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Effect of phosphate electrolyte buffer on the dynamics of water in tendon and cartilage

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Abstract

A number of NMR spectroscopic and microscopic MRI (μ MRI) techniques were used to study proton dynamics in canine tendon and articular cartilage immersed in normal saline solution (NaCl solution) and high-concentration phosphate-buffered saline (PBS) solution. In a proton CPMG experiment on tendons, the T_2 relaxation of the tissue was found to be anisotropic and had two populations. When immersed in saline, the T_2 values were short and their relative populations were anisotropic. When immersed in PBS, the T_2 values increased and their relative populations became isotropic. These phenomena, also verified by proton double-quantum-filtered (DQF) NMR spectroscopy, were interpreted as the catalyzing effect of phosphate ions on proton exchange between water molecules. In the experiment on articular cartilage, the immersion of cartilage–bone blocks in PBS resulted in a significant reduction in the laminar appearance of cartilage on MRI (the magic angle effect). The quantitative T_2 anisotropy by μ MRI at 13 μ m pixel resolution and DQF NMR spectroscopy confirmed the substantial effect of PBS on the water dynamics in cartilage tissue blocks. This preliminary study has two important implications. For *in vitro* cartilage research, this work confirms the importance of the salt solution in which the specimen is stored – not all salts have the same effect on the measurable quantities in NMR and MRI. For *in vivo* cartilage diagnosis, especially using whole-body MRI scanners, this work suggests the possibility of using a suitable electrolyte as a novel contrast agent to assess the ultrastructural changes in cartilage due to tissue degradation.

Keywords

cartilage; phosphate; magic angle; T_2 relaxation; anisotropy; microscopic MRI; double-quantum filtered spectroscopy

INTRODUCTION

Several NMR and MRI properties of connective tissues (e.g. tendon and articular cartilage) are known to be anisotropic (i.e. depend on the orientation of the specimen in the polarizing magnetic field B_0) (1,2). As early as 1962, Berendsen (3) reported that the proton NMR signal had an isotropic central peak and an angular-dependent doublet in a partially dehydrated tendon. In a fresh bovine tendon, Krasnosselskaia *et al.* (4) measured the

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orientational dependence of the free induction decay of proton signals and showed that the dipolar coupling was responsible for a sixfold variation in the signal intensity with the specimen orientations between that parallel with the external magnetic field and the one at the magic angle. These anisotropic phenomena in tendons have been attributed to the direct dipolar interaction between intramolecular water protons in the fibrillose specimens (5–13).

Compared with the morphological structure of tendon, which is densely packed bundles of type-I collagen fibers, the morphological structure of articular cartilage is much more complex. In particular, non-calcified cartilage has a depth-dependent structure of type-II collagen fibrils, where the tissue is commonly divided into three structural zones across its thickness (depth). Starting from the surface, these three structural zones are: (I) the superficial (tangential) zone, where the collagen fibrils are parallel to the surface; (II) the transitional (intermediate) zone, where the collagen fibrils are mostly randomly oriented; and (III) the radial (deep) zone, where the collagen fibrils are oriented perpendicular to the surface (14,15). This depth-dependent fibril structure of articular cartilage results in a laminar appearance in the intensity images from MRI, the so-called magic angle effect in MRI of cartilage (2,16–26). The experimental fact (2) that the anisotropic characteristics of T_2 relaxation in articular cartilage quantitatively follow the geometric factor in the dipolar spin Hamiltonian, $(3\cos^2\theta - 1)$, promotes the idea that T_2 anisotropy in cartilage is mainly influenced by the dipolar interaction between the water protons and the collagen fibril network (2,18,22,25).

