AN INVESTIGATION OF THE EFFECTS OF EXOGENOUS CROSSLINKING OF BOVINE ANNULUS FIBROSUS TISSUE

A Thesis

by

JONATHAN MICHAEL GOLIGHTLY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Biomedical Engineering

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Approved by:

Chair of Committee,	Thomas Hedman
Committee Members,	John Criscione
	Harry Hogan
Head of Department,	Gerard Coté

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ABSTRACT

An Investigation of the Effects of Exogenous Crosslinking of Bovine Annulus Fibrosus Tissue. (May 2009)

Jonathan Michael Golightly, B.S., Texas Tech University

Chair of Advisory Committee: Dr. Thomas Hedman

This study investigates the changes due to crosslinking treatment in stiffness, permeability, and glycosaminoglycan (GAG) content of bovine intervertebral discs.

The objective of this study was to determine the mechanical and biochemical effects of crosslinking treatment on lumbar bovine tissue.

Previous studies have found that crosslinking can increase stiffness and permeability in the intervertebral disc. These changes have not yet been investigated by confined compression, stress-relaxation tests of young bovine tissue.

Eleven lumbar motion segments were harvested from calf spines and soaked in a saline solution or one of four crosslinking treatments (genipin, methylglyoxal, proanthrocyanidin, and EDC). Five mm diameter samples were removed from the mid-annulus region at anterior / anterior-lateral locations, confined in a saline bath, swelled to equilibrium, and tested in confined compression stress-relaxation to 15% strain in 5% increments. Radial samples were also harvested, treated with saline solution and EDC, and tested in the same manner. The aggregate modulus and hydraulic permeability were calculated using the nonlinear biphasic theory. Swelling pressure was calculated as the

load at swelling equilibrium. GAG content was measured using the dimethylmethylene blue assay. Differences with P value < 0.05 were considered significant.

In the axial orientation, all crosslinking treatments except methyglyoxal at least doubled the aggregate modulus relative to soaked controls ($P < 0.05$). Genipin treatment resulted in 78% lower axial permeability, proanthrocyanidin (PA) 50% lower, and EDC treatment 84% lower relative to soaked controls ($P < 0.05$). GAG content measured in the methyglyoxal treatment group was 25% lower than in soaked control group. Genipin (G), proanthrocyanidin (PA), and EDC treatment increased the swelling pressure by at least 65% ($P < 0.05$). In the radial orientation, EDC treatment increased the stiffness by 75%, and did not significantly affect the permeability or swelling pressure.

Some crosslinking treatments proved effective in increasing the stiffness and swelling pressure of the disc. The increased swelling pressure in G, PA, and EDC treatment groups relative to soaked controls suggests reduced GAG leaching during soaking treatment, further confirmed by the reduction in permeability in these groups.

DEDICATION

To my wonderful wife, Melissa, and my family, Amy, Margaret, and Steven.

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CHAPTER I

INTRODUCTION: THE PROBLEM OF LOW BACK PAIN

Low back pain is the second leading cause for doctor visits and costs an estimated $50 billion annually in medical costs and lost time and labor[1]. This condition's cause is not yet precisely known, but many experts point to degeneration of the intervertebral disc (IVD).

The IVD consists of three distinct materials: the nucleus pulposus (NP), the annulus fibrosus (AF), and the cartilaginous end plates. In a juvenile, non-degenerated disc, the nucleus is a gelatinous substance which bears much of the spinal load through swelling. This swelling is restrained by the surrounding annulus fibrosus[2]. The annulus consists of alternating layers of collagen fibers known as lamellae; at the outer annulus, lamellae are oriented at 60° from vertical, whereas at the inner annulus this angle is reduced to 45°[3]. Disc tissue is composed of a gel containing proteoglycans (PG) - glycosaminoglycans (GAG) attached to a protein core - which are loosely fixed in a collagenous network known as the extra-cellular matrix that "[govern] the hydration [and] the rate of fluid transport"[4]. Since the IVD is relatively isolated from blood flow, the permeability of the annulus and cartilaginous end plates is also a determining factor in nutrient supply and waste removal.

There are many common mechanical and biochemical changes associated with both age and degeneration, as evidenced by the statement of one author: "degeneration-

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This thesis follows the style of *Spine*.

related changes in material properties also correlate with age, making a distinction difficult"[5].

Both are characterized by a loss of proteoglycans, especially in the nucleus, and decreased water content[5]. The loss of proteoglycans reduces the swelling pressure of the nucleus which, along with the reduced compressive stiffness, increases load bearing in the annulus[2]. Both are associated with a decreased number of annulus layers and thicker collagen fibers[5]. In both processes, the nucleus becomes stiffer in shear[6], less stiff in compression[7], and more elastic[6]. Studies of the changes in disc properties with age and degeneration have also shown increased stiffness, increased axial permeability, and decreased radial permeability[5].

Delamination, a common failure mode of the disc, often "occurs in the presence of high interlaminar shear stresses that in turn are increased after initial radial and circumferential tears in the annulus"[5]. As noted there, the increased layer thickness associated with degeneration and aging can increase shear stresses, and thus the potential for delamination[5].

One notable difference between aging and degeneration is the nature of collagen crosslinks. Duance found that with aging and mild degeneration (up to Grade 4) there is an increase in pentosidine or "age-increasing crosslinks"; however with severe degeneration, there are lower quantities of these "age-increasing" crosslinks present[8]. As suggested by this author, the lack of these crosslinks in highly degenerative discs may be part of the problem in degeneration.

With these changes in mind, I propose that age related changes are a natural mechanical response to the loading our intervertebral discs experience in our lifetimes, and that, to a point, degeneration may be an adaptive mechanism to prevent further damage to an excessively loaded disc by an accelerated onset of such age related changes. Therefore inducing these types of age related changes could prevent overloading of the disc, and thus disc damage and low back pain.

Previous work in has shown the efficacy of exogenous collagen crosslinking using certain reagents in improving disc and intervertebral joint properties[9-11]. With this in mind, adding to our understanding of the changes in disc properties by exogenous treatment with crosslinking reagents could aid in determining a proper treatment to prevent progression of the disease.

Previous studies by Iatridis, Mow, and Ateshian have shown the usefulness of confined stress-relaxation testing to evaluate intervertebral disc properties[4, 6, 12, 13]. These studies establish governing equations for the IVD stress response in confined compression, and solutions of these equations using finite differencing techniques have repeatedly been used to quantify the tissue aggregate modulus and hydraulic permeability[6, 12, 13]. These studies have noted that both the permeability and aggregate modulus depend on the biochemical environment (the density of glycosaminoglycans and water content, especially) and the structure of the extracellular matrix.

Previous studies have measured the changes associated with crosslinking treatments, and have almost uniformly found that crosslinking increases stiffness and permeability[11, 14-18].

One of these studies found increased permeability, implied from fluid flow measurements, with genipin treatment[11]. From these changes and other studies[14, 16], crosslinking was proposed to increase the mean fiber diameter of the extra-cellular matrix by drawing collagen fibers together, thus structurally increasing permeability; however, no GAG measurements were taken, so the role and magnitude of biochemical changes with crosslinking treatments remains unclear.

It has been reported that certain crosslinking treatments such as glycation[19-22], and glutaraldehyde[23, 24], cause increased loss and / or decreased synthesis of proteoglycans. Other crosslinkers such as Transglutaminase[25], genipin[26], and EDC[27] seem to reduce proteoglycan loss. Note that changes in fixed charge density by changes in proteoglycan content can have significant effects on hydraulic permeability[28-32]; specifically increases in fixed charge density may cause a decrease in the hydraulic permeability of a tissue. This is particularly relevant in the intervertebral disc as glycation is the process by which age-increasing crosslinks occur in the disc[8]. The associated loss of proteoglycans with these crosslinks may help explain the increase in axial permeability with age and mild degeneration.

Increased permeability with crosslinking by glycation has been reported by Boyd-White, Cochrane, and Hunter[14-17]. As stated above, glycation can affect increased permeability through biochemical means. However in Boyd-White's study[14], crosslinking was performed on a non-biological specimen so that proteoglycan concentration would not have been changed, therefore the effect in this study is anticipated to be solely structural. In all of these studies, the crosslinking treatment is

expected to increase permeability by structural means, as noted above, and biochemical means, by the loss of proteoglycan content, when present in the tissue.

With this basis in mind, we hypothesize that treatment with crosslinking reagents will increase both stiffness and permeability, and may alter the amount of GAG lost, depending on the nature of the crosslinker, during the treatment process. Specifically, crosslinks that work by glycation – methylglyoxal – are anticipated to increase the leaching of GAG content during prolonged free swelling or soaking periods without protease inhibitors, while the other crosslinking reagents – genipin and EDC – are expected to reduce the GAG leaching. These parameters, specifically the aggregate modulus (Ha) and hydraulic permeability (ko), will be measured in bovine annulus tissue by way of confined compression, stress-relaxation tests, and the GAG content by the dimethylmethylene blue (DMMB) assay[33].

This work will add to the knowledge of intervertebral disc material properties by quantifying meaningful parameters of a novel tissue type. By investigating the effects of crosslink augmentation using various crosslinkers, additional information will be available to differentiate between crosslinking strategies based on effects that may correlate to clinical benefits.

CHAPTER II

CONFINED COMPRESSION STRESS RELAXATION

Materials and Methods

Calf spines (~4-6 months old) were obtained from an abattoir. A band saw was used to cut through vertebral bodies, yielding 11 bone-disc-bone complexes or motion segments (MS) which were immediately frozen and stored at -20 C. Previous studies have shown that frozen storage has negligible effect on mechanical properties [34-37].

Prior to treatment, a motion segment was removed from the freezer, wrapped in a paper towel soaked with phosphate buffered saline solution (PBS) (0.14 M), and thawed for 2 hours at room temperature in a sealed plastic bag. A scalpel was then used to remove excess tissue, and one of the remaining vertebral bodies was removed, so that a bone-disc complex remained.

These complexes were then soaked in one of 5 solutions: Control – PBS(C), 10 mM Genipin (0.2%) in a 0.1 M Tris solution, pH 8.0 (G), 20mM Methylglyoxal in a 0.1 M *N*-(2-hyroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) solution, pH 6.0 (MG), 0.1% proanthrocyanidin in a 0.1 M Tris solution, pH 8.0 (PA), or 0.5 % 2-(*N*-morpholino)ethanesulfonic acid in a 0.1 M MES buffer solution, pH 8 (EDC). From these treatment groups the sample sizes were as follows: PBS – ten samples from four discs, Genipin – eight samples from two discs, Methyglyoxal – six samples from two discs, Proanthrocyanidin – five samples from one disc, EDC – five samples from one disc. A limited number of non-soaked specimens were also tested, primarily in the development of the test protocol described below, and are denoted in figures by NSC.

Given the small sample size of this group (N=3), no statistical analysis was performed with these results, however they are included in the results section for reference. The complexes were placed in a 1 L beaker, solution was added to completely cover the disc and remaining vertebral body; the beaker was then sealed and incubated for 4 hours at 37°C. Discs treated with Genipin were gently stirred hourly to ensure a homogenous mixture. Prior testing in our lab provided optimal incubation times and concentrations for each reagent to diffuse through the tissue and form collagen crosslinks.

After incubation, the complex was rinsed and soaked in PBS for 30 minutes three consecutive times to remove active crosslinker. Each soaking was performed with fresh solution. These complexes were then frozen at -20 C.

Specimens were harvested in both axial and radial orientations. Axial specimens were harvested as follows: while frozen, sections were removed in the transverse plane from the top of a frozen disc until the disc surface was flat using a Lipshaw No. 80-1 Sledge Microtome. Slices of uniform thickness were then cut and stored at -20 C on large microscope slides. On the evening prior to testing, a 3/16" punch was used to remove cylindrical samples from the middle or inner annulus fibrosus region (AF) at anterior and anterio-lateral locations of a disc section. Each punch was stored at -20 C in a plastic vile until testing.

Radial specimens were harvested as follows: after treatment, each disc was removed from the remaining bone segment so that only the disc remained. On the day of testing, a disc was removed from frozen storage and, while frozen, two parallel cuts were made in a circumferential orientation in the inner annulus fibrosus region in the anterio-

lateral portion of the disc. Two cuts perpendicular to these were then made so that a circumferential portion of the disc could be removed from within the disc. A 3/16" punch was then used to remove a single radially oriented specimen from each circumferential portion removed from the disc.

Disc specimens were placed on a stainless steel, 5 mm diameter porous platen inside a stainless steel specimen confining ring with an inner diameter of 5.38 mm. A second stainless steel porous platen, attached to a load cell, was then lowered into the confining ring to contact with the disc, as seen in Figure 1.

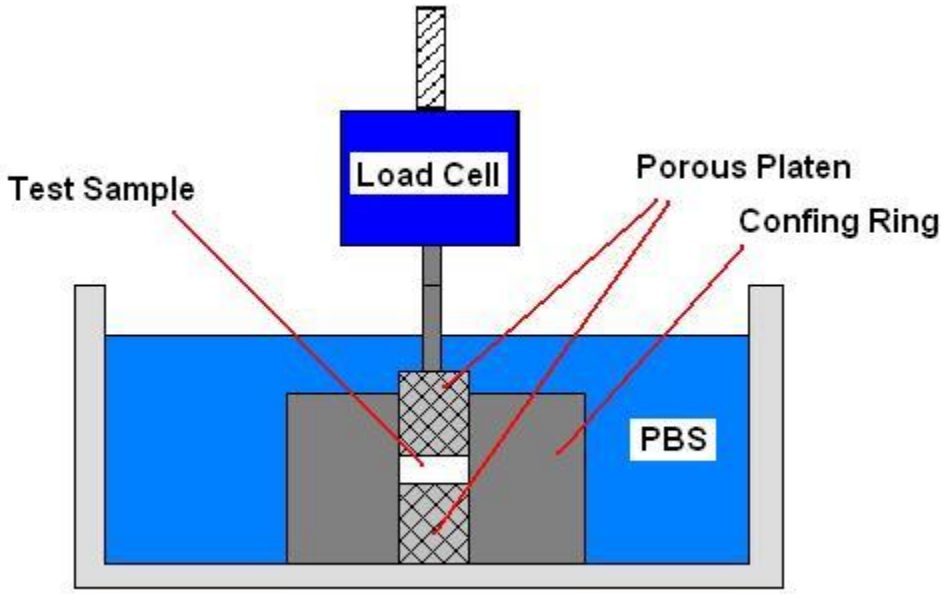


Figure 1. Confined Compression Test Setup.

Pilot tests revealed that the optimal method for sample insertion was placing the disc on top of the lower porous platen, and then placing this platen-disc complex inside

the confining ring. Therefore distance measurement was zeroed on the TestResources uniaxial hydraulic testing system (Test Resources, MN) by measuring distance from the top of the confining ring to the top of the lower porous platen. This ensured an accurate zero point from which to begin testing after removing and replacing the lower porous platen during test setup.

Prior to removing the sample from frozen storage, distance measurement was zeroed by measuring the distance from the top of the confining ring to the top of the lower porous platen on which the specimen would be placed. This was measured by lowering the upper platen to the top of the confining ring until a load of 0.5 N was reached. Displacement was zeroed, the upper platen raised ~0.1 mm, and the ring centered beneath the upper porous platen. The lower platen was not in place at this time. With the upper platen raised, the lower platen was then inserted into the confining ring, and the upper platen lowered to contact the lower platen until a load of 0.5 N was reached. This displacement was recorded. The upper platen was raised, the ring and lower platen removed, and the ring replaced. These steps were repeated until the standard deviation of three consecutive measurements was less than 0.02 mm. The distance from the top of the confining ring to the top of the lower platen was then calculated as the average of these three measurements greater one standard deviation.

To ensure that the axes were aligned and that no frictional interactions would skew data, the upper platen was then lowered into the confining ring beyond the expected position for the top of the disc. If frictional load was observed, the ring was realigned and the process repeated.

Confined compression testing of the inner annulus specimens was performed with uniaxial hydraulic testing equipment, a 10 N load cell (Interface Force, Scottsdale, AZ), calibrated to within 0.001 N (0.1% of read-out value), and a set of custom porous platens. Due to equipment repairs, it became necessary to use a 10 lbf load cell (Test Resources) calibrated to within 0.01 N for the radial tests; however, the weight of the upper platen assembly was recorded for each test and was within 0.002 N of the weight measured with the 10 N load cell for all tests, therefore this is not believed to have caused a significant effect.

