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An *In Vivo* Model of Reduced Nucleus Pulposus Glycosaminoglycan Content in the Rat Lumbar Intervertebral Disc

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Abstract

Study Design—An *in vivo* model resembling early stage disc degeneration in the rat lumbar spine.

Objective—Simulate the reduced glycosaminoglycan content and altered mechanics observed in intervertebral disc degeneration using a controlled injection of chondroitinase ABC (ChABC).

Summary of Background Data—Nucleus glycosaminoglycan reduction occurs early during disc degeneration; however, mechanisms through which degeneration progresses from this state are unknown. Animal models simulating this condition are essential for understanding disease progression and for development of therapies aimed at early intervention.

Methods—ChABC was injected into the nucleus pulposus, and discs were evaluated via micro-CT, mechanical testing, biochemical assays, and histology 4 and 12 weeks after injection.

Results—At 4 weeks, reductions in nucleus glycosaminoglycan level by 43%, average height by 12%, neutral zone modulus by 40%, and increases in range of motion by 40%, and creep strain by 25% were found. Neutral zone modulus and range of motion were correlated with nucleus glycosaminoglycan. At 12 weeks, recovery of some mechanical function was detected as range of motion and creep returned to control levels; however, this was not attributed to glycosaminoglycan restoration, because mechanics were no longer correlated with glycosaminoglycan.

Conclusion—An *in vivo* model simulating physiologic levels of glycosaminoglycan loss was created to aid in understanding the relationships between altered biochemistry, altered mechanics, and altered cellular function in degeneration.

Keywords

intervertebral disc; degeneration; nucleus pulposus; glycosaminoglycan; animal model; chondroitinase ABC

Intervertebral disc degeneration is a complex progression of structural, biochemical, and biological alterations that contribute to compromised mechanical function and in some instances discogenic low back pain. Despite the prevalence of the condition, with billions of dollars in associated health care costs paid annually in the United States,¹ treatments for disc degeneration and the associated pain are limited, due in part to the lack of a thorough understanding of the mechanisms at play. Among the early degenerative changes is a

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The manuscript submitted does not contain information about medical device(s)/drug(s).

breakdown of large aggregating proteoglycans reducing the sulfated glycosaminoglycan content in the nucleus pulposus.²⁻⁴ This reduction of glycosaminoglycan content has an impact on the mechanical function of the disc: the ability to imbibe and bind water is diminished, pressure within the nucleus is decreased, and ultimately, the mechanical function of the nucleus and the entire motion segment is altered.⁵⁻⁸ It is plausible that progressive degeneration follows this reduced glycosaminoglycan content and altered mechanics, yet this ultimately remains a hypothesis, and knowledge of specific mechanisms and interactions is limited. To this end, *in vivo* animal models simulating the condition of decreased nucleus pulposus glycosaminoglycan content as observed in early degeneration would be of great utility. Such models would allow the study of the links between a relevant altered biochemistry, altered mechanical function, and altered cellular function, further allowing for the development of specific therapies targeted at bolstering anabolic and inhibiting catabolic cellular function, ultimately restoring disc structure and mechanical function.

The experimental reduction of the glycosaminoglycan content in the nucleus is not a new concept; however, to date this has not been performed in a controlled setting where a reduction of a physiologically relevant quantity of glycosaminoglycan has been achieved as an isolated factor. Chemonucleolysis studies using the enzyme chymopapain date back over 40 years, and were developed as a treatment for disc herniations through a reduction in nucleus pulposus pressure.⁹ Negative outcomes from chymopapain led to the later introduction of chondroitinase ABC (ChABC) as a herniation treatment as this enzyme selectively degrades chondroitin-4 sulfate, chondroitin-6 sulfate, and dermatan sulfate glycosaminoglycan chains^{10,11} and is less aggressive than chymopapain. More recently, after the observations of a degeneration-like loss of pressure and disc height,^{12,13} ChABC has been specifically used to induce degenerative changes using *in vivo* animal models.^{14,14a} Common limitations in previous ChABC injection studies include the utilization of excessive quantities of enzyme sufficient to render discs devoid of glycosaminoglycan chains and the use of large needle diameters relative to overall disc heights, which has been shown to induce degenerative changes.^{15-17a}

