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# Alterations in Magnetic Resonance Imaging $T_2$ Relaxation Times of the Ovine Intervertebral Disc Due to Non-enzymatic Glycation

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## Abstract

**Study Design—**An *in vitro* study using ovine intervertebral discs to correlate the effects of advanced glycation end-products (AGEs) with disc hydration evaluated by magnetic resonance imaging (MRI).

**Objective—**To determine the relationship between the level of AGEs and tissue water content in intervertebral discs using $T_2$ relaxation MRI.

**Summary of Background Data—**AGEs result from nonenzymatic glycation, and AGEs have been shown to accumulate in the IVD tissue with aging and degeneration. AGEs can alter biochemical properties, including the hydrophobicity of the extracellular matrix. Since one of the degenerative signs of the IVD is the reduced hydration, it was hypothesized that increased levels of tissue AGEs may contribute to disc hydration. $T_2$ relaxation MRI has been shown to be sensitive to the hydration status of the disc, and may be valuable in detecting the changes in the IVD mediated by the increase of AGEs.

**Methods—**Thirty-eight IVDs were obtained from 4 ovine spines, and the annulus fibrosis (AF) and nucleus pulposus (NP) tissues were isolated from these discs. The tissues were incubated in either a ribosylation or control solution for up to 8 days to induce the formation of AGEs. These tissues were subsequently analyzed for tissue water content and concentration of AGEs. $T_2$ relaxation times were obtained from these tissues after ribosylation.

**Results—**Ribosylation led to the increased accumulation of AGEs and reduced water content in both the AF and NP in a dose-dependent manner. When analyzed by MRI, ribosylation significantly altered the mean $T_2$ relaxation times in the NP ($p=0.001$), but not in the AF ($p=0.912$). Furthermore, the mean $T_2$ values in the NP significantly decreased with increasing periods of incubation time ($p<0.001$).

**Conclusion—**This study demonstrates that levels of AGEs in the IVD may affect the tissue water content. Moreover, these ribosylation-mediated changes in tissue hydration were detectable

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using $T_2$ relaxation MRI. $T_2$ relaxation MRI may provide a non-invasive tool to measure *in vivo* changes in disc hydration that are negatively correlated with the accumulation of AGEs.

### Keywords

Non-enzymatic glycation; advanced glycation end products; magnetic resonance imaging; intervertebral disc; degenerative disc disease

## Introduction

Degenerative disc disease is a major public health problem in the United States with significant economic and social costs [1,2]. Degeneration of the intervertebral disc (IVD) is characterized by a loss of cellularity, degradation of extracellular matrix, and dehydration of disc tissue leading to the loss of structural integrity in the functional spine segment [3]. Advanced glycation end-products (AGEs) are formed through the non-enzymatic glycation (NEG) of amino residues and oxidation of fatty acids [4,5]. Tissues that undergo relatively low levels of biological turnover containing long-lived structural proteins, such as collagen, are known to accumulate AGEs [5]. Thus, the modest vascularity combined with the limited tissue remodeling that occurs within the IVD make it particularly susceptible towards accumulating AGEs. This accumulation of AGEs has been associated with the narrowing of intradiscal space [6,7]. At the tissue level, increased AGEs affect the mechanical behavior of disc tissues including reduced strain energy dissipation and increased stiffness of the IVD matrix [8]. The loss disc hydration is a common finding in disc degeneration although the exact mechanisms are not known. The formation of AGEs is known to cause changes in the charge-density characteristics, membrane permeability, and tissue hydrophobicity in the extracellular matrix [4,9,10]. Since AGEs are known to accumulate in the degenerating disc, and increased AGEs can alter proteoglycan charge density and hydrophobicity of tissues, we thus hypothesized that the accumulation of AGEs in the IVD tissue may affect its tissue water content.

Magnetic resonance imaging (MRI) is a valuable noninvasive clinical tool for assessing the morphology of the IVD [11–13]. The Pfirmann and Modic grading systems use $T_1$ and $T_2$ weighted MRI sequences to classify the level of IVD degeneration in the lumbar spine [12–16]. $T_2$ relaxation MRI has been shown to correlate with IVD tissue water content [11]. Furthermore the $T_2$ signal intensity, a surrogate measure for hydration, has been shown to be a useful indicator of disc degeneration [12,16]. The ability to measure disc hydration non-invasively makes $T_2$ relaxation MRI an ideal tool to monitor the AGEs-mediated changes in tissue water content, and may be useful in monitoring therapies targeted towards the rehydration of the IVD. We thus further hypothesize that the AGEs-mediated changes in IVD tissue water content is detectable by $T_2$ relaxation MRI. Using *in vitro* ribosylation system on ovine discs, we investigate the effects of AGEs on the water retention characteristics of the IVD and the ability of MRI to detect these changes.