Cylindrical disc specimens were removed from the freezer, briefly allowed to thaw, and weighed. Dry weight was estimated using previous tests, and approximate water content was estimated. Using this estimate of water content, samples were dehydrated using Polyethylene Glycol (PEG) to approximately 80% water content prior to all testing. Residual PEG was rinsed off with PBS, the disc gently patted dry to remove excess water, and weighed. Sample height was then measured using a non-contact laser measuring system (Keyence LK-081), calibrated biweekly to within 0.02 mm (1% of readout value).

The sample was then placed on a porous platen, and placed inside the confining ring. Using the data from laser measurements, a porous platen (attached to the load cell with a custom fixture) was lowered to 95% of the disc height less one standard deviation (i.e. 0.95* (H-SD)) at 0.1% / s[38]. All strain rates were calculated as a percentage of this height so that an equivalent strain rate was applied for all specimens. This acted as a

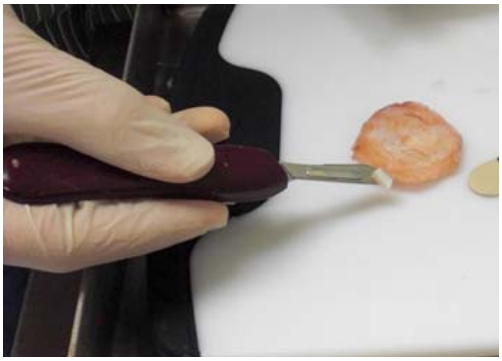
preload to define an equilibrium point from which to begin confined compression testing.

Approximately 30 seconds after this strain was applied, PBS was added until the ring was completely covered. The load was recorded until the average change in load was less than 0.5% / min. This typically took 4000-5000 s. This step was added to emulate previous studies[32, 39].

Other studies[6, 12, 13, 32, 39] have primarily used a preload method rather than a prestrain method as described here, therefore a series of validation tests were performed with axially oriented oxtail specimens less than 1 year old by applying a preload of 0.15 N in two phases – loaded to 0.06 N at 0.001 mm / s and further loaded to 0.15 N at 0.0005 mm / s. This preload was selected on the basis of a prior study by Best[32]; the loading rate was selected so as to be similar to the rate applied during the prestrain method (nominally 0.001 mm / s) without causing excess efflux of fluid. The intermediate point at which the loading rate changed was arbitrary, and is not anticipated to have caused a significant change in the tissue response. Note that a prior study[6] found no significant difference due to a change in strain rate from 0.001 / s to 0.0001 / s with nucleus tissue; given the proximity of the harvested specimens to the nucleus this is expected to hold true for the loading rates described above. As before, approximately 30 seconds after this preload was reached, PBS was added until the ring was completely covered and the disc allowed to swell to equilibrium as described above. The height of the disc was calculated based on the distance traveled to reach this preload and the previously measured zero point from the top of the confining ring to the lower porous

platen. On average this method amounted to a nominal strain of 30%, as opposed to the 5% strain applied in the method above.

A series of pictures appear below in Figure 2 demonstrating the sample preparation and test setup process. For a scale reference, in Figure 2 c) the platen on which the disc is resting is approximately 6.3 mm tall.

a)  b) 

c)  d) 

Figure 2. Photographs of Specimen Harvesting and Test Setup. The sample being harvested from a disc (a, b); on a porous platen next to the confining bowl with confining ring and upper porous platen visible (c); and placed inside the confining ring with load cell and servo arm visible (d).

From this swelling equilibrium point, confined compression testing was performed, consisting of 5%, 10%, and 15% compressive strains at 0.01% / s with

intermediate relaxation periods[6, 12, 13]. These strains were calculated from the height of the disc after the prestrain was applied or, in the case of the validation experiments, after the preload was applied. Pilot tests demonstrated that relaxation periods of 3000, 3500, and 4000 s were sufficient to allow equilibration. A picture of the typical stress response appears below in Figure 3.

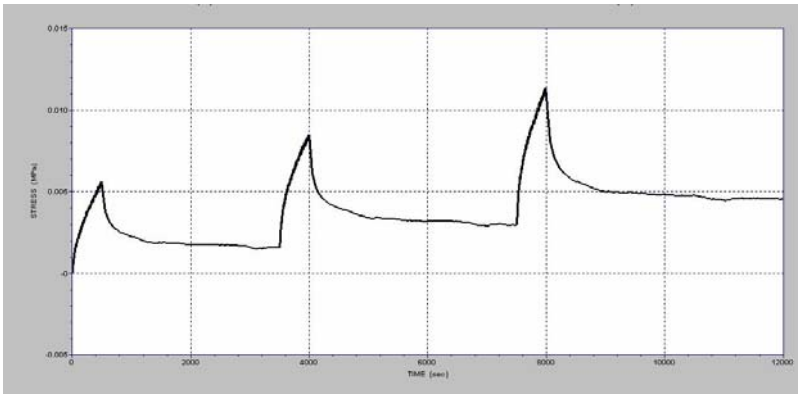


Figure 3. A Normal Stress Response During a Stress-Relaxation Experiment.

After each confined compression test was completed, the bathing solution was removed from the confining chamber to prevent unconfined swelling of the sample. The sample was then removed, gently patted dry to remove excess water, and weighed. To calculate the dry weight, samples were dried at 90 C for at least 11 hours. Pilot testing indicated that additional drying had negligible effect on the dry weight.

Analysis was performed in accordance with previous papers outlining the nonlinear biphasic theory[4, 6, 12, 39].

The behavior of the disc in confined compression is governed by Eq. 1:

$$\frac{\partial \sigma^e}{\partial \lambda}\frac{\partial^2 U}{\partial^2 Z} = \frac{\lambda}{k}\frac{\partial U}{\partial t} \quad 0 < Z < Z_{top}, \quad t > 0 \qquad (1)$$

where stretch is calculated as:

$$\lambda = 1 + \frac{\partial U}{\partial Z} \qquad (2)$$

Elastic stress, σ^e, is governed by Eq. 3:

$$\sigma^e = \frac{1}{2} H_{Ao}\left(\frac{\lambda^2 - 1}{\lambda^{2\beta+1}}\right) e^{\beta\left(\lambda^2 - 1\right)} \qquad (3)$$

and permeability, k, is governed by Eq. 4:

$$k = k_o\left(\frac{\lambda - \phi_o^s}{1 - \phi_o^s}\right)^2 e^{\left(M\frac{\left(\lambda^2 - 1\right)}{2}\right)} \qquad (4)$$

To quantify the aggregate modulus, H_{Ao}, and the associated non-linearity term, β, equilibrium stress values at 5%, 10%, and 15% compressive strains were calculated as the average of the last 1500 s of each respective stress-relaxation period. A 10 x 10 mesh of H_{Ao} values ranging from 0 to 0.25 MPa and β values ranging from -0.49 to 15 was created. Note that any value of β less than -0.5 is excluded since this implies a finite amount of stress at infinite compression. The coefficient of correlation of the fit was calculated at each node. The combination of H_{Ao} and β which yielded the optimal coefficient of correlation were then defined as the center point of a new mesh. Note that if the optimal value of β was -0.49 this was assumed to be the optimal value for β, and further meshes only included this β value. Successive iterations were performed until the change in H_{Ao} was less than 0.0001 MPa and the change in β was less than 0.001.

A third order function was used to define displacement, U:

$$U(z,t) = Az^3 + Bz^2 + Cz + D; \quad 0 < z < z_{top}; \quad t > 0 \qquad (5)$$

This function was subject to the following boundary conditions and constraints:

$$U(0,t) = 0$$

$$U(z_{top},t) = u_{top} \qquad (6)$$

$$U(\frac{z_{top}}{2},t) = \frac{u_{top}}{2}$$

By further defining the A and B coefficients as exponentially decaying functions, and applying these boundary conditions, the function becomes:

$$U(z,t) = A_0 e^{\frac{-2(t-t_0)}{\tau}}\left(z^3 - 1.5z_{top}z^2 + 0.5z_{top}^2 z\right) + \frac{u_{top}}{z_{top}}z; \; 0 < z < z_{top}; t > 0 \quad (7)$$

where t_0 refers to the time at the beginning of each relaxation period.

Subsequent attempts with fourth and fifth order functions showed insignificant improvements in curve fitting capabilities (<0.01%).

To obtain the parameters A_0 and τ, a 10 x 10 mesh was created with values of A_0 ranging from 1 to 1000001 and values of τ ranging from 1 to 4001 to be sure to include all possible solutions. This solution was constrained such that the minimum value of compressive strain in the disc was no greater than 0% for the 5% stress-relaxation period, 5% for the 10% stress-relaxation period, and 10% for the 15% stress-relaxation period. The coefficient of correlation of the fit was calculated at each node. The combination of A_0 and τ which yielded the optimal coefficient of correlation were then defined as the center point of a new mesh. Successive iterations were performed

until the change in A_0 was less than 1 $\frac{1}{m^2}$ and the change in τ was less than 0.02 s. This procedure was repeated for each stress-relaxation period to obtain an (A_0 , τ) set for each period. Preliminary results yielded poor fits of the 5% stress response, therefore this data was excluded from permeability analysis.

Only the permeability parameters, k_o and M , then remained to be quantified. With the values for the displacement function, all other quantities in the PDE could then be calculated at each point in time and in the disc. The disc was divided into 40 points over its height. The permeability, k , was calculated using these known values in the PDE. Since the top, bottom, and middle of the disc yielded trivial solutions in the PDE, all permeability analysis was performed with a point 25% of the sample height from the top surface of the disc sample.

For all permeability calculations, the post test water content was used for the water content ϕ_o^s . This was used since it was anticipated that a larger amount of water was imbibed during swelling than was exuded during stress-relaxation testing, therefore the weight throughout stress-relaxation testing should be closer to the post test weight. Steady state permeability for each compressive strain level was calculated as the average of k values from the last 1000 s of each stress-relaxation period. The permeability function parameters k_o and M were calculated for each compressive strain level using a custom nonlinear regression code in Matlab for data over the full stress-relaxation period at each strain level. A full range k_o and M were also calculated based on the data from

the 10% and 15% stress-relaxation periods with a custom nonlinear regression code using the "fminsearch" function in Matlab.

Using only the data from the 5% compressive strain, four parameters were calculated. A linear fit was made to this response, yielding a Full Modulus. The stress response was biphasic, therefore linear fits were made to the response at the beginning and end of this period. Slopes of these lines were treated as Low and High Modulus, respectively. Using the intercept of these two linear fits and their slopes, a Shift Point was calculated. These are referred to as "Neutral Zone" parameters as this type of analysis bears some resemblance to the Neutral Zone calculation as described by Yerramalli[26]; however, the Neutral Zone is typical of tension-compression tests, so our use of the terminology here is strictly for reference and does not indicate a different testing method.

Statistical analysis was performed using a Mann-Whitney non-parametric test in Matlab (Mathworks). Due to the large number of treatments and parameters, statistical tests were only performed in cases where a significant difference seemed likely. This was to maximize the efficiency and validity of the statistical analysis. To compare the hydraulic permeability at 10% and 15% for a particular disc, a paired test was used; in all other cases a non-paired test was used. Note that due to the small sample size in the radial orientation, no statistical analysis was performed on these parameters. Results with a p-value of less than 0.05 were considered to be significant.

Results

Initial analysis of all results was performed as follows: any point more than 2 standard deviations from the mean in more than 3 parameters was considered an "outlier" and removed from the data set. This process resulted in the omission of three data points. Any results in which protocol was not exactly followed or results were skewed by disturbance during testing, for example bumping the test setup, were also removed from the data set.

First let us briefly compare the validation experiments performed with a preload of 0.15 N to the 5% prestrain results. Table 1 shows the values for aggregate modulus, 10% steady state permeability, 15% steady state permeability, and the permeability coefficients for the 10%, 15%, and composite relaxation periods from our validation study, our typical study using a prestrain method, and mean values from an independent study by Perie and Iatridis performed with approximately 4 year old oxtail discs[7].

Table 1. Nonlinear Biphasic Analysis of Non-Soaked Discs: Preload vs. Prestrain.

		Hao (MPa)	10% SS K (e-15)	15% SS K (e-15)	10% Ko (e-15)	15% Ko (e-15)	Full Ko (e-15)
Validation Study	**Avg**	0.122	0.911	0.781	1.29	1.47	1.27
	S D	0.018	0.444	0.386	0.717	0.889	0.796
Prestrain Method	**Avg**	0.078	1.22	1.29	1.68	2.73	0.959
	S D	0.027	0.367	0.284	0.760	2.30	0.562
Published Values	**Avg**	0.740	-----	-----	-----	-----	0.420

Table 2 provides a comparison of soaked control specimens from our study and from the same independent study noted above[7]. Note that soaked specimens in our study were soaked for a period of 4 hours, whereas in the previous study this period was limited to 1 hour.

Table 2. Aggregate Modulus and Permeability Coefficient Comparison: Present Study vs. Previous Studies.

	Hao (MPa)	**Full Ko (m^4/N-s)**
Present Study	0.029	2.57E-15
Published Values	0.06	2.50E-15

Table 3 shows coefficient of correlation values for the modulus parameters, 10% and 15% compressive strain surface stress-relaxation response and permeabilities, and the full range permeabilities in the axial orientation from all treatment groups (N=37).

Table 3. Coefficient of Correlation for Nonlinear Biphasic Curve Fits of Axial Data.

	Hao (MPa)	**10% S**	**15% S**	**10% K**	**15% K**	**FR K**
Mean	0.979	0.885	0.917	1.000	1.000	0.967
St. Dev.	0.019	0.111	0.036	0.000	0.000	0.054

Table 4 reports similar data for the radial orientation (N=6).

Table 4. Coefficient of Correlation for Nonlinear Biphasic Curve Fits of Radial Data.

	Hao (MPa)	**10% S**	**15% S**	**10% K**	**15% K**	**FR K**
Mean	0.993	0.576	0.731	1.000	1.000	0.895
St. Dev	0.008	0.288	0.207	0.000	0.000	0.164

In the axial orientation, the aggregate modulus was increased by approximately 150% by genipin (p=0.002057), 330% by proanthrocyanidin (p=0.00067), and 220% by EDC treatment (p=0.00066) relative to soaked controls. Non-soaked controls were 167% stiffer than soaked controls. Proanthrocyanidin treatment increased the aggregate modulus 75% relative to genipin (p=0.02953) and 200% relative to methylglyoxal (p=0.004329). Aggregate modulus was also increased by 125% with EDC treatment relative to methylglyoxal (p=0.004329). Non-soaked controls were 87% stiffer than methylglyoxal treated specimens. These data are displayed in Figure 4 below.

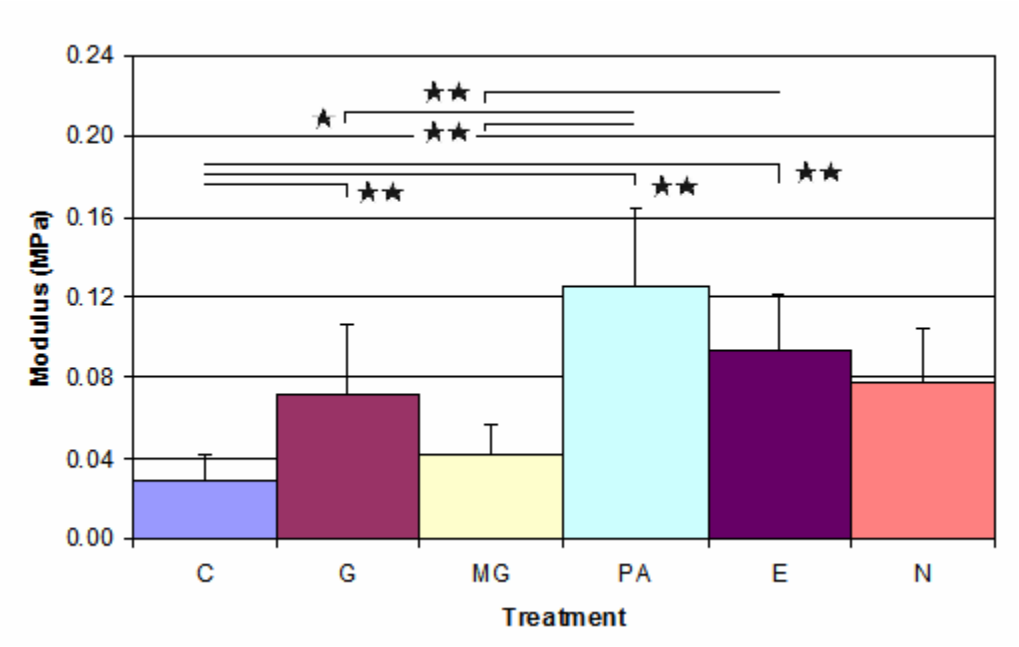


Figure 4. Axial Aggregate Modulus vs. Treatment. (C – PBS Soaked Controls, G – Genipin Treated, MG – Methylglyoxal Treated, PA – Proanthrocyanidin Treated, E – EDC Treated, N – Non-Soaked Controls; a Single Star indicates a p-value < 0.05, a Double Star indicates a p-value < 0.01.)