The objective of the current study was to establish an *in vivo* model in the rat lumbar spine simulating the diminished glycosaminoglycan and altered mechanics observed in early disc degeneration using a controlled injection of ChABC via a sufficiently small needle. The rat lumbar intervertebral disc was selected for this model, as this disc has shown many similarities to human lumbar discs in terms of biomechanics, biochemistry, cellularity, and geometry.¹⁸⁻²¹ The rat also has the advantage over other larger models by providing a relatively inexpensive platform for the future implementation of many previously established biologic and genetic assays and treatments. Moreover, we have previously shown *in vitro* that an injection with a 33-gauge needle does not cause alterations in mechanical function in the rat lumbar disc, and that glycosaminoglycan can be reduced to a physiologic level through ChABC injection.⁵ For the present study, we hypothesized that the injection procedure itself would not alter disc properties, and further, that changes in disc height, mechanics, biochemistry, and histologic structure consistent with human degeneration would be observed due to glycosaminoglycan degradation. Additionally, we hypothesized that degeneration-like changes would be evident at 4 weeks after glycosaminoglycan reduction and that these changes would progress at 12 weeks. Specifically, we hypothesized that glycosaminoglycan reduction and the altered biochemical profile would result in progressively decreased disc height, hypermobility (increased axial range of motion and decreased neutral zone modulus), and increased viscoelastic creep strain.

Materials and Methods

Study Design

Twenty-six male retired breeder Sprague-Dawley rats (age 7–9 months, weight 523 ± 43 g) were included in this IACUC approved study. Animals were assigned to either a 4-week or 12-week time point. Those in the 4-week group were assigned to receive 1 of 2 injection volumes (1 μ L: $n = 8$; or 2.5 μ L: $n = 5$), whereas the rats in the 12-week group received 1- μ L injection volumes ($n = 13$). Within each animal, L4L5 and L5L6 were randomly assigned to receive an injection of ChABC (Seigaku Corp., Tokyo, Japan) or a sham injection of 1 $\times$ phosphate-buffered saline (PBS) with 0.1% bovine serum albumin. Levels L3L4 and L6S1 served as unaltered control levels. Each ChABC injection contained 0.00025 U of enzyme, an amount selected to decrease the nucleus pulposus glycosaminoglycan content by $\sim 50\%$.⁵ ChABC was prepared to the appropriate concentration in 1 $\times$ PBS with 0.1% bovine serum albumin: 0.25 or 0.1 U/mL for the 1- or 2.5- μ L injections, respectively.

Surgery

After ~ 1 week of facility acclimation, rats were operated on using aseptic technique. Rats were anesthetized via inhalation of 1.5% to 3% isoflurane with an oxygen carrier. On reaching appropriate depth of anesthesia, animals were placed in a supine position on a heated pad, and an anterior approach to the lumbar spine was performed.^{22,23} The lumbar spine from L3 to S1 was exposed, and a custom 33-gauge needle attached to a gas tight microsyringe (Hamilton Company, Reno, NV) was inserted through the anterior of the appropriate discs to a controlled depth of 2.5 mm. This insertion depth places the needle tip approximately in the center of the nucleus pulposus. Musculature adjacent to the ChABC and sham PBS injection sites was labeled with a metallic marker for postoperative level identification. The abdominal wall was closed, and animal recovery was monitored for adverse symptoms under heated lamp for 45 minutes. Animals were returned to normal housing and received food and water *ad libitum*. Postoperative analgesics (0.05 mg/kg buprenorphine) were given subcutaneously once per day for the first 3 days. Animals were killed via CO₂ inhalation at 4 or 12 weeks after surgery.

