Development of a Biomimetic, Collagen-Based Scaffold for the Repair and Regeneration of the Annulus Fibrosus

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DEVELOPMENT OF A BIOMIMETIC, COLLAGEN-BASED SCAFFOLD FOR THE REPAIR AND REGENERATION OF THE ANNULUS FIBROSUS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Ryan Andrew Borem
December 2018

Accepted by:
Dr. Jeremy Mercuri, Committee Chair
Dr. Ken Webb
Dr. John DesJardins
Dr. Sanjitpal Gill
ABSTRACT

Annually, over 5.7 million Americans are diagnosed with two IVD-associated pathologies: IVD herniation (IVDH - a mechanical disruption of the concentric fibrous layers of the annulus fibrosus (AF))) and/or degeneration (IVDD- a multifactorial process which initiates within the inner gelatinous core (NP), and results in a biochemical degradation of NP tissue), with over 2.7 million requiring surgical interventions.\textsuperscript{1,2} Although both underlying pathologies are different, quite often they both lead to a decrease in IVD height, impaired mechanical function, and increased pain and disability. These pain symptoms affect approximately 80% of the adult population during their lifetime with estimated expenditures exceeding $85.9 billion.\textsuperscript{3,4}

Current surgical procedures for IVDH and IVDD are palliative and suffer from drawbacks. While they are performed to address patient symptoms, they fail to address the underlying pathology of a focal defect remaining within the subsequent outer layers of the AF. It is hypothesized that an effective AF closure/repair device in conjunction with a less aggressive discectomy for IVDH and/or NP arthroplasty for IVDD may result in improved patient outcomes, decreased pain, and provide fewer revision surgeries via lower re-herniation and expulsion rates.\textsuperscript{5,6} Therefore, an intact AF must be re-established to prevent implant expulsion or re-herniation, thus addressing the two major spinal pathologies directly associated with an IVD.

Currently, within the medical device market, no tissue engineering biomaterials are available for AF closure/repair. Current market AF closure devices (Intrinsic Barricaid\textsuperscript{®}, Anulex X-Close\textsuperscript{®} Tissue Repair System, and Anulex Inclose\textsuperscript{®} Surgical Mesh System) are
synthetic materials focused solely on preserving and reinforcing the native tissue and lack effective strategies for implantation, fixation, and regeneration. Therefore, there has been an increase in tissue engineering and regenerative therapeutic approaches aiming for structural and biological AF repair investigated over the last decade using in vitro and in vivo experimentation. It is proposed that the optimum AF tissue engineering scaffold should reproduce the native AF microarchitecture and native mechanical properties. Recent articles illustrate several novel sutures, sealants, and barrier techniques currently under development, resulting in an increasing attention at scientific workshops and conferences.

To develop a tissue engineering biomaterial that is suitable for AF closure we propose it must first meet the following criteria: (1) mimic the structural angle-ply architecture of the native AF, (2) fundamentally demonstrate mechanical properties mimicking the native functional characteristics, and (3) demonstrate cytocompatibility while promoting tissue regeneration. Current biomaterials gaining attention in the tissue engineering academic field, electrospinning, polymers, glue, silk scaffolds, and honeycomb-scaffolds, require complex manufacturing procedures and typically work to address two of the three criteria (mimicking the biological or structural characteristics). Therefore, the use of a decellularized tissue from a xenogeneic source may be ideal due to its advantage of maintaining native extracellular matrix (ECM) while also removing all potential harmful xenogeneic factors. Although, the mechanical advantage of closing annular focal defects to retain NP material seems intuitive, only recently have AF closure
devices begun to examined in human cadaveric or animal tissues for their ability to withstand *in situ* IDP or flexibility testing.\textsuperscript{16}

We propose to address all three criteria with the development of a biomimetic, collagen-based angle-ply annulus fibrosus repair patch (AFRP) comprised of the decellularized porcine pericardium. The porcine pericardium was chosen due to its innate type I collagen content, mechanical strength, and cytocompatibility. The objectives of this research were to investigate the development of this biomimetic AFRP to biologically augment AF repair by (1) mimicking and characterizing the micro-architecture of the multi-laminate angle-ply AFRP, (2) mechanically evaluating the AFRP’s mechanical properties and attachment strength *in situ*, (3) evaluating the ability of the AFRPs to support AF tissue regeneration in the context of a healthy and inflammatory environment, and (4) evaluating the *in vivo* mechanical strength, biocompatibility, and tissue regeneration capacity of the AFRP in a large animal model for intervertebral disc degeneration/herniation.
DEDICATION