The aggregate modulus data from tests in the radial orientation appear below in Figure 5.

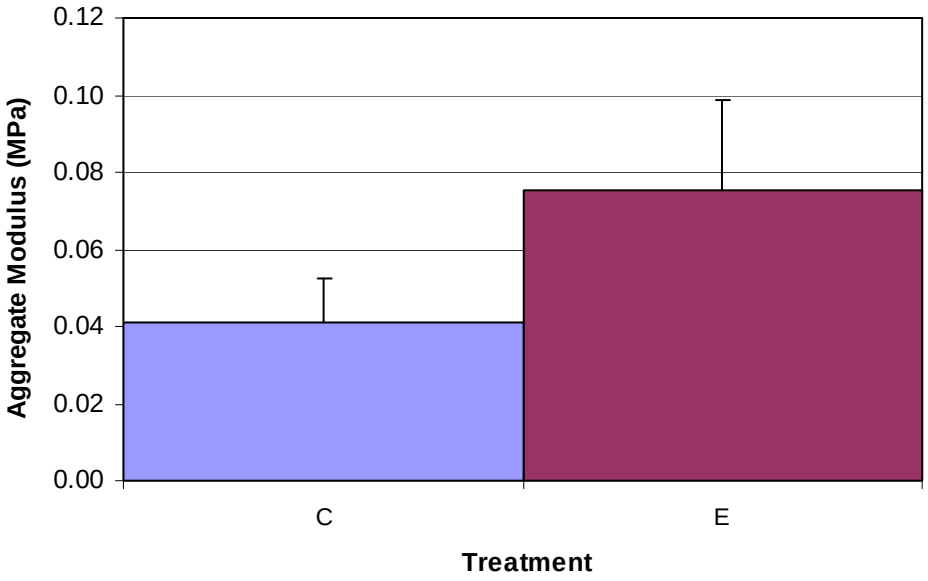


Figure 5. Radial Aggregate Modulus vs. Treatment.

Values for the nonlinear stiffening parameter, β, for each treatment group in the axial orientation are displayed in Table 5.

Table 5. Nonlinear Stiffening Data for Treatment Groups in Axial Data.

	C	G	MG	PA	E	NSC
Mean	1.40	0.25	0.24	-0.49	-0.49	0.61
St. Dev.	2.58	2.10	1.79	0.00	0.00	2.33

Similar data for the tests in the radial orientation are presented in Table 6.

Table 6. Nonlinear Stiffening Data for Treatment Groups in Radial Data.

	C	**E**
Mean	3.01	0.41
St. Dev.	6.06	1.55

Given the variance of these data, no statistical analysis was performed.

Considering steady-state permeability at 10% compressive strain in the axial orientation, genipin treatment reduced permeability by 61% relative to methylglyoxal treated specimens ($p=0.04515$). EDC treated specimens were 73.5 % less permeable than methylglyoxal treated specimens, 69% less permeable than soaked controls and 50% less permeable than non-soaked controls; these results were not statistically significant, but are included to document the observed trend. These data are displayed below in Figure 6.

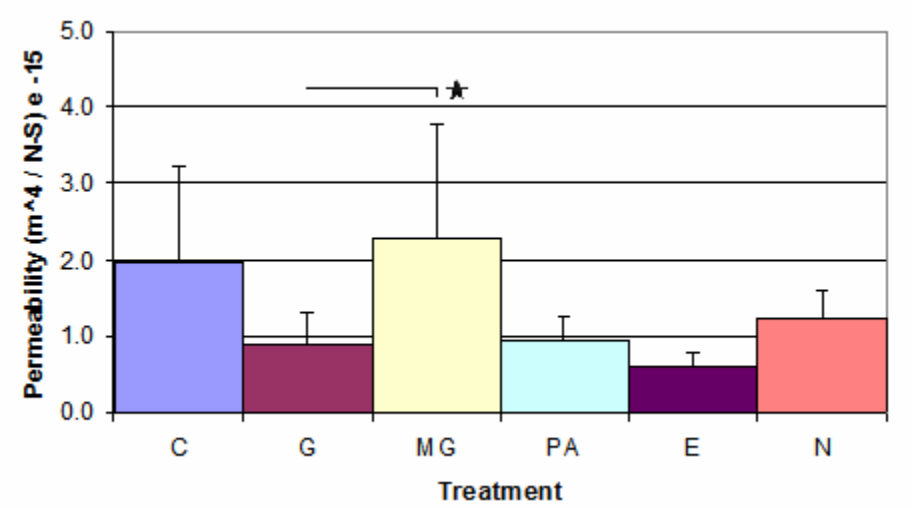


Figure 6. 10% Steady State Axial Permeability vs. Treatment.

The steady-state permeability at 10% compressive strain in the radial orientation increased by 98% in EDC treated specimens relative to soaked controls. These data are shown below in Figure 7.

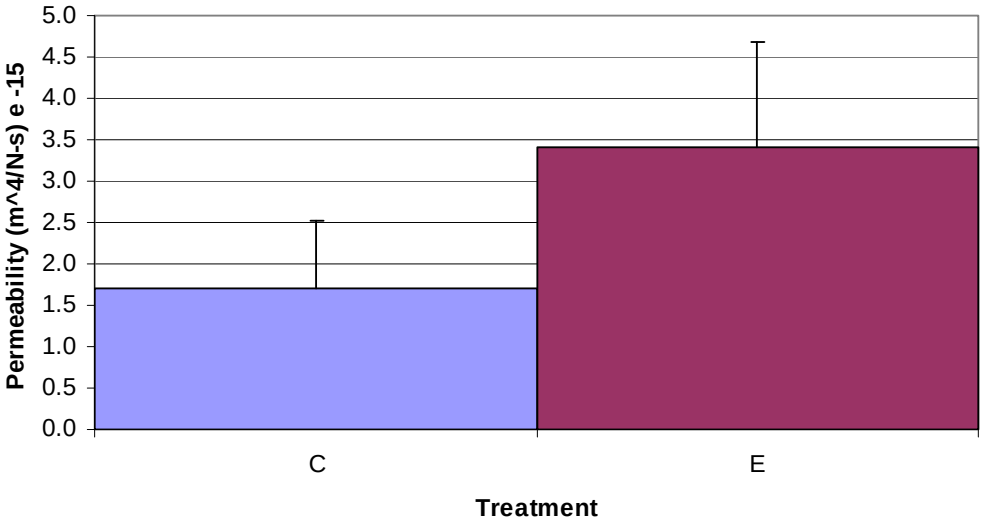


Figure 7. 10% Steady State Radial Permeability vs. Treatment.

Relative to soaked controls over the full 10% compressive strain relaxation period, EDC treatment reduced the axial permeability coefficient by 75% (p=0.02797). Methylglyoxal treated specimens were 147% more permeable than genipin treated specimens (p=0.04515) and 284% more permeable than EDC treated specimens (p=0.0303). All other reported results were not statistically significant, but reflect trends observed in the data. Genipin tests were 62% less permeable than soaked controls. EDC treated specimens were 36% less permeable than proanthrocyanidin treated specimens, 35.6% less permeable than genipin treated specimens, and 58% less permeable than non-soaked control specimens. In the radial orientation, the permeability coefficient increased by 71% with EDC treatment relative to soaked controls. These data sets are displayed below in Figures 8 and 9, respectively.

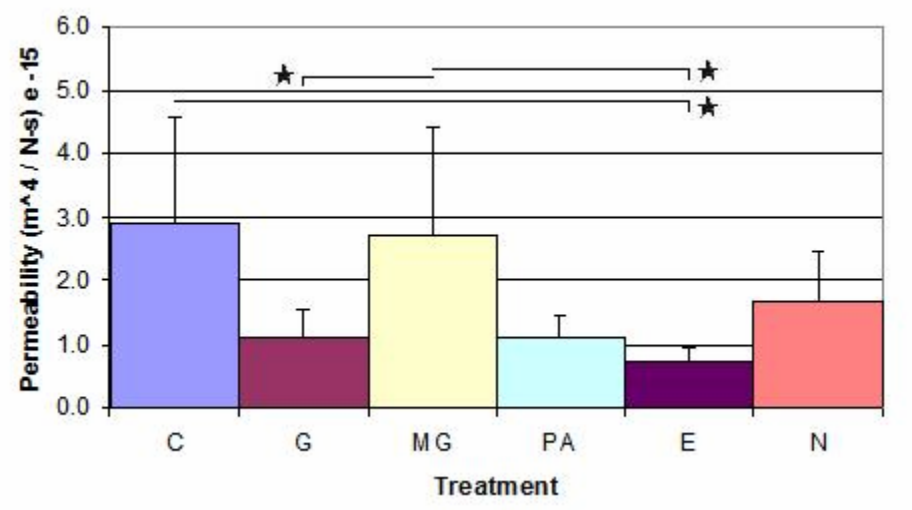


Figure 8. 10% Full Relaxation Axial Permeability vs. Treatment.

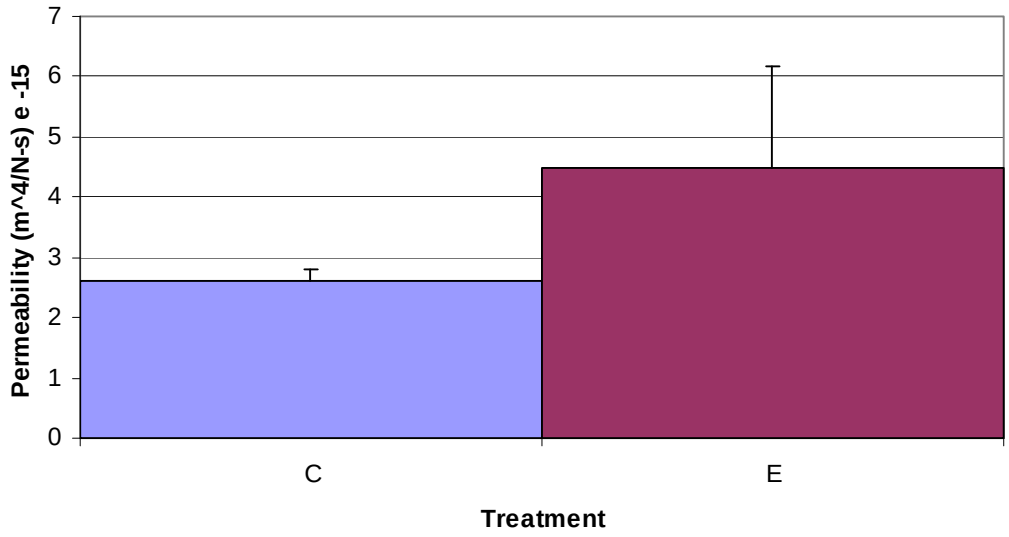


Figure 9. 10% Full Relaxation Radial Permeability vs. Treatment.

In the axial, steady-state case at 15% compressive strain, methglyoxal treatment significantly increased the permeability relative to genipin treated specimens by 310% ($p=0.0293$). Methyglyoxal treated specimens also had a 230% higher permeability than proanthrocyanidin treated specimens and 412% higher permeability than EDC treated specimens. These results were not statistically significant, but are referenced to note the trends observed in this parameter. In the radial orientation, EDC treatment increased the 15% steady state permeability by 89% relative to soaked controls. These data sets are provided below in Figures 10 and 11, respectively.

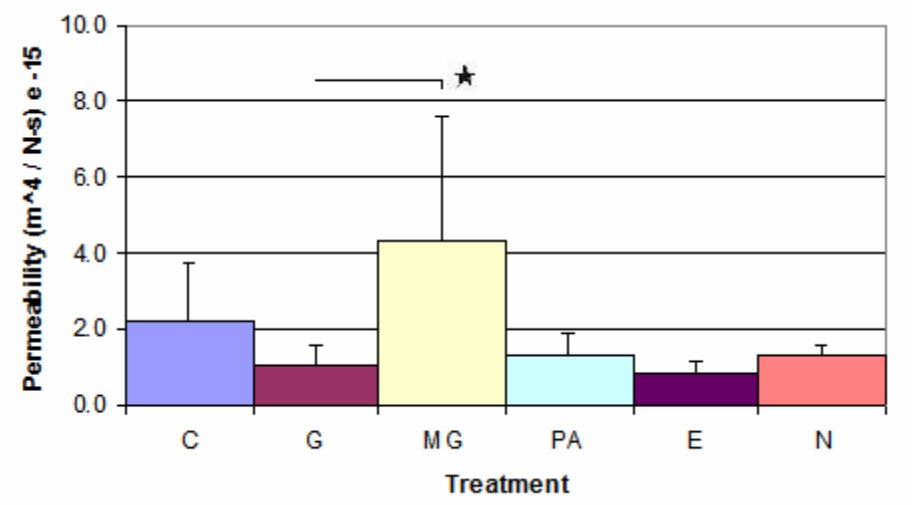


Figure 10. 15% Axial Steady State Permeability vs. Treatment.

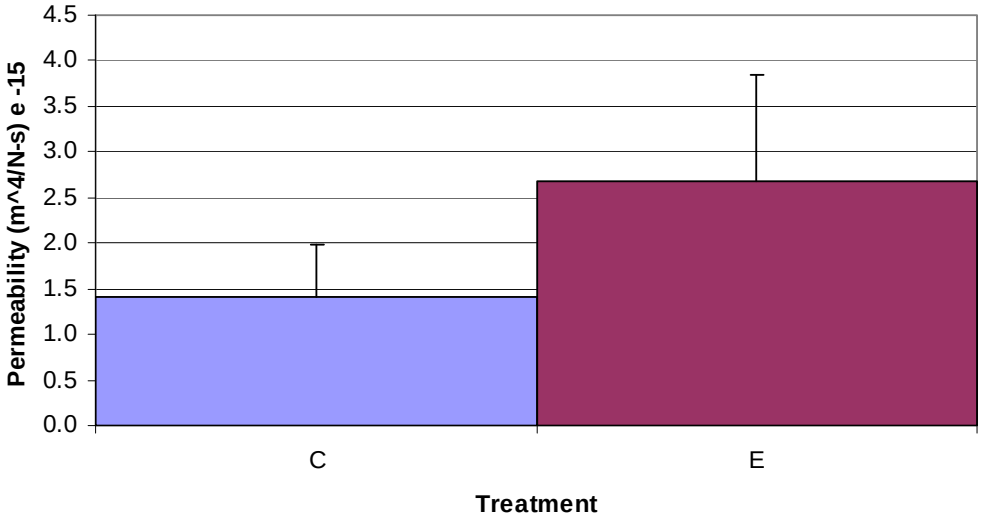


Figure 11. 15% Radial Steady State Permeability vs. Treatment.

Over the full 15% compressive strain relaxation period, methylglyoxal treated specimens had a significantly higher axial permeability by 90% relative to genipin treated specimens ($p=0.04262$), by 180% relative to proanthrocyanidin treated specimens

(p=0.008658), and by 209% relative to EDC treated specimens (p=0.01732). EDC treated specimens had a 63% lower permeability than genipin treated specimens and 188% lower permeability than non-soaked controls specimens in the axial orientation. These results were not statistically significant. In the radial orientation, soaked controls had a 30% higher permeability than EDC treated specimens. These data sets are shown below in Figures 12 and 13, respectively.

