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Authors

Ma, Ya-Jun
Chang, Eric Y
Bydder, Graeme M
et al.

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Can ultrashort-TE (UTE) MRI sequences on a 3-T clinical scanner detect signal directly from collagen protons: freeze-dry and D₂O exchange studies of cortical bone and Achilles tendon specimens

Ya-Jun Ma^{a†}, Eric Y. Chang^{a,b†}, Graeme M. Bydder^a and Jiang Du^{a*}

Ultrashort-TE (UTE) sequences can obtain signal directly from short- T_2 , collagen-rich tissues. It is generally accepted that bound and free water can be detected with UTE techniques, but the ability to detect protons directly on the collagen molecule remains controversial. In this study, we investigated the potential of UTE sequences on a 3-T clinical scanner to detect collagen protons via freeze-drying and D₂O–H₂O exchange studies. Experiments were performed on bovine cortical bone and human Achilles tendon specimens, which were either subject to freeze-drying for over 66 h or D₂O–H₂O exchange for 6 days. Specimens were imaged using two- and three-dimensional UTE with Cones trajectory techniques with a minimum TE of 8 μ s at 3 T. UTE images before treatment showed high signal from all specimens with bi-component T_2^* behavior. Bovine cortical bone showed a shorter T_2^* component of 0.36 ms and a longer T_2^* component of 2.30 ms with fractions of 78.2% and 21.8% by volume, respectively. Achilles tendon showed a shorter T_2^* component of 1.22 ms and a longer T_2^* component of 15.1 ms with fractions of 81.1% and 18.9% by volume, respectively. Imaging after freeze-drying or D₂O–H₂O exchange resulted in either the absence or near-absence of signal. These results indicate that bound and free water are the sole sources of UTE signal in bovine cortical bone and human Achilles tendon samples on a clinical 3-T scanner. Protons on the native collagen molecule are not directly visible when imaged using UTE sequences. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: UTE; freeze-drying; D₂O–H₂O exchange; collagen

INTRODUCTION

Collagen is the most abundant protein in the human body and is the main component of connective tissue (1). The collagen molecule is composed of a three-stranded arrangement of α -helices which are stabilized by a ladder of hydrogen bonds, with additional support from stereoelectronic effects and post-translational modifications, such as hydroxylation and cross-linking (2). Alterations of collagen structure, amount and type are of clinical interest in nearly all biological systems, including musculoskeletal, neurological, cardiovascular and respiratory (3–6).

Of the clinically utilized imaging modalities, MRI has the potential to provide the most information about collagen. However, the highly anisotropic structure of collagen causes short transverse relaxation times, which result in little or no signal when imaged using conventional MR sequences. Ultrashort-TE (UTE) sequences, with TEs that range from 0.008 to 0.50 ms, have been increasingly used on clinical MRI systems to directly image collagen-rich short- T_2 tissues or tissue components, such as cortical bone, calcified cartilage, menisci, ligaments and tendons (3,7,8).

Although UTE sequences can obtain signal directly from collagen-rich tissues, the precise origin of this signal remains controversial. Potential candidates include: (i) protons on the collagen molecule; (ii) collagen-associated water with various amounts of restricted motion (also known as 'bound' water);

and (iii) unrestricted water surrounding collagen (also known as 'free' or 'bulk' water). With regard to restricted collagen-associated water, three distinct compartments have been identified, including water bridges (single and double bridges between α -helices too distant to allow direct hydrogen bonds), cleft water (residing in the groove-like depressions of the triple helix) and interfacial monolayer (also known as surface or hydration layer) water, in order of increasing mobility and energy (9,10). Using NMRI at different levels of hydration, Fullerton *et al.* (10) proposed a mono-exponential decay pattern of all

* Correspondence to: J. Du, University of California, San Diego, Department of Radiology, 200 West Arbor Drive, San Diego, CA 92103-8226, USA.
E-mail: jiangdu@ucsd.edu

a Y.-J. Ma, E. Y. Chang, G. M. Bydder, J. Du
Department of Radiology, University of California, San Diego, CA, USA

b E. Y. Chang
Radiology Service, VA San Diego Healthcare System, San Diego, CA, USA

† These authors contributed equally to this work.