## Materials and Methods

### Sample Preparation

Four 6-month old ovine spines were obtained from Colorado State University in accordance to the Institutional Animal Care and Use Committee (IUCAC) protocols. Thirty-eight IVDs were obtained from the thoracic and lumbar regions of these spines. Care was taken to maintain the intact tissue integrity during the dissection process. The nucleus pulposus (NP=38) and the annulus fibrosus (AF=38) were separated from each sample, resulting in a total of 76 specimens (Figure 1). Specimens were dissected in the transverse direction from the nucleus pulposus and along the anterior region of the annulus fibrosis (Figure 1). Tissues

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were stored in a 0.15M phosphate buffered solution (PBS) with protease inhibitors at 4°C to minimize swelling.

## In vitro ribosylation of IVD tissues

The specimens were paired by disc level and incubated in ribosylation solution for 0, 2, 4, 6, or 8 days in 37°C. Once the tissues have undergone the designated incubation time points, it is then removed from the ribosylation solution, placed into a control solution, and placed back at 37°C until all the specimens have undergone 8-days of incubation. The ribosylation solution contained 0.6M ribose, 25mM e-amino-n-caproic acid, 5mM benzamidine, 10mM N-ethylmaleimide, and 30mM Hepes in Hanks buffer [17]. The control solution had the same composition as the ribosylation solution but without ribose. In preliminary studies, these ribosylation parameters achieve a four to five-fold increase in AGEs of the IVD tissue that is comparable to the levels observed in aging and degeneration [6]. After all specimens have been subjected to an 8 day incubation period, the samples were then stored back in 0.15M PBS at 4°C.

## Biochemical analyses of the IVD matrix

Forty samples of disc tissues (NP-20, AF-20) were massed before and after speedvac dessication. The tissues were first digested by papain (Sigma Aldrich, 18 mg/ml, 26 U/mg) for 16 hours, and then assayed using 1,9-dimethylmethylene blue dye-binding assay (DMMB) to determine the normalized glycosaminoglycan (GAG) concentration. The remaining papain-digested tissue lysates were hydrolyzed in 6N HCl at 60°C for 24 hours and the autofluorescence of the hydrolysates, a measure of AGEs [5, 17], was determined at an excitation wavelength of 370 nm and an emission wavelengths of 440 nm and normalized to a quinine standard. The collagen content, determined by a colorimetric assay for hydroxyproline, was determined from these same lysates [18]. The final AGEs measurement is then given by the fluorescence in nanograms quinine and divided by the collagen content of the tissue. The GAG content is calibrated by a standard curve (optical density read at a 525 nm) of chondroitin sulfate C from shark cartilage (chondroitin 6-sulfate; Sigma-Aldrich) and normalized by the detected hydroxyproline mass present in an equal volume of tissue. The collagen content is reported as the assayed collagen amount over the dry tissue weight.

## Magnetic Resonance Imaging

The remaining 36 specimens were subjected to the same ribosylation protocol and then analyzed by MRI. The disc samples were placed in conical tubes and immersed in PBS. Imaging was performed on a clinical 3.0 T GE Excite MRI scanner (GE Medical Systems, Milwaukee, WI) using a 7-turn solenoidal small animal coil built in-house. Multi-echo spin echo $T_2$-weighted images were acquired using a 4 cm x 2 cm field of view, 256 x 128 matrix, 1 mm slice thickness and 2000 ms repetition time ($T_R$). Individual echo times ($T_E$) were varied at intervals of 20 ms between 20 and 160 ms for samples. Histogram analysis of the AF and NP MRI data was performed using in-house software written with IDL 7.0 (ITT Visual; Boulder, CO). Nonlinear fits containing additive Gaussians were used separate background and tissue voxels. The means for $T_2$ values were averaged over the tissue voxels.

## Statistical Analyses

The analysis of variance (ANOVA) was used to determine the effects incubation time on the resulting AGEs concentration and tissue water content in the IVDs; and the effects of *in vitro* ribosylation on $T_2$ relaxation times. The Fisher's Least Squares Differences test was used to determine post-hoc comparisons between groups. Pearson's correlation coefficient

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between AGEs and water content of IVD was determined for both the AF and NP. Statistical analyses were conducted using MiniTab (MiniTAB Inc. State College, PA).

