Combined nucleus pulposus augmentation and annulus fibrosus repair prevents acute intervertebral disc degeneration after discectomy

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Tissue-engineered approaches for the treatment of early-stage intervertebral disc degeneration have shown promise in preclinical studies. However, none of these therapies has been approved for clinical use, in part because each therapy targets only one aspect of the intervertebral disc’s composite structure. At present, there is no reliable method to prevent intervertebral disc degeneration after herniation and subsequent discectomy. Here, we demonstrate the prevention of degeneration and maintenance of mechanical function in the ovine lumbar spine after discectomy by combining strategies for nucleus pulposus augmentation using hyaluronic acid injection and repair of the annulus fibrosus using a photocrosslinked collagen patch. This combined approach healed annulus fibrosus defects, restored nucleus pulposus hydration, and maintained native torsional and compressive stiffness up to 6 weeks after injury. These data demonstrate the necessity of a combined strategy for arresting intervertebral disc degeneration and support further translation of combinatorial interventions to treat herniations in the human spine.

INTRODUCTION

Intervertebral disc (IVD) degeneration has an estimated lifetime prevalence of 90% in the United States and is largely implicated as the cause of back and neck pain (1). IVD degeneration is a broad definition encompassing multiple graded conditions affecting the IVD (2), the cartilaginous tissue connecting adjacent vertebrae in the spine that cushions loads and allows movements such as bending and twisting (3). Early stages of degeneration involve the loss of proteoglycans in the nucleus pulposus (NP), leading to reduced hydration of the IVD (4), as well as disorganization of the lamellar collagen fiber network within the annulus fibrosus (AF) (5). As degeneration progresses, micro-fissures and subsequent tears accumulate in the AF, enabling the gelatinous NP to herniate and compress neighboring structures such as spinal nerve roots, leading to pain and motor and sensory deficits (2, 6). Commonly known as herniated or slipped discs, IVD herniations are a global cause of morbidity and disability and are often associated with chronic pain, limited mobility, and a decreased quality of life (7–10). Discectomy is effective in relieving symptoms arising from nerve root compression, but it does not replenish the NP lost from herniation and often leaves a large defect through which the NP can continue to herniate (11). As such, in the estimated 480,000 lumbar discectomy cases occurring annually (12, 13), reherniation rates from 5 to 26% have been reported (14–17). Even in the absence of reherniation, discectomized IVDs are highly likely to progress to a degenerative state (18).

There is an obvious unmet goal of successfully repairing the IVD after herniation, yet clinical treatments have been relatively stagnant over the past decade (19). Recent work has demonstrated the potential for biomaterials and tissue engineering strategies to treat late-stage IVD degeneration (20–22). However, the application of these approaches to target early-stage disease and prevention of degeneration has not been effectively demonstrated. Discectomy is the current standard intervention for early-stage disease, which relieves clinical symptoms but does not address the underlying disease. Likewise, attempts using sutures or rigid implants to prevent reherniation after discectomy have shown success over acute time periods, yet these devices do not promote tissue healing and thus are unable to prevent degeneration in long-term clinical trials (23–26). Consequently, with the goal of preventing further herniation after discectomy and promoting healing and remodeling of the degenerated IVD, tissue-engineered and biomaterial approaches are of interest (27–31). Injectable hydrogels for AF repair have been shown in large animal models to successfully patch AF defects after discectomy, but do not restore lost hydration to the NP (27, 28, 32, 33). NP hydration is critical for the mechanical function of the IVD; thus, multiple injectable biomaterial and cell-based therapies have been developed to restore NP hydration in degenerated IVDs (34–40). NP augmentation therapies have restored hydration and biochemical content to degenerated IVDs in clinical trials, but without a method of healing the AF after injection, these methods are prone to reherniation and further degeneration (41, 42). As such, long-term prevention of degeneration has yet to be achieved after discectomy.

To address these challenges, we developed an injectable repair strategy composed of two distinct biomaterials to target both the NP and AF after discectomy. To rehydrate and restore herniated material to the NP, a modified hyaluronic acid (HA) is injected into the NP space (43, 44). After the HA NP injection, a high-density collagen patch is injected into the AF lesion and photocrosslinked in situ to fill the size and shape of the defect (30, 32, 33, 45–47). We have previously shown the efficacy of the collagen AF patch in small (30, 47–49) and large animal models in vivo (32, 33) and evaluated the combined repair strategy in an ex vivo rat tail spine model (50). These results...
demonstrated that, in vivo, the collagen AF patch becomes infiltrated by host cells and integrates with the surrounding AF to prevent further herniation. In addition, the combined repair strategy improved IVD hydration and mechanics after discectomy compared to individual repairs applied alone. This and other similar approaches demonstrate the potential immediate benefit of combined NP augmentation and AF repair ex vivo (31, 50), but the ability of such an approach to provide enduring benefit in vivo has not been investigated.

