Annulus Fibrosus Repair Using High-Density Collagen Gel: An In vivo Ovine Model

Brenton Pennicooke, MD, MS¹, Ibrahim Hussain, MD¹, Connor Berlin BS¹, Stephen R. Sloan, BS², Brandon Borde, PhD², Yu Moriguchi, MD, PhD¹, Gernot Lang, MD¹,³, Rodrigo Navarro-Ramirez, MD¹, Jonathan CheethamVetMB, PhD⁴, Lawrence J. Bonassar, PhD⁵, Roger Härtl, MD¹

¹Department of Neurological Surgery, Weill Cornell Brain and Spine Center, New York-Presbyterian Hospital, New York, NY
²Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY
³Department of Orthopedic and Trauma Surgery, Freiburg University Medical Center, Freiburg, Germany
⁴Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY
⁵Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY

Corresponding author:
Roger Härtl, MD
Department of Neurological Surgery
Weill Cornell Brain and Spine Center
New York-Presbyterian Hospital
525 East 68th Street, Box 99
New York, NY 10065
Telephone: (212) 746-2152
Fax: (212) 746-8947
Email: roh9005@med.cornell.edu

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

AO Foundation funds were received in support of this work (Project no. S-14-123Y).

Relevant financial activities outside the submitted work: grants, patents, stocks.
ABSTRACT

**Study Design:** Ovine *in vivo* study.

**Objective:** To perform lateral approach lumbar surgery in an ovine model to administer an injectable riboflavin (RF) cross-linked high-density collagen (HDC) gel and to assess its ability to mitigate intervertebral disc (IVD) degeneration after induced annulus fibrosus (AF) injury.

**Summary of Background Data:** Biological-based injectable gels have shown efficacy in restoring biomechanical, radiographic, and histological parameters in IVD-injured animal models. RF cross-linked HDC gel has previously demonstrated retention of nucleus pulposus (NP) tissue, reduced loss of disc height, and prevention of terminal cellular degenerative changes in rat-tail spines. However, this biological therapy has never been tested in large animal models.

**Methods:** 40 lumbar IVDs were accessed from 8 sheep via lateral approach surgery. IVDs were randomly assigned to healthy control, injury and HDC treatment, or negative control with injury and no treatment. IVD injury was carried out using a drill-bit through the AF followed by needle puncture of the NP. Sheep were followed for 16 weeks and underwent qualitative/quantitative MRI, X-ray, and histological analyses of collagen and proteoglycan content.

**Results:** The lateral approach to the ovine lumbar spine to deliver HDC gel proved to be safe and reproducible. IVDs treated with the HDC gel revealed less degenerative changes at the microscopic level based on AF and NP histology. However, mean Pfirrmann grade, T2 relaxation time, NP voxel size, and disc height index were not significantly different between the two injury groups.
Conclusions: Injectable HDC gel can be administered safely via lateral approach surgery in an ovine AF injury model. IVDs treated with HDC gel demonstrated less degeneration at the microscopic level though radiographic changes were slight when comparing treated to untreated IVDs. Future studies will need to elucidate the role of injury technique and time frame for follow-up in correlating histological and radiographical outcomes.

Key Words: annulus fibrosus; annular repair; sheep model; ovine model; intervertebral disc; high-density collagen; tissue-engineering; disc degeneration; lumbar spine; disc herniation; riboflavin cross-linking

Level of Evidence: N/A
INTRODUCTION

The intervertebral disc (IVD) is an avascular structure comprised of an aggrecan-rich nucleus pulposus (NP) surrounded by concentric layers of collagen forming the annulus fibrosus (AF). The pathophysiology of IVD degeneration involves disorganization of the extracellular matrix (ECM) and loss of proteoglycan content leading to dehydration of the NP. This leads to microfissure formation, which coalesce into tears extending to the AF. Annular tears can result in herniation of NP contents and compression of neighboring neurologic structures.\(^1\) Lumbar discectomy is performed in 300,000 individuals annually, and while predominantly successful in relieving acute symptoms, the procedure does not address the progressive disc degeneration that ensues.\(^2\) Furthermore, the annular defect is not treated and exacerbated during surgery. Persistent annular defects increase the risk of recurrent disc herniation, which requires reoperation in 6-13% of cases.\(^3-8\)