NMR/MRI study of relaxation anisotropy in tendon and cartilage is important because it provides a quantitative mechanism for monitoring the structure and macromolecular characteristics of the tissue. This approach has been used to study rehabilitation of ruptured tendons (12,27) and early changes in the tissue due to osteoarthritis (28,29). To filter out the large isotropic signal, Navon and his colleagues have been using multiple-quantum-filtered techniques to obtain NMR signal that is exclusively from macromolecules experiencing anisotropic motion (27,32–44). As the laminar appearance of cartilage in MRI is mainly due to the non-vanishing intramolecular dipolar interaction, which may complicate the clinical interpretation of the images, several dipolar refocusing schemes (e.g. solid echo and spin lock) have been proposed to eliminate the dipolar interaction and to reduce the laminar appearance of cartilage in clinical MRI (30,31).

Berendsen and Migchelsen (45,46) in the 1960s made the interesting observation that the addition of certain salts that contain proton-donating or proton-accepting ions to hydrated collagen influences the proton NMR spectra in the same way as raising the temperature. This phenomenon was explained by the fact that these salts increase the rate of proton exchange between the water molecules, thus limiting the average lifetime of proton pairs (6,7). The purpose of this preliminary study is therefore twofold: (a) to investigate the influence of phosphate-buffered saline (PBS) on the dynamics of water molecules in tendons and cartilage through T_2 relaxation and proton double-quantum-filtered (DQF) NMR spectroscopy, and (b) to investigate this effect on the laminar appearance of articular cartilage in MRI.

EXPERIMENTAL

Solutions of normal saline and PBS

All solutions of normal saline and PBS used in our experiments were prepared in our laboratory. A solution of normal saline is simply 0.9% NaCl in deionized water. A solution of PBS was prepared as follows. First, 276 g sodium phosphate monobasic (monohydrate) was added to deionized water. The volume of the phosphate buffer was then brought to 1 liter after adjustment of the pH to 7.3 with NaOH. Then 9 g NaCl was added to 50 mL of the phosphate buffer and diluted to 1 liter with deionized water. The final PBS solution contained a phosphate concentration of 100 mM and a sodium concentration of 150 mM, and had a pH corresponding to physiological pH (~7.4).

Tendon and cartilage samples

Fresh Achilles tendons and humeral heads were harvested within 4 h of death from dogs sacrificed for unrelated cardiovascular research. The dogs were 1–2 years old and musculoskeletally healthy. The tendons were immersed in saline solution and frozen at -80°C until used. Before the NMR measurement was taken, a tendon was thawed at 4°C in saline or the PBS solution for 2 days for stabilization. Then the surface of the specimen was wiped dry and the specimen was sealed in a 5 mm NMR tube with Fluorinert FC-77 (3M Co., St Paul, MN, USA). Three pieces of tendon (from three different animals) were used in this investigation. For cartilage samples, tissue slices of 1.75 mm thickness were cut from the humeral head using a table diamond saw. Three or four cartilage–bone plugs were cut from each slice, and the plugs, all with relatively flat surface, were used in the experiments. A total of five cartilage specimens (from three dogs) were used in this study. Before the NMR or MRI experiment, the cartilage–bone plugs were bathed in saline or PBS and kept at -20°C until used. All cartilage–bone samples were sealed in NMR tubes with an internal diameter of 2.34 mm (Wilmad Glass, Buena, NJ, USA) for NMR and MRI experiments.

NMR spectroscopy and microscopic MRI (μMRI)

NMR spectroscopic and μMRI experiments were performed at room temperature on a Bruker Avance II 300 NMR spectrometer equipped with a 7T/89 mm-wide vertical-bore superconducting magnet and micro-imaging accessory (Bruker Instruments, Billerica, MA, USA). A Bruker 5 mm birdcage coil with rotation device was used for the tendon experiments, where the long axis of the tendon sample was varied in the magnetic field. A home-made 3 mm solenoid coil was used for cartilage–bone experiments, where the orientation of the collagen fibrils in the deep zone of the cartilage block with respect to the static magnetic field can be adjusted (26).