Abbreviations used: 2D/3D, two-/three-dimensional; 3D Cones, three-dimensional UTE with Cones trajectory; PBS, phosphate-buffered saline; ROI, region of interest; SNR, signal-to-noise ratio; UTE, ultrashort TE; UTE-MT, UTE magnetization transfer.

three restricted water compartments due to fast exchange. Using clinical MR scanners, quantitative UTE techniques have been employed with bi-component T_2^* analysis to evaluate collagen-associated bound and free water compartments (11–13).

More recently, a study by Siu *et al.* (14) has suggested that UTE sequences can detect signal directly from protons on the collagen molecule, with a reported mean short T_2^* of 0.75 ± 0.05 ms and a mean chemical shift of -3.56 ± 0.01 ppm relative to water at 7 T. The purpose of this study is to further investigate whether UTE sequences can detect signal from collagen protons via freeze-dry and D_2O exchange studies of cortical bone and Achilles tendon specimens at 3 T.

METHODS AND MATERIALS

Sample preparation

Two bovine cortical bone samples ($2 \times 2 \times 6$ mm³) and two cadaveric human Achilles tendon samples (1 cm in length) were prepared for this study. The bone samples were sectioned from two fresh femoral mid-shaft bovine specimens obtained from a local slaughterhouse using a low-speed diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA) with constant water irrigation. The two tendon samples were sectioned from two human ankle specimens obtained from our institutional anatomical laboratory using a scalpel. All samples were stored in phosphate-buffered saline (PBS) solution for 24 h prior to use.

Pulse sequences and imaging protocol

All samples were imaged with two-dimensional (2D), non-slice-selective, UTE sequences and three-dimensional UTE with Cones trajectory (3D Cones) sequences on a GE 3-T Signa TwinSpeed MR scanner (GE Healthcare Technologies, Milwaukee, MI, USA) which had a maximum gradient strength of 40 mT/m and a maximum slew rate of 150 mT/m/ms. The non-slice-selective 2D UTE sequence employed a short rectangular pulse excitation (duration, 32 μ s), followed by 2D radial ramp sampling with a minimal nominal TE of 8 μ s. The 3D UTE Cones sequence employed a short rectangular pulse (duration, 32 μ s) for non-slice-selective excitation, followed by 3D spiral sampling on the Cones sequence (15). A home-built 1-in-diameter birdcage T/R coil was used for signal excitation and reception for both 2D and 3D UTE imaging.

For 2D UTE morphological imaging, the following parameters were used: TR = 100 ms; flip angle, 10°; bandwidth, 62.5 kHz; field of view, 4 cm; 403 projections; reconstruction matrix of 128×128 for cortical bone and 256×256 for Achilles tendon. The 3D Cones sequence used similar imaging parameters, except for a shorter TR of 20 ms, 20 slices, a slice thickness of 4 mm and a reconstruction matrix of $192 \times 192 \times 16$ for cortical bone and $256 \times 256 \times 16$ for Achilles tendon. Furthermore, T_2^* was measured with 2D non-selective UTE imaging with 17 TEs (8 μ s, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12 ms) for cortical bone and 17 TEs (8 μ s, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 4, 6, 8, 12, 16, 20, 25, 30 ms) for Achilles tendon. For T_2^* measurement, the image flip angle was low (i.e. 10°) and the pulse repetition time (i.e. 100 ms) was sufficiently long to eliminate the T_1 dependence, especially for cortical bone with a short T_1 (16,17). The total scan time for T_2^* measurement was 11 min and 25 s for both cortical bone and Achilles tendon.

Experimental procedures

The bovine cortical bone and human Achilles tendon samples were blot-dried and then imaged with the above morphological and quantitative UTE imaging protocols. After the initial 2D and 3D UTE imaging, one bovine bone sample and one human Achilles tendon sample were lyophilized using a Labconco Lyph-Lock 4.5-L freeze-dry system (model 77510-00, Labconco Corp., Kansas City, MO, USA) for over 66 h. The weight of each sample was measured before and after freeze-drying using a Mettler-Toledo AL-104 digital balance (Mettler-Toledo, Schwerzenbach, Switzerland) with a precision of 0.1 mg. After freeze-drying, each sample was stored in a sealed tube and warmed to room temperature, and then imaged again with the same protocol as described above.