Here, we demonstrate the prevention of IVD degeneration and maintenance of mechanical function in the ovine lumbar spine after discectomy by combining strategies for NP augmentation using HA injection and repair of the AF using a photocrosslinked collagen patch. This combined approach healed defects in the AF, restored water content to the NP, and maintained native mechanical properties out to 6 weeks after injury. The results presented here demonstrate that a combined repair strategy is necessary to prevent IVD degeneration after discectomy and support further translation of combinatorial interventions to treat IVD herniations in the human spine.

RESULTS

Combined NP augmentation and AF repair in the ovine lumbar spine

To assess the efficacy of combined NP and AF therapies, injections of modified HA and photocrosslinked collagen were delivered to the ovine lumbar spine after discectomy and followed for 6 weeks. With the intent to ultimately translate this technology to humans, the sheep lumbar spine was chosen for this study because of its similar size, range of motion, and intradiscal pressure as the human lumbar spine (51–53). Sheep do not share the upright nature of the human spine; however, the musculature of sheep provides adequate tension such that the pressure within lumbar IVDs has been reported to be equal to if not slightly higher than human intradiscal pressure (54).

A total of 40 IVDs in eight sheep were used in the study, with five lumbar IVDs in each spine subjected in a randomized fashion to various treatments as described below. To model clinical discectomy, IVDs were subjected to a large (3 mm by 10 mm) annular defect and removal of NP tissue (~200 mg). IVDs receiving the discectomy injury were subjected to a 3 mm by 10 mm box annulotomy followed by removal of ~200 mg of NP tissue (B). IVDs receiving discectomy either were treated with NP augmentation, AF repair, or combined NP augmentation and AF repair, or were not treated (Fig. 1). One IVD per animal served as an intact control, which did not receive discectomy or treatment. NP augmentation involved injection of modified HA (8 mg/ml dissolved in PBS) into the NP void to restore hydration and lost material (43, 44). The HA is a formulation modified with C16 repeating side chains at a degree of substitution of ~3%, with similar charge density and swelling ability to natural HA. The hydrophobic side chains create a stable hydrogel at 10× lower concentrations than native HA (44). The modified HA is approved for clinical use as injections into the knee and other joints to prevent osteoarthritis (55–57). AF repair consisted of collagen gel (15 mg/ml) mixed with 0.06 mM riboflavin that was photocrosslinked in situ with blue light to patch the discectomy defect and prevent further herniation.

Through a lateral approach between the psoas and peritoneum, the sheep lumbar spine was amenable to both the partial discectomy and biomaterial injections (Fig. 1, A to D). All injected biomaterials remained localized to their target sites upon surgical closing (Fig. 1, C and D).

Fig. 1. Individual and combined NP and AF repair strategies in an in vivo sheep lumbar spine model. Schematics and intraoperative images depicting the tissue-engineered approach to IVD repair. All intraoperative images are oriented with the cranial side of the sheep on the left, caudal on the right, ventral on the top, and dorsal on the bottom. Through a pre-psoas lateral approach to the lumbar spine, IVDs were exposed (A), and those receiving the discectomy injury were subjected to a 3 mm by 10 mm box annulotomy followed by removal of ~200 mg of NP tissue (B). IVDs receiving the biomaterial repair strategies were injected with the modified HA into the NP space (C), had a collagen patch injected and cross-linked in the AF defect (D), or had both treatments. X-linking, cross-linking.
Four adjacent IVDs in each sheep underwent discectomies; however, previous sheep lumbar spine studies evaluated over longer time points have shown that there are negligible or no effects of injury on adjacent segments (28, 32, 33). The entire cohort successfully survived to the defined 6-week endpoint and showed no signs of pain or neurological deficits as determined by veterinary care staff.

**Combined NP and AF repair prevented clinical signs of degeneration**

Upon inspection of the lumbar spine during harvest 6 weeks after surgery, there were observable differences in gross appearance of the IVDs that had received discectomy alone versus those treated with the biomaterial repairs (Fig. 2, A to J). Whereas the AF appeared white and homogeneous in the intact controls (Fig. 2F), those IVDs that had received discectomy with no treatment showed disrupted AF with persistent defects, leaving the inside of the IVD exposed (Fig. 2G). Such persistent defects resemble those seen clinically in discectomy patients (51, 58). All three treatment groups showed improved healing via tissue infill at the discectomy site (Fig. 2, H to J). IVDs that had received the HA NP injections alone had visible tissue in the defect, but the defect boundaries were still clear. IVDs that had received the collagen AF patch with or without HA NP injections had robust tissue ingrowth, resulting in a more continuous covering at the defect site.