## Results

Increasing durations of ribosylation incubation times resulted in the increased concentration of AGEs in both the AF ($p<0.001$; ANOVA) and NP tissues ($p<0.001$; ANOVA; Figure 2A). The water content in these tissues also decreased significantly with incubation time in both the AF ($p<0.001$; ANOVA) and NP ($p<0.001$; ANOVA; Figure 2B). The reduction of tissue water content in the intervertebral disc correlated negatively with the concentration of AGEs ($p<0.001$; Pearson's correlation; Figure 3). The GAG content and collagen content were not statistically different between the tissues that underwent ribosylation and the 0-day controls (Table I).

*In vitro* ribosylation significantly altered the mean peak $T_2$ relaxation times (Table II; Figure 4) in the NP ($p=0.001$; ANOVA), but not in the AF ($p=0.912$; ANOVA) when compared with the respective 0-day controls. Furthermore, the mean $T_2$ values of the NP samples significantly decreased ($p<0.001$; Pearson's correlation) in a dose-dependent manner with increasing periods of incubation time (Figure 5).

## Discussion

We demonstrate for the first time that the loss of water content in IVD can be mediated through the accumulation of AGEs by nonenzymatic glycation, and these changes in tissue water content were detectable by $T_2$ magnetic resonance imaging. The results of this study suggest that the increases of AGEs observed in aging and degenerating intervertebral disc may be contributing to the loss of hydration in the tissue.

The Maillard reaction that occurs with NEG creates AGEs that structurally crosslink the amino residues of matrix components including collagen [5, 6] and proteoglycans [4]. At the molecular level, these crosslinks inhibit stretching of the collagen network resulting in reduced energy dissipation capabilities of the tissue [5,10]. The increased accumulation of AGEs also alters the molecular characteristics of proteoglycans by reducing the buoyant density of the aggrecan subfractions [4], causing a shift in the balance between hydrophobic and hydrophilic interactions that are necessary for optimal permeation of fluids [4,15]. Because the intervertebral disc is a biphasic material consisting of matrix proteins and ionic fluids whose deformation resistance is based on the intricate interactions between the two phases [19], it is likely that the changes in hydration due to AGEs observed in this study would have adverse consequences for IVD tissue mechanical behavior [20]. Because AGEs do not directly imbibe water, the loss of water content may also be a consequence of the changes in the extracellular matrix due to alterations in the inability of the collagen network to swell under crosslinked conditions [21], or the reduced ability of the proteoglycans to reversibly bind to water due to AGEs-mediated crosslinking with collagen [22]. Furthermore, it is likely that the permeability of the intervertebral disc, which is critical towards the nutrition and viability of the disc tissue [20], affects the tissues' general health and ability to retain water [23].

The detrimental effects of AGEs on the IVD extend beyond tissue hydration and material properties of the disc. AGEs have pleotropic effects on cellular function through advanced glycation end-product receptors (r-AGEs) that are found on macrophages, mesangial, and endothelial cells. The activation of r-AGEs have been implicated in inflammatory pathways including those of cytokines TNF alpha, IL-1 alpha, growth factors, endocytosis, and proteolytic enzymes [24]. Tsuru *et al.* showed that AGEs cause macrophage apoptosis that

leads to further disc extrusion during human disc herniation [25]. An *in vitro* study by Yokosuka *et al.* showed that culture of nucleus pulposus cells in an oxidative environment with AGEs resulted in the reduced cellular protein expression of proteoglycan and aggrecan [3].

Given these pathologic effects of AGEs on the degeneration of the intervertebral disc, treatment strategies for intervertebral disc rehydration may require, at least in part, the reduction or cleavage of AGEs concentration in the extra-cellular matrix. There are several compounds that can inhibit the formation or breakdown of these advanced glycation end-products. One such compound, aminoguanidine has been shown to both decrease AGE levels through inhibition of NEG and reverse the characteristic damage to tissue such as crosslinking of collagen fibers and increased permeability of cellular basement membrane [26, 27]. Other compounds such as aspirin and pyridoxamine have also been shown to reverse AGEs formation [21, 28].