The other bovine bone sample and the other human Achilles tendon sample were subjected to a D_2O - H_2O exchange study. Each sample was placed in a 20-mL syringe filled with D_2O solution for exchange in the refrigerator for 6 days. Each syringe was flushed with fresh D_2O every 2 days in order to achieve more complete D_2O - H_2O exchange. Finally, the same UTE MRI protocol was applied to the D_2O -exchanged samples.

Data analysis

A bi-component model was used for the quantification of both bound and free water components, which assumes that both bound and free water contribute to the UTE signal. The following bi-component analysis model was employed to analyze the T_2^* values of both bound and free water and their relative fractions:

$$S(t) = S_{bw} \times e^{-t/T_{2s}^*} + S_{fw} \times e^{-t/T_{2l}^*} + \text{noise} \quad [1]$$

where a constant noise term was fitted to account for background noise. S_{bw} and S_{fw} represent the magnetization of the bound and free water components, respectively, and T_{2s}^* and T_{2l}^* are their T_2^* relaxation times.

The analysis algorithm was written in Matlab (The MathWorks Inc., Natick, MA, USA) and was executed offline on the DICOM images obtained by the protocols described above. The program allowed the placement of regions of interest (ROIs) on the first UTE image of the series, which was then copied onto each of the subsequent images. The mean intensity within each of the ROIs was used for subsequent curve fitting. The bi-component exponential fitting model shown in Equation [1] was used to fit the UTE T_2^* images. Noise was estimated automatically using a maximum likelihood estimation algorithm.

RESULTS

Freeze-drying resulted in a significant loss of sample weight. Bone weight was reduced from 0.3544 to 0.1759 g, corresponding to 50% weight loss. The Achilles tendon sample weight was reduced from 1.9640 to 0.3934 g, corresponding to 80% weight loss. Figures 1, 2 show UTE images and T_2^* bi-component analysis of the blot-dried bovine cortical bone sample and human Achilles tendon sample, respectively. High signal intensity and excellent bi-component decay behavior were observed from UTE imaging of both samples before freeze-drying. The image signal-to-noise ratios (SNRs) of cortical bone and Achilles tendon acquired with the 2D UTE sequence with TE = 8 μ s were 327 and 193, respectively. The bovine cortical bone sample showed two distinct water components: a shorter T_2^* of 0.36 ms and a longer

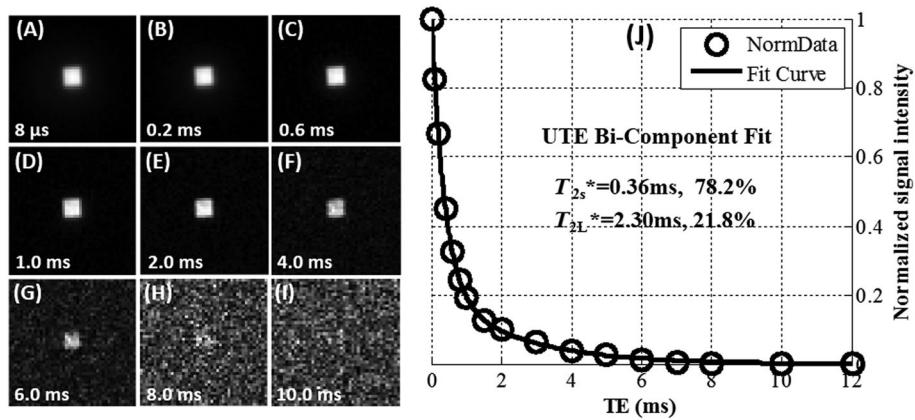


Figure 1. Selected two-dimensional ultrashort-TE (2D UTE) imaging of a bovine cortical bone sample with TEs of 8 μ s (A), 0.2 ms (B), 0.6 ms (C), 1.0 ms (D), 2.0 ms (E), 4.0 ms (F), 6.0 ms (G), 8.0 ms (H) and 10.0 ms (I), as well as bi-component fitting of the UTE images (J), which shows a shorter T_{2s}^* of 0.36 ms and a longer T_{2L}^* of 2.30 ms with fractions of 78.2% and 21.8% by volume, respectively. The region of interest was located in the cortical bone center with a size of 4 \times 4.