T2-weighted magnetic resonance imaging (MRI) is the clinical imaging modality frequently used to evaluate IVD health in human patients. This modality probes the structure and hydration of the IVD. Higher-intensity regions correlate with greater water content and tissue hydration (59). In this study, axial sections of T2 MRI allowed for the visualization of the AF and NP, including the defect site and effects of the injected biomaterials (Fig. 2, K to O). T2 MRI revealed that the discectomized IVDs without treatment had severely disrupted morphology, including decreased hydration and heterogeneity in the NP region compared with the intact controls (Fig. 2, K and L). The discectomy group showed lesions present in the AF, and the border between the AF and the NP was not well defined (Fig. 2L). Treatment with the HA NP injection alone or in combination with the collagen AF patch appeared to restore the hydration of the NP and preserve the native NP geometry (Fig. 2, M to O). The collagen AF patch alone did little to improve NP morphology over the discectomy control (Fig. 2N) but, in combination with the HA NP injection, resulted in IVDs with the most similar morphology to the intact controls (Fig. 2O). Mid-sagittal cross sections of the T2 MRI similarly demonstrated that discectomy disrupted the homogeneity of the intact NP, and the collagen AF patch with the HA NP injection preserved native NP hydration and morphology (Fig. 2, P to T). The mid-sagittal T2 MR images were reviewed for injury and changes to the intervertebral endplates, but no evidence of alterations from the intact IVDs was observed.

Quantitative image analyses further supported the qualitative MRI outcomes. Pfirrmann grading is a rank-based approach that evaluates the severity of IVD degeneration using axial and sagittal T2 MR images (60). Pfirrmann grade considers the NP intensity, NP homogeneity, distinction of the NP/AF border, and disc height to rank IVD degeneration from 1 to 5. All intact controls showed no signs of degeneration and received Pfirrmann grades of 1, whereas the discectomy-only controls showed significantly increased degeneration from intact controls ($P<0.01$) with a median grade of 3 and an interquartile range of 1 (Fig. 2U). The individual repairs showed increased Pfirrmann grades 6-week endpoint and showed no signs of pain or neurological deficits as determined by veterinary care staff.

**Fig. 2. Six-week gross morphological and MRI assessment of individual and combined NP and AF repair.** (A to E) Axial schematic diagrams of the intact, discectomy only, and treated IVDs. (F to J) Representative sagittal gross dissection images showing the AF at the site of discectomy and biomaterial injections (yellow arrows) after 6 weeks in vivo. (K to O) Representative axial T2 MR images of the IVDs obtained at 3 T showing the AF, NP, and treatment site (yellow arrows). (P to T) Representative mid-sagittal T2 MR images showing differences in the NP across treatment groups. High-intensity regions in T2 MR images correlate with greater water content. (U) Pfirrmann grading of IVD degeneration and (V) DHI from the T2 MR images compared between all groups; bars denote significance ($P<0.05$). Pfirrmann grades are shown as median ± interquartile range, whereas DHI is means ± SD ($n=8$). Significant differences were assessed with a Kruskal-Wallis with Mann-Whitney $U$ tests for Pfirrmann grades and a general linear mixed model with Tukey’s post hoc tests for DHI.
grades from the intact controls, with the HA NP injection leading to significantly increased degeneration \( (P < 0.05) \). The Pfirrmann grades of IVDs that had received the combined therapy were significantly lower than the discectomy group \( (1 \pm 1, P < 0.01) \) and were similar to intact controls \( (P = 0.86) \). Similar trends were observed in the disc height index (DHI), a measure of disc height relative to the adjacent vertebrae, quantified from the MR images (Fig. 2V) \((61)\). In the clinic, degenerating IVDs are observed to lose disc height over time because hydration and tissue are lost from the NP, resulting in a reduced capacity to resist compressive loads in the spine. Discectomy without treatment and HA NP injection alone yielded a lower DHI than the intact controls \( (P < 0.01 \text{ and } P < 0.01) \). IVDs that received the collagen AF patch with or without the HA NP injection had a similar DHI to the intact controls \( (P = 0.50 \text{ and } P = 0.10) \).

These clinically relevant outcome measures indicate that the combined NP augmentation and AF repair reduced degeneration after injury over the 6-week period in vivo. The HA NP injection is critical to restoring lost hydration and T2 MRI signal, whereas the collagen AF patch is necessary to maintain integrity of the AF and retain tissue inside of the IVD. Discectomy without treatment is akin to the current standard intervention for symptomatic IVD herniations, which leaves the IVD prone to further degeneration and herniation. All treatment groups showed fewer signs of degeneration than discectomy only, with the combined NP and AF repair best preserving native IVD morphology.