Proton DQF spectroscopic signals were acquired using the following pulse sequence (37–39):

$$90^{\circ}_{\phi_1} - \frac{\tau}{2} - 180^{\circ}_{\phi_2} - \frac{\tau}{2} - 90^{\circ}_{\phi_1} - t_1 - 90^{\circ}_{\phi_3} - (\text{acquisition}) \quad (1)$$

A set of phase cycles with $\phi_1 = 0, 1, 2, 3$; $\phi_2 = 1, 2, 3, 0$; $\phi_3 = 0, 0, 0, 1, 1, 1, 1, 2, 2, 2, 3, 3, 3, 3$, and $\phi_{\text{receiver}} = 0, 2, 0, 2, 3, 1, 3, 1, 2, 0, 2, 0, 1, 3, 1, 3$ was used in the DQF

experiments. Other critical parameters were: $TR = 5$ s; 16 dummy scans; $\tau = 400$ μ s; $t_1 = 4$ μ s; a 90° radiofrequency excitation pulse of 5 μ s and 7.75 μ s for cartilage and tendon samples in solenoid and birdcage coils, respectively.

Spectroscopic measurements of T_2 relaxation were performed using a standard CPMG pulse sequence:

$$90^\circ - (\tau - 180^\circ - \tau)_n - (acquisition) \quad (2)$$

A τ value of 500 μ s was used in the spectroscopic experiments to avoid the spin-locking effect (47). Only even echoes were used in the experiments, where the last number of echoes was 820. The 90° radiofrequency excitation pulse had a duration of 7.95 μ s, the TR was 5 s, and the number of dummy scans was 4. As tendons have at least two populations of water molecules – free water, which has a long T_2 relaxation (tens of milliseconds), and bound water, which has a short T_2 relaxation (a few milliseconds) (1,8–13) – the T_2 decay curves of proton signals in tendon were fitted by a double-exponential function:

$$S(t) = A[P_a \times \exp(-t/T_{2a}) + (1 - P_a) \times \exp(-t/T_{2b})] + B \quad (3)$$

where A is the overall signal intensity, B accounts for any offset of the signal, and P_a is the population of one component (T_{2a}).

Quantitative T_2 -imaging experiments were performed using a magnetization-prepared T_2 -imaging sequence (2), with a TE of 8.6 ms in the imaging segment (TEi). The TE of the leading contrast segment (TEc) had five increments (0.002, 4, 10, 30, 60 ms) for cartilage in saline at 0° relative to B_0 , four increments (0.002, 20, 50, 100 ms) for cartilage in saline at the magic angle ($\sim 55^\circ$) and the cartilage sample in PBS at both 0° and 55° . The TR of the imaging experiment was 2 s. The T_2 relaxation time in imaging was calculated by fitting the data on a pixel-by-pixel basis with a single exponential equation. The in-plane pixel resolution, which was across the depth of the cartilage tissue, was 13.0 μ m, and the slice thickness was 1 mm.

RESULTS

Tendon in saline and PBS

The spectroscopic results for the tendon from the proton CPMG experiments were fitted with the double-exponential function (eqn 3) and are shown in Fig. 1, where the R values of the exponential fitting were better than 0.998. Four distinguishable features were found. Firstly, the T_2 values of water from the tendon immersed in PBS were longer than those from the tendon immersed in saline at all orientations from 0° to 90° relative to the main magnetic field B_0 . The range of the average T_2 values in tendon was 4.6–11.4 ms when immersed in saline, and 10.2–25.2 ms when immersed in PBS. Secondly, both the long and short T_2 components were orientational-dependent, no matter whether the tendon was immersed in saline or PBS. Thirdly, the longest T_2 values occurred when the specimen was oriented at the magic angle ($\sim 55^\circ$) to B_0 . Finally, the two T_2 populations in the tendon in saline were strongly anisotropic, whereas in the tendon in PBS they were isotropic.