Magnetic resonance imaging operates on the principle of proton realignment of water molecules within a shifting magnetic field, and the spin-echo relaxation times (such as $T_{1\rho}$ and $T_2$) are commonly used as surrogates for tissue hydration in the intervertebral disc [11]. The observed decreases in the $T_2$ relaxation times in the nucleus pulposus suggests that the AGEs-mediated changes in water content of the tissue may be detectable by non-invasive biomedical imaging [11–14]. The decreasing $T_2$ relaxation times observed in the ribosylated nucleus pulposus tissues are consistent with the trends observed in the pathologic degeneration of the IVD [12, 14], and the increased AGE concentration reduced tissue water content in the NP in a dose-dependent manner. On the other hand, the changes in AF hydration were not detectable by MR imaging, confirming the findings by Chatani et al [29]. The lack of detectable difference by MR in AF hydration may be a limitation of the sensitivity range of MR imaging [11,29].

Individuals with elevated glycemic levels, such as those afflicted with diabetes mellitus, are known to have increased accumulations of AGEs in their tissues [5,10,26,27]. These individuals are three times as likely to have disc herniation even after adjusting for age and weight, suggesting that AGEs may increase the risk of disc degeneration [30]. Discs from diabetic animal models have reduced levels of disc hydration, fixed charged density, and osmotic resistance [31], all of which are consistent with AGEs-mediated modifications of the extra-cellular matrix. These changes in the disc are coupled with reduced buoyant density in the disc proteoglycans and increased under-sulfated glycosaminoglycans [32]. Our findings presented here demonstrate the causal relationship between increased AGEs and the reduced ability of the IVD matrix to retain water.

There are several notable limitations to this study. First, although the ovine spine is a commonly used model for the human spine biomechanics [33, 34], subtle differences exist in the matrix components of the intervertebral disc, including the species of glycosaminglycans with different molecular confirmations [35]. However, because nonenzymatic glycation does not target specific proteins, but rather exposed amino residues, it is likely that the formation kinetics and resulting species of AGEs within human and ovine tissues are quite similar. Secondly, the current study examines only the effects of AGEs within the IVD tissue, and does not account for other age-related and pathological changes such as matrix degradation and cellular function. With these additional changes occurring in the degenerating disc, it is likely that the changes in water content and $T_2$ relaxation times will be more pronounced in tissues that exhibit all of the degenerative changes.

The current results suggest that AGEs may directly mediate the tissue water content, and these changes are detectable using magnetic resonance imaging. Because AGEs may have a



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multifactorial role in the pathogenesis of degenerative disc disease, it is critical to investigate and understand the role of AGEs on the intervertebral disc, and develop therapeutic strategies targeted towards the inhibition of AGEs. Clinical MR shows great promise in its ability to monitor these AGEs-mediated changes.

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**Key Points**

- Increased incubation time in ribose solution causes reduced water retention in the ovine IVD.
- AGEs accumulation is significantly correlated with tissue water content of IVD.
- AGEs accumulation reduces $T_2$ relaxation time in the nucleus pulposus.

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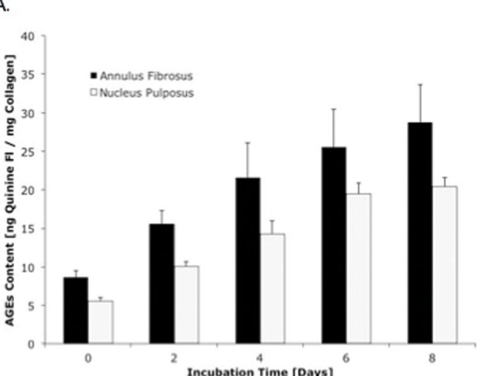


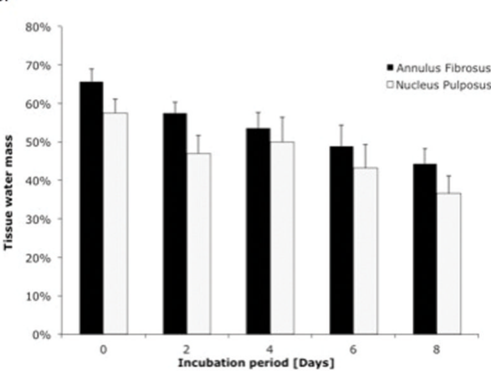


**Figure 1.**
(A) The intervertebral disc tissues were dissected from the annulus fibrosus (AF) and nucleus pulposus (NP). (B) A schematic illustrating the allocation of sample sizes in each experiment.