**Combined NP and AF repair preserved native IVD morphology after discectomy**

The outcomes observed through clinically relevant methods of measuring IVD health were supported by histological analyses. Alcian blue histology of the IVDs sectioned through a mid-coronal plane enabled visualization of IVD morphology and tissue health (Fig. 3, A to J). The intact control IVDs demonstrated typical native morphology, with a homogeneous NP that was rich in proteoglycans and intact lamellar bundles in the AF that were rich in collagen (Fig. 3F). Six weeks after discectomy, IVDs that had not received treatment showed heterogeneity in the NP, lesions in the AF, and increased proteoglycan staining in the AF (Fig. 3G). The HA NP injections resulted in healthy AF lamellar bundles but a distinct and disrupted NP morphology (Fig. 3H). The collagen AF patch showed disrupted NP morphology similar to the discectomy alone, but had less proteoglycan staining in the AF than discectomy alone (Fig. 3I). The combined NP and AF repair resulted in a disrupted NP with healthy AF lamellae (Fig. 3J). Although some gaps were observed

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**Fig. 3. Six-week histological evaluation of individual and combined NP and AF repair to show overall IVD morphology.** (A to E) Axial schematic diagrams of the intact, discectomy only, and treated IVDs showing the approximate mid-coronal section through the IVD for histological analyses. \((F \text{ to } J)\) Representative alcian blue--stained histological sections imaged with bright-field microscopy. Proteoglycans are stained blue, and collagen is stained pink. \((K \text{ to } O)\) Representative second harmonic generation (SHG) multiphoton images showing collagen orientation and alignment in the IVDs with higher-magnification images to show AF lamellae \((P \text{ to } T)\). White arrows point to AF lamellae.
between the AF and NP, this is seen in other histological sections and may be an artifact from histological sectioning. The mid-coronal histological sections were evaluated for injury and changes to the endplates, but there was no evidence of alterations compared to the intact condition. Additional Safranin O histology (fig. S1) and alcian blue histology of the most and least damaged IVDs can be found in the Supplementary Materials to better illustrate the range of degeneration associated with each treatment (fig. S2).

Second harmonic generation (SHG) microscopy is a multiphoton imaging modality that enables visualization of collagen structure and alignment and was performed on unstained mid-coronal histology sections to further evaluate IVD morphology (Fig. 3, K to T). SHG confirmed the morphological differences observed through safranin-O histology, with greater detail observable in the lamellar structure of the AF. In intact IVDs, the NP was fully pressurized within the AF, and thus, AF lamellae were curved around the NP (Fig. 3, K and P). Discectomy led to buckling and inversion of the AF lamellae (Fig. 3, L and Q), which was improved with the collagen AF patch (Fig. 3, N and S) and to a greater extent with HA NP injections (Fig. 3, M and R). Combined NP augmentation and AF repair resulted in AF lamellae that curved around the NP and did not show signs of buckling or inversion (Fig. 3, O and T). Although the AF lamellar bundles in Fig. 3O appear similar to the native control, there is visible increased fibrosity on the contralateral side of the IVD that may be due to the repair strategies. High-magnification images of the outer AF showed similar morphology in all experimental conditions (fig. S3).

**Combined repair locally restored NP content and prevented herniation**

Histological sections were also taken through the defect site to assess reherniation and evaluate the local effects of each treatment (Fig. 4, A to E). Alcian blue histology showed intense proteoglycan staining in the NP region and collagen in the AF and vertebrae of the intact control IVDs, as expected (Fig. 4F). Under high magnification, native AF appeared as aligned, fibrous collagen with little observable cellularity (Fig. 4K). After 6 weeks in vivo, the discectomy defect was still present without treatment (Fig. 4G), allowing herniation of the NP as demonstrated by proteoglycans and vacuolated chondrocyte-like cells outside of the IVD (Fig. 4M). The collagen AF patch resulted in IVDs with newly remodeled tissue in the defect site staining for both proteoglycans and collagen (Fig. 4I). Under higher magnification, this tissue appeared fibrous with little cellularity, similar to intact controls (Fig. 4N). Combined NP augmentation and AF repair led to rich proteoglycan content in the NP and tissue infill at the defect site, which under higher magnification appeared as aligned, fibrous tissue. Additional alcian blue histology of the most and least damaged IVDs can be found in the Supplementary Materials (fig. S4). Most histological sections of the endplates in the defect sites appeared similar to intact IVDs, except for a few samples that showed minor endplate thinning near the defect that was not a result of direct injury during surgery.

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**Fig. 4. Six-week histological evaluation of the discectomy site demonstrating herniation in IVDs without collagen AF repair.** (A to E) Axial schematic diagrams of the intact, discectomy only, and treated IVDs showing the approximate section through the defect location for histology. (F to J) Representative alcian blue-stained histological sections through the defect location imaged with bright-field microscopy, with higher-magnification images (K to O) showing the intact AF (K), vacuolated chondrocyte-like cells in herniated NP (L and M), and fibrous tissue where the collagen patch was injected (N and O). Alcian blue stains proteoglycans blue and collagen pink, black arrows show direction of collagen alignment in the AF, and white arrows point to chondrocyte-like cells in herniated regions.
The histological sections of the defect site were also evaluated for signs of chronic inflammation and immune reaction to the implanted materials through hematoxylin and eosin staining (fig. S5). There was no evidence of immune infiltrate in the NP, AF, or endplate at the injury site or adjacent regions in any of the treatment groups. Histological sections through the defect site yielded information about the local effects of each treatment group and showed how the collagen gel prevented herniation, whereas groups without the collagen gel had persistent lesions in the AF.