Several attempts have been made to mechanically repair AF defects using suture and annuloplasty devices. However, none of these techniques significantly alter annular healing in animal models or demonstrate long-term benefits in recent clinical trials.\(^9-12\) To this end, biological approaches for annular repair and prevention of progressive DDD have become of increasing interest.\(^13-17\) Iatridis et al. engineered an injectable fibrin-genipin adhesive hydrogel sealant which prevented IVD height loss and remained well-integrated with native AF tissue following extended compression cycles in vitro, as well fully restoring compressive stiffness in vivo.\(^18\) Mesenchymal progenitor cells combined with pentosanpolysulfate embedded in a gelatin/fibrin scaffold restored IVD height, morphology, and NP proteoglycan content in ovine models six months after simulated discectomy.\(^19\)

We have previously reported the development of a riboflavin (RF) cross-linked high-density collagen (HDC) gel for the treatment of annular injuries.\(^20-22\) Collagen prepared with RF has the
advantage of maintaining a low, injectable viscosity until exposed to blue light, which photo-initiates cross-linking of collagen fibrils to form a hydrogel with increased mechanical properties. Using an in vivo rat-tail model, HDC gel helped retain 70-80% of NP tissue 18 weeks post-injury. In contrast, negative control IVDs showed complete NP extrusion and terminal degenerative changes by 5 weeks.

Although these results were promising, rat-tail spine outcomes have limited clinical translation. Ovine lumbar spines have comparable anatomical, cellular, and biomechanical features to humans, and are frequently used as translational in vivo models for spine disease. Like humans, sheep are among the few mammals that rapidly lose notochordal cells in the IVD following birth. Persistence of these cells, as seen in rats, rabbits, and pigs, can influence proteoglycan metabolism and progenitor cell differentiation. Physiological loading properties are similar to human IVDs and experience comparable mechanical alterations after annular injury. As such, annular injury in sheep models induces histological and radiographical degenerative changes similar to those seen in humans.

The objectives of the present study were as follows. First, to validate a modification of a previously described drill-bit injury technique to induce IVD degeneration in an ovine model. Second, to perform a lateral access, extraperitoneal approach to the sheep lumbar spine to deliver an injectable HDC gel after induced AF injury. Third, to assess the histological and radiographical changes in injured sheep IVDs treated with the HDC gel as compared with negative controls over a 16-week period.

**MATERIALS AND METHODS**

*Study Groups*

This study was approved by the Cornell University Institutional Animal Care and Use Committee. Eight skeletally mature sheep aged 4-7 years old were obtained through the Cornell University Ruminant Center. A total of 40 disc segments from L1/2 to L5/6 were randomized into one of three groups. The first group included healthy discs that were not manipulated (healthy control) (N=10), the second group
contained injured IVDs treated with HDC gel (N=15), and the third group consisted of injured IVDs with no treatment (negative control) (N=15). Randomization across all levels was implemented to mitigate focal segmental variance. All sheep were euthanized at 16 weeks following standard protocol from the American Veterinary Medical Association.

High-Density Collagen Gel Preparation
Collagen was harvested and reconstituted from rat-tail tendon as previously described (see Supplemental Digital Content 1 for detailed methodology).

Annular Drill Injury Surgical Technique
All surgical procedures were performed in accordance with the Weill Cornell Medical College Research Animal Resource Center guidelines. A technique modified from Oehme et al. was utilized (see Figure 1A-F and Supplemental Digital Content 1 for detailed surgical approach).

Histology
All disc segments were fixed with 10% neutralized formalin supplemented with 1% cetylpyridinium chloride, then decalcified using 5% nitric acid for 1-2 months. They were then cut in the mid-coronal plane along the trajectory of the drilled annular defect, and transferred to 75% ethanol. Segments were embedded in paraffin, cut to 5-μm thickness, and stained with Picrosirius Red and Safranin-O.

MRI Analysis
3T MRI (Siemens, Erlangen, Germany) was performed on all lumbar spines at 16 weeks. For qualitative analysis, two blinded observers used a modified Pfirrmann scale to classify discs between eight grades of degeneration taking NP size, degree of hyperintensity, clarity of the AF/NP border, and
disc height into account. For quantitative analysis, a previously described method developed by our group was modified for the ovine spine to quantify NP mid-sectional volume and average T2-relaxation time (T2-RT). This algorithm filters out all MRI voxels not representing NP tissue according to their respective T2-RT. Briefly, the algorithm applies a Gaussian mixture model to a rectangle of voxels spanning two adjacent vertebrae from the first-echo T2 image (Figure 2A-B) to separate intensities into two distinct populations of either NP or surrounding bone and soft tissue. NP is segmented from surrounding tissues by thresholding voxels at three standard deviations higher than the average surrounding tissue relaxation time (Figure 2C-E). The NP mask segmented from the first-echo image is then applied to the T2 mapped image to calculate NP average T2-RT and mid-sectional volume (Figure 3). The mean T2-RT and mid-sectional volume of experimental segments were compared with proximal adjacent healthy discs.