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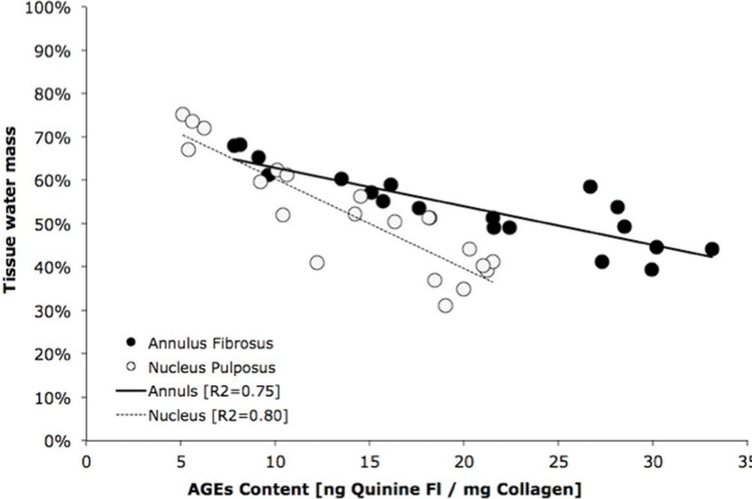


**Figure 2.**

(A) The accumulation of AGEs increased with ribosylation incubation time in both the annulus fibrosis ($p<0.001$; ANOVA) and the nucleus pulposus ($p<0.001$; ANOVA). (B) The increases in AGEs also corresponded with the decrease in the tissue water mass in these IVD tissues. Water content in the intervertebral disc tissues decreased significantly with incubation time in both the annulus fibrosis ($p<0.001$; ANOVA) and the nucleus pulposus ($p<0.001$; ANOVA).

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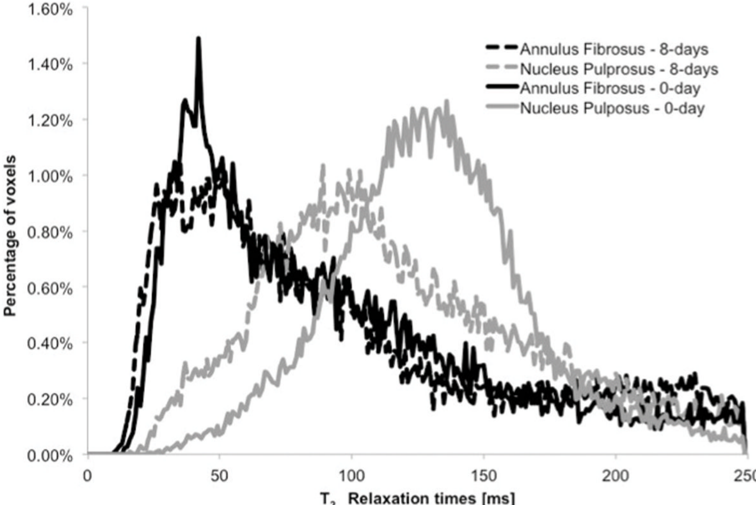


**Figure 3.**

The loss of water content in the intervertebral disc tissues was significantly correlated with the accumulation of AGEs in both the annulus and the nucleus.

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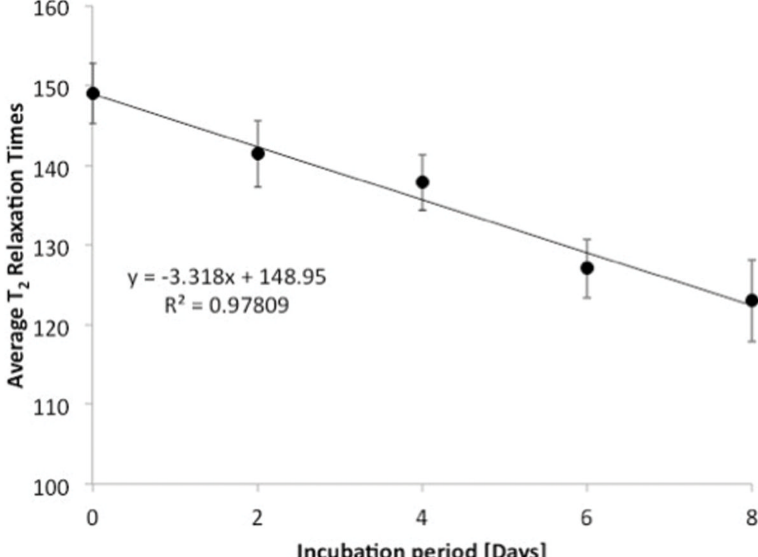


**Figure 4.**

Representative $T_2$ relaxation distributions of AF and NP samples at 0 days of ribosylation (control) and at 8 days of ribosylation. Ribosylation has little effect on the MR $T_2$ relaxation times of the annulus fibrosus samples, while reducing the MR $T_2$ relaxation times of the nucleus pulposus samples.