SHG multiphoton imaging was performed on the defect site histology to better understand the AF collagen structure and alignment near the defect site (fig. S6). SHG imaging showed that the discectomy group and the HA NP injection group had reduced collagen alignment in the AF compared to the intact controls, with no apparent lamellar structure. The collagen AF patch and combined treatment IVDs did not show lamellar collagen, but had increased aligned collagen in the direction of native lamellar bundles.

**Combined repair maintained IVD functional mechanical properties**

To assess the functional impact of the treatments, half of the IVDs were subjected to mechanical testing to evaluate torsional, compressive, and tensile properties (Fig. 5A). Ideal repair after discectomy should restore native mechanical properties to maintain proper spine function and reduce the risk of degeneration in adjacent IVDs. Full motion segments composed of vertebra-IVD-vertebra were isolated, stripped of the spinous and transverse processes, and potted in polymethyl methacrylate (PMMA) for a consistent grip (Fig. 5, B to D) (62, 63).

To evaluate compressive and tensile properties, a cyclic axial loading protocol was applied (25 cycles of compression/tension, 0.1 Hz, −0.5 to 0.25 MPa) followed by a stress relaxation protocol (four steps of 5% compressive strain, for 45 min). Torsional properties were then investigated through a cyclic torsion protocol while maintaining a 0.5 MPa compressive stress (25 cycles of torsion, 0.1 Hz, from −4° to +4°). The peak compressive stress during the imposed cyclic loading is equivalent to loads measured in the human lumbar spine during unsupported standing, whereas ±4° torsion is a typical range of twisting seen by human IVDs under voluntary motion (64, 65).

Torsional testing yielded hysteresis curves that showed clear effects of injury and treatment (Fig. 5, E to L). Qualitatively, injury caused torsional loading curves to be shallower in slope with less energy dissipation per cycle. NP augmentation or AF repair alone did not change this loading pattern, but combined repair generated curves that were similar to the intact controls. To quantify these effects,
torsional stiffness was calculated as the average slope of the upper and lower 20% of the loading curves, and torque range was evaluated as the torque difference measured over the +4 to −4° angular displacement (Fig. 5J). IVDs that received discectomy alone had significantly reduced torsional stiffness and torque range relative to the intact control from the same spine (P < 0.05 and P < 0.05; Fig. 5, K and L). Similarly, individual NP and AF repairs resulted in significantly decreased torsional mechanics (P < 0.05 and P < 0.05). Collectively, these torsion mechanics indicate that after discectomy or individual repair, IVDs are more compliant than intact controls and have more freedom to displace, which may lead to further degeneration. IVDs treated with combined NP and AF repair had similar torsional stiffness and torque range to the intact controls (P > 0.05 and P > 0.05). Furthermore, the combined repair group had significantly greater torsional stiffness than the collagen AF patch alone (P < 0.05), indicating the key role of NP hydration in IVD torsional mechanics. Torsional mechanical properties are highly sensitive to IVD injury, making them an important indicator of function for IVD repair (62, 66, 67).

IVD function under compression was not significantly impaired by discectomy or the biomaterial injections (P > 0.05). Hysteresis curves obtained from tensile/compressive loading showed similar trends to the torsional hysteresis curves, wherein the tensile regimen of the injury and repair groups had shallower slopes than the intact control (Fig. 6, A to E). Compressive stiffness and range of motion did not vary across the treatment groups; however, all experimental groups had significantly lower tensile stiffness compared to the intact controls (P > 0.05, P > 0.05, and P < 0.05; Fig. 6, F to I). Equilibrium and instantaneous moduli, which measure the elastic and viscoelastic responses, respectively, of the IVD under compressive stress relaxation, did not show differences across treatment groups (P > 0.05 and P > 0.05; Fig. 6, J to L). Hydraulic permeability was increased in IVDs receiving the HA NP injection with or without the collagen AF patch (P < 0.05; Fig. 6M), which is not desired but may be due to the difference in viscosity between the sheep NP and the modified HA. Cumulatively, the mechanical results support that the combined NP and AF repair restored healthy IVD function after discectomy, which was not achieved by individual repairs.