**Disc Height Measurements**

XRs were performed in all sheep at 16 weeks for disc height analysis. Care was taken to achieve true lateral radiographs of the segment. The IVD height was measured using a modified method previously described by Lu et al., which determines disc height index (DHI) by dividing the disc height by the adjacent vertebral body height.

**Statistics**

All quantitative values from XRs and MRIs represent the proportion of experimental to adjacent healthy control measurements, and were expressed as mean ± SD. A two-way ANOVA was used to analyze statistical differences between treatment groups and disc levels, and comparisons between treatment groups were made with one-way student’s t-tests. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed with IBM SPSS Statistics 22 (SPSS, Chicago, IL, USA).
RESULTS

Histology

Picrosirius Red stain for collagen was used to delineate the structure of the AF. Healthy IVDs demonstrated a clear AF/NP interface and vertically organized lamellar layers that were symmetric to the contralateral side. Injured IVDs treated with HDC gel had only mild reduction in NP area on representative coronal cuts compared with healthy controls as well as early reorganization of lamellar layers on the side of injury. Staining intensity extended into a fibrous cap overlying the defect, which approximated the underlying AF structure. The contralateral AF maintained vertical lamellar organization. In contrast, negative controls demonstrated severe disorganization of lamellar structure on the side of injury and over 50% reduction in NP area on representative coronal cuts. There was infolding of the contralateral AF with further reduction in NP dimensions. Decreased staining intensity and disorganization of the subjacent cartilaginous layers were noted, along with new border irregularities signifying early endplate destruction (Figure 4A-C).

In healthy discs, Safranin-O the AF/NP border was clearly defined, with evenly diminishing transitions into the deep layers of the AF. In treated IVDs, mild reduction in NP height with small regions of breakdown of the ipsilateral border was observed. Again noted was a fibrous cap which was clearly demarcated from the underlying AF and displayed moderately organized structure. There was less NP vacuolization and endplate destruction compared to untreated IVDs. In contrast, untreated IVDs demonstrated marked loss of NP height and islets of vacuolization within the disc signifying advanced stages of degeneration. A substantial loss of the ipsilateral AF border was observed, which signified persistent rupture and connective scar tissue along the tract of injury extending into the disc (Fig. 4D-F).
Qualitative MRI Assessment

All uninjured segments demonstrated a Pfirrmann grade of 1, whereas injured segments demonstrated varying degrees of degeneration. IVDs treated with HDC gel demonstrated Pfirrmann grades ranging from 2 to 3, with a mean of 2.5 (± 0.5). Negative control IVDs had Pfirrmann grades ranging from 2 to 4, with a mean of 2.6 (± 0.7) (Figure 5). There was no statistically significant difference between these two groups (P=0.78).

Quantitative MRI Analysis

The average T2-RT was calculated from the mid-sagittal cut of the T2 map and was quantified from the segmented NPs. This yielded average T2-RT of 52.9±17.8ms, 38.1±5.4ms, and 35.4±3.6ms for healthy, treated, and untreated IVDs, respectively (Figure 6). The difference between treated/untreated IVDs approached but did not reach statistical significance (P=0.054).

The size of the NP was assessed using the voxel count within a mid-sagittal cut of the T2 first-echo image. The average NP voxel count was found to be 71.3±14.9, 69.3±24.4, and 71.5±21.7 voxels for healthy, treated, and untreated IVDs, respectively (Figure 6). There was no statistically significant difference between healthy and injury groups or between injury groups with and without treatment (P=0.395 and P=0.424, respectively).

Disc Height Measurements

The mean DHI was 0.043±0.001, 0.037±0.001, and 0.039±0.005 for healthy, treated, and untreated IVDs, respectively (Figure 7). There was no statistically significant difference between treated and untreated discs (P=0.6156).
DISCUSSION

The present study is the first in vivo assessment of a RF cross-linked HDC gel in a large animal model of induced IVD degeneration to date. We safely performed lateral approach surgeries to the ovine lumbar spine in a reproducible manner without short- or long-term complications. Based on histological findings, HDC gel application prevented features of severe degeneration at 16 weeks. Radiographic findings suggested that application of HDC gel slightly improved NP hydration, but no statistically significant differences between treated and untreated IVDs were observed.