Figure 2 shows the DQF spectroscopic signals for a tendon specimen immersed in saline and PBS, where the splitting and intensity of the ^1H DQF signal are clearly anisotropic. At the 0° orientation where the long axis of the tendon specimen was parallel with B_0 (spectrum 1), the dipolar splitting was ~ 690 Hz for the tendon immersed in saline (a), which agrees with the result in the literature (37). The same splitting was reduced to ~ 150 Hz when the specimen was immersed in PBS (b). In addition, the intensity of the DQF signal for the tendon in PBS at 0° became less than one-twentieth of that in saline. At the 55° orientation (spectrum 4), the smallest ^1H dipolar splitting and the weakest ^1H dipolar DQF intensity were found for the tendons in both solutions. The dipolar splitting at 55° was ~ 125 Hz for the tendon in saline, and appeared to contain multiple peaks (from 80 Hz to 200 Hz) for the tendon in PBS. The intensity of the dipolar signal for the tendon in PBS at 55° was only about one-fiftieth of that in saline. The full width of the dipolar splitting and the half-height of the dipolar signal are plotted in Fig. 3, which summarizes the trends observed in these orientational-dependent DQF signals.

Cartilage in saline and PBS

As the collagen organization in articular cartilage is more complex than that in tendons, μMRI was used to investigate the effect of PBS on the anisotropy of T_2 relaxation in different structural zones of cartilage at 0° and the magic angle at $13\ \mu\text{m}$ pixel resolution. Figure 4 shows the proton intensity and T_2 images of a cartilage–bone specimen immersed in saline and PBS at $\sim 0^\circ$ and 55° . The reduction in the laminar appearance of cartilage in MR images when the specimen is immersed in PBS is clear. The one-dimensional profiles of T_2 relaxation in cartilage from these images are shown in Fig. 5. Whereas the T_2 profiles for cartilage in saline clearly show the well-documented characteristics of a depth-dependent anisotropy (2,22,26), the anisotropy of the T_2 profiles for cartilage in PBS appears much weaker. In addition, the T_2 values for the tissue in PBS were reduced.

The proton DQF spectroscopic method was also used to study the cartilage specimens at 0° and 55° . The results are shown in Fig. 6. Although the tissue block was immersed in either saline or PBS, the protons in the solutions made no contribution to the DQF signal because of their isotropic motions. The DQF signal came exclusively from the residual dipolar coupling, originating from the macromolecules in the tissue that were experiencing anisotropic motions. It is clear from this figure that a strong proton DQF spectrum with a splitting of 120 Hz can only be observed when the specimen is immersed in saline at the 0° orientation. The elimination of the DQF signal for tissue in saline at 55° verifies the prevailing influence of the dipolar coupling, which is minimized at the magic angle. The disappearance of the DQF signals for cartilage in PBS at 0° clearly illustrates the influence of phosphate ions in the PBS solution. The fact that a strong proton DQF signal can only be observed when the sample is immersed in saline at 0° offers vital confirmation that the observed signal in cartilage is from the intended DQF mechanism which is exclusively from anisotropic molecular motion, and not from any leakage of normal (single-quantum) NMR signal. Work investigating other issues that might affect the dynamics of water in tendon and cartilage (e.g. the effects of pH and the concentration of ions) is being performed in our laboratory.

DISCUSSION

The T_2 relaxation in NMR/MRI of tendons and articular cartilage is known to be strongly anisotropic (1,2,8–10,12,13,18–20,22,24–26) because of the non-zero averaging of the dipolar interaction between the molecules in the tissues. This non-zero averaging originates from the organized structures of macromolecules at the morphological scales in these tissues, which results in a motional anisotropy in the dynamics of water protons. For articular cartilage, as the tissue has multiple histological zones across its thin depth (thickness), the T_2 anisotropy also becomes depth-dependent (2). At the magic angle, minimization of the dipolar interaction restores the T_2 relaxation to approximately isotropic, which results in loss of the laminar appearance of articular cartilage in MRI (25).