Sample Preparation

Immediately after sacrifice, animals were imaged using a fluoroscope (FluoroScan 50700, FluoroScan Imaging Systems, Northbrook, IL) for visualization of the metallic site markers and subsequent identification of the ChABC and sham PBS levels. Next, lumbar spines were removed *en bloc* with the ligamentous structures and posterior bony anatomy remaining intact. Spines designated for mechanics and biochemistry were wrapped in PBS-soaked gauze and frozen at -20°C until micro-computed tomography (μ CT) scanning could be performed. Spines designated for histology were immediately dissected further into bone disc bone motion segments and placed in CalEx-2 (Fisher Chemical, Fairlawn, NJ) solution for a period of 7 days (changed every other day) for concurrent fixation and decalcification.

Micro-CT Imaging

Spines frozen at -20°C were removed for the mechanics/biochemistry assays and allowed a 1-hour thaw period followed by 1 hour of submersion in room temperature PBS. Spines then underwent μ CT scanning in room temperature PBS at a 42 μ m isotropic resolution (General Electric, London, Ontario, Canada). After scanning, all spinal elements were removed and spines were cut into individual bone disc bone motion segments. Each motion segment was refrozen at -20°C until mechanical testing. Average disc height, disc area, and total disc volume were calculated from the μ CT volumetric data using a custom written MatLab program. A height map of each disc was created (Figure 1A), after which a regional height map was created by dividing a disc into 25 equally sized regions using a rectangular coordinate system and grid

(Figure 1B). Regional heights, calculated as the median height within each rectangular disc region, allowed for direct quantitative comparison of anterior, lateral, nucleus, and posterior heights between all discs.

Mechanical Testing

Each motion segment was thawed in room temperature PBS for 1 hour. Mechanical testing was performed as described previously.^{5,19,24} Compression-tension testing was performed at a frequency of 0.1 Hz and ranged from 4.5 N ($\sim 0.9 \times$ body weight) compression to 3 N tension. Compressive creep testing was performed with an applied load of 4.5 N for 45 minutes. After mechanical testing, each motion segment was rehydrated for 1 hour in room temperature PBS and then returned to storage at -20°C until biochemical analysis. Data from the 20th cycle of compression-tension was analyzed using a trilinear fit as described previously,^{5,25,26} where the loading curve was divided into compressive, neutral zone, and tensile regions. Stiffness from each region and total range of motion were obtained, and normalized by area and average disc height to obtain apparent modulus and strain values.

Biochemistry

Discs were isolated from the vertebral bodies via sharp scalpel dissection while frozen. The entire nucleus pulposus was removed from the isolated disc using a hollow 1.5-mm-diameter biopsy punch. The entire inner annulus fibrosus was then isolated using a hollow 3.6 mm $\times$ 2.5 mm elliptical biopsy punch. The remaining peripheral disc tissue was considered to be the outer annulus. Tissue wet weight for each region was obtained, and each specimen dried at 65°C for 24 hours. Dry weight was then obtained, followed by tissue digestion in 5 mg/mL proteinase K solution at 65°C for 18 hours. After digestion, each aliquot was analyzed for glycosaminoglycan content using the 1–9 dimethylmethylene blue binding assay.²⁷ The remaining aliquot was hydrolyzed for 18 hours in 6 N HCl, followed by detection of hydroxyproline content using a standard colorimetric assay.²⁸ Glycosaminoglycan and hydroxyproline contents were normalized by tissue wet weight.

Histology

Histological analysis was performed by embedding the disc in paraffin after fixation and decalcification. Sagittal sections were created at a $5\text{ }\mu\text{m}$ thickness up to the disc midline, after which, specimens were re-embedded in a reoriented position such that axial sections of the remaining half of the disc could be created, again at a $5\text{ }\mu\text{m}$ thickness. Slides were stained with either hematoxylin and eosin or alcian blue and picrosirius red and were qualitatively analyzed for disc organization and composition, cellularity, and the presence of matrix damage by 2 independent researchers blinded to treatment.

