# **Immersion of Achilles tendon in phosphate buffered saline influences T1 and T2\* relaxation times: an *ex vivo* study**

M. Krämer1,\*, M. R. Kollert2,3, N. M. Brisson2, M. B. Maggioni1,   
G. N. Duda2,3, J. R. Reichenbach1

1 Medical Physics Group, Institute of Diagnostic and Interventional Radiology, Jena University Hospital, Friedrich Schiller University Jena, Germany

2 Julius Wolff Institute and Center for Musculoskeletal Surgery, Charité – Universitätsmedizin Berlin, Germany

3 Berlin-Brandenburg Center and School for Regenerative Therapies, Charité – Universitätsmedizin Berlin, Germany

Corresponding Author:

Dr. rer. nat. Martin Krämer

Medical Physics Group, IDIR, Jena University Hospital

Philosophenweg 3, D-07443 Jena, Germany

Phone: +49 3641 9 390731

Fax: +49 3641 9 390728

E-Mail: [martinkraemer84@gmail.com](mailto:martinkraemer84@gmail.com)

Authors’ Names, Degrees, and E-Mails:

* Martin Krämer, Dr. rer. nat.: [martinkraemer84@gmail.com](mailto:martinkraemer84@gmail.com)
* Matthias R. Kollert, M.Sc.: [matthias.kollert@charite.de](mailto:matthias.kollert@charite.de)
* Nicholas M. Brisson, Ph.D.: [nicholas.brisson@charite.de](mailto:nicholas.brisson@charite.de)
* Marta B. Maggioni, M.Sc.: [marta.maggioni@uni-jena.de](mailto:marta.maggioni@uni-jena.de)
* Georg N. Duda, Dr.-Ing.: [georg.duda@charite.de](mailto:Georg.Duda@charite.de)
* Jürgen R. Reichenbach, Dr. rer. nat.: [Juergen.Reichenbach@med.uni-jena.de](mailto:Juergen.Reichenbach@med.uni-jena.de)

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**Abstract**

Robust relaxation parameter mapping in *ex vivo* tissue requires hydration to be closely controlled to ensure tissue integrity and consistent measurement conditions over long durations. One way to maintain the hydration of *ex vivo* tendon tissue is immersing samples in a buffer solution. To this end, various buffer solutions have been proposed; however, many appear to influence the tissue relaxation times, especially over prolonged exposure. In this work, we investigated the effect of immersing ovine Achilles tendon tissue in phosphate buffered saline (PBS) on T1 and T2\* relaxation times. *Ex vivo* samples were measured at 0 h (baseline), 30 h and 67 h after immersion in PBS. Ultra-short echo-time (UTE) imaging was performed using variable flip angle and echo-train shifted multi-echo imaging for T1 and T2\* estimation, respectively. Compared to baseline, both T1 and T2\* relaxation times increased significantly after 30 h of immersion in PBS. T2\* continued to show a significant increase between 30 h and 67 h. Both T1 and T2\* tended to approach saturation at 67 h. These findings demonstrate the importance of applying stringently controlled tissue preparation and preservation techniques, both before and during MRI experiments, as an experimental step as basic as immersing tissue samples in PBS can strongly influence relaxation parameter measurements.

**Introduction**

Tendons are crucial structures of the musculoskeletal system, as their primary mechanical role is to transmit muscle forces to bones, thereby allowing locomotion and enhancing joint stability [1]. Knowledge of basic structural and mechanical functions of healthy tendons is essential to understanding and treating injured or pathological tendons. Nonetheless, tendons have thus far been understudied with magnetic resonance imaging (MRI), likely due to challenges that arise with parameter quantification, notably, rapid signal decay caused by ultra-short T2\* relaxation times ranging between 0.5 ms and 2.5 ms [2, 3, 4]. Direct imaging of tendons by means of MRI requires the application of ultra-short echo-time (UTE) imaging sequences, which utilize two-dimensional half radiofrequency (rf) pulses [5, 6] or non-selective short rectangular rf pulses [7, 8, 9], which are not readily available on most clinical MRI systems. UTE imaging sequences can achieve echo-times (TE) below 1 ms, thus enabling direct imaging of tendons and parameter quantification simultaneously.

Being a non-invasive method, MRI has the advantage of yielding detailed examinations of *ex vivo* tissue while preserving its structural and mechanical properties. This not only enables longitudinal analysis but also direct comparison to histological or biomechanical measurements. Importantly, tissue parameters quantified in such samples could aid in linking structural or biomechanical properties from *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 unwanted susceptibility artifacts that can be reduced by immersing the samples in fluids that closely match susceptibility differences. Second, to achieve high spatial resolutions or to quantify tissue parameters of interest, the samples (which are typically small) must often be measured over a longer period of time: from several hours to more than a day. During such prolonged periods, sample integrity and hydration must be controlled as dehydration is known to strongly affect structure and mechanical properties of tendon tissue [10, 11]. For this reason, many experiments are performed after having stored the samples in water-free substances such as perfluoropolyether (Fomblin), with or without additional formalin fixation [12, 13]. Nevertheless, such approaches can influence tissue structural and mechanical properties, as well as quantitative MRI parameters [14, 15, 16]. To address these potential complications, an alternative method is to immerse the samples in saline or phosphate-buffered saline (PBS) solutions. These mimic the physiological osmolarity of interstitial fluid, for the measurement duration, especially when imaging fresh and unfixed samples.