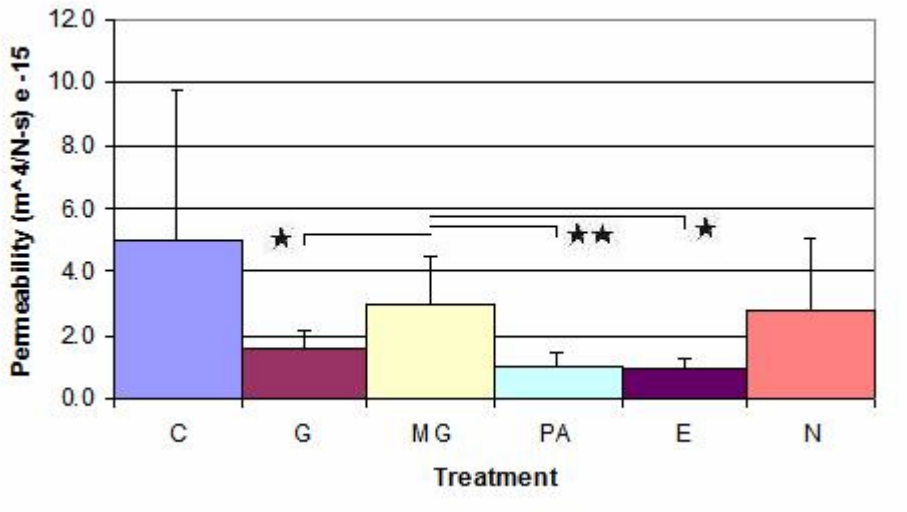


Figure 12. 15% Full Relaxation Axial Permeability vs. Treatment.

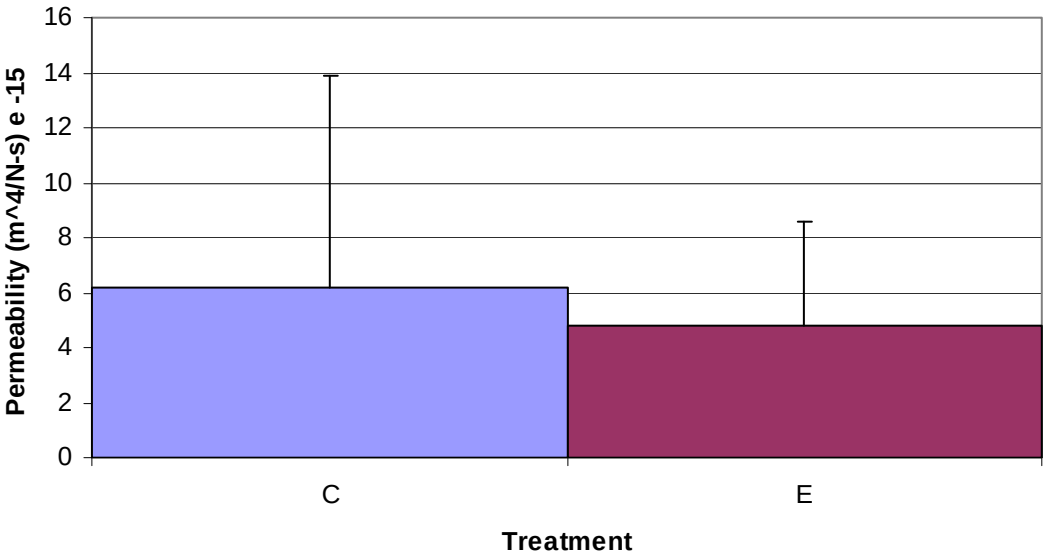


Figure 13. 15% Full Relaxation Radial Permeability vs. Treatment.

In the axial orientation, a paired analysis of the steady state permeability at 15% relative to 10% compressive strain showed an increase in permeability of 89% in methylglyoxal treated, 38% in proanthrocyanidin treated, and 40% in EDC treated specimens; however, these results were not statistically significant.

With respect to the full range permeability fit, methylglyoxal treated specimens were 460% more permeable than EDC treated specimens ($p=0.01732$) and 316% more permeable than genipin treated specimens. Proanthrocyanidin treatment significantly increased permeability by 109% relative to genipin treated specimens ($p=0.02953$). Soaked control specimens had a 516% higher permeability than EDC treated specimens ($p=0.01265$) and 358% higher than genipin treated specimens ($p=0.02052$).

In the radial orientation, the full range permeability coefficient was relatively unaffected - a decrease of only 2.6% - in EDC treated specimens relative to PBS soaked specimens.

These data are given below in Figures 14 and 15.

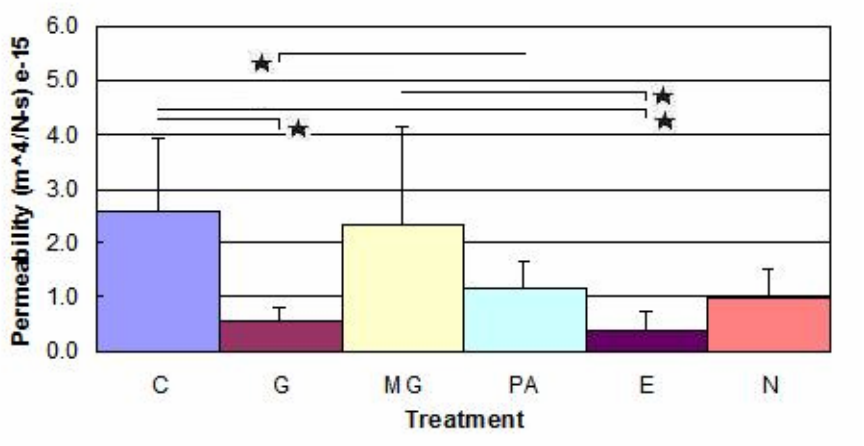


Figure 14. Full Strain Range Relaxation Axial Permeability vs. Treatment.

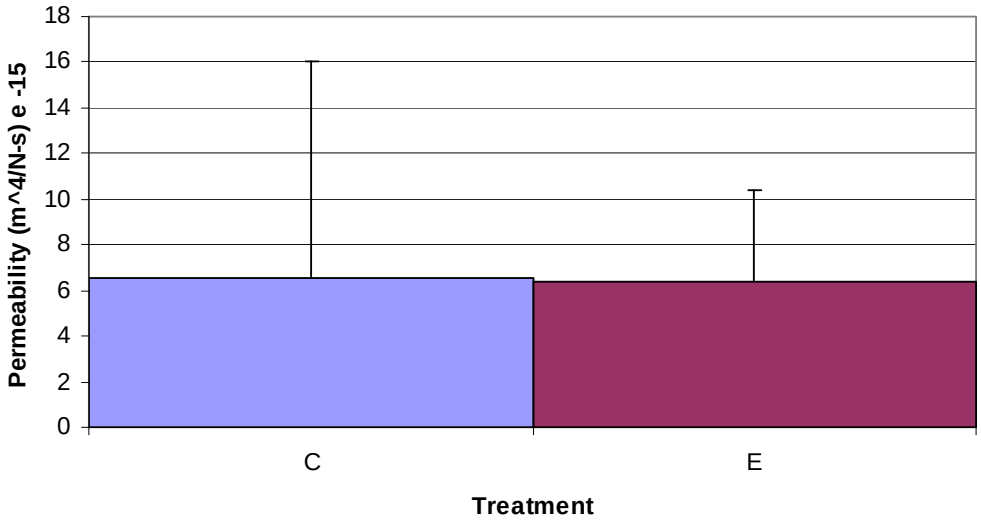


Figure 15. Full Strain Range Relaxation Radial Permeability vs. Treatment.

Values for the nonlinear permeability parameter, M, for 10% relaxation, 15% relaxation, and Full Range Permeability fits for axial and radial data are given in Tables 7 and 8, respectively.

Table 7. Nonlinear Axial Permeability Term Data for Treatment Groups.

	10%		**15%**		**FR**	
	Mean	**St. Dev**	**Mean**	**St. Dev**	**Mean**	**St. Dev**
C	1.32	2.99	2.04	4.46	-1.39	8.08
G	-0.03	2.47	-0.02	3.63	-7.76	3.34
MG	-0.03	2.13	0.02	3.23	-3.11	4.24
GS	-1.22	0.11	-1.69	0.14	-1.02	3.13
EDC	-0.88	0.07	-1.28	0.08	-8.15	5.36
NSC	0.51	2.32	0.85	3.57	-5.96	3.95

Table 8. Nonlinear Radial Permeability Term Data for Treatment Groups.

	10%		**15%**		**FR**	
	Mean	**St. Dev**	**Mean**	**St. Dev**	**Mean**	**St. Dev**
C	2.90	6.57	4.03	9.22	0.40	17.12
EDC	0.20	1.95	0.43	3.02	3.22	6.38

As previously, given the variance of these data, no statistical analysis was performed.

In the axial orientation, non-soaked controls had a higher stiffness (as measured by the Neutral Zone Full Modulus parameter) by 123% relative to methylglyoxal treated specimens and 112% relative to soaked controls. These results were not subject to a statistical analysis due to the small sample size of the non-soaked controls group, but are reported to reflect the observed changes relative to this reference point. Proanthrocyanidin treatment significantly increased the modulus by 144% relative to soaked controls ($p=0.001332$), 157% relative to methylglyoxal treated specimens ($p=0.004329$), and 51% relative to genipin treated specimens ($p=0.04507$). Treatment with EDC significantly increased the stiffness by 103% relative to soaked controls ($p=0.004662$) and by 114% relative to methylglyoxal treated specimens ($p=0.004329$). EDC treatment also increased the full range modulus by 26% relative to genipin treated specimens. Genipin treatment increased the full range modulus by 61% relative to soaked controls and by 70% relative to methylglyoxal treated specimens. These results were not statistically significant. In the radial orientation, EDC increased the stiffness by 71% relative to soaked controls in the full range modulus. These data sets are displayed below in Figures 16 and 17, respectively.

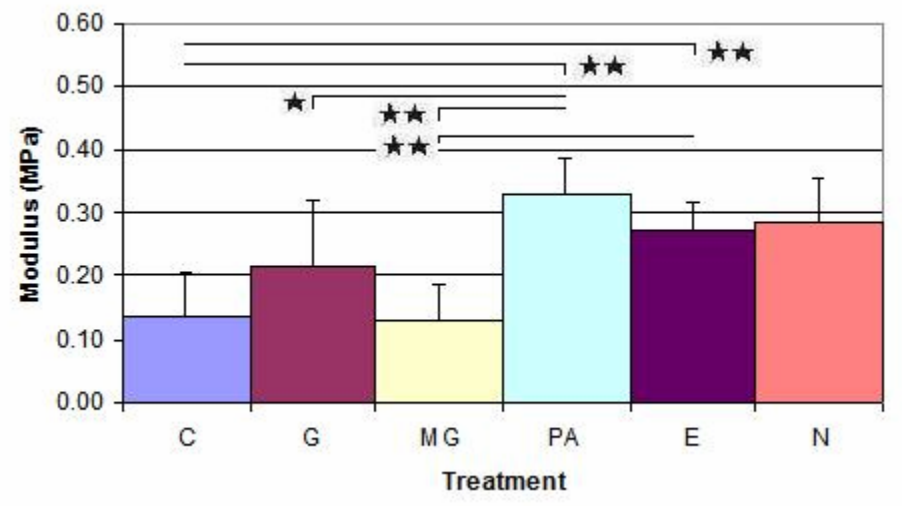


Figure 16. Axial Neutral Zone Full Modulus vs. Treatment.

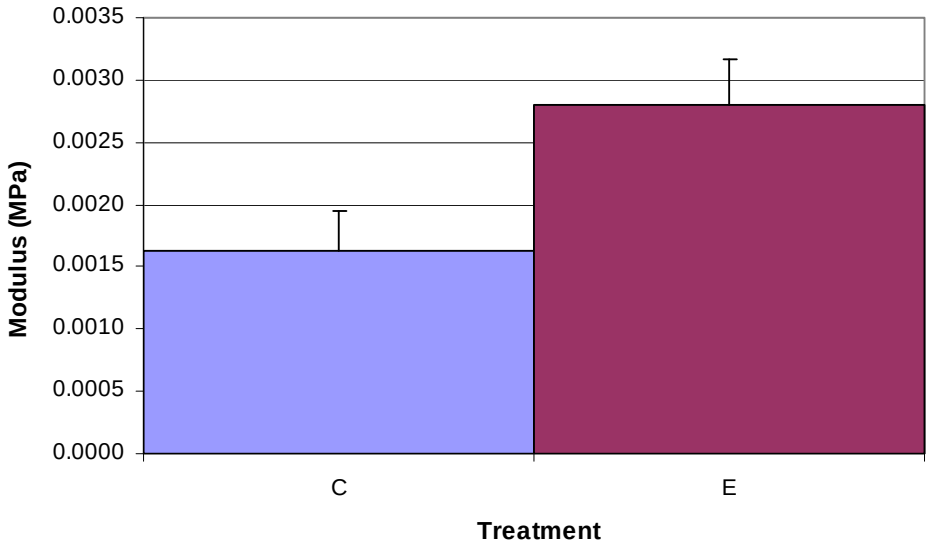


Figure 17. Radial Neutral Zone Full Modulus vs. Treatment.

In the axial orientation, the Neutral Zone low modulus parameter was higher in non-soaked controls by 186% relative to methylglyoxal treated specimens, by 151% relative to soaked controls, and by 49% relative to proanthrocyanidin treated specimens.

Genipin treated specimens were significantly stiffer by 113% relative to methylglyoxal treated specimens (p=0.004662) and by 86% relative to soaked controls (p=0.002057). EDC treatment yielded significantly higher low modulus by 86% relative to soaked controls (p=0.01265) and by 113% relative to methylglyoxal treated specimens (p=0.0303). Proanthrocyanidin treated specimens were significantly stiffer than soaked controls by 68% (p=0.02797). Proanthrocyanidin treatment also increased the low modulus by 93% relative to methylglyoxal treated specimens; however, this result was not statistically significant. In the radial orientation, EDC treatment increased the stiffness by 87% relative to soaked controls. These data sets are provided below in Figures 18 and 19, respectively.

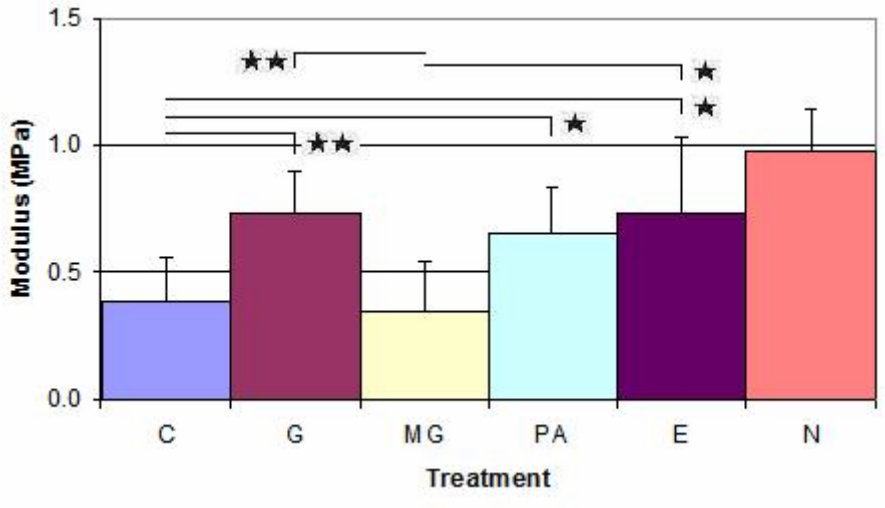


Figure 18. Axial Neutral Zone Low Modulus vs. Treatment.