Statistics

An initial statistical analysis was made to determine the effect, if any, of injection volume on height, mechanical, and biochemical parameters. A 1-factor ANOVA (*factor*: treatment, *levels*: intact control, 1 μL PBS, 2.5 μL PBS) was performed on the data from the 4-week animals. Also at the 4-week time point, a Student *t* test was performed comparing the 1- μL ChABC group *versus* the 2.5- μL ChABC group. As there was no difference between 1- and 2.5- μL treatments at 4 weeks (see Results), samples were pooled. After these analyses, a 2-factor ANOVA (*factor 1*: treatment, *levels*: intact control, PBS, ChABC; *factor 2*: time, *levels*: 4 weeks, 12 weeks) was performed for the entire data set. When a significant difference was detected in treatment, contrasts within each time point were made comparing across treatments (ex. 4-week ChABC *vs.* 4-week PBS and 4-week intact control). When a significant difference was detected in time, contrasts comparing 4 weeks *versus* 12 weeks within each treatment were made (ex. 4-week ChABC *vs.* 12-week ChABC). Pearson correlation

coefficients were used to determine univariate relationships between mechanics (range of motion and neutral zone modulus) and nucleus biochemistry (glycosaminoglycan and hydroxyproline) separately at 4 and 12 weeks. Statistical analyses were performed using SYSTAT 10.2 (SYSTAT Software Inc., San Jose, CA), and significance was set at $P < 0.05$.

Results

Surgical procedures were tolerated well with the exception of 1 animal from the 12-week time point, which died from unknown causes after the first week of recovery. All other animals appeared healthy and body weight was either maintained or increased during the study.

Injection Volume Effect

Nested within the study design was the evaluation of the effect of injection volume on disc parameters at 4 weeks. The 2.5- μ L PBS injection resulted in parameters that were no different from the 1- μ L PBS injection, and neither PBS injection parameters were any different from those of the intact control discs (Table 1); therefore, the PBS groups were pooled to form the 4-week dataset. Consistent with the sham injection observations, 1- μ L ChABC discs were no different from the 2.5- μ L ChABC discs (Table 1), and the ChABC groups were pooled to form the 4-week data set. It was concluded that the injection procedure used in this study did not alter any quantitative nor qualitative properties. The 1- μ L volume corresponded to ~40% of nucleus volume and 10% of disc volume, whereas 2.5 μ L corresponded to ~100% of nucleus volume and 25% of disc volume. The 33-gauge needle diameter corresponded to 15% of anterior disc height and 25% of average disc height.

Disc Height and Volume

Average disc height was significantly reduced by 12% in the ChABC discs compared with sham PBS 4 weeks postsurgery; however, at 12 weeks postinjection, no significant differences were found between any treatment groups (Figure 2A). Additionally, a significant increase in control disc average height was observed between 4 and 12 weeks (Figure 2A). Total disc volume was not significantly altered by treatment at either time point, remaining near the 4-week control volume ($9.16 \pm 1.93 \text{ mm}^3$) in all groups at all times. A detailed analysis of the disc height profile revealed localized alterations in disc height 4 weeks after ChABC injection (Figures 2B–E): a significant 20% decrease was observed in the posterior of ChABC discs relative to both control and sham and a 10% decrease in the lateral portion of ChABC discs relative to sham. At 12 weeks, both sham and ChABC discs had a 20% reduced posterior height compared with control (Figure 2E), and nucleus height significantly increased with time in control discs and ChABC discs by 13% and 24%, respectively (Figure 2C), perhaps indicating continued growth despite the rats being of a skeletal mature age.

Disc Mechanics

The neutral zone modulus of the ChABC injected discs was significantly reduced by ~40% compared with both intact control and sham PBS at 4 weeks postinjection. At 12 weeks, neutral zone modulus was significantly reduced by 30% in ChABC discs compared with control, however, no longer significantly different from the sham PBS (Figure 3A). Tensile modulus was 25% lower in the 12-week ChABC group compared with intact control (Figure 3B), whereas compressive modulus was not significantly altered by the ChABC-induced GAG depletion (Figure 3C). Total range of motion increased in the ChABC discs by ~40% over both intact control and sham PBS at 4 weeks postinjection (Figure 3D). This difference was no longer significant at 12 weeks, because the ChABC discs experienced a reduction in range of motion with time, returning to levels not significantly different from control discs. Consistent with the range of motion parameter, creep strain was significantly increased by 25% at 4 weeks in the ChABC discs compared with intact control and to sham PBS (Figure 3E). By 12 weeks,

the creep strain in the ChABC discs returned to levels not significantly different from both intact control and sham PBS, whereas creep strain reduced 15% over time from 4 to 12 weeks in the control discs.