**DISCUSSION**

Individual NP and AF repair therapies have previously been explored as means to prevent IVD degeneration and promote healing after discectomy. Both therapies effectively treated their respective target tissue; however, the composite nature of the IVD makes a single treatment approach for IVD degeneration inadequate. The present study demonstrates the efficacy of a combined NP and AF repair strategy in a sheep lumbar spine model of IVD degeneration. Simultaneously targeting the NP and AF preserved native morphology and mechanical function after 6 weeks in vivo by both restoring hydration to the IVD and preventing herniation through tissue remodeling at the discectomy site.

Discectomy without treatment, the current clinical standard of care for IVD herniations, led to degeneration over the 6-week period,
including loss of disc height and NP hydration, persistent lesions in the AF, NP herniation, and decreases in IVD mechanical properties. Individual repairs targeting the NP or AF successfully treated their respective tissue, where the HA NP injection restored hydration to the NP, and the collagen AF patch prevented herniation through new tissue formation at the defect site. However, because neither of the individual repairs addressed both the hydration of the NP and the integrity of the AF, only treatment with the combined therapy resulted in IVDs similar to the intact controls. The combined therapy filled defects in the AF, restored water content to the NP, maintained disc height and native IVD morphology, and yielded functional mechanical properties similar to intact controls.

The results from this study support the translation of combined strategies for IVD repair. None of the treated animals showed signs of pain or neurological deficits as assessed by the veterinary care staff, nor was there histological evidence of chronic inflammation at the site of the biomaterial repairs. The sheep lumbar spine model was chosen for this study because sheep have comparable lumbar IVD biomechanics and geometry to humans (51, 54), progressive degeneration can be induced with IVD injury (33), and sheep spines are amenable to clinical analyses such as Pfirrmann grading and disc height analysis. Other large animal models have been used for preclinical studies of IVD repair and regeneration including pigs (25, 68), goats (22), and dogs (21, 69), all of which have potential merit for modeling specific disease processes. The sheep spine is particularly amenable to simulating the human spine after discectomy. Intradiscal pressure of sheep lumbar spines has been reported to be 0.7 MPa while standing, slightly greater than the 0.5 MPa measured in human lumbar spines (54). None of the collagen AF patches were observed to herniate in the 16 treated IVDs even at these high pressures. Furthermore, no animal showed neurologic deficits, consistent with the observation that these patches did not migrate over the course of the study. Unlike defects observed clinically, the lateral approach used here is not in proximity to nerve roots, and thus, impingement of such structures is not likely.

The discectomy used in this study has been extensively validated to approximate the state of human IVDs after discectomy (28, 32, 33, 41, 70). Previous work has demonstrated the need for an aggressive annulotomy with removal of NP to induce measureable and consistent degeneration in vivo (32, 33, 71). The amount of injury to facilitate degeneration depends on features of the animal model including disc height, NP fibrosity, and presence of notochordal cells (41, 72, 73). Because of the similarities of discectomy in sheep lumbar spine model and the human lumbar spine, we anticipate that the AF collagen patch and NP HA injection will have reparative effects similar to those observed in this study when applied to human patients after discectomy.

Critical to the function of the spine is its ability to respond under complex and varied mechanical loads. As mentioned above, sheep are excellent models to evaluate mechanics after repair because sheep lumbar IVDs experience similar loads to those seen in human lumbar IVDs. When individual NP and AF repairs were applied after discectomy, they somewhat prevented degenerative changes as seen through MRI, histology, and biochemical analyses; however, the individual repairs did not preserve native IVD mechanical properties. Similar outcomes have been observed through in vivo studies with different materials for both NP and AF repair (27, 28, 35, 64, 74). Proper IVD mechanical function cannot be achieved with dehydrated NP tissue and lesions present in the AF because in healthy IVDs, compressive loads pressurize the NP that is contained within an intact AF. The combined NP augmentation and AF repair restored hydration to the NP and filled lesions in the AF. Thus, when the combined strategy was applied after discectomy, native IVD morphology and functional mechanical properties were maintained after 6 weeks in vivo. The torsional stiffness and torque range were similar to intact values. Torsional mechanical properties have been shown to be highly sensitive to discectomy injuries and difficult to restore with biomaterial repair strategies (31, 62, 66). Also, the hydraulic permeability data suggest that the collagen patch functionally heals AF lesions, at least in part. These results collectively demonstrate the need to both restore hydration to the NP and heal the AF after discectomy to prevent degenerative changes that lead to altered IVD morphology and mechanical function.