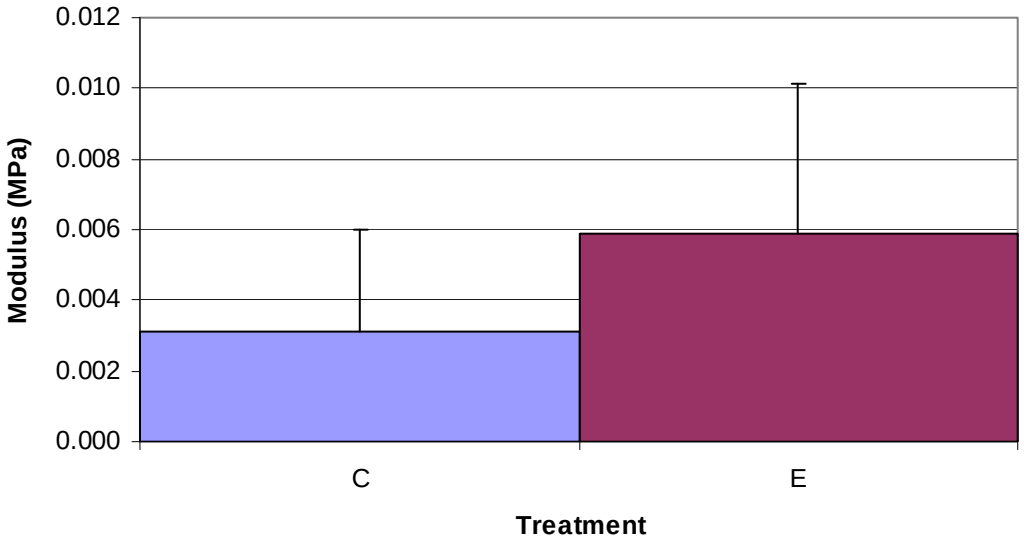


Figure 19. Radial Neutral Zone Low Modulus vs. Treatment.

Considering the Neutral Zone high modulus in the axial orientation, methylglyoxal treatment significantly reduced the stiffness by 154% relative to proanthrocyanidin treated specimens (p=0.004329), and by 102% relative to EDC treated specimens (p=0.004329). Non-soaked controls also had a 99% higher stiffness relative to methylglyoxal treated specimens. Proanthrocyanidin treatment significantly increased the stiffness by 149% relative to soaked controls (p=0.001332) and by 63% relative to genipin treated specimens (p=0.005051). Non-soaked controls were 95% stiffer than soaked controls. EDC treatment significantly increased the stiffness by 30% relative to genipin treated specimens (p=0.04798) and by 98% relative to soaked controls (p=0.007992). In the radial orientation, EDC treatment increased the stiffness by 85%

relative to soaked controls in the high strain modulus. These data are shown below in Figures 20 and 21.

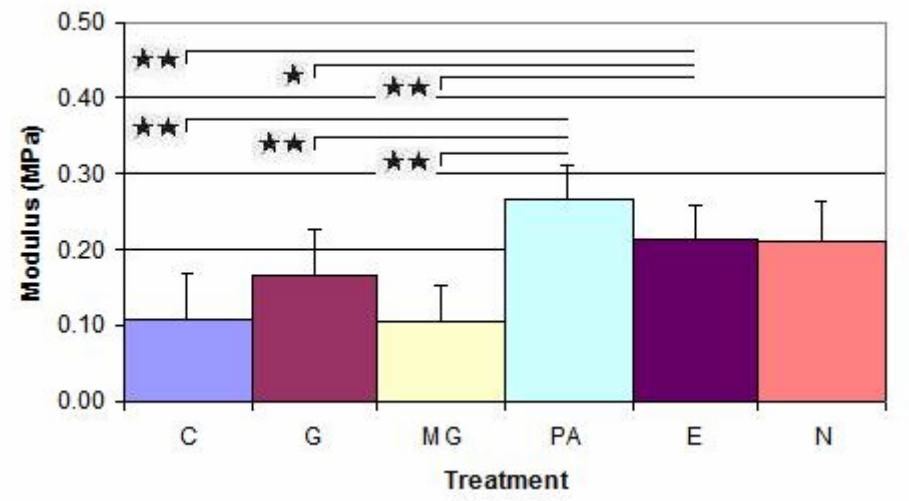


Figure 20. Axial Neutral Zone High Modulus vs. Treatment.

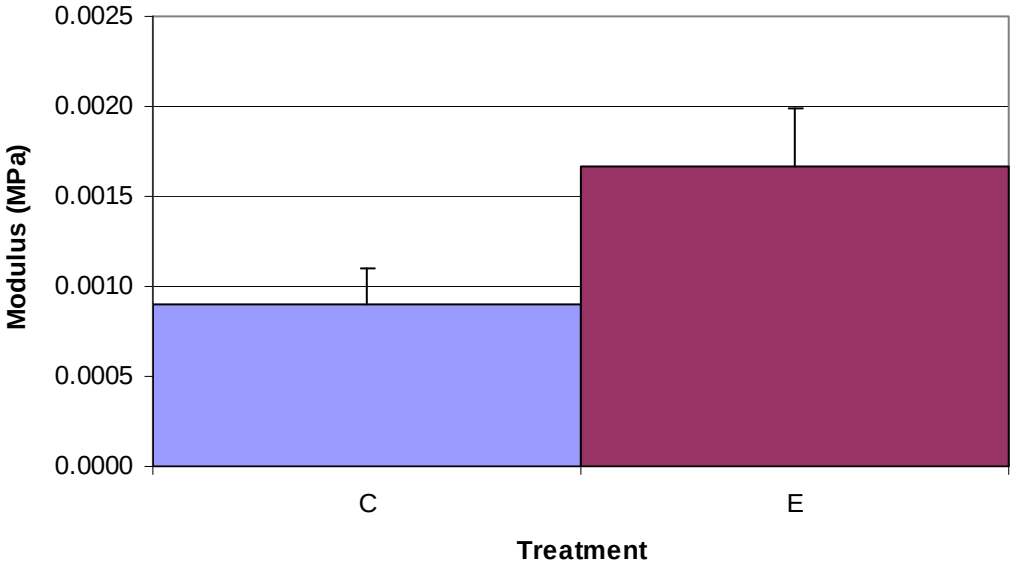


Figure 21. Radial Neutral Zone High Modulus vs. Treatment.

The shift point between the Neutral Zone Low and high Modulus was significantly shifted by genipin treatment to a point 81% sooner than soaked controls (p=0.04309), 143% sooner than proanthrocyanidin treated specimens (p=0.005051), and 106% sooner than EDC treated specimens (p=0.04798). In radially oriented specimens, the EDC group shift point was 38% earlier than that of soaked control specimens. These data are presented below in Figures 22 and 23, respectively.

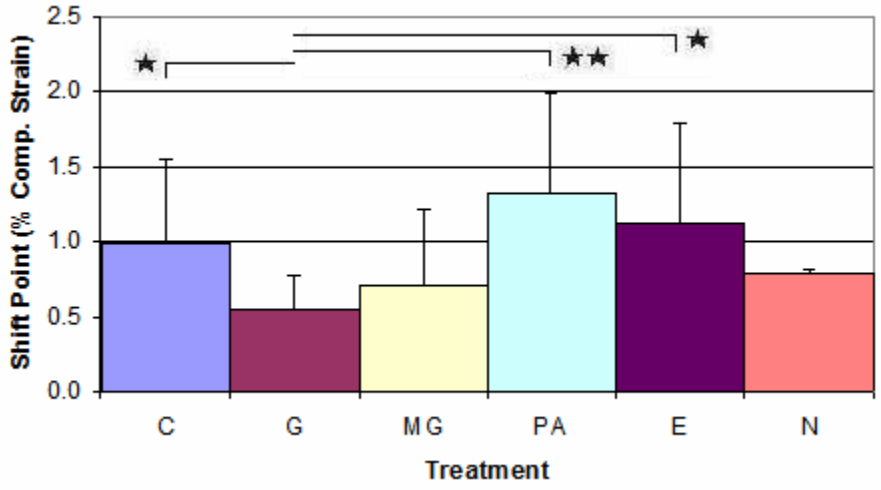


Figure 22. Axial Neutral Zone Shift Point vs. Treatment.

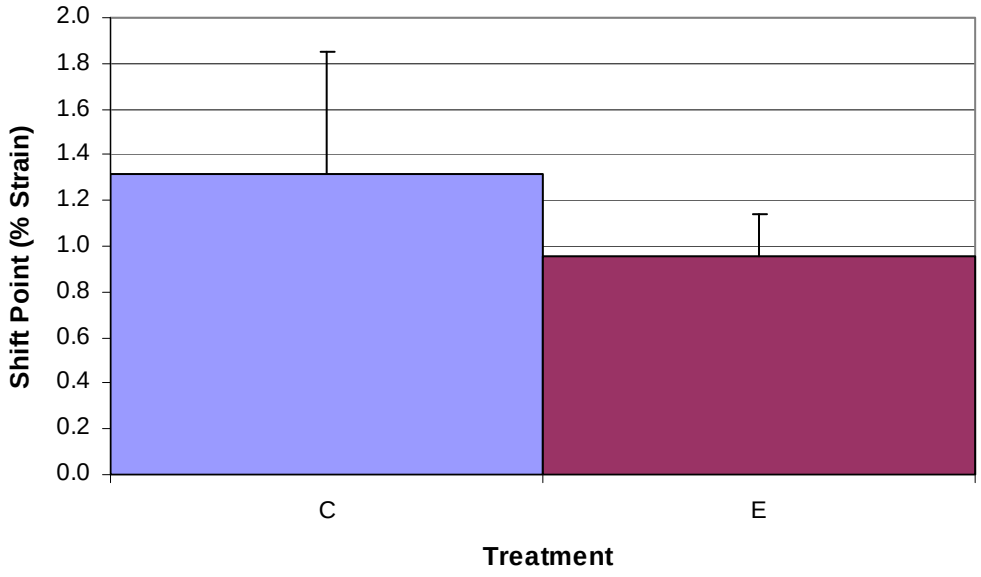


Figure 23. Radial Neutral Zone Shift Point vs. Treatment.

Since previous studies[6,32] have investigated the relationship between water content and stiffness and permeability as measured in this study, but not that between water content and the shift point as noted above, the potential correlation between the shift point and water content was measured using a least squares regression technique. In all treatment groups except for soaked controls the coefficient of correlation was less than 0.15, therefore only the soaked control data has been included here. In soaked controls the coefficient of correlation was 0.38; a plot of these results along with the fitted linear curve appear below in Figure 24. The equation for this curve is also provided below in Equation 8.

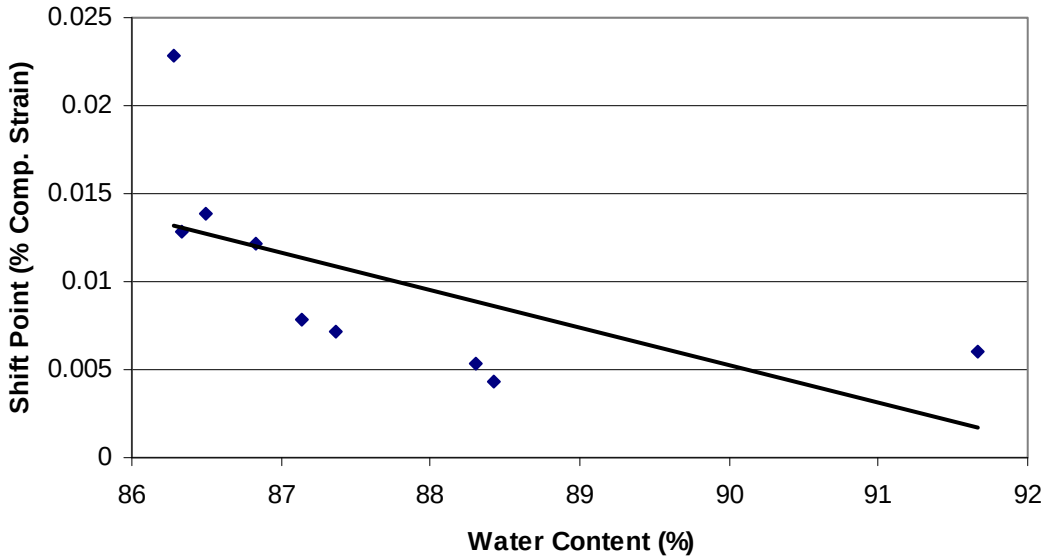


Figure 24. Neutral Zone Shift Point vs. Water Content in Soaked Control Specimens.

$$\text{Shift Point (\% compressive strain)} = -0.0021 * \text{WC (\%)} + 0.1966 \quad (8)$$

Proanthrocyanidin treatment resulted in a significantly lower post treatment water content by 9.1% relative to soaked controls ($p=0.01898$), by 8.8% relative to genipin treated specimens ($p=0.01865$), and by 10.6% relative to methylglyoxal treated specimens ($p=0.004329$). These data are presented below in Figure 25. Since radial specimens were not dehydrated with PEG, only pre- and post-test data are pertinent to that group.

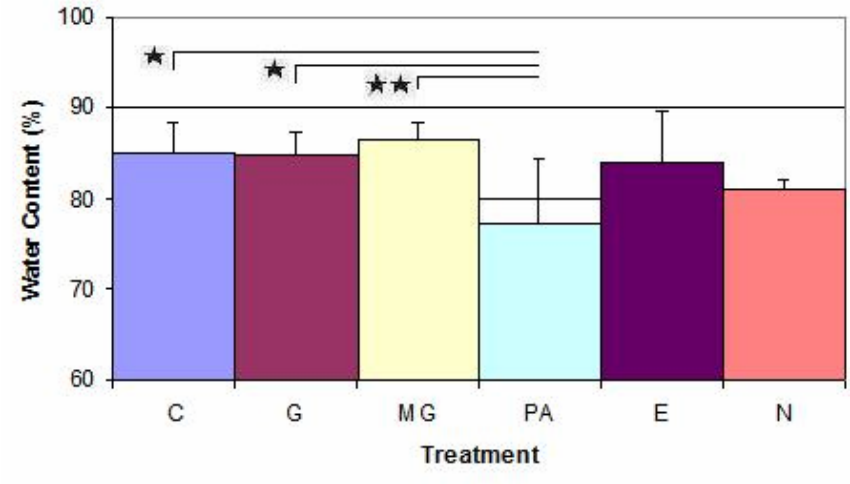


Figure 25. Axial Post Treatment Water Content vs. Treatment.

In the axial orientation, proanthrocyanidin treatment resulted in a significantly lower pre test water content by 11% relative to soaked controls ($p=0.000999$), by 9.8% relative to genipin treated specimens ($p=0.02953$), and by 12.4% relative to non-soaked controls. EDC treatment did not affect pre test water content relative to soaked controls in either axially or radially oriented specimens. These data are presented below in Figures 26 and 27, respectively.

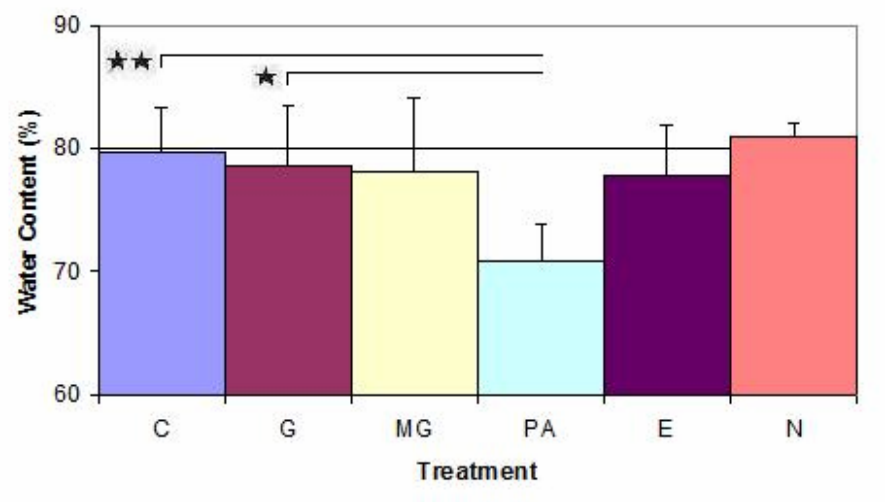


Figure 26. Axial Pre Test Water Content vs. Treatment.