Safe and reproducible surgical approach to the ovine lumbar spine

We were able to successfully perform and standardize the lateral, extraperitoneal approach to the ovine lumbar spine. None of the 8 sheep experienced intraoperative complications such as excessive blood loss (>500mL), durotomy, vertebral body fractures, or spinal cord injury. The HDC gel was easy to deliver with minimal dissection, exposure, and added time. There were no instances of postoperative neurologic deficits, wound complications, adverse immune response, or infections. All collagen patches stayed in place through the 16 weeks of the study.

Histological and imaging analysis

Histological examination of between treated and untreated IVDs revealed marked differences. The formation of a fibrous cap resulted in decreased AF and NP disorganization, analogous to the rat-tail model. Hallmarks of DDD including loss of disc height and endplate destruction were more prominent in the untreated group. Staining intensity of proteoglycan content in treated IVDs approached that of healthy IVDs compared to minimal intensity on the injured side in the untreated
group. Increased vacuolization and persistent scar tissue along the tract of the drill-bit demonstrated more advanced stages of degeneration in the untreated group.

In patients with corneal ectasia due to connective tissue disorder for example, application of RF cross-linked collagen increased biomechanical strength and reduced permeability to water.\textsuperscript{41} Concordantly, the fibrous cap formed by the HDC gel observed at the annular injury site served not only as a mechanical buttress to prevent herniation of NP contents, but possibly also prevented the diffusion of water molecules bound to proteoglycans from the NP. This reduction in dehydration allowed the NP to maintain volume compared to untreated IVDs. Collectively, these findings demonstrate that the RF cross-linked HDC gel prevents the rate at which degeneration occurs in injured IVDs at the microscopic level.

Imaging studies did not reveal statistically significant differences, but showed that none of the treated IVDs achieved Pfirrmann grade 4, which was the most severe stage of degeneration observed. Likewise, the mean T2-RT suggested slightly increased retention of NP hydration in the treated segments. No differences were noted between each of the tested groups in regards to NP voxel size or DHI.

\textit{Discrepancy between histological and radiographic outcomes}

There are a number of possibilities to explain the marked differences observed in at the histological and radiographic levels. First, an extended period of time may be required for the repaired AF to have reached the point where it would not allow further degeneration of the NP. Second, the stable NP voxel size and DHI observed between healthy and injury groups suggests that the drill-bit injury technique leads to changes that are apparent on histology, but not substantial enough to translate into imaging endpoints. Due to less disruption of the AF and NP milieu, induction of degenerative changes required to induce loss of disc height or decrease in NP volume was not met, thus the ability to assess the efficacy of the HDC gel was diminished. Third, aging sheep are known to develop calcified
deposits in the transitional zone between the AF and NP, leading to progressively denser IVDs.\textsuperscript{28} Our sheep were aged 4-7 years old (average life span is 10-12 years), these microcalcifications may have restricted the ability of the injury technique to inflict significant damage beyond the tract of injury. This is in contrast to rat-tail IVDs, which have a liquid consistency that allows the rapid extrusion of NP following injury.\textsuperscript{42} Fourth, while MRI is the gold standard for assessing NP hydration and structure, its ability to delineate the fine anatomical organization of the AF are unclear. Notably, clear structural differences that were noted in collagen organization as demonstrated by polarized light microscopy were not manifested in MRI analysis. As mentioned above, there is an undetermined lag time between NP reorganization and annular repair, therefore focusing imaging endpoints around the NP at too early of a time point may be misleading. The use of 7T MRI may circumvent these obstacles by providing more advanced spatial resolution which correlates more accurately with cellular features.\textsuperscript{43,44} However, 7T MRIs are limited by economic and logistical constraints in the research arena at this time.

Other groups have described similar discordant results between histological and radiographic studies in animal models. Rabbits demonstrate decreased gene expression of NP collagen and dramatic microscopic changes related to loss of proteoglycan content with normal aging despite relatively modest age-related MRI changes.\textsuperscript{45} Nonchondrodystrophoid dogs display histological evidence of degeneration that predate radiological and biomechanical changes.\textsuperscript{46} Goats subjected to drill-bit injury likewise show significant degenerative changes in cell density, morphology, and ECM appearance at 2 months, but no correlative findings on MRI.\textsuperscript{33} In contrast, partial thickness annular incision techniques for IVD injury in ovine models demonstrated significant changes in imaging concordant with histological loss of morphology and proteoglycan content, within three months of injury.\textsuperscript{29,47} To what extent the injury technique, age, and time frame for post-mortem assessment factor into the relationship between histological and radiographical parameters in a sheep model remains unclear.