Disc Biochemistry

Glycosaminoglycan content (Figure 4) was significantly reduced by 43% in the nucleus pulposus and by 25% in the inner annulus of ChABC-treated discs 4 weeks postinjection. Glycosaminoglycan content was not significantly altered with time between the 4-week and 12-week groups; however, at 12 weeks, ChABC discs displayed glycosaminoglycan profiles that were not significantly different from intact or PBS controls. Outer annulus fibrosus glycosaminoglycan content was unaltered by either time or treatment (Figure 4C). Hydroxyproline content was not significantly altered by either time or treatment remaining approximately equal to the 4-week control values of $14.4 \pm 10.0 \mu\text{g}/\text{mg}$ in the nucleus, $32.0 \pm 9.5 \mu\text{g}/\text{mg}$ in the inner annulus, and $41.3 \pm 11.6 \mu\text{g}/\text{mg}$ in the outer annulus.

Correlations Between Mechanics and Biochemistry

At 4 weeks postinjection, nucleus pulposus glycosaminoglycan content was significantly correlated ($P < 0.05$) with both range of motion ($r = -0.61$) (Figure 5) and neutral zone modulus ($r = 0.55$). Further, at 4 weeks, no correlations existed ($P > 0.05$) between nucleus hydroxyproline content and either range of motion or neutral zone modulus. At 12 weeks postinjection, no correlation was detected ($P > 0.05$) between nucleus glycosaminoglycan and either range of motion (Figure 5) or neutral zone modulus; however, at 12 weeks, nucleus hydroxyproline content was significantly correlated ($P < 0.05$) with both range of motion ($r = 0.34$) and neutral zone modulus ($r = -0.35$).

Disc Histology

Qualitative analysis of the histology sections confirmed that the sham PBS injection was not altering the disc morphology compared with unaltered controls at either 4 or 12 weeks (Figures 6A, B). These discs showed intense alcian blue staining for glycosaminoglycans in the nucleus pulposus and in between the lamellae of the inner annulus fibrosus. The annular lamellae were well organized and stained strongly for picrosirius red. The nucleus region had abundant vacuolated cells (Figure 6E). End-plates were all intact with no large bony protrusions into the disc.

At 4 weeks, 2 of the 3 discs that received ChABC injections had degeneration-like changes (Figure 6C), whereas the third was not distinguishable from either control or sham PBS discs. The 2 ChABC discs with different morphology had a decreased staining for alcian blue throughout the entire disc. The nuclear regions of these discs were populated by very few cells, and were stained by picrosirius red. The annulus fibrosus in both discs appeared increasingly disorganized and nuclear material had protruded into the disrupted posterior annulus fibrosus of 1 disc. At 12 weeks, a similar finding in the ChABC discs relative to 4 weeks was observed, as 2 discs were different from controls (Figure 6D) and 1 was indistinguishable from control. ChABC discs with properties different from control had fibrous protrusions into the disc near the nucleus region. Additionally, while restoration of some alcian blue staining was apparent, the nucleus pulposus appeared reduced in size relative to control and comprised relatively few, clustered, chondrocyte-like cells (Figure 6F). The nucleus material appeared to be protruding into a severely disrupted posterior annulus in both discs, although herniation of nucleus material outside of the disc was not evident.