The physical origin of the proton DQF signal in connective tissues is similar to that of T_2 anisotropy, also coming from the residual dipolar interaction associated with the anisotropic averaging of the molecular motions. The proton DQF signal, however, is more sensitive to the degree of molecular orders in biological tissue because it comes exclusively from molecules experiencing anisotropic motion and has no contribution from the large pool of molecules experiencing isotropic motion. In contrast, the anisotropic signal of T_2 relaxation in cartilage contains multiple components, both isotropic and anisotropic. Spectroscopic studies of collagen fibrils have found the DQF signal and the splitting of the NMR spectra to be temperature-sensitive (3,6,7,9–11,45), which has been ascribed to proton exchange between water molecules, the rate of which is increased at increasing temperature.

In the 1960s, Berendsen and Migchelsen (45,46) noticed that the addition to hydrated collagen of certain salts that contain proton-donating or proton-accepting ions can influence the proton NMR spectra in the same way as raising the temperature or increasing the water content. This phenomenon can be explained by the fact that the average lifetime of proton pairs can become limited with the increase in the rate of proton exchange between the water molecules (6,7). Berendsen and Migchelsen (45,46) also found that salts that stabilize native conformations and have hydrogen-bonding properties can increase the exchange rate between water molecules; phosphate and ammonium ions are the most effective and to a lesser extent sulfate ions, but salts such as NaCl do not exert such an effect.

Therefore, our experimental results must be evaluated on the basis of the difference in water dynamics in the specimens when they are immersed in different salt solutions. The mechanism of proton exchange in normal water has been proposed by Meiboom (48)

where k_1 and k_2 are the rate constants of proton exchanges. For the mechanism of proton exchange in aqueous solution of phosphate buffer, Luz and Meiboom (49) suggested a series

of proton transfer reactions between the buffer components through one or more water molecules. Of these reactions, the most efficient was:

The second-order rate constant k in eqn (6) was found to be $1.45 \times 10^9 \text{ mol}^{-1} \cdot \text{l} \cdot \text{s}^{-1}$ at 25° . It is obvious that H_2PO_4^- and HPO_4^{2-} act as proton-donating and proton-accepting groups, respectively, for indirect proton exchange in this phosphate buffer solution.

These proton exchange mechanisms can be used to explain our results qualitatively as follows. *In vivo* or when immersed in saline, there are approximately two populations of water molecules in tendon and cartilage: bound water and free water. The former has a longer time scale in the anisotropic reorientation than the latter population, both contribute to the final signal in the experiments. The direct proton exchange between the two populations of water molecules must be slow for the tendon sample, which results in the observation of approximately two T_2 components. In contrast, when the tissue is immersed in PBS, the phosphate ions can work as a bridge for proton exchange between bound or free water molecules or even between bound and free water molecules, which results in the increase in the T_2 value of water in tendon and cartilage.

Although the effect of phosphate ions on the dynamics of water is similar in both tendons and cartilage, small differences between the two tissues can be observed from this study. For instance, a residual dipolar interaction can be seen clearly in the DQF NMR signal in tendons immersed in PBS (Fig. 2b), but the same is not the case for cartilage in PBS (Fig. 6c,d). We think that this difference is caused by the difference in water concentration and ultrastructure of these two tissues: water only occupies about 10% of the wet weight in tendons compared with ~70% in cartilage. So the dipolar interaction in cartilage is weaker than in tendon. In addition, because of the tightly bound collagen fibers in tendons, it is possible that some of the bound water cannot be accessed by the ions in PBS, which results in insufficient exchange between this subpopulation of protons through phosphate ions. In this case, immersion in PBS has no significant effect on this population of water molecules, and, as a result, the DQF NMR signal can still be clearly observed, as seen in Fig. 2. A similar result was found in a previous study (6), in which the protons that give rise to the center line in the H_2O collagen spectra below 100% relative humidity did not exchange with H_2O or D_2O . The authors pointed out that these protons might come from the OH groups on the collagen that are shielded from contact with water.