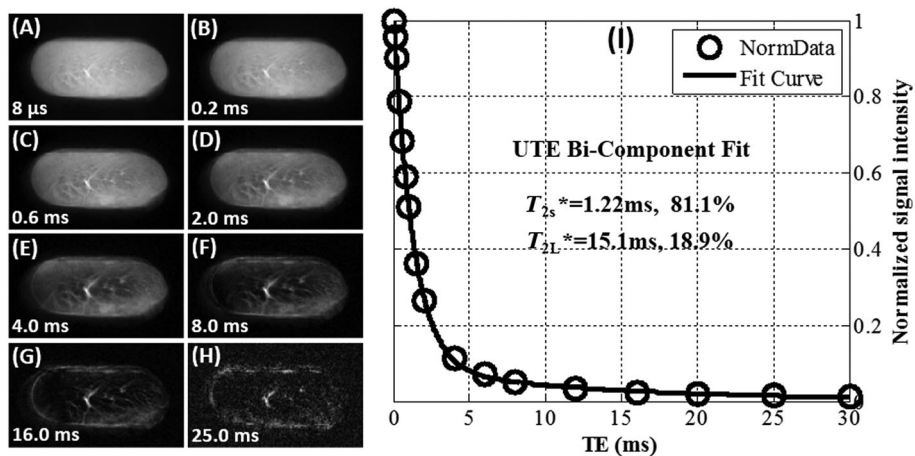


Figure 2. Selected two-dimensional ultrashort-TE (2D UTE) imaging of an Achilles tendon sample with TEs of 8 μ s (A), 0.2 ms (B), 0.6 ms (C), 2.0 ms (D), 4.0 ms (E), 8.0 ms (F), 16.0 ms (G) and 25.0 ms (H), as well as bi-component fitting of the UTE images (I), which shows a shorter T_{2s}^* of 1.22 ms and a longer T_{2L}^* of 15.1 ms with fractions of 81.1% and 18.9% by volume, respectively. The region of interest was located in the Achilles tendon center with a size of 8 \times 24.

T_{2L}^* of 2.30 ms with fractions of 78.2% and 21.8% by volume, respectively. The human Achilles tendon sample also showed two distinct water components: a shorter T_{2s}^* of 1.22 ms and a longer T_{2L}^* of 15.1 ms with fractions of 81.1% and 18.9% by volume, respectively. The fitting quality can be represented by residual = $\sqrt{\frac{\sum_i (S_{i,fit} - S_i)^2}{\sum_i S_i^2}}$, where S_i and $S_{i,fit}$ ($i = 1, \dots, N$, where N is the total number of data points in one multiple-TE dataset) were the experimental and fitted data points. For the bi-component fitting of cortical bone and Achilles tendon, the residuals were 0.4% and 2.2%, respectively. The residuals of bi-component fitting were very low, which demonstrates that the bi-exponential model describes the observed signal decay well.

Figures 3, 4 show 2D UTE and 3D Cones imaging of the bovine cortical bone sample and human Achilles tendon sample, respectively, before and after freeze-drying. High signal was observed for both samples before freeze-drying. The fascicular pattern in the blot-dried Achilles tendon was nicely depicted.

However, after freeze-drying, near-zero signal was observed for bovine cortical bone, and only a few bright spots were observed near the central region of the Achilles tendon sample. The bright spots are probably from residual water surviving the freeze-drying of 66 h. Longer freeze-drying may be necessary to remove all detectable water in the Achilles tendon sample. These results suggest that freeze-drying removes the source of the MR signal, namely both bound water and free water. Collagen protons are expected to survive the freeze-drying process and, when imaged using 2D and 3D UTE sequences, show zero signal. Therefore, collagen protons in cortical bone and the Achilles tendon are 'invisible' with UTE sequences on clinical MR scanners.