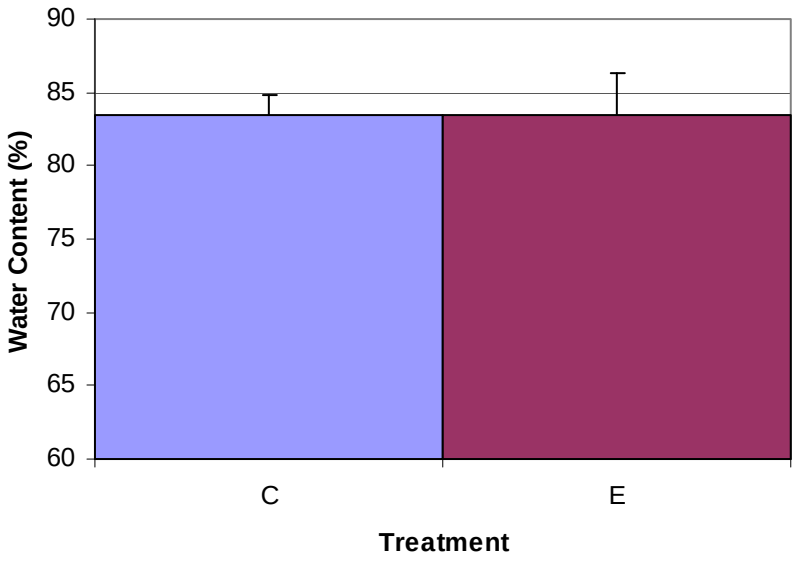


Figure 27. Radial Pre Test Water Content vs. Treatment.

In axial specimens, proanthrocyanidin treated discs had an 8.8% lower post test water content relative to soaked controls (p=0.000999), 9% lower than genipin treated specimens (p=0.001554), 8.8% lower than methylglyoxal treated specimens

(p=0.004329), 9.4% lower than EDC treated specimens (p=0.007937), and 11% lower than non-soaked controls. EDC treatment did not affect post test water content relative to soaked controls in either axially or radially oriented specimens. These data are presented below in Figures 28 and 29, respectively.

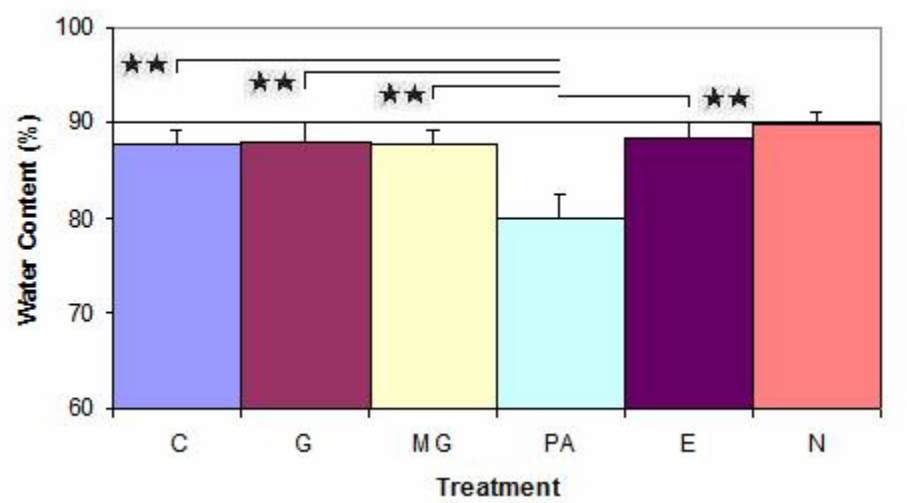


Figure 28. Axial Post Test Water Content vs. Treatment.

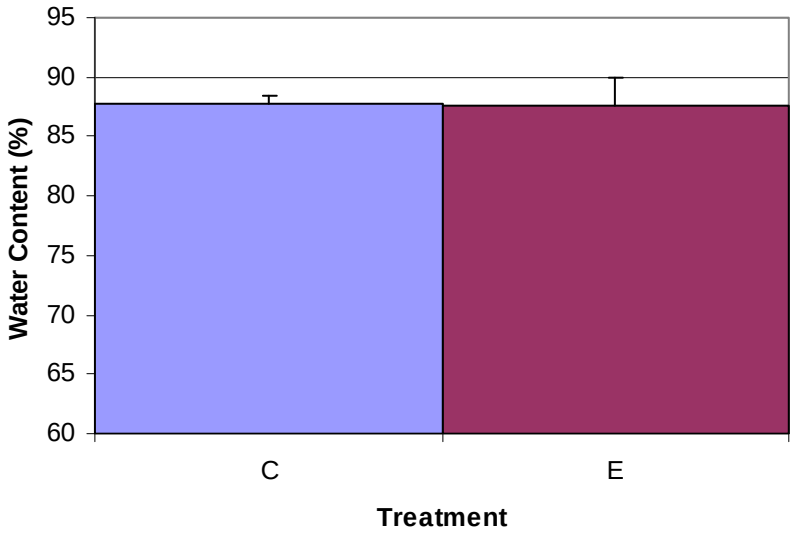


Figure 29. Radial Post Test Water Content vs. Treatment.

In axial specimens, proanthrocyanidin treatment significantly increased the dry density by 87% relative to soaked controls (p=0.003319), by 71% relative to genipin treated specimens (p=0.001554), by 72% relative to methylglyoxal treated specimens (p=0.00797). Proanthrocyanidin treatment also increased the dry density by 47% relative to non-soaked controls. EDC treated specimens had a 16% lower dry density than non-soaked controls. EDC treatment increased dry density relative to soaked controls by 82% in radially oriented specimens. These data are presented below in Figures 30 and 31, respectively.

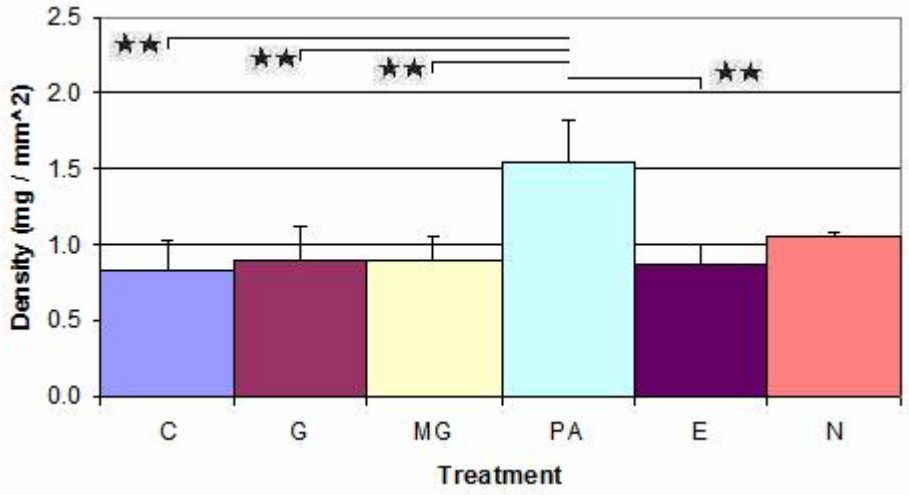


Figure 30. Axial Dry Density vs. Treatment.

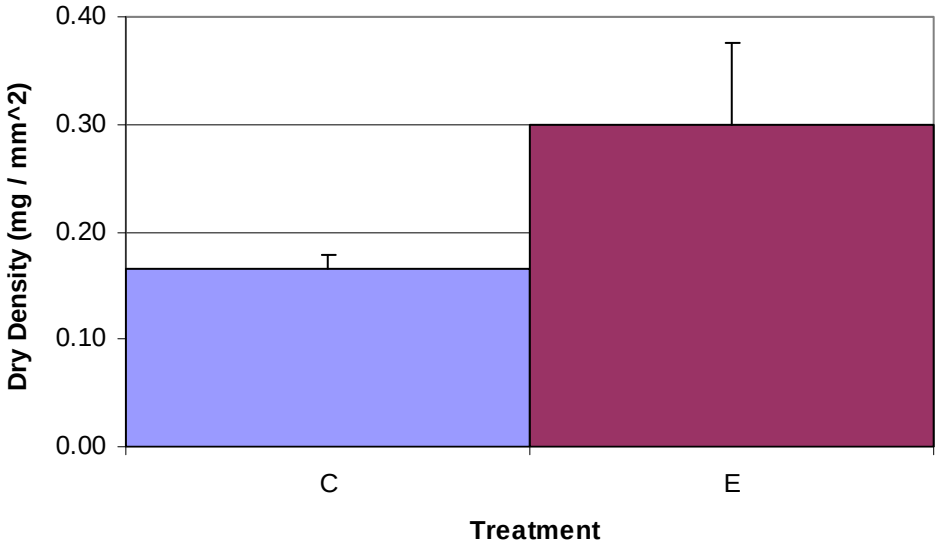


Figure 31. Radial Dry Density vs. Treatment.

In axial specimens, the swelling pressure was significantly increased relative to soaked controls by 67% with genipin treatment (p=0.04342), 106% with

proranthrocyanidin treatment (p=0.02797), and 135% with EDC treatment (p=0.004662). Non-soaked controls had an 84% higher swelling pressure than soaked controls, but this result was not statistically significant. Methglyoxal treatment significantly reduced the swelling pressure by 40% relative to genipin treated specimens (p=0.0293) and by 51% relative to proanthrocyanidin treated specimens (p=0.0303). EDC treated specimens had a 135% higher swelling pressure relative to methylglyoxal treated specimens and 41% higher swelling pressure than genipin treated specimens; however, these results were not statistically significant. In the radial orientation, swelling pressure was not affected by EDC treatment relative to soaked controls. These data are presented below in Figures 32 and 33, respectively.

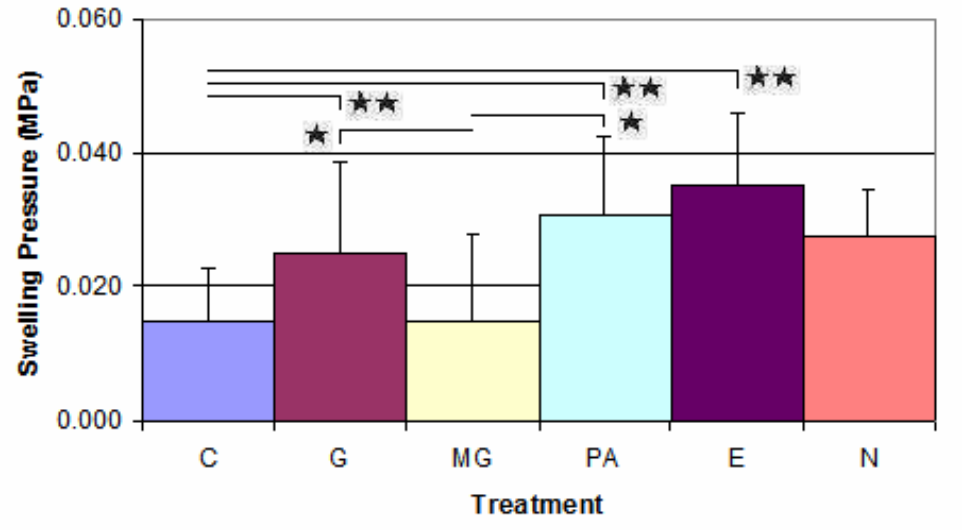


Figure 32. Axial Swelling Pressure vs. Treatment.

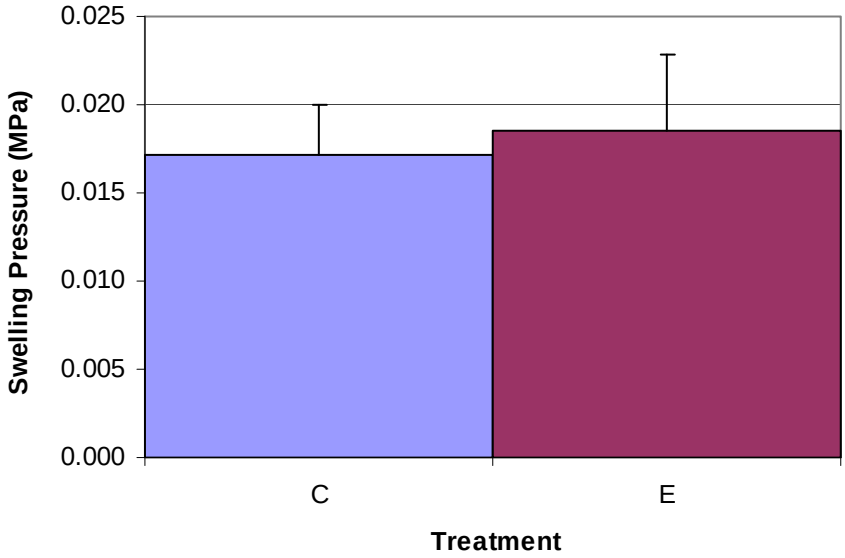


Figure 33. Radial Swelling Pressure vs. Treatment.

Discussion

Confined stress relaxation experiments of bovine lumbar inner annulus fibrosus, both treated and control, revealed significant changes in both aggregate modulus and hydraulic permeability.

Note first that the results of the validation study with an initial preload agree closely with the results of the method used in all other experiments, i.e. using a prestrain instead of a preload. The observed differences may be explained in part by the difference in tissue location, i.e. caudal vs. lumbar discs, and perhaps the age difference of the samples. This suggests that the difference in these methods did not significantly affect the results of this study. The low stiffness and high permeability values of the samples from the validation study relative to those of *in situ* specimens in a study by Iatridis[6] which used a 0.1 N preload are likely indicative of changes in these properties

with age. The specimens used in Iatridis' study[6] were approximately four years of age, whereas the samples used in our study were less than one year old. It is well documented in the human intervertebral disc that stiffness increases and permeability decreases in the axial orientation with age[5], which fits well with the difference between our findings and the published values noted. Further, note that our findings in the soaked control specimens using a prestrain are similar to those reported in Iatridis' study[6]. The lower modulus values in the present study again are likely attributable to age, although water content may also have been a contributing factor as the present study had on average 5% higher water content which, by correlations provided by Iatridis[6], would account for a reduction in stiffness of approximately 0.165 MPa. Collectively, this suggests that the methods used in this study are comparable to those used in previously published studies.

The variance in the nonlinear terms from the nonlinear biphasic model are due some consideration. The nonlinear stiffening term, β, values ranged from -0.49 to 5.8 whereas values in the literature have typically ranged from approximately -0.2 to 3.5[39]. To this author's knowledge the only restriction that should be placed on this parameter is a lower limit of -0.49 as any value less than or equal to -0.5 results in a finite amount of stress at infinite compression; but the question remains whether the higher values for β observed in this study affected the mechanical properties. With that in mind, I have plotted the stress response over a 20% compressive strain using the aggregate modulus of soaked control specimens with 5 different values for β as indicated in the plot legend below in Figure 34.

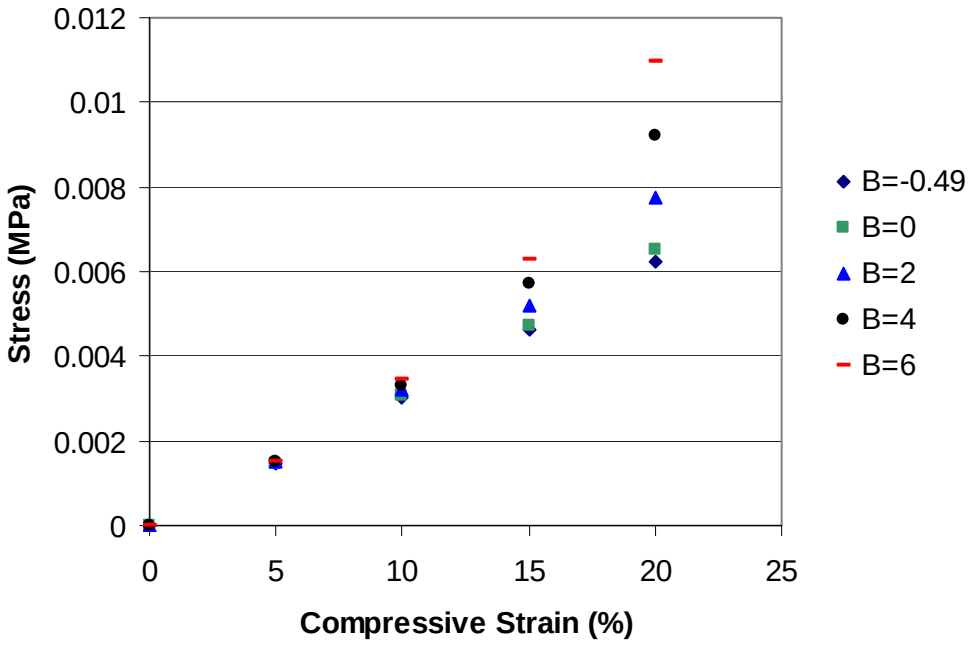


Figure 34. Influence of Nonlinear Stiffening Term B on Stress Response.

