Immersion of Achilles tendon in phosphate-buffered saline influences T₁ and T₂* relaxation times: An ex vivo study

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Robust mapping of relaxation parameters in ex vivo tissues is based on hydration and therefore requires control of the tissue treatment to ensure tissue integrity and consistent measurement conditions over long periods of time. One way to maintain the hydration of ex vivo tendon tissue is to immerse the samples in a buffer solution. To this end, various buffer solutions have been proposed; however, many appear to influence the tissue relaxation times, especially with prolonged exposure. In this work, ovine Achilles tendon tissue was used as a model to investigate the effect of immersion in phosphate-buffered saline (PBS) and the effects on the T₁ and T₂* relaxation times. Ex vivo samples were measured at 0 (baseline), 30 and 67 hours after immersion in PBS. Ultrashort echo time (UTE) imaging was performed using variable flip angle and echo train-shifted multi-echo imaging for T₁ and T₂* estimation, respectively. Compared with baseline, both T₁ and T₂* relaxation time constants increased significantly after 30 hours of immersion. T₂* continued to show a significant increase between 30 and 67 hours. Both T₁ and T₂* tended to approach saturation at 67 hours. These results exemplify the relevance of stringently controlled tissue preparation and preservation techniques, both before and during MRI experiments.

KEYWORDS
Achilles tendon, ex vivo, phosphate-buffered saline, relaxation time constants, tissue swelling, ultrashort echo time

INTRODUCTION

Tendons are critical structures of the musculoskeletal system, because their primary mechanical role is to transmit muscle forces to the bones, which enables locomotion and improves joint stability. Knowledge of the basic structural and mechanical functions of healthy tendons is essential for understanding and treating injured or pathological tendons. Nevertheless, tendons have thus far been understudied with magnetic resonance imaging (MRI), which is probably due to challenges in parameter quantification, in particular due to rapid signal decay caused by ultrashort T₂* relaxation times ranging between 0.5 and 2.5 ms. Direct imaging of tendons by means of MRI therefore requires the application of ultrashort echo time (UTE) imaging sequences.

As a noninvasive method, MRI has the advantage that it enables detailed examinations of ex vivo tissues while preserving their structural and mechanical properties. This enables not only a longitudinal analysis but also direct comparison with histological or biomechanical measurements.
Importantly, tissue parameters quantified in such samples could help to link structural or biomechanical properties of ex vivo experiments to disease-related changes observed in in vivo measurements. However, imaging of ex vivo tissue samples can be challenging for several reasons. First, air-tissue boundaries can cause undesirable susceptibility artifacts, which, however, can be reduced by immersing the samples in fluids that closely match the differences in magnetic susceptibility. Second, to achieve high spatial resolutions or to quantify tissue parameters of interest, the (typically small) samples often must be measured over a long period of time, that is, from several hours to longer than 1 day. During such prolonged periods, the integrity and hydration of the sample must be controlled since it is known that dehydration has a strong effect on the structure and mechanical properties of tendon tissue. For this reason, many experiments are performed after the samples have been stored in water-free substances such as perfluoropolyether (Fomblin) with or without additional formalin fixation. Nevertheless, such approaches can influence the tissue's structural and mechanical properties as well as the quantitative MRI parameters. To address the abovementioned challenges, an alternative method is to immerse the samples in saline or phosphate-buffered saline (PBS) solutions. These mimic the physiological osmolality of interstitial fluid for the duration of the measurement, especially when fresh and unfixed samples are imaged.

To date, the influence of buffer solutions on tendon MRI relaxation parameters has only been investigated to a very limited extent. Chang et al. investigated the effect of different fluids (saline solution, Fomblin and perfluorooctyl bromide) on immersed ex vivo human Achilles tendons and found no influence on T2 values. However, their six tendon samples were obtained from tissue banks and had high inter-donor variability. Moreover, the samples underwent a deep freeze–thaw cycle before the measurements, which could have affected the swelling behavior of the tissue. For example, Pownder et al. reported that exposure of fresh tendon tissue to such a freeze–thaw cycle reduces T2*. Furthermore, Safa et al. described that exposing tendon tissue to a buffer solution such as PBS influences tissue hydration and mechanics, and leads to solute diffusion into the tissue. Accordingly, these swelling effects may alter MRI relaxation parameters and confound data interpretation. Yet it remains unclear how exposure of fresh tendon tissue to PBS affects T1 and T2* measurements.