\textit{Limitations}
There are limitations of our study that should be acknowledged. The small sample size of 40 IVDs may not be powered enough to accurately discern changes between experimental groups. Notably, the lack of statistical difference of the effect of the collagen patch on T2-RT ($P=0.0539$) is likely the result of type II statistical error. We also did not use a quantitative histological method for characterizing the degree of IVD degeneration due to several sections that were not orthogonal to the drill-bit tract. Finally, biomechanical testing was not performed, therefore the functional implications and ability of the collagen gel to stay in the defect under higher pressures remains undetermined.

**CONCLUSION**

Injectable HDC gel can be delivered in a safe and reproducible manner using lateral access surgery in the ovine model. HDC gel application mitigated AF disorganization and reduced NP degeneration at the microscopic level. Radiographic changes were slight when comparing treated to untreated IVDs. These results indicate that HDC gel is a potential option for annular repair but requires further analysis in large animal models. Further work is currently being conducted using a more aggressive injury model and longer follow-up period.
References


Supplemental Digital Content 1

**High-Density Collagen Gel Preparation**

Collagen was harvested and reconstituted from rat-tail tendon as previously described. Fibers were digested in 0.1% acetic acid, frozen for 48 hours, lyophilized, and reconstituted at 20 mg/ml in 0.1% acetic acid. Immediately before delivery, acidic collagen solutions were mixed with working solutions consisting of 10x Dulbecco’s phosphate buffered saline (DPBS), 1N NaOH and 1x DPBS to initiate polymerization of final collagen gels at 15mg/ml. RF was added to the 1x DPBS to yield a final RF concentration of 0.06 mM in the injectable gel. Gels were exposed to blue light with a 480 nm wavelength for 40 seconds after injection to cross-link in situ.

**Annular Drill Injury Surgical Technique**

All surgical procedures were performed in accordance with the Weill Cornell Medical College Research Animal Resource Center (RARC) guidelines for large animal surgery, including sterilization of surgical supplies, aseptic techniques, preoperative care, monitoring and supportive care, surgical procedures, and postoperative care. For the surgical approach to the spine, a technique modified from Oehme et al. was conducted. Once anesthetized and intubated, the sheep were placed in left lateral decubitus position. An intraoperative X-ray was taken to confirm adequate localization of the incision and to identify the respective index levels of the lumbar spine. The surgical site was prepared by clipping wool and then scrubbing with a combination of chlorhexidine and betadine scrub solutions. Monitoring equipment including pulse oximetry, arterial blood pressure line, and temperature probes were connected. All sheep were given a dose of ceftiofur prior to incision. Iodine adhesive tape and sterile surgical drapes were then placed over the planned incision site. A longitudinal incision was made from the most caudal rib to the iliac crest 1-cm ventral to the transverse processes (Figure 1A).
Using a combination of sharp dissection and monopolar cautery, the skin, subcutaneous layers, and paraspinal musculature were dissected. Once the psoas muscle was identified, it was retracted posteriorly, allowing the operating surgeon direct visualization of the anterolateral aspect of the lumbar spine. A characteristic convex bulge in relation to the concave vertebral bodies identified the IVD, and levels were confirmed an intraoperative radiograph. For IVDs requiring injury, a 3.2 mm drill bit was inserted through the AF (Figure 1B). This was followed by insertion of an 18-gauge needle into the NP at a depth of 9-10 mm (Figure 1C). For segments randomized to receive treatment, 1mL of 0.06mM RF cross-linked HDC was injected using a sterile syringe with stopcock (Figure 1D). Gels were exposed to blue light with a 480nm wavelength for 40 seconds after injection to initiate cross-linking (Figure 1E). Once treatment was completed (Figure 1F), the surgical cavity was thoroughly irrigated with antibiotic solution and hemostasis was achieved with a combination of monopolar and bipolar cautery. The fascia and deep and superficial subcutaneous layers were closed in standard fashion. Staples were used to close the skin and the wound was covered with topical antibiotic gel and gauze dressing.

References