Figure 5 shows 2D UTE and 3D Cones images of the other Achilles tendon sample before and after D_2O exchange. High signal was observed before D_2O exchange, but the signal dropped to near zero after D_2O exchange, confirming that the signal foci in the tendon images after freeze-drying were generated from residual water which survived the lyophilization process. Similar results were observed for cortical bone (not

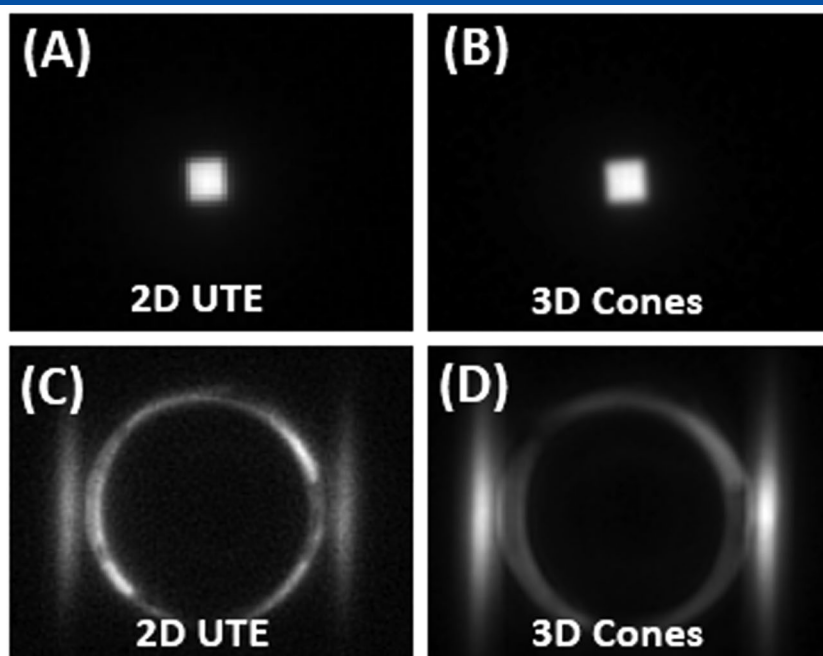


Figure 3. Normal bovine cortical bone sample imaged with two-dimensional ultrashort-TE (2D UTE) (A) and 3D Cones (B) at 3 T, as well as 2D UTE (C) and 3D Cones (D) imaging of the same bone specimen after freeze-drying for over 66 h. Abundant signal is seen before freeze-drying (A, B), but no signal is seen from the specimen after freeze-drying (C, D). The high signal ring in (C, D) represents the coil with the 'invisible' specimen in the center.

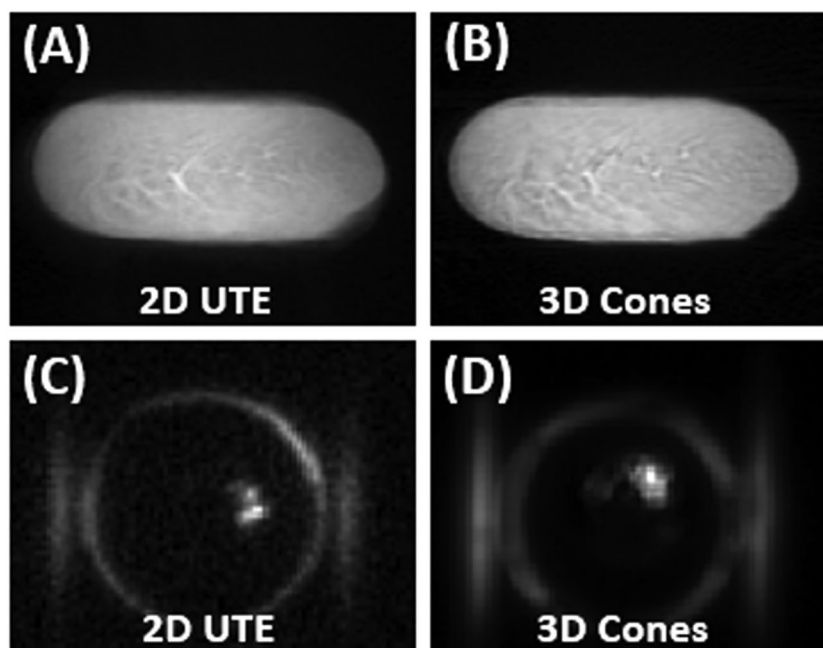


Figure 4. Normal Achilles tendon imaged with two-dimensional ultrashort-TE (2D UTE) (A) and 3D Cones (B) at 3 T, as well as 2D UTE (C) and 3D Cones (D) imaging of the same tendon specimen after freeze-drying for over 66 h. The normal tendon is visible before freeze-drying (A, B), but only a small focus of signal is present after freeze-drying (C, D), consistent with residual water which survived the lyophilization process.