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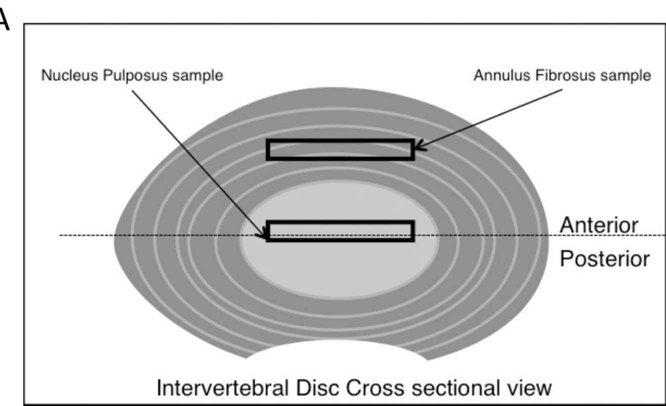


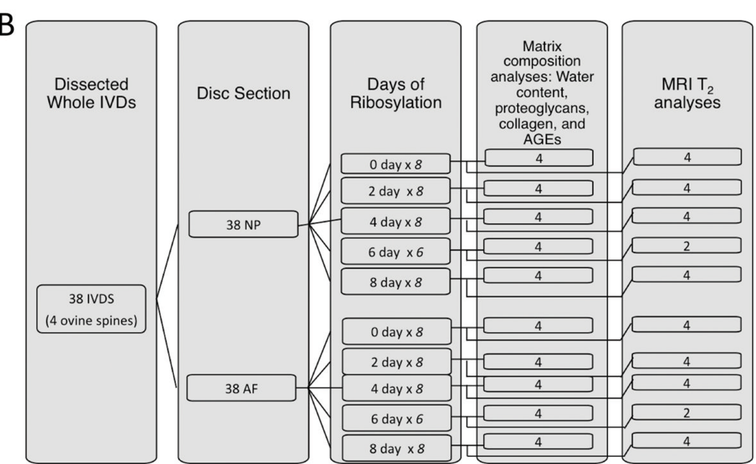


**Figure 5.**

NP tissues exhibited a loss in $T_2$ relaxation times in a dose-dependent manner with increasing ribosylation times ($p<0.001$; Pearson's correlation).

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## Table I

Average glycosaminoglycan (GAG) and collagen content (standard deviations in parenthesis) of interverbral disc samples after *in vitro* ribosylation.

| | Ribosylation incubation period | | | | | p-values |
|---|---|---|---|---|---|---|
| | **Control** | **2 days** | **4 days** | **6 days** | **8 days** | **Ribosylated vs 0-day control** |
| AF GAG content [GAG/hydroxyproline] | 8.22 (2.13) | 7.14 (1.96) | 6.98 (1.74) | 8.42 (2.01) | 7.85 (1.65) | $p = 0.472$ |
| AF Collagen content [assayed/dry mass - %] | 65.3 (11.0) | 69.4 (9.11) | 63.2 (12.3) | 61.2 (13.6) | 67.5 (8.87) | $p = 0.562$ |
| NP GAG content [GAG/hydroxyproline] | 34.2 (7.87) | 33.8 (5.45) | 29.4 (6.51) | 30.1 (8.13) | 28.3 (9.35) | $p = 0.627$ |
| NP Collagen content [assayed/dry mass - %] | 25.1 (11.0) | 28.2 (9.11) | 22.8 (12.3) | 21.2 (13.6) | 24.5 (8.87) | $p = 0.366$ |

AF = Annulus fibrosus; NP = Nucleus pulposus.

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## Table II

Average MR $T_2$ relaxation times (standard deviations in parenthesis) of interverbral disc samples after *in vitro* ribosylation.2

| | Ribosylation incubation period | | | | | p-values |
|---|---|---|---|---|---|---|
| | **Control** | **2 days** | **4 days** | **6 days** | **8 days** | **Ribosylated vs 0-day control** |
| Annulus fibrosus [ms] | 97.6 (11.1) | 99.5 (3.47) | 99.7 (13.3) | 103 (11.5) | 83.3 (9.96) | $p = 0.912$ |
| Nucleus pulposus [ms] | 149 (5.35) | 141 (7.19) | 137* (6.01) | 127* (3.73) | 123* (8.87) | **$p < 0.001$** |

*Denotes significantly lower than the control as determined by the Fisher's LSD test.