Discussion

A reduction in glycosaminoglycan content is one of the earliest changes in human intervertebral disc degeneration.²⁹ In the earlier stages of degeneration, glycosaminoglycan levels in the

nucleus pulposus and inner anulus fibrosus decrease by ~50% and 30%, respectively.^{2-4,30} In the present study, it was hypothesized that a controlled depletion of nucleus pulposus glycosaminoglycan, by levels consistent with early degenerative decreases, would produce degenerative changes in disc height, mechanics, biochemistry, and histologic structure at 4 and 12 weeks. At 4 weeks postinjection significant changes in discs height, mechanics, biochemistry, and histology resembled early human intervertebral disc degeneration. These changes were consistent with previous *in vitro* data using the same ChABC dose.⁵ At 12 weeks, there were no significant alterations compared to 4 weeks for many parameters, including disc height, neutral zone modulus, and nucleus glycosaminoglycan content; however, these parameters at 12 weeks were also not significantly different from the sham control. This difference likely resulted from variability in biologic responses among specimens and in the assays applied. The secondary hypothesis was that the injection procedure itself would not induce degenerative changes. Importantly, the sham injection was not different from intact control for any parameter, confirming that the glycosaminoglycan depletion was in fact isolated from the injection procedure.

Disc height declines during degeneration, although the exact time course of this height reduction is a debated topic. Pfirrmann *et al* have shown a stronger association between disc volume loss and degeneration in the elderly population compared with younger patients,³¹ and others have similarly shown that disc height losses are not necessarily observed in the early stages of degeneration.^{32,33} The detection of a relatively small decrease of 12% in average disc height at 4 weeks in our study indicates changes consistent with early degeneration. Utilization of three-dimensional μ CT volumes instead of planar radiograph allowed for a more accurate measurement of height. This technique revealed heights in the anterior and nucleus regions of the disc that were over twice the posterior height, and further showed that the height loss was primarily in the posterior regions of the disc, likely due to the loss of pressure and swelling capacity of the posteriorly located nucleus pulposus. This loss of posterior height may increase the mechanically induced damage to this region due to increased strains. The anterior of the disc was not observed to demonstrate a height loss, which can be attributed to unaltered GAG levels and normal structure observed histologically in that region of the disc.

The mechanical function of an intervertebral disc is compromised during the progression of degeneration. Specifically, studies have shown hypermobility at low loads³⁴ and altered viscoelastic properties including increased creep rate and deformation³⁵ in early degeneration, mechanical changes that are attributed to the reduction in nucleus glycosaminoglycan.^{5,7,8,36} At 4 weeks postinjection, neutral zone modulus, range of motion, and creep were all altered in a manner consistent with the expected changes in a degenerating disc, and were altered to the same degree as observed after an equivalent ChABC injection *in vitro*. Further, both neutral zone modulus and range of motion were linearly correlated with nucleus glycosaminoglycan content 4 weeks postinjection—relationships that were identical to those determined in the absence of biologic activity.⁵ This indicates that throughout the 4-week period following ChABC injection, discs were exposed to an altered stress and strain environment, and further suggests that neither substantial matrix remodeling nor large scale matrix damage, which both may alter disc mechanics, had occurred by 4 weeks postinjection.

An interesting finding in our study is the apparent return of many parameters toward control levels at 12 weeks postinjection. An ability of the disc to mount a small recovery after ChABC injection, suggesting cells which maintain an ability for a remodeling response, has been shown previously when young rabbit nucleus pulposus restored matrix structure after 12 weeks.^{11,37} Considering our prior *in vitro* data⁵ as “time zero” for the present study, it is apparent that neither glycosaminoglycan content nor mechanics was recovered in the ChABC discs by 4 weeks. Statistical analyses revealed that no significant increase in glycosaminoglycan was observed over time from 4 to 12 weeks after ChABC injection; however, in contrast, these

same analyses revealed that nucleus glycosaminoglycan content was not significantly reduced ($P = 0.11$) at 12 weeks postinjection relative to controls (Figure 4). Qualitatively, at 12 weeks the mean nucleus glycosaminoglycan content for the ChABC discs was 30% less than control levels, and the scatter plot distribution was similar to the 4-week data (Figure 5). Further, the observed reduced nucleus glycosaminoglycan staining in the histology indicates that nucleus glycosaminoglycan levels are not recovered and remained reduced in the ChABC injected discs. The difference may indicate a type II statistical error due to lower statistical power at the 12-week time point for nucleus glycosaminoglycan content, where, due to the high standard deviations, 25 samples per treatment would have been required to detect a 30% difference with power of 0.8. Thus, while it is possible that partial recovery of nucleus glycosaminoglycan occurred between 4 and 12 weeks, it seems unlikely, and the high standard deviations in all treatment groups may have played a role in not detecting a significant result.