shown) with high signal when imaged with 2D UTE and 3D Cones before D_2O exchange, but near-zero signal after D_2O exchange.

From the results, the water protons can be effectively removed from the cortical bone and Achilles tendon by both freeze-drying and D_2O - H_2O exchange. The cortical bone

showed an absence of signal after both freeze-drying and D_2O - H_2O exchange. Although there was a small focus of signal intensity in the central region of the Achilles tendon after freeze-drying, the D_2O exchange experiment confirmed that this focus of signal in the tendon images was generated from residual water after freeze-drying.

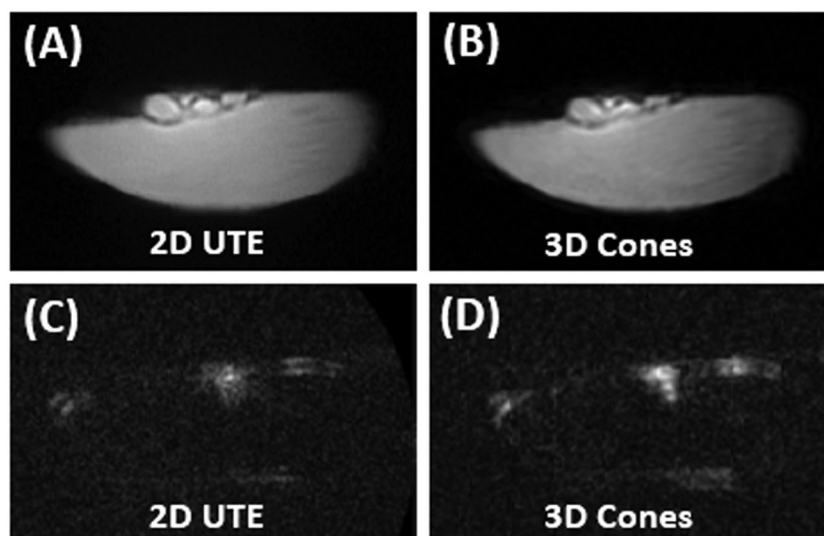


Figure 5. Normal Achilles tendon imaged with two-dimensional ultrashort-TE (2D UTE) (A) and 3D Cones (B) at 3 T, as well as 2D UTE (C) and 3D Cones (D) imaging of the same tendon specimen after D₂O exchange for 1 week. The normal tendon is visible before D₂O exchange (A, B), but invisible after D₂O exchange (C, D).

DISCUSSION

Short- T_2 structures that were previously invisible when imaged with conventional MR sequences, including bone and tendon, can now be visualized using UTE techniques. However, an understanding of the source of signal is important for image interpretation, particularly in the setting of *in vivo* translation, where MRI may be a surrogate for more invasive techniques. In our study, we have shown that bound and free water are the sources of UTE signal in bovine cortical bone and human Achilles tendon samples on a clinical 3-T scanner. Specifically, the absence of signal in both bone and tendon samples after freeze-drying indicates that protons on the native collagen molecule are not directly visible using UTE sequences.

Our results contrast with those recently published by Siu *et al.* (14), who suggested that collagen protons could be detected and quantified using UTE sequences at 7 T. However, there are a number of differences between our studies. Siu *et al.* (14) performed their experiments on a hydrolyzed collagen solution, which causes cleavage of the molecule into smaller peptides. This results in an amorphous state of collagen, lacking either partially or completely the tertiary, secondary and primary structure of native collagen (18). The destruction of the highly ordered, 3D structure of collagen would be expected to alter the T_2^* values of the collagen protons. Presumably, the collagen proton T_2^* values would increase, and this could result in their detection using UTE sequences, in a similar manner to macromolecular protons on myelin which may be detectable using UTE techniques (19,20). The effect of this on the stability and accuracy of bi-component fitting, which requires sufficient separation of the two modeled pools, is unclear (21,22). In addition, at higher field strengths, such as 7 T, oscillations in signals resulting from non-water off-resonance spins further degrade bi-exponential fitting (23).