Note that with this relatively large range of values in β, the stress values at 15%, the peak compressive strain applied in this study, differ at the most by about 25%. With this in mind and the relative similarity between our range and the previously published study, it does not appear that the variation in β will cause much distortion of the aggregate modulus data.

To the nonlinear permeability parameter, M, values observed in this study ranged from -14.6 to 11, whereas previous studies have measured values in the range of 1.1 to 4.5[39]. The potential seems to exist here for more variability in our results due to a larger range that is far outside of previous studies. It seems that these prior studies have primarily limited themselves to values of M which do not allow for increasing

permeability with increasing strain due to the typical phenomenon of compressing proteoglycans causing an increased fixed charge density that does not allow electrochemically for increased permeability with increasing compressive strains. However, it was desirable to allow for increases in permeability due to other mechanical effects during the testing, such as bowing as described later, therefore the nonlinear permeability term was not restricted as it has been in prior studies. With that in mind, I have plotted the permeability at compressive strains up to 20% using the average full range permeability coefficient from the soaked control group and M values ranging from -10 to +10 as described in the legend for Figure 35 below to demonstrate the effect of the M parameter.

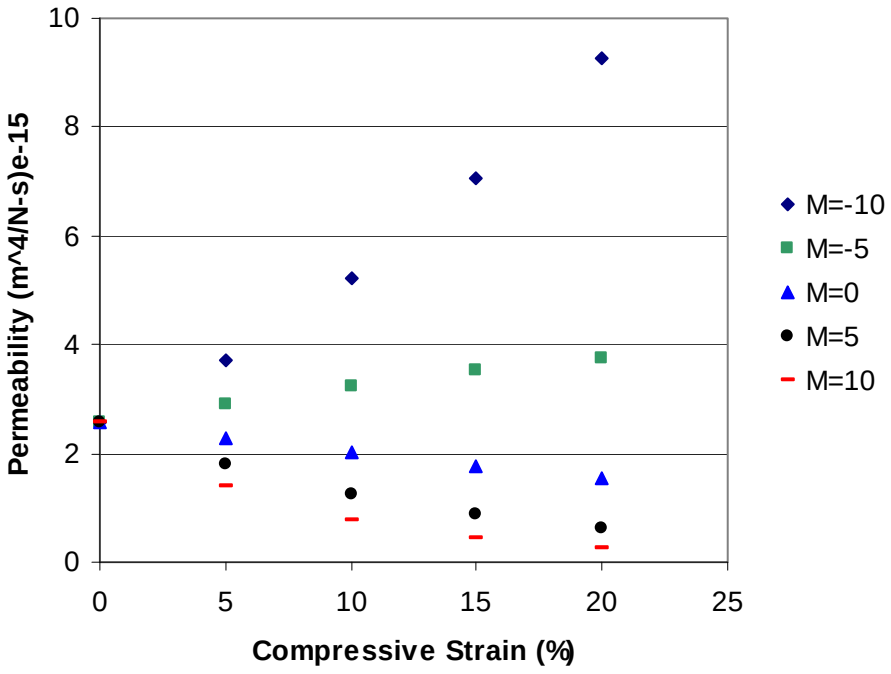


Figure 35. Influence of Nonlinear Permeability Term M on Permeability.

The range of permeability values suggests that the range of M values can account for a variety of permeability trends with increasing compressive strain, as desired. It should also be noted that in only 7 of the nearly 40 samples tested were values in the range of -10 or +10; so while the variation in M may have potential to skew the data, large values are relatively uncommon, and thus unlikely for the data to be significantly skewed. It is also partly for this reason that the steady state permeability at 10% and 15% are provided independently, as they are determined directly from the stress response, so that there can be little doubt in these results. With all of this in mind, the effect of the large variation in M is concluded to be significant in quantifying the true composite mechanical - electrochemical response of the tissue and its effect on permeability, but not in artificially skewing the permeability data.

With all treatments except methylglyoxal the aggregate modulus in axial specimens was at least doubled compared to soaked controls, indicating a significant combination of structural and biochemical changes. Using values from the correlations provided between aggregate modulus and GAG and water content of the disc[6] suggests that the relatively minor changes in water content in genipin and EDC treatment groups relative to soaked controls likely account for only one third of the increased stiffness in crosslinked specimens. The influence of GAG content will be discussed further in the next chapter, but suffice it to say at this time that higher stiffness in crosslinked specimens is anticipated to be a result primarily of strengthening of the extra cellular matrix by way of collagen crosslinking and an expected higher GAG content in these treatment groups relative to soaked controls.

The apparent increase in aggregate modulus and other stiffness parameters in the radial orientation, although not significant, are important to interpret in the context of the experiment. Applying a radial compressive strain is similar to a bulging experiment in which the strain is primarily resisted by the intact ring of annular tissue as hoop strain. In an excised portion of this annular ring, resistance to such a load can come from the drag of fluid through the matrix, resistance from compressing the collagen matrix and proteoglycans closer together, or resistance from compression of the lamella together. The first seems unlikely given that EDC treated specimens had a higher permeability than saline soaked specimens which would suggest reduced ability to resist load by drag through the matrix. In the radial orientation, the swelling pressures of the EDC specimens differ very little from the soaked control specimens, and based on a previous study by Best[32] it is reasonable to conclude that proteoglycan content was not significantly changed; therefore the proteoglycan resistance argument also seems unlikely. The other possibilities of intralamellar compression of the collagen matrix and some increased level of interlamellar crosslinks relative to soaked controls seem the most likely, the latter of which seems further evidenced by the near doubling of the tissue dry density.

The low values of the aggregate modulus in both axial and radial soaked controls relative to previously published values may likely be attributed to a combination of reduced water content relative to non-soaked controls and leaching of GAG content during the treatment and rinse incubation periods, as the modulus data are on the order of values reported in a “free swelling” tissue group in Iatridis’ study[6]. The significantly

higher axial moduli (Aggregate, Neutral Zone Full Range, Neutral Zone Final, and Neutral Zone Initial Modulus) in non-soaked controls relative to soaked controls seems to confirm this hypothesis.

Increased axial stiffness has been observed with crosslinking, both by degeneration and treatment, by other researchers[9, 10, 39], therefore our findings are not surprising. The permeability results, however, are more difficult to interpret. Previous reports have established that compression increases fixed charge density and decreases porosity[29, 30], therefore permeability should decrease with increasing compressive strain; however in the methylglyoxal, proanthrocyanidin, and EDC treatment groups the steady state permeability increased from the 10% to 15% compressive strain levels in axially oriented specimens. This may indicate that these crosslinking treatments, particularly proanthrocyanidin and EDC, allowed the matrix to become more resilient and be engaged more quickly, thus causing a bowing phenomenon that would increase structural porosity with increasing compression.

Additionally, previous studies have almost uniformly found that crosslinking resulted in a higher hydraulic permeability[11, 14-18, 40], whereas we observed lower permeability in the axial orientation and increased permeability in the radial orientation relative with crosslinking treatment relative to soaked controls. There are several possible explanations for this difference.

First, by freeing one surface of the disc, loose axially directed fibers could be drawn together during crosslinking treatment, causing a structural decrease in porosity and permeability relative to controls. There seems to be evidence for this in the

proanthrocyanidin treatment where the dry density of the tissue was significantly increased relative to soaked and non-soaked controls. This treatment group also had a water content less than 80% by weight. Attempts were made to soak specimens prior to testing to swell the tissue to 80% water content, but were unsuccessful. This increase likely contributed to the highest aggregate modulus existing in this group and one of the highest swelling pressures. This change had little effect on any of the permeability parameters; however, the effective increase in permeability by reduced water content may be offset by the reduced porosity associated with increased tissue density.

Second, other studies that documented increased permeability have primarily not measured fluid flow parallel to collagen fibers[14-17], whereas much of the present study measured flow in the plane of the fibers. (In two studies by Cochrane[15, 16], films of glomerular basement membrane were prepared in part by being minced and repeatedly "smeared through a sieve"; such a preparation may have altered the extra cellular matrix.) The finding of increased radial permeability in this study with EDC treatment lends some credence to this factor.

Third, the altering of GAG content due to crosslinking treatment seems to be an important factor.

Gu found that radial permeability slightly decreased with degeneration, but concluded that this was most strongly related to loss of water content with degeneration[41]. In the same study, Gu further observed a slight increase in axial permeability with degeneration where: 1) porosity should be increased by crosslinks, 2) fixed charge density should be reduced by proteoglycan loss, and 3) water content is

decreased, the first two of which would increase axial permeability. Given these observations, decreased axial permeability in this study with little change in water content in treated specimens, except in the proanthrocyanidin group, may simply be a result of higher GAG content in treated specimens relative to controls outweighing the effect of structural changes associated with crosslinking treatments.

As noted earlier, it has been reported that certain crosslinking treatments such as glycation[19-22], and glutaraldehyde[23, 24], cause increased loss and / or decreased synthesis of proteoglycans, while other crosslinkers such as Transglutaminase[25], genipin[26], and EDC[27] seem to reduce proteoglycan loss. With the exception of a study that used creep loading and unloading to induce fluid flow[11], studies that observed an increase in permeability[14-18, 40] did not report a change in water content. The hydration change in the creep loading study was only 2-3% which is not expected to have a large effect on the permeability. Therefore the influence of proteoglycan retention and water content on permeability is consistent across all of these studies.

A study by Hedman concluded an increased permeability due to genipin treatment by measurements of loading induced water influx and efflux at different regions of the disc[11]. The increased influx of water to the inner annulus and the increased fluid efflux from the outer annulus seem consistent with the results of a study by Iatridis in which an increased fixed charge density was simulated using FEA[31], which is analogous with a genipin treatment which may reduce proteoglycan loss. The increased inflow and outflow from the nucleus pulposus was not reported in the fixed charge density study, so some explanation is in order.

The increased inflow of fluid to the nucleus is most probably explained by the increase of structural porosity of the matrix at rest by collagen crosslinking. As cited in the study by Hedman[11], crosslinking has been shown to increase mean fiber diameter, which is reasonably interpreted as increasing structural porosity. At rest this would allow for a greater influx of fluid. The increased efflux of fluid during loading requires some additional explanation.

The simplest explanation may be derived from the radial studies noted in this chapter. Although EDC treatment, not genipin, was used, these two treatments manifested similar effects of similar magnitude in the axial orientation, particularly in the permeability. It may well be that genipin crosslinking would have a similar effect of increasing radial permeability, thus allowing increased inflow and outflow of fluid, although further studies would clearly be required to confirm such an assertion.

Another possible explanation is that previous studies have observed that collagen fibers reorient towards the loading direction in compression, but that collagen crosslinks restrain this reorientation[42]. It may be that the restraint of fiber reorientation allows for an increased structural porosity at higher tensile strain levels, which could allow increased outflow from the nucleus. This would explain increased efflux from the nucleus during loading. At a resting state these crosslinks may also have increased the size of pores in the extracellular matrix, thus allowing greater influx. In other words, collagen crosslinking may have increased the inherent structural porosity of the extracellular matrix, thus increasing influx at rest, and resisting reorientation that would reduce structural porosity during loading, thus increasing influx.

It is also possible that the bowing effect in genipin treated discs described above may have increased permeability at high compressive strains, again causing an increased permeability relative to controls that could allow increased nuclear efflux.

As stated before, it seems likely that GAG was leached from the soaked control specimens during the soak-rinse protocol in the present study. The decrease in permeability in certain treated specimens relative to soaked controls seems to indicate that these treatments reduced proteoglycan loss relative to soaked controls. It also appears that the significantly higher swelling pressure in genipin and EDC treatments relative to soaked controls adds weight to this theory, as previous studies show a direct relationship between swelling pressure and GAG content[32].

The effect of soaking on permeability, however, was negligible in soaked relative to non-soaked controls. This is certainly in part due to the variability in the data, but may also be a result of the effects of increased water content, which could cause a reduction in permeability, offsetting the effects of GAG leaching, which could cause an the increased permeability. As in the stiffness discussion, an examination of the changes in light of published relationships[6] between permeability and GAG and water content suggests that particularly in genipin and EDC treated specimens the slight change in water content may only contribute 25-30% of the total reduction in permeability. The remainder is likely an effect of the change in GAG content which, particularly at low densities, can manifest as relatively large changes in permeability[28].

Thus the combination of factors: preferential leaching of GAGs from soaked control discs, permeability being measured along the axes of the fibers and in the plane

of the lamella, and the interfibrillar crosslinking of unattached fibers at the cut surface could all have contributed to greater permeability in the soaked controls relative to some treated specimens. It is the author's opinion that the loss of fixed charge density resulting from preferential loss of control specimen GAGs in the initial free swelling treatments was the dominant effect.

CHAPTER III

BIOCHEMICAL CONTENT QUANTIFICATION

Materials and Methods

As described previously, calf spines (~4-6 months old) were obtained from an abattoir. A band saw was used to cut through vertebral bodies, yielding 4 bone-disc-bone complexes or motion segments (MS) which were immediately frozen and stored at -20 C. Previous studies have shown that frozen storage has negligible effect on mechanical properties[34-37].

Prior to treatment, a motion segment was removed from the freezer, wrapped in a paper towel soaked with phosphate buffered saline solution (PBS) (0.14 M), and thawed for 2 hours at room temperature in a sealed plastic bag. A scalpel was then used to remove excess tissue, and one of the remaining vertebral bodies was removed, so that a bone-disc complex remained.

These complexes were then soaked in either Control – PBS(C) or 0.5 % 2-(*N*-morpholino)ethanesulfonic acid (EDC). The complexes were placed in a 1 L beaker, solution was added to completely cover the disc and bone ; the beaker was then sealed and incubated for 4 hours at 37 C. Prior testing in our lab provided optimal incubation times and concentrations for each reagent to form crosslinks.

After incubation, the complex was rinsed and soaked in PBS for 30 minutes three consecutive times to remove active crosslinker. Each soaking was performed with fresh solution. These complexes were then frozen at -20 C.

A previous study by Farndale[33] documents a method of estimating GAG content of the intervertebral disc through a spectrophotomeric assay. As described in that study, attempts were made at digesting disc tissue removed from the middle annulus region with a scalpel with a papain digest solution; however, these efforts proved unsuccessful. An alternative digestion method using collagenase (Sigma Chemical Company) in a buffer of 100 mM Tris pH 7.5 and 1 mM $CaCl_2$ was subsequently and successfully used to digest disc tissue.

Samples were removed from the middle annulus region of previously tested discs to estimate GAG content in tested tissue. After removing a sample from a disc, each sample was weighed wet, dried overnight at approximately 80 C, and weighed dry to allow determination of water content. These dried discs were then digested in a collagenase buffer solution. Discs soaked in PBS and non-soaked discs proved the easiest to digest, requiring up to 48 hours of incubation at approximately 40 C in 1 ml of a 5 mg / ml collagenase buffer solution. Attempts were made to digest crosslinked disc tissue using 1 ml of collagenase buffer solutions up to a concentration of 25 mg / ml for periods of 72 hours; however these incubations proved unsuccessful at digesting crosslinked tissue with the exception of the methylglyoxal treated group where a 5 mg / ml collagenase buffer solution provided some digestion.

To estimate GAG content, standards were made using shark GAG, specifically chondroitin sulfate (Sigma Chemical Company), in concentrations of 0.05 – 0.5 mg / ml. Given that the 5 mg / ml collagenase buffer solution had a visible reddish-brown tint, the standards were measured in this solution. The primary solvent for the spectrophotomeric

tests was dimethylmethylene blue (DMMB) dye, consisting of 95 ml of 0.1 M HCl, 3.04 g Glycine, 2.37 g NaCl, and 16 mg DMMB dye in 1 L of deionized water.