In this work, we investigated the effect of immersing ex vivo tendon tissue in PBS on MRI relaxation parameters. Since immersing excised tendon tissue in PBS is a common standard practice before further analysis is performed, it is important to understand any induced changes in tissue properties that can occur with such a treatment. The results of such a study also enable assessment of the suitability of PBS as a buffer for tissue storage/preservation. On the other hand, knowing the influence of PBS on relaxation time constants could be specifically used to change their values in tendon samples, which in turn could be used to potentially mimic tendon disease and their MRI characteristics. We applied variable flip angle and multi-echo 3D-UTE imaging to quantify T1 and T2*, respectively. Soleus portions of fresh ovine Achilles tendons were immersed in PBS for 67 hours and relaxation parameters were estimated after 0 (baseline), 30 and 67 hours.

2 | MATERIALS AND METHODS

2.1 | Sample preparation

Fresh Achilles tendons from eight healthy sheep (Merino, female, aged ≥4 years) were provided from another research project after euthanasia in compliance with the ethical guidelines of legal, local animal rights protection authorities (Landesamt für Gesundheit und Soziales, Berlin; G 0424/17). The tendons were excised close to the calcaneus and before the onset of the calf muscle, and thus contained a mid-tendon portion along with adjoining fibrocartilaginous enthesis tissue. The samples were enveloped in plastic wrap and stored for 4 days at 4°C in a moist environment to avoid dehydration. The moist environment was achieved by placing the samples in individually sealed 50 ml polypropylene centrifuge tubes with moistened cotton gauze at the conical tip of the tubes. On the fourth day, any remaining surrounding tissues were removed, and the denuded soleus portions of the Achilles tendons were enveloped in plastic wrap and stored for 3 additional days at 4°C in a moist environment. Due to the unavailability of a clinical MR scanner, the samples had to be stored for a total of 7 days. Each denuded tendon sample was between 8 and 9 cm long with a mid-tendon diameter of ~ 0.7 cm (Figure 1). For the MRI experiments, the samples were removed from the plastic wrap and transferred from the centrifuge tubes to similar tubes filled with PBS (Dulbecco's phosphate-buffered saline, Sigma-Aldrich, Taukirchen, Germany), then were positioned in the MRI scanner within 10 minutes for imaging.

2.2 | Relaxation parameter mapping

To estimate T2*, a single-echo 3D-UTE acquisition with an echo time (TE) of 0.15 ms was repeated using variable flip angles (VFA) of 34, 26, 19, 14, 11 and 5°. To calculate T1 relaxation time maps from these six datasets, a two-parameter fit of the signal equation for fast low-angle shot (FLASH) gradient-echo MRI sequences was applied. To estimate T2*, an echo train-shifted double-echo 3D-UTE imaging sequence was used with TEs of 0.15, 0.35, 0.60, 1.77, 1.97 and 2.22 ms. TE was defined as the time from the center of a rectangular RF pulse with 260 μs duration to the start of the readout. The first three echoes were acquired by echo-shifting, that is, using three excitation pulses with different delays between excitation and acquisition. The latter
three echoes were acquired as second echoes in a monopolar fashion within the corresponding readout train using rephasing gradients. To map $T_2^*$ from these six echoes, a voxel-wise squared exponential fit to the corresponding power images was computed from the reconstructed magnitude data, including an additional offset parameter to account for potential noise bias.25,26

Example relaxation time maps for one tendon sample are shown in Figure 1.

2.3 | MRI protocol

All tendon samples were measured simultaneously, with the laboratory tubes positioned next to one another and wrapped between the two elements of a 16-channel NORAS variety flex measurement coil (NORAS MRI Products, Höchberg, Germany). Measurements were performed with a clinical 3 T whole-body MRI scanner (Magnetom PRISMA, Siemens Healthineers, Erlangen, Germany). For 3D-UTE imaging, nonselective hard pulse excitation and spiky-ball trajectories were used.27 Echo train-shifted, double-echo and VFA acquisitions were performed with the same geometrical orientation and gradient parameters described above, using a 192 x 148 x 58 acquisition matrix size and a 180 x 139 x 55 mm$^3$ field of view that resulted in an isotropic spatial resolution of 0.95 x 0.95 x 0.95 mm$^3$. The echo train-shifted, multi-echo acquisition used a flip angle of 15° and a repetition time (TR) of 5.5 ms, whereas the VFA acquisition used a longer TR of 9.4 ms to allow the application of larger flip angles without violating the limits of the specific absorption rate. Three blocks of VFA and echo train-shifted, multi-echo data acquisition, each of 65 minutes duration, were collected at baseline, 30 and 67 hours after filling the laboratory tubes with PBS. A comprehensive list of acquisition and sequence parameters is provided in Appendix A.