Currently, a paucity of literature is available on the influence of buffer solutions on the quantification of MRI relaxation parameters in tendons. Chang *et al.* investigated the influence of immersing *ex vivo* human Achilles tendons in different fluids (saline solution, Fomblin and perfluorooctyl bromide) and found no influence on T2 and T2\* values [17]. However, their results were based on six tendon samples obtained from tissue banks, which had high inter-donor variability [17]. Moreover, those tendon samples underwent a deep-freeze-thaw cycle before being measured, which could have affected tissue swelling behavior. Pownder *et al.* reported that exposing fresh tendon tissue to such a freeze-thaw cycle decreases T2\* [18]. 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 [19]. 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. Variable flip angle [20] and multi-echo [21] 3D-UTE imaging was applied to quantify T1 and T2\*, respectively. Soleus portions of fresh ovine Achilles tendons were immersed in PBS for 67 h, and relaxation parameters were estimated after 0 h (baseline), 30 h and 67 h.

**Material and methods**

### **Sample preparation**

Fresh Achilles tendons from eight healthy sheep (Merino, female, 4+ years old) were excised after euthanasia in a different research project in compliance with the ethical guidelines of legal, local animal rights protection authorities (Landesamt für Gesundheit und Soziales Berlin). 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 individual 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. 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, Taufkirchen, Germany), and immediately (within 10 minutes) positioned in the MRI scanner for imaging.

### **Relaxation parameter mapping**

To estimate T1, a single-echo 3D-UTE acquisition with TE of 0.15 ms was repeated using a variation of the flip angle (VFA) [22] of 34°, 26°, 19°, 14°, 11°, and 5°. To calculate T1 relaxation time maps from the six data sets with different flip angles, a two-parameter fit of the signal equation for fast low-angle shot (FLASH) gradient-echo MRI sequences was applied [20].

To estimate T2\*, an echo-train shifted [23, 24] multi-echo 3D-UTE imaging sequence [20] was used with TE of 0.15 ms, 0.35 ms, 0.60 ms, 1.77 ms, 1.97 ms, and 2.22 ms. The multi-echo acquisition during one readout train was carried out in a monopolar fashion using re-phasing gradients between echoes. To map T2\* from the 6 echoes acquired, 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].

### **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 GmbH, Höchberg, Germany). Measurements were performed with a clinical 3 T whole-body MRI scanner (Magnetom PRISMA, Siemens Healthineers, Erlangen, Germany). For 3D-UTE imaging, non-selective hard pulse excitation and spikey-ball trajectories were used [27]. Echo-train shifted multi-echo and VFA acquisition were performed with the same orientation and gradient parameters described above, using a 192 x 148 x 58 acquisition matrix size and a 180 x 139 x 55 mm³ field of view that resulted in an isotropic spatial resolution of 0.95 x 0.95 x 0.95 mm³. Furthermore, 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 use 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 65 minutes in duration, were collected at baseline, 30 h and 67 h after filling the laboratory tubes with PBS.

Images were reconstructed offline in MATLAB (The MathWorks, Inc., Natick, Massachusetts, United States of America) using re-gridding with iterative sampling density compensation and an optimized kernel [28]. Data analysis and non-linear curve fitting were also performed in MATLAB. After calculation of relaxation parameter maps, regions-of- interest (ROI) were drawn manually in mid-tendon and enthesis regions of all samples. To this end, 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 T1 and T2\* relaxation parameters of all voxels were averaged for each ROI. To test the significance of changes in T1 and T2\* between 0 h (baseline), 30 h and 67 h of immersion in PBS, a two-sided Wilcoxon rank-sum test was performed in MATLAB. Statistical significance was set at p<0.05.

## **Results**

Typical FLASH signal curves were observed for the VFA acquisitions (Figure 1), with a peak between flip angles of 5° and 15° followed by a fall-off for larger flip angles. Since the individual peak positions change along the x-axis, the curves qualitatively indicate different T1 relaxation times between mid-tendon (Figure 1, left) and enthesis (Figure 1, right) regions, and between different immersion durations. After fitting the data to the FLASH signal model, the comparison of the extracted T1 relaxation times averaged over all samples (Figure 2) demonstrated a significant increase from baseline to 30 h for both the mid-tendon (p = 0.004) and enthesis (p = 0.002) region. Changes in T1 relaxation times from 30 h to 67 h were not significant for both regions (p>0.05). The change of T1 over the entire immersion duration was significantly greater (p = 0.004) in the enthesis (T1 = 231 ± 44 ms) compared to the mid-tendon region (T1 = 110 ± 20 ms).