To understand the potential impact of combined NP augmentation and AF repair, the proper context must be given in terms of the clinical and health care impact. Current state-of-the-art treatment for lumbar disc herniations is microdiscectomy. This procedure’s goal is to remove the herniated NP to decompress the impinging surrounding neural structures. Although this provides temporary symptomatic relief, the AF defect remains untreated, and the underlying degenerative process is not affected. Each year in the United States, there are an estimated 480,000 lumbar discectomies (12), with each operation costing an average of $24,000 (17). It is also estimated that for every 100 lumbar discectomy operations, about $300,000 is spent on revision surgeries because of recurrent pain and reherniation (17). Thus, the economic burden on the U.S. health care system each year for revision surgeries after lumbar discectomy can be conservatively estimated as $1.4 billion. Discectomy procedures provide a potential therapeutic window for intervention that arrests the process of IVD degeneration. The injectable nature of HA and collagen therapies can be rapidly applied in a non-invasive manner during a discectomy procedure. This is in contrast to barrier devices that require invasive implantation procedures that have been shown to induce endplate lesions and osteolysis in vertebrae (75). Further, the HA formulation used here is approved for treatment in the knee in the United States. Collagen hydrogels have been approved for various therapeutic applications, and the riboflavin crosslinker is used clinically for the treatment of keratoconus (76). Collectively, these attributes suggest that this approach could be rapidly translated to the clinic.

The NP augmentation and AF repair therapies in this study were shown to be effective as used; however, the chosen biomaterials are also platforms amenable to delivery of cells and signaling factors. Cell delivery for NP regeneration is in clinical trials with multiple studies supporting the safety and feasibility of cellular injections into the NP (77, 78). These therapies attempt to restore biological and mechanical function to degenerated IVDs by augmenting native cell populations with stem cells and other potent cell types that will synthesize proteoglycans to rehydrate the NP. Most of the reported clinical trials injected cells suspended in aqueous solutions; however, without scaffold materials for localization, the cell suspensions are free to migrate away from the injection site (77). Injectable HA formulations have been widely used as cell carriers and would be amendable for the process for IVDs as well (79–81). In addition to its hydrating and mechanical benefits, the modified HA used in this study can deliver cells to the NP in a localized fashion. The collagen patch used to treat AF defects in this study can also be laden with cells (33, 47). Although there are no clinical studies attempting to repair AF defects with cell delivery, our group and others have...
demonstrated in preclinical models how cell-laden collagen gels can heal AF defects more quickly and in a more robust manner than acellular scaffolds \((33, 47)\). Cells were not administered with the biomaterial repairs in the present study because they would introduce another source of variability to the data, and acellular materials have a clearer regulatory pathway that is more amenable to human translation. Last, growth factors have been shown to lead to new extracellular matrix production and tissue remodeling in AF defects \((82)\). Thus, the combined NP and AF approach demonstrated here represents a platform technology that can serve to deliver other regenerative therapies.

This study yielded valuable insight for the translation of injectable IVD repair; however, there are limitations we must address. The sheep lumbar spine is an excellent animal model that has been used for a number of investigations on IVD degeneration and repair, yet discoveries in this model must be contextualized to understand how therapies can be applied to humans. Although sheep lumbar IVDs have been reported to have similar intradiscal pressure to humans, the IVD size is slightly smaller than human lumbar IVDs, and sheep NP is more fibrous. The present study evaluated coronal histology sections though the middle of the IVD and the defect site to assess repair strategies and overall disc morphology. On the basis of the anatomy and curvature of the sheep IVD, coronal sections may not necessarily sample both the AF and NP defects or repair biomaterials. As such, it was difficult to directly visualize the repair process in both the AF and NP simultaneously. In terms of our analytical methods, six-axis mechanical loading may yield useful insight into the function of the injured and repaired IVDs; however, we chose biaxial loading because it yields straightforward results that can be more easily interpreted and compared to previous work. In addition, our functional analyses focused on motion segment mechanical properties, not whole spine kinematics that might indicate how repairs affect adjacent IVDs. Evaluating our study design, time points longer than 6 weeks would enable us to determine how IVD health is affected long term.

In summary, we demonstrated that combined NP augmentation and AF repair successfully prevented degeneration after discectomy in vivo. As such, this approach has great potential to maintain IVD health and prevent subsequent progressive degeneration in the spine. The results from this study warrant further investigation of combined repair strategies to fully characterize their effects before human application.

**MATERIALS AND METHODS**

**Study design**

The objectives of this study were (i) to prevent IVD degeneration after discectomy in a large animal model by injecting a combined NP augmentation and AF repair into discectomized IVDs, (ii) to demonstrate the feasibility of the injectable repair strategy in a preclinical large animal model, and (iii) to demonstrate that a combined repair strategy is superior to individual repairs alone. We hypothesized that a combined repair strategy would maintain healthy IVD morphology and mechanical function after discectomy compared to individual AF or NP repair and discectomy alone. The in vivo study was approved by the Cornell University and the Barton West End Farms Institutional Animal Care and Use Committee and was performed according to the guidelines recommended by these committees. The lumbar spine in female Finn sheep \((n = 8)\) was exposed with each IVD randomly assigned to receive one of the following treatments: intact control, discectomy control, HA NP injection, collagen AF patch, or combined HA NP injection with collagen AF patch. After 6 weeks in vivo, all spines were harvested and grossly inspected, subjected to 3-T MRI, and sectioned for histological inspection. Biaxial testing was performed on half of the samples \((n = 4)\) before histology. One IVD was omitted from the mechanical analyses because of poor grip during testing, and one outlier was removed from the hydraulic permeability analysis for being 1 standard deviation away from the group average. This work was a randomized, nonblinded, controlled laboratory experiment. Raw data are provided in data file S1.