Images were reconstructed offline in Matlab (MathWorks, Natick, MA) using regridding with iterative sampling density compensation and an optimized kernel.28 Data analysis and nonlinear curve fitting were also performed in Matlab. After calculation of relaxation time maps, regions of interest (ROIs) were drawn manually in the mid-tendon and enthesis regions of all samples. For this purpose, transverse slices in the middle of the tendon and close to the enthesis end were used to define the respective mid-tendon and enthesis positions. The extracted $T_1$ and $T_2^*$ relaxation parameters of all voxels were averaged for each ROI. To test the significance of changes in $T_1$ and $T_2^*$ between 0 (baseline), 30 and 67 hours of immersion in PBS, a two-sided Wilcoxon rank-sum test was performed in Matlab. Statistical significance was set at $P < 0.05$.

3 | RESULTS

Typical FLASH signal curves were observed for the VFA acquisitions (Figure 2), with a peak between the flip angles of 5 and 15°, followed by a fall-off for larger flip angles. Since the individual peak positions change along the flip angle axis, the curves indicate already qualitatively different $T_1$ relaxation times between mid-tendon (Figure 2, left) and enthesis regions (Figure 2, right), and between different immersion durations. After fitting the data to the FLASH signal model, the extracted $T_1$ relaxation times, averaged over all samples (Figure 3), demonstrated a significant increase from baseline to 30 hours for both the mid-tendon ($P = 0.004$) and enthesis ($P = 0.002$) regions. Changes in $T_1$ relaxation times between
30 and 67 hours were not significant for either region \( (P > 0.05) \). The change of \( T_1 \) over the entire immersion duration was significantly greater \( (P = 0.004) \) in the enthesis \( (\Delta T_1 = 231 \pm 44 \text{ ms}) \) compared with the mid-tendon region \( (\Delta T_1 = 110 \pm 20 \text{ ms}) \).

Signals plotted as a function of echo time displayed rapid decays for both the mid-tendon (Figure 4, left) and enthesis (Figure 4, right) regions. Qualitatively, the signal in the enthesis region decayed more slowly. Quantitatively, the \( T_2^* \) relaxation times, averaged over all samples (Figure 5), increased significantly between baseline and 30 hours \( (P = 0.002) \) and between 30 and 67 hours for the mid-tendon region \( (P = 0.004) \). Furthermore, a significant increase was found for the enthesis region from baseline to 30 hours \( (P = 0.002) \) and from 30 to 67 hours \( (P = 0.002) \). The total change in \( T_2^* \) over the entire immersion duration was significantly higher \( (P = 0.001) \) in the enthesis \( (\Delta T_2^* = 0.31 \pm 0.03 \text{ ms}) \) compared with the mid-tendon \( (\Delta T_2^* = 0.18 \pm 0.02 \text{ ms}) \) region.

Tables 1 and 2 summarize the absolute relaxation times \( T_1 \) and \( T_2^* \) for baseline and after immersion in PBS for each of the eight samples.

### DISCUSSION

In this study, significant increases in \( T_1 \) and \( T_2^* \) were observed after immersing ovine Achilles tendon samples in PBS. This finding is in contrast to that of Chang et al.,\(^{17}\) who reported no increase in \( T_2^* \) for ex vivo human Achilles tendon after immersion in PBS. The latter study,\(^{17}\) however, investigated samples that were deep-frozen and thawed. This process can disrupt the microstructure of the extracellular matrix and alter the
**FIGURE 4** Decays of the transverse magnetization and T₂* curve-fitting results (dashed lines) for the mid-tendon and enthesis regions, respectively, for one tendon sample at baseline (blue) and after 30 (red) and 67 hours (yellow) immersion in PBS. To simplify data visualization, the signals were normalized to the individual maximums of the decay curves. For the displayed curves, the average normalized root mean square error for the fit was 0.86 ± 0.03 and 0.92 ± 0.02 for mid-tendon and enthesis, respectively.