Direct detection of collagen protons is possible using spectroscopic techniques at high field strengths. This has been shown by Siu *et al.* (14) and Kflak-Hachulska *et al.* (23) on collagen solutions at 7 T and on powdered collagen at 19.6 T, respectively. Although short T_2^* values broaden the measured spectral

linewidths, the liquid solution used by Siu *et al.* (14) and the magic-angle spinning technique used by Kflak-Hachulska *et al.* (23) allowed for narrower linewidths. However, these spectroscopic techniques are not compatible with *in vivo* imaging. Fortunately, collagen protons can be detected indirectly via UTE magnetization transfer (UTE-MT) imaging (24,25). Modeling of UTE-MT images acquired with a series of MT frequency offsets and power may provide quantitative assessment of protons in free water, bound water and collagen in short- T_2 tissues, such as cortical bone and the Achilles tendon. The precise relationship between these three proton pools in the native *in vivo* condition and with regard to healthy *versus* pathological tissue remains to be elucidated. UTE-MT imaging combined with bi-component analysis would provide information from all three pools and will be the focus of future work. The answer to the question of which proton pool is most sensitive and discriminative of pathological change would be of great clinical significance. Along the lines of clinical translation, our study utilized the 3D UTE Cones sequence with the correction of linear eddy current components rather than the 3D radial UTE sequence. Although the 3D radial UTE sequence may be more immune to hardware imperfections with a shorter readout window with a linear acquisition trajectory, the 3D UTE Cones sequence demonstrates higher scan time efficiency with a spiral acquisition trajectory. The 3D Cones sequence also allows anisotropic spatial encoding with lower slice resolution than in-plane resolution to achieve higher SNR and less scan time (26).

Our study has several limitations. First, freeze-drying is an elegant way to remove water whilst largely preserving structure, but does not result in a completely anhydrous state. Previous studies using collagen-rich tissues have shown residual water measuring less than 3% (27). This is confirmed by the central hyperintensities present in the Achilles tendon sample after lyophilization. However, this may be considered to be the most economic method achievable, whilst largely maintaining structure. Second, freeze-drying results in some minor degree of protein denaturation. The process of drying removes water bridges which play a role in stabilization (28). However, the expected

change in structure is probably small (2) and we would not expect large changes in collagen proton T_2^* values. Third, D_2O exchange for 6 days, even in the refrigerator, may lead to significant tissue degradation. Fourth, as a large amount of collagen protons are not exchangeable with D_2O (e.g. $-CH_2-$, $-CH_3$), the absence of any signal after D_2O exchange probably remains a valid indicator that collagen protons are not directly visible with UTE sequences on clinical MR scanners. Fifth, both freeze-drying and D_2O exchange may change the mobility of the collagen molecule, as it interacts closely with the surrounding water; in this case, the two treatments themselves may alter the T_2^* value of the proton signal specifically associated with collagen. Future high-field spectroscopic studies are needed on samples before and after freeze-drying and D_2O flushing to show whether or not the collagen signals observable under these conditions are affected. Sixth, all samples in this study were maintained at room temperature during scanning. Although it is likely that quantitative relaxation measurements would be affected by differences between room and body temperature, we do not anticipate that the main results of our study, which show that protons on the native collagen molecule are invisible with UTE sequences, would be significantly affected. Finally, it would be of interest to investigate whether other imaging techniques, such as zero-echo-time (ZTE) imaging or sweep imaging with Fourier transformation (SWIFT), could conceivably be able to detect the signals of collagen protons directly (29,30).

CONCLUSIONS

We have demonstrated that bound and free water, but not collagen protons, are the sources of signal in cortical bone and Achilles tendon samples when imaged with UTE sequences on a clinical 3-T MR scanner.

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