Signals plotted over the six acquired echoes displayed rapid decays for both the mid-tendon (Figure 3, left) and enthesis (Figure 3, right) regions. Qualitatively, the signal in the enthesis region decayed more slowly. Quantitatively, the T2\* relaxation times averaged over all samples (Figure 4) increased significantly between baseline and 30 h (p = 0.002) and between 30 h and 67 h (p = 0.004) for the mid-tendon region. Furthermore, a significant increase was found for the enthesis region from baseline to 30 h (p = 0.002) and from 30 h to 67 h (p = 0.002). The total change in T2\* over the entire immersion duration was significantly higher (p = 0.001) in the enthesis (T2\* = 0.31 ± 0.03 ms) compared to mid-tendon region (T2\* s = 0.18 ± 0.02 ms).

## **Discussion**

In this study, significant increases in T1 and T2\* were observed after immersing ovine Achilles tendon samples in PBS. This finding is in contrast to that from Chang *et al.* [17], who reported no increase in T2\* for *ex vivo* human Achilles tendon following immersion in PBS. The latter study, however, investigated samples that were deep-frozen and thawed. This process could disrupt the microstructure of the extracellular matrix and change water binding properties within the tendon [18, 29], thereby altering its ability to interact with PBS. In addition, it appears that T1 and T2\* came close to saturation after 67 h of tendon immersion in PBS. However, confirmation of a true saturation behavior (i.e., to extract saturation time constants for the increases in T1 and T2\* following tendon immersion in PBS) requires more than three time points for reliable fitting of 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 through tissue swelling. Furthermore, solute molecules may diffuse into the tissue [19] and interact with its structure, which could also affect MRI measurements.

A comparison of T1 and T2\* relaxation times from the current study to those reported in the literature is challenging because there are currently no known data for ovine Achilles tendons. For human tendons, T2\* relaxation times of 2.4 ms [30] and 0.9 ms [31] have been reported for *ex vivo*, and 1.5 ms [3] and 0.4 ms [32] for *in vivo* studies. With baseline T2\* relaxation times of 0.2 ms 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 3T. Furthermore, increased T2\* values have been reported for the enthesis compared to the mid-tendon region [31, 32], consistent with our findings. Nevertheless, a comparison of the 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 *vs*. quadrupedal nature of locomotion [33]. Moreover, discrepancies across studies may be due to magic angle effect [34, 35], which is known to have a strong influence on T2\*. In the present study, the samples were fully excised from surrounding tissue, thus allowing 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, T2\* values reported in the literature may vary due to orientation issues.

Reference values for T1 relaxation times in human Achilles mid-tendons have been reported to range between 610 ms and 640 ms, both in *in vivo* [3, 4] and *ex vivo* studies [30, 31]; lower values (i.e., 530 ms) were found for an *ex vivo* rabbit Achilles tendon [36]. In humans, one study found a higher T1 value in the enthesis compared to the mid-tendon [31], while another study found no T1 difference between these regions [3]. In the current study, the observed baseline T1 values of 390 ± 38 ms for the mid-tendon and 600 ± 64 ms for the enthesis are lower than those indicated above. In addition to the above-mentioned differences between human and animal tendons, B1 inhomogeneities [37, 32], different tissue sample sizes and temperature effects [38] 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 effects were deemed negligible as the samples were placed in a temperature-controlled MRI system with an ambient temperature of 23°C for the entire duration of the 67 h measurement.

In conclusion, we have shown that an experimental step as fundamental as immersing tendon samples in PBS for measurement or preservation can have significant effects on both T1 and T2\* relaxation parameters. The strong effect of swelling of tendon tissue in PBS on the measured relaxation times underscores the importance of closely controlling the handling and hydration of samples not only before, but also during the MRI of *ex vivo* tissue experiments to yield valid and reliable results. More studies are needed to obtain reproducible and robust relaxation parameters through improved protocols for *ex vivo* tendon imaging. 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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## **Figure legends**

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| Figure 1: Dependence of the MR signal on the flip angle and the curve-fitting results (dashed lines) used to estimate the T1 relaxation time. Results are shown for one sample at baseline (blue) and after 30 h (red) and 67 h (yellow) of immersion in PBS. To simplify data visualization, the signals were normalized to the individual maximum of the curves. |

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| Figure 2: Box plots showing the estimated T1 relaxation times for a region-of-interest in the mid-tendon (*left*) and enthesis (*right*) at baseline, 30 h and 67 h after immersion in PBS. Statistically significant differences (p<0.05) are denoted by \*. |

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| Figure 3: 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 h (red) and 67 h (yellow) immersion in PBS. To simplify data visualization, the signals were normalized to the individual maximums of the decay curves. |

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| Figure 4: Box plots showing the estimated T2\* relaxation times for a region-of-interest in the mid-tendon (*left*) and enthesis (*right*) at baseline, 30 h and 67 h after immersion in PBS. Statistically significant differences (p<0.05) are denoted by \*. |