**FIGURE 5** Box plots showing the estimated T₂* relaxation times for a region of interest in the mid-tendon (left) and enthesis (right) at baseline, 30 and 67 hours after immersion in PBS. The differently colored dots represent data from individual samples. *Statistically significant differences (P < 0.05)

**TABLE 1** T₁ relaxation times of all samples for mid-tendon and enthesis portions, and different immersion times in PBS. All values ± standard deviation are given in milliseconds

| Sample | Mid-tendon | Enthesis | Enthesis |
|--------|------------|-----------|-----------|
|        | 0 h  | 30 h | 67 h | 0 h  | 30 h | 67 h |
| 1      | 374 ± 41 | 482 ± 37 | 496 ± 21 | 713 ± 74 | 835 ± 99 | 866 ± 78 |
| 2      | 362 ± 37 | 468 ± 47 | 463 ± 23 | 539 ± 29 | 775 ± 92 | 803 ± 85 |
| 3      | 363 ± 59 | 458 ± 30 | 462 ± 27 | 597 ± 111 | 856 ± 159 | 870 ± 170 |
| 4      | 380 ± 86 | 483 ± 49 | 468 ± 23 | 580 ± 69 | 781 ± 49 | 822 ± 88 |
| 5      | 464 ± 100 | 613 ± 157 | 608 ± 134 | 610 ± 136 | 762 ± 91 | 823 ± 94 |
| 6      | 393 ± 52 | 505 ± 54 | 501 ± 39 | 600 ± 136 | 778 ± 104 | 814 ± 159 |
| 7      | 557 ± 190 | 638 ± 156 | 623 ± 121 | 542 ± 109 | 742 ± 118 | 786 ± 147 |
| 8      | 410 ± 58 | 571 ± 139 | 560 ± 91 | 633 ± 69 | 924 ± 119 | 945 ± 157 |
| mean   | 413 ± 75 | 527 ± 84 | 523 ± 60 | 602 ± 104 | 807 ± 104 | 841 ± 135 |
with other methods of tissue analysis, such as histology and biomechanics, and may lead to improved interpretation of in vivo measurements. Ultimately, such methodological refinements will improve the effectiveness of combining MRI relaxation parameter mapping of ex vivo tendon water-binding properties within the tendon, thereby altering its ability to interact with PBS. In addition, it appears that $T_1$ and $T_2^*$ came close to saturation after 67 hours of tendon immersion in PBS. However, confirmation of a true saturation behavior (ie, to extract saturation time constants for the increases in $T_1$ and $T_2^*$ following tendon immersion in PBS) requires more than three time points to reliably fit the corresponding data. The underlying mechanism causing the increases in relaxation parameters in the mid-tendon and enthesis regions is likely related to an increase in weakly bound water or free water in the extracellular matrix due to tissue swelling. Furthermore, solute molecules may diffuse into the tissue and interact with its structure, which could also affect MRI measurements.

It is difficult to compare the $T_1$ and $T_2^*$ relaxation times from the current study with those given in the literature, since no data for sheep Achilles tendons are currently known. For human tendons, ex vivo $T_2^*$ relaxation times of 2.4 and 0.9 ms have been reported, and 1.5 and 0.4 ms for in vivo studies (all at 3 T). With baseline $T_2^*$ relaxation times of 0.2 and 0.5 ms for the mid-tendon and enthesis regions, respectively, our results fall within the lower range of values reported for human tissue at 3 T. Furthermore, increased $T_2^*$ values have been reported for the enthesis compared with the mid-tendon region, consistent with our findings. However, comparing our current results with data from human studies is not straightforward. For instance, tendons from animals and humans exhibit molecular differences and are exposed to distinct mechanical loading patterns and magnitudes due to the bipedal versus quadrupedal nature of locomotion. Moreover, discrepancies between studies may be due to the magic angle effect, which is known to have a strong impact on $T_2^*$. In the current study, the samples were fully excised from the surrounding tissue, which allowed a parallel alignment with the scanner’s main magnetic field. With larger samples, still containing surrounding tissue, alignment of the tendons with the magnetic field may be more difficult and possibly imprecise. Consequently, $T_2^*$ values reported in the literature may vary due to such orientation issues.