**Statistical analyses**

Statistical analyses were performed in R (RStudio Inc.), with significance defined as \(P < 0.05\). DHl and all mechanical outcome values were normalized to the intact segment of the same sheep. All data shown are means \pm SD. A general linear mixed model was used to analyze statistical differences in DHl and mechanical outcomes between treatment groups and IVD location in the spine, and comparisons between groups were made with two-tailed Tukey’s honest significant difference post hoc tests. Kruskal-Wallis analysis of variance followed by two-tailed Mann-Whitney \(U\) tests were used to determine statistical differences of the nonparametric Pfirrmann grades.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig. S1. Safranin O histology of individual and combined NP and AF repair.

Fig. S2. Mid-coronal alcian blue histology of the most and least damaged IVDs from each experimental group.

Fig. S3. SHG imaging of the outer AF from mid-coronal histology sections.

Fig. S4. Alcian blue histology through the defect site of the most and least damaged IVDs from each experimental group.

Fig. S5. Hematoxylin and eosin staining of the NP, AF, and endplate near the discectomy site.

Fig. S6. SHG imaging of histology sections through the defect site.

Materials and Methods

Statistical analyses were performed in R (RStudio Inc.), with significance defined as \(P < 0.05\). DHl and all mechanical outcome values were normalized to the intact segment of the same sheep. All data shown are means \pm SD. A general linear mixed model was used to analyze statistical differences in DHl and mechanical outcomes between treatment groups and IVD location in the spine, and comparisons between groups were made with two-tailed Tukey’s honest significant difference post hoc tests. Kruskal-Wallis analysis of variance followed by two-tailed Mann-Whitney \(U\) tests were used to determine statistical differences of the nonparametric Pfirrmann grades.

**REFERENCES AND NOTES**


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Acknowledgments: We would like to thank C. Chlebek for technical assistance with mechanical analyses and R. R. Arbona for assistance with organizing surgical facilities.

Funding: This study was supported by Cornell University CTSC NIH/NCATS Grant (T1L-TR-002386) and the Colin MacDonald Fund. Imaging data were acquired through the Cornell University Biotechnology Resource Center, with NYSTEM (CO29155) and NIH (S10OD018516) funding for the shared Zeiss LSM880 confocal/multiphoton microscope. Author contributions: S.A.S.: study design, data acquisition and analysis, interpretation of results, and manuscript preparation. C.W.: study design, performed surgeries, data acquisition and analysis, interpretation of results, and manuscript preparation. S.K.: performed surgeries, data acquisition and analysis, and manuscript preparation. R.N.-R.: performed surgeries. F.S.: data acquisition. D.M.: data acquisition. T.P.: interpretation of results. A.S.: study design, interpretation of results, manuscript preparation. R.H.: study design, interpretation of results, and manuscript preparation. R.J.H.: study design, interpretation of results, and manuscript preparation. C.J.M.: study design, interpretation of results, manuscript preparation. Competing interests: A.S. is an employee of Fidia Pharmaceuticals. R.H. is a consultant for AO Spine, Brainlab, Depuy-Synthes, and Lanx and receives research funding for the shared Zeiss LSM880 confocal/multiphoton microscope. A.S. is an employee of Fidia Pharmaceuticals. R.H. is a consultant for AO Spine, Brainlab, Depuy-Synthes, and Lanx and receives research funding for the shared Zeiss LSM880 confocal/multiphoton microscope. 

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials.

Submitted 6 August 2019
Accepted 10 February 2020
Published 11 March 2020
10.1126/scitranslmed.aay2380

Combined nucleus pulposus augmentation and annulus fibrosus repair prevents acute intervertebral disc degeneration after discectomy

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Sci Transl Med 12, eaay2380.
DOI: 10.1126/scitranslmed.aay2380

Strategic lumbar support

Discectomy is a common treatment for herniated or “slipped” intervertebral discs that can help alleviate symptoms but does not prevent reherniation or progression of disc degeneration. Sloan et al. developed a two-part, acellular tissue-engineered therapy to prevent degeneration after discectomy. Injecting hyaluronic acid into the inner region of the disc (nucleus pulposus) and applying a photocrosslinked collagen patch to the outer annulus fibrosus healed disc defects and maintained biomechanical support in the lumbar spines of sheep for 6 weeks after discectomy. Results support further investigation of this combined approach to potentially treat herniation while preventing disc degeneration after discectomy in humans.