Restoration of mechanical properties in ChABC-injected discs toward control levels was observed at the 12-week time point, suggesting a matrix remodeling response. Interestingly, this recovery of mechanical function could not be attributed to a restoration of glycosaminoglycan content, because at 12 weeks postinjection, no correlations existed between nucleus glycosaminoglycan content and either range of motion or neutral zone modulus. Instead, both mechanical parameters were found to be weakly correlated with nucleus collagen (hydroxyproline) content, a relationship that did not exist at the earlier time point. The presence of only a weak correlation further suggests that other matrix changes not investigated in this study, such as an increase in matrix protein crosslinking^{38,39} or other matrix proteins, may be influencing the mechanical behavior and the restoration of mechanical properties. Restoration of mechanical properties without recovery of the original matrix composition was confirmed through histology, because at 12 weeks the nucleus region maintained an altered morphology, relatively low staining for glycosaminoglycan, and altered cell population while annular disruption remained present in the disc posterior. It is plausible that the attempts at disc remodeling are indicative of a degenerative response similar to that observed in human discs, because in late stage degeneration motion segments also have a reduced level of hypermobility compared with early degeneration.³⁴ However, further work needs to elucidate the cellular responses and matrix changes leading to the recovered mechanical properties and attribute the response to be either regenerative or degenerative.

A common limitation of animal models used in disc research is that the nucleus pulposus cell population may not be representative of the human disc cell population. Humans lose their nucleus notochordal cell population at an earlier time point relative to lifespan and skeletal maturity,⁴⁰ though data regarding the functional role of notochordal cells in disc repair and degeneration are needed. Rat lumbar discs have been suggested to lose their notochordal cell population by around 16 months of age,²⁰ and the rats in our study were near this age threshold. Interestingly, the cells within the nucleus pulposus of the glycosaminoglycan reduced discs morphologically resembled chondrocytes, indicating a similarity with degenerating discs. Future work will investigate this model on a cellular level. An additional limitation inherent to all ChABC studies is that the activity of ChABC on glycosaminoglycan chains is not identical to the degradation as observed *in vivo*. We were not concerned about the actual process of the glycosaminoglycan reduction, instead focused on the resulting levels of sulfated glycosaminoglycan after the decrease has occurred, and thus selected 4 weeks postinjection, well after enzymatic cleavage of the glycosaminoglycan chains, as our earliest time point. Further work will characterize the matrix in more detail and relate it to known changes within a degenerating human nucleus.

In summary, we have presented an *in vivo* model that reproduces the decrease in nucleus pulposus glycosaminoglycan content as observed in early human degeneration. A physiologically relevant level of glycosaminoglycan reduction resulted in hypermobile discs

with altered height profiles at 4 weeks postinjection, exposing the posterior region of the disc to an elevated risk for damage progression. At 12 weeks, disc structure and biochemistry remained unaltered relative to the 4-week changes, though mechanical function had returned toward control levels. As this return toward normal was not correlated with glycosaminoglycan content, it is unclear as to whether or not the response can be categorized as degenerative or regenerative, and future work will investigate this finding. A foundation for understanding the role of nucleus glycosaminoglycan loss in early degeneration and in the degenerative cascade has been set, and future work will further investigate in detail the changes within the extracellular matrix and within the cells themselves following the reduction in glycosaminoglycan content. Ultimately this model will be of utility in development and testing of novel biologic treatments.

