascular area ratio (VAR) which presents the area occupied by micro-vessels within the selected regions of interest (ROI), e.g., Achilles tendon, was used as the quantitative vascularity index. Assume that the colored area within the selected ROI in the combined photoacoustic and ultrasound images in Figs. 2 and 3 represent its vascularity. The VAR can be calculated by dividing the number of colored pixels by the number of demarcated Achilles tendon pixels [24]. Here, VAR represents vascular density in the selected Achilles tendon. Figure 4 shows the VAR of the controlled and injured Achilles tendons before the injection of saline and collagenase and on the 12th and 23rd days post injection. Note that five successive combined photoacoustic and ultrasound images of each tendon were used to estimate the mean and standard deviation of the VAR. The VAR values in the controlled tendon are 48.86 ± 3.68%, 47.26 ± 4.53% and 48.75 ± 5.09%, indicating no specific vascular changes. In the injured one, the neovascularization is confirmed by a rise of VAR from 50.36 ± 4.67% to 61.74 ± 5.68% during the collagenase-induced inflammation. The VAR then reduces to 44.46 ± 5.56% after the self-healing process, which is in correspondence to the findings in the literature [9].
Fig. 3. Images of the Achilles tendons acquired on the 23rd day post injection. Left panels are images of the controlled tendon and right panels are images of the injured tendon. (A) and (B): ultrasound images; (C) and (D): photoacoustic images; (E) and (F): combined photoacoustic and ultrasound images; (G) and (H): H&E stained histological sections. The terms C, AT, and V in (G and (H) represent calcaneus, Achilles tendon, vessels around the tendon, respectively.
5. Concluding remarks

In this study, we have demonstrated the potential of PAM in exploring the microvascular and morphological changes in injured Achilles tendons of mice \textit{in vivo}. Although the spatial resolution provided by our 25-MHz PAM is not good enough to resolve single microvessels smaller than 68 μm, micro-vascular changes during the inflammation and self-healing process of injured Achilles tendons can still be sensitively detected, observed in the provided PAM B-mode images, and quantitatively analyzed because of high sensitivity and high contrast nature of PAM to blood vessels. It is also demonstrated that the necessity and advantage of the dual-modal – photoacoustic and ultrasound – imaging for identifying the contour of the Achilles tendon and distinguishing the vessels inside the tendon from the other vasculatures around it. PAM shows promise in identifying the microvascular changes inside the tendons, and thus can potentially be served as a good and sensitive alternative of high-frequency ultrasound Doppler imaging to monitor neovascularization of injured Achilles tendons in small animals [23]. It is suggested that applying PAM to small-animal models will be an invaluable aid in explorations of the role of neovascularization in the tendinopathy and assessment of performance of treatment. Future work will focus on time-course assessments of microcirculation changes in tendinitis before and after treatment.

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