A Hitachi U-4100 UV-Vis-NIR spectrophotometer was used to measure the absorbance and % Transmittance of a sample, consisting of 1.4 ml of the DMMB dye solution, 0.098 ml of de-ionized water, and 0.002 ml (2 ul) of the sample or standard solution, relative to 1.5 ml of the DMMB dye solution. This high dilution factor was necessary in order for the absorbance of a volume of digested disc to be within the linear range measured using the standards. All measurements were made as a wavelength scan from 400 to 800 nm. The peak absorbance of each standard was recorded, typically at or near 525 nm as found in other studies, and these standard points allowed a linear fit of absorbance vs. mass of GAG to be made from which the GAG content of digested disc samples could be estimated.

A typical method for reporting GAG content is in μg of GAG / mg of dry weight. To calculate this parameter, the estimated amount of GAG based on absorbance measurements was multiplied by a factor of 500, since each disc sample was dissolved in 1 ml of collagenase buffer solution and the absorbance of 0.002 ml of this solution measured. This total estimated GAG content was then be divided by the dry weight of the tissue in milligrams.

Results

The absorbance data for the standards prepared with shark chondroitin sulfate appear below in Figure 36, as well as the correlation in Equation 9, with a linear function fit to the data using a least squares method:

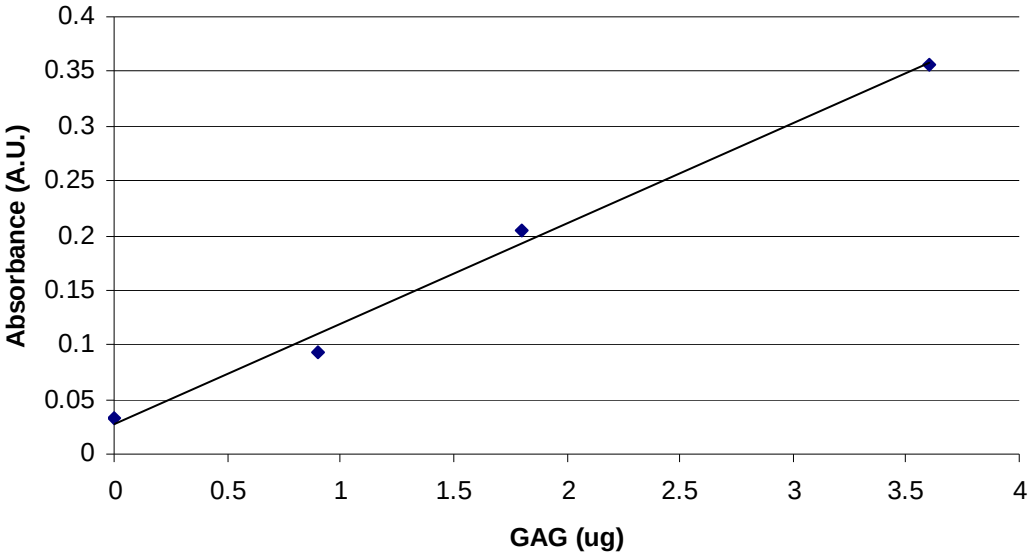


Figure 36. Absorbance Measurements as a Function of GAG Content.

$$\text{Absorbance (A.U.)} = 0.0919 * \text{GAG } (\mu g) + 0.0272 \qquad (9)$$

The extrapolated GAG content, in µg of GAG / mg of dry weight, as well as water content data appear below in Table 9 for PBS soaked and non-soaked intervertebral discs from this study along with values from a previously published study[6].

Table 9. Water Content and Estimated GAG Data.

		Water Content (%)	GAG Content (ug GAG / mg dry weight)
Soaked Controls (N=12)	**Average**	81.62	137.09
	St. Dev.	5.65	44.03
Previous Study: Soaked	**Average**	74.00	60.00
Non-Soaked Controls (N=9)	**Average**	56.31	86.13
	St. Dev.	13.06	53.97
Previous Study: Non-Soaked	**Average**	68.00	120.00
Methylglyoxal Treated (N=7)	**Average**	84.91	118.44
	St. Dev.	5.30	42.88

Soaked control specimens and methylglyoxal treated specimens had a significantly higher water content than non-soaked specimens by 45% and 52%, respectively ($p = 0.0484$, 0.0333, respectively), but were not significantly different from one another.

Correlations were also made of the GAG content with water content for each of the three treatment groups, and appear below in Figures 37, 38, and 39, respectively. These correlations had R^2 values of 0.918, 0.866, and 0.249 respectively.

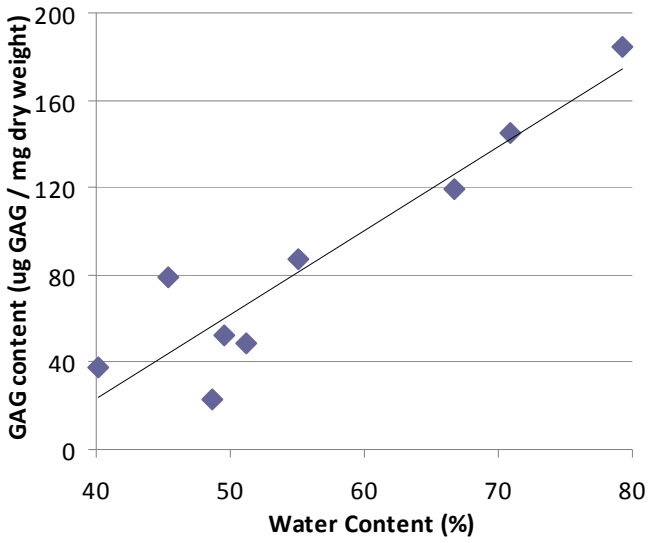


Figure 37. GAG Content vs. Water Content Plot for Non-Soaked Control Specimens.

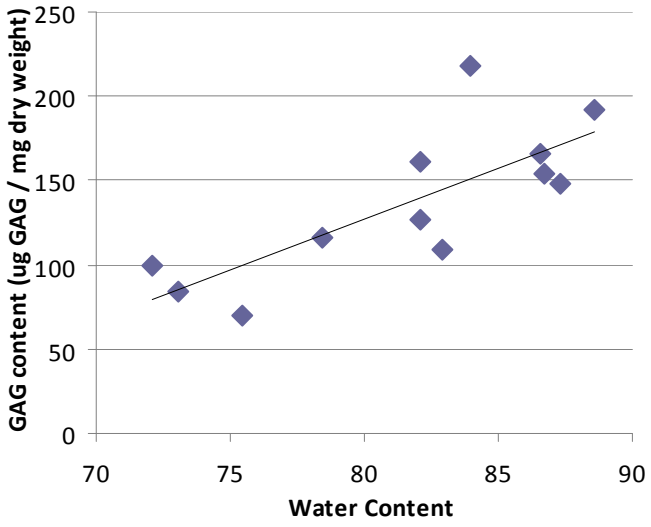


Figure 38. GAG Content vs. Water Content Plot for Soaked Control Specimens.

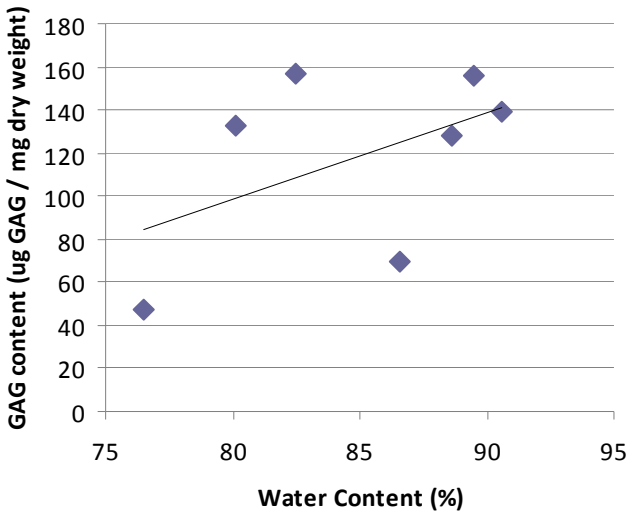


Figure 39. GAG Content vs. Water Content Plot for Methylglyoxal Treated Specimens.

Equations for the correlations between GAG content and water content for non-soaked controls, soaked controls, and methylglyoxal treated specimens were, respectively, as follows in Equations 10 - 12:

GAG content = 3.8455 * Water Content (%) – 130.41	(Non-soaked)	(10)
GAG content = 6.035 * Water Content (%) – 355.47	(Soaked)	(11)
GAG content = 4.0352 * Water Content (%) – 224.19	(Methylglyoxal)	(12)

Using these correlation equations, GAG contents were estimated at the mean water content levels noted from the previous study[6] referenced above in Table 9. These calculated GAG values appear as the underlined values below in Table 10.

Table 10. Extrapolated and Experimental GAG Data Comparison.

	Water Content (%)	GAG Content (µg GAG / mg dry weight)
Soaked Controls	74.00	91.12
Previous Study: Soaked	74.00	60.00
Non-Soaked Controls	68.00	131.08
Previous Study: Non-Soaked	68.00	120.00

Based on the above correlation equations, GAG contents from the axial confined compression testing specimens were calculated based on average post test water contents for the soaked controls, non-soaked controls, and methylglyoxal treated groups. These data appear below in Table 11.

Table 11. Extrapolated and Experimental GAG Data Comparison for Axial Tests.

Treatment Group	Water Content (%)	GAG Content (µg GAG / mg dry weight)
Soaked Controls	87.6	173.20
Non-Soaked Controls	89.8	214.91
Methylglyoxal Treated	87.6	129.29

Discussion

In all soaked and non-soaked control specimens, the disc sample was completely digested or digested thoroughly enough that no interference in the GAG data is anticipated. This was observed visually as the remaining tissue appeared to be a highly porous matrix, likely the thicker grouping of fibers from the collagen matrix that was unable to be dissolved completely. Additionally, there was visible particulate present in solution that could readily be dissolved by mechanical agitation which is anticipated to be GAG released from the tissue by the enzymatic digestion of the collagenase as the shark chondroitin sulfate material used to measure standards was similarly soluble. This may indicate that the incubation temperature for the digestion process was not idealized to allow for complete digestion of more resilient groups of fibers, but enough to provide for release of the majority of tissue GAG content; however this indicates a need for improved control of the incubation temperature in future studies. Methylglyoxal samples were less digested than either control group (soaked or non-soaked).

The close agreement of our results with those of previous studies[6, 26, 32] in estimating the GAG content of non-soaked control specimens in the study by Iatridis[6], suggests that our methods are nearly equivalent with those used in previous. The slightly higher values for non-soaked specimens in this study as compared to the results tabulated above are consistent with the expected differences due to age of the specimens, i.e. less than 1 year old compared to 4 years old, as well as the location, i.e. middle annulus in this study as compared to outer annulus in the previous study.

The further loss of GAG content in methylglyoxal treated specimens is in agreement with studies that suggest that glycation is a possible mechanism for loss of proteoglycans[19-21]. However, the similarities in swelling pressure, aggregate modulus, and permeability coefficients between soaked controls and methylglyoxal treated specimens in the axial data set suggest that a loss of GAG content of this magnitude does not significantly affect such parameters.

The anticipated loss of GAG with soaking in PBS is likely to have occurred, as manifested by the estimate of GAG content in axial confined compression groups based on average post-test water content in the non-soaked and soaked control groups. Making use of this GAG loss data and correlation equations described by Iatridis[6] for both the aggregate modulus and permeability coefficient with GAG content and water content, some explanation of the difference between soaked controls relative to non-soaked controls may be offered. Given that the location of our specimens is intermediate between the annulus and nucleus, values from Iatridis' study for the respective influences of GAG content and water content for annulus and nucleus were averaged for an estimate of their respective influence in the present study. With such values for their respective influences, the differences in GAG content and water content between soaked and non-soaked specimens as observed in this study may result in a decrease of approximately 0.08 MPa in the aggregate modulus and an increase of approximately 1.5 m^4 / N-s in the full range hydraulic permeability coefficient in soaked controls relative to non-soaked controls. While these values are only estimates, they are consistent with the axial confined compression results of the present study and lend at least a possible

explanation of the mechanism for the observed changes in mechanical response. Again based on the correlations described in a previous study[6], the changes in GAG content are anticipated to be responsible for at least half of the increased stiffness and reduction in permeability with genipin and EDC treatment relative to soaked controls.

While we were unable to digest genipin, proanthrocyanidin, or EDC treated specimens, it was noted previously that these treatments may reduce loss of proteoglycans. With this in mind, using the correlation equations noted from Iatridis' study[6] and the post-test water content for the treated tissues in the present study, the difference in the axial full range permeability coefficient between non-soaked control specimens and genipin and EDC treated specimens may be explained primarily by reductions in water content that overshadowed the structural effects of these treatments. This suggests that these treatments may have maintained an amount of GAG content nearly that of non-soaked control specimens in agreement with previous studies[26,27].

Thus, the anticipated and observed loss of GAG content with soaking[6] and glycation crosslinking is likely responsible for the apparent loss of stiffness and increase in permeability in the soaked and methylglyoxal treatment groups relative to non-soaked and other crosslink treated specimens. While GAG content changes were not measured in more strongly crosslinked tissue, using data from this study and findings from previous studies it is reasonable to conclude that the other crosslinkers used in this study did in fact reduce the loss of proteoglycans and GAG content relative to soaked control specimens.

CHAPTER IV

CONCLUSIONS

Stress-relaxation testing of bovine annulus tissue in confined compression proved to be a useful method in determining the stiffness and permeability of control and crosslinked specimens. As hypothesized, treatment with exogenous crosslinking reagents significantly increased stiffness in all groups except for methylglyoxal. Likewise with all crosslinking treatment except methylglyoxal, permeability was observed to be lower than soaked controls. Where GAG content was able to be measured by a DMMB assay, the results agreed with our hypothesis that free swelling of control specimens resulted in a leaching of GAG content, and that treatment with a glycation reagent like methylglyoxal may have further increased this leaching.

Comparison of results with a correlation study by Iatridis[6] also suggests that reduced water content in genipin and EDC groups relative to non-soaked controls may have contributed more significantly than reduced GAG leaching to the reduction of permeability in these treatment groups relative to non-soaked controls. The significant increase in dry density and significant decrease in water content of the proanthrocyanidin group relative to soaked controls suggests that the reduced permeability in that group is primarily a result of loss of structural porosity and water content.

Future studies are needed to better quantify the material properties observed in this study, as well as the effects of different methods of treatment. The variance in the data suggests that larger experimental groups are likely needed, however the sample

sizes are comparable to published studies incorporating this testing method. Improved digestion methods and larger data sets also hold value in quantifying the changes in GAG content due to crosslinking treatment.

As noted, the method of treating discs by soaking has distinct disadvantages, particularly in exposing the disc to a GAG leaching effect and allowing changes in the macro-structure due to one surface of the disc being free from the vertebral body. With this in mind, future tests should also be performed with specimens treated by injection so as to better understand the effects of this method of treatment on tissue mechanical and biochemical properties. Given the intended clinical relevance of this type of treatment, injection offers a further benefit in a better understanding of the effect of crosslinking in a more realistic environment.

Since none of the observed effects were significant relative to non-soaked controls which are anticipated to be closer to living tissue, particularly in GAG content, little can be said from this study on the possible clinical effects of crosslinking treatment. However, in view of selecting a clinical treatment, all treatments except methylglyoxal seem appropriate for stiffening the disc to compensate for increased loading during the lifetime of the intervertebral disc. The permeability data suggests two somewhat opposing clinical effects. A decreased permeability is anticipated to help maintain GAG content throughout aging as well as increasing the ability of the disc to bear load by swelling. However, a decreased permeability is also anticipated to reduce nutritional and waste flow throughout the disc which is clearly undesirable to support biological regeneration. The only caveat to this observation that can be offered at this time is again

noting the effects of a previous study that observed increased flow throughout the disc with genipin treatment. Again, with this in mind, future studies with specimens treated by injection can help illuminate the effects of these crosslinking treatments. If the lower permeability observed with certain crosslinking treatments in this study is primarily an effect of lost GAG and water content in the soaked control group, and injection with crosslinking reagents can reduce the loss of GAG content with age and degeneration as well as increasing nutritional flow throughout the disc, the results of future studies can further aid in selecting an optimal reagent for clinical benefit.

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