Key Points

- An *in vivo* model recreating the percent decrease in nucleus pulposus glycosaminoglycan content observed in early human degeneration was established in a rat lumbar disc.
- Alterations in disc structure, biochemistry, and mechanical function were present 4 weeks after glycosaminoglycan reduction via ChABC injection. At 12 weeks, disc structure and biochemistry remained consistent with the 4-week observations, though mechanical function returned toward control levels through a mechanism other than glycosaminoglycan restoration
- This model will be of utility for understanding the role of glycosaminoglycan reduction in the degenerative cascade and the relationships between altered biochemistry, altered mechanics, and altered cellular function, though further work is required evaluating consistencies with the process of degeneration.

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Figure 1.

A, Representative disc height profile demonstrating varying heights (in mm) across the disc.

B, Regional disc height map with anterior (ANT), lateral (LAT), nucleus (NP), and posterior (Post) regions labeled.

Figure 2.

A, Average disc height. **B**, Anterior region height. **C**, Nucleus region height. **D**, Lateral region height. **E**, Posterior region height. Results for all graphs presented as mean $\pm$ standard deviation. □ = intact control; △ = sham PBS; ● = ChABC; x = significantly different from control; # = significantly different from sham PBS; lines represent significant difference between 4 and 12 weeks.

Figure 3. Mechanical properties. **A**, Neutral zone modulus. **B**, Tensile modulus. **C**, Compressive modulus. **D**, Range of motion. **E**, Creep. □ = intact control; △ = sham PBS; ● = ChABC; x = significantly different from control; # = significantly different from sham PBS; lines represent significant difference between 4 and 12 weeks.

Figure 4. Glycosaminoglycan (GAG) content in the (A) nucleus pulposus (NP), (B) inner anulus fibrosus (IAF), and (C) outer anulus fibrosus (OAF). □ = intact control; △ = sham PBS; ● = ChABC; x = significantly different from control; # = significantly different from sham PBS.

Figure 5.

A, Range of motion (ROM) is correlated with nucleus glycosaminoglycan (NP GAG) content at 4 weeks following ChABC injection ($r = -0.61$, $P < 0.05$); however, at 12 weeks no such relationship exists ($r = -0.09$, $P > 0.05$). **B,** At 4 weeks following ChABC injection, no significant correlation between range of motion and nucleus hydroxyproline (NP OHP) existed ($r = 0.10$, $P > 0.05$); however, at 12 weeks, range of motion is correlated with nucleus hydroxyproline ($r = 0.34$, $P < 0.05$).

Figure 6.

Histology: Picrosirius red and alcian blue reveals no differences between (A) control discs and (B) sham PBS discs at 4 weeks (shown) and 12 weeks (not shown). Reduced glycosaminoglycan staining and a fibrous nucleus (arrow) are evident at (C) 4 weeks after ChABC injection. D, 12 weeks after ChABC injection, nucleus glycosaminoglycan staining and size are still reduced. Fibrous protrusions (arrow) into the disc from the end-plates are evident as well as posterior anulus disruption and nucleus herniation (arrow). Higher magnification pictures hematoxylin and eosin stained sections shows morphologic differences in nucleus pulposus cells from (E) control and (F) 12-week ChABC discs.

Table 11- and 2.5- μ L PBS Injections Do Not Alter Any Properties Relative to Control or to Each Other

Group	NZMod	ROM	NPGAG	AVGHT
Control	1.84 (0.63)	0.22 (0.05)	60.0 (13.0)	0.73 (0.06)
1 μ L PBS	1.93 (0.64)	0.22 (0.07)	66.2 (12.0)	0.72 (0.10)
2.5 μ L PBS	2.30 (0.51)	0.19 (0.03)	60.6 (4.2)	0.82 (0.04)
1 μ L ChABC	1.16 (0.51)	0.30 (0.10)	41.6 (23.2)	0.68 (0.08)
2.5 μ L ChABC	1.15 (0.81)	0.28 (0.07)	24.27 (17.2)	0.68 (0.06)

1- and 2.5- μ L ChABC injections are not significantly different from one another ($P > 0.05$ for all).