Finally, Fig. 1 shows that both the value and population of the short and long T_2 components were anisotropic when the tendon was immersed in saline, but the population of T_2 values became isotropic when the tendon was in PBS. Similar results have been reported (10,12) for fresh tendon not immersed in saline or PBS. Peto and Gillis (10) explained this phenomenon as the influence of cross-relaxation which moves the orientation-dependence of the relaxation times towards the initial values of the decay modes. Takamiya *et al.* (12) assumed that the magic angle effect had obscured the distinction between the bound and free water fractions. Although we can cautiously ascribe this phenomenon to the proton

exchange between bound and free water, a deeper understanding clearly awaits further investigation.

We would like to emphasize that the results in this report were obtained from specimens that were immersed in PBS with a phosphate concentration of 100 mM. We show in this study that phosphate ions at this concentration have strong effects on the dynamics of water molecules in tendons and cartilage. Further study of the concentration-dependence may provide further insights into the mechanism that governs the water dynamics of biological tissues.

CONCLUSION

To the best of our knowledge, this is the first report that documents the effect of an electrolyte buffer (limited to a high concentration of phosphate ions in this report) on the dynamics of water molecules in articular cartilage observed using microscopic imaging. The μ MRI result and spectroscopic NMR results show clearly that the phosphate ion has strong effects on the dynamics of water molecules in tendons and cartilage. When immersed in the PBS solution, the effect of T_2 anisotropy in articular cartilage was greatly reduced and the laminar structure almost disappeared. This result has two significant implications. First, in *in vitro* cartilage studies, it is important to choose the soaking/preservation solution carefully. Some of the ions in the salt solution may have vital effects on the water dynamics of the tissue. Second, in *in vivo* cartilage studies and clinical diagnosis, if a suitable electrolyte that has strong effects on proton exchange can be found, manipulation of the laminar appearance of cartilage in clinical MRI could become a new image contrast approach for monitoring ultrastructural changes in cartilage degradation due to osteoarthritis and other joint diseases.

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Abbreviations used:

μ MRI	microscopic MRI
DQF	double-quantum-filtered
PBS	phosphate-buffered saline

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Figure 1.

The double-exponential fitting of proton spectroscopic results from tendon. (a) and (b) are the results for tendon immersed in saline, and (c) and (d) are the results for tendon immersed in PBS. The average of two T_2 relaxation times (T_{2Ave}) was calculated using $1/T_{2Ave} = P_a/T_{2a} + (1 - P_a)/T_{2b}$.

Figure 2.

The DQF signals for tendon samples immersed in saline (a) and PBS (b). The labels 1–6 indicate the orientation of the specimens at 0° (parallel with B_0), 17° , 34° , 55° (the magic angle), 74° , and 90° , respectively. The amplitudes of the signals in (b) were increased by 20 times.

Figure 3.

The full width (a) and half-height (b) of the proton DQF signals, measured from the signals in Fig. 2. The splitting for tendon in PBS was not measured because the splitting was small and the signal was noisy, as seen in Fig. 2b.

Figure 4.

The proton intensity images and T_2 images of a cartilage–bone specimen immersed in saline and PBS, at $\sim 0^\circ$ and 55° , respectively. The proton images were acquired with the shortest T_2 contrast ($TE_c = 0.002$ ms and $TE_i = 8.6$ ms). All T_2 images were displayed using the maximum and minimum of 60 and 0 ms, respectively.

Figure 5.
The T_2 profiles of articular cartilage extracted from the two-dimensional images shown in Fig. 4. The weakening of T_2 anisotropy when the tissue is immersed in PBS is clear.

Figure 6.
Proton DQF spectroscopic results from cartilage–bone blocks, in saline (a,b) and PBS (c,d).