Reference values for $T_1$ relaxation times in human Achilles mid-tendons at 3 T have been reported to range between 610 and 640 ms, both in vivo and ex vivo studies; lower values (ie, 530 ms) were found for an ex vivo rabbit Achilles tendon (at 2 T). In humans, one study found a higher $T_1$ value in the enthesis compared with the mid-tendon, while another study found no $T_1$ difference between these regions. In the current study, the observed baseline $T_1$ values of 390 ± 38 ms for the mid-tendon and 600 ± 64 ms for the enthesis are lower than those indicated above. Apart from that, when comparing reported relaxation times between studies, it is important to consider the main magnetic field strength $B_0$, since both $T_1$ and $T_2^*$ are influenced by field strength. However, it is expected that $B_0$ will only affect the extent of the change, not the overall effect that the relaxation times of the tendons will cause by immersion in PBS. In addition to the abovementioned differences between human and animal tendons, $B_1$ inhomogeneities, different tissue sample sizes and temperature effects are another possible source of variation, especially when comparing values obtained from small excised tendons, intact cadaveric limbs and in vivo studies. In our experiments, temperature changes were considered negligible because the samples were placed in a temperature-controlled MRI system with an ambient temperature of 23°C for the entire duration of the 67-hour measurement.

In conclusion, we have shown that an experimental step as fundamental as immersing tendon samples in PBS for storage or preservation can have a significant impact on both $T_1$ and $T_2^*$ relaxation parameters. The strong effect of tendon swelling in PBS on the measured relaxation times underlines the importance of precise control of sample handling and hydration, not only before but also during the MRI of ex vivo tissue experiments, to yield valid and reliable results. Future studies using PBS for immersion of tendons should include detailed information regarding the immersion time and keep it as short as possible. Further studies are needed to obtain reproducible and robust relaxation parameters through improved protocols for ex vivo tendon imaging. Such studies should also include investigations on the effects of fresh versus frozen samples, because in many experimental instances it is necessary to freeze the samples for later evaluation. For such situations, it would be valuable to know the effects of freezing on the tissue properties since there are reports that frozen tendon samples have displayed alterations in tissue properties. Ultimately, such methodological refinements will improve the effectiveness of combining MRI relaxation parameter mapping of ex vivo tendon with other methods of tissue analysis, such as histology and biomechanics, and may lead to improved interpretation of in vivo measurements.
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APPENDIX A

The following table lists the acquisition parameters in alphabetical order.

| Acquisition parameter                        | Value                                                                 |
|-----------------------------------------------|----------------------------------------------------------------------|
| Bandwidth                                     | 1108 Hz/Pixel                                                         |
| Coil                                          | NORAS Variety (16 channels)                                          |
| Delay between ADC and readout gradient        | 20 μs                                                               |
| Dummy cycles                                  | 200                                                                 |
| Echo time (T<sub>1</sub> mapping)             | 0.15 ms                                                             |
| Echo times (T<sub>2</sub>* mapping)           | 0.15 ms, 0.35 ms, 0.60 ms, 1.77 ms, 1.92 ms, 2.22 ms                |
| Fat saturation                                | Off                                                                 |
| Field strength                                | 2.9 T                                                               |
| Flip angles (T<sub>1</sub> mapping)           | 5°, 11°, 14°, 19°, 26°, 23°                                       |
| Flip angle (T<sub>2</sub>* mapping)           | 15°                                                                 |
| Matrix size                                   | 192 x 148 x 58                                                       |
| Spatial resolution                            | 0.95 x 0.95 x 0.95 mm<sup>3</sup>                                   |
| Readout gradient flat top time                | 370 μs                                                              |
| Readout gradient ramp down time               | 170 μs                                                              |
| Readout gradient ramp up time                 | 170 μs                                                              |
| Readouts/spokes per fully sampled k-space     | 53 064                                                              |
| Repetition time (T<sub>1</sub> mapping)       | 9.4 ms                                                              |
| Repetition time (T<sub>2</sub>* mapping)      | 5.5 ms                                                              |
| RF-pulse type                                 | Rectangular                                                         |
| RF-pulse duration                             | 260 μs                                                              |
| RF-spoiling phase increment                   | 50°                                                                 |
| Spoiling                                      | RF + constant z-gradient                                             |
| Spoiling gradient moment                      | 3 times readout moment                                              |
| Trajectory                                    | 3D radial spike ball                                                |