This work is dedicated to mi hermosa angel. I would like to express my sincerest gratitude for her love and continued support throughout the years. Thank you, Rose, for all the encouragement and assistance you have provided to me along the way. I greatly appreciate everything you have done.
ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Jeremy Mercuri, for his trust in my capabilities to conduct research within our laboratory (The Laboratory of Orthopaedic Tissue Regeneration and Orthobiologics). His expertise, guidance, support, and constant encouragement proved to be a great motivator and large influence in my work and life.

I would also like to thank all my past and present committee members; Dr. Sanjitpal Gill, Dr. Ken Webb, Dr. John DesJardins, and Dr. Dan Simionescu for their insight, constructive criticisms, and advice in support and development of my research project.

I would also like to thank all my family, friends, and loves ones for their support throughout the years and for their constant guidance, unconditional love, and questioning of when I will ever be done. Especially my mother, brother, sister, and in-laws.

I would also like to thank all my fellow past and present researchers of the OrthO-X lab. Their comradery made this experience exciting and memorable, and I wish you all the best of luck in your future endeavors. More specifically, I want to send a special thank you to Allison Madeline for her unwavering dedication and assistance on this project, in addition, to her friendship. The amount of research that we have completed over these past few years would not have been possible without her.

I would like to make a special mention to administrative staff (especially: Ms. Maria Torres and Dr. Martine Laberge), fellow peers, and professors of the Bioengineering Department of Clemson.

Lastly, I would like to thank my funding sources for this work: National Science Foundation Graduate Research Fellowship and The National Institutes of Health COBRE.
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CHAPTER I

‘Part of the research contained within this chapter has been published as Borem, Ryan, Characterization of a Multi-Laminate Angle-Ply AF Patch for Annulus Fibrosus Repair, Clemson Master’s Thesis, December 2015’

LITERATURE REVIEW

BACKGROUND

Low back pain (LBP) affects approximately 80% of the adult population during their lifetime with estimated expenditures exceeding $85.9 billion.³ ⁴ Patients suffering from LBP often experience a diminished quality of life. Within the United States of America, LBP is the most common cause of activity limitation in people under the age of 45, the 2nd most frequent reason for doctor visits, the 5th leading cause of hospital admission, the 3rd most common surgical procedure, and the most common cause of job-related disability leading to days missed on the job.² ¹⁷–¹⁹ Although LBP is multifactorial; LBP can originate from intervertebral disc (IVD) pathologies. Annually, over 5.7 million Americans are diagnosed with two IVD-associated pathologies: IVD herniation (IVDH- a mechanical disruption of the concentric fibrous layers of the annulus fibrosus (AF))) and degeneration (IVDD- a multifactorial process which initiates within the inner gelatinous core (NP), and results in a biochemical degradation of NP tissue), with over 2.7 million requiring surgical intervention.¹ ² Although both underlying pathologies are different, quite
often they both lead to a decrease in IVD height, impaired mechanical function, and increased pain and disability.

**Intervertebral Disc Anatomy, Physiology, And Pathology**

An IVD links adjacent vertebral bodies together to provide mobility and stability to the spine. The IVD’s function is to support compressive loads arising from body weight and muscle forces while allowing for spinal motion. There are 23 IVD’s in the human spine: 6 cervical, 12 thoracics, 5 lumbar. The IVD is divided into three distinct regions as seen in **Figure 1**: nucleus pulposus (NP), annulus fibrosus (AF), and hyaline cartilaginous endplates.²⁰

![Figure 1: Intervertebral Disc Overview](image)

During early development, IVD’s have some vascular supply to the cartilage endplates and AF, but these supplies quickly deteriorate leaving nearly no direct blood supply in healthy adults.²² Thus, making these the largest, avascular structures in the human body. IVD’s are composed of three major constituents: water, fibrillar collagens, and aggrecan (cartilage-specific proteoglycan). The proportion and organization of these
components vary considerably with position across the IVD, for example, the NP has a higher concentration of aggrecan and water, but a lower collagen content than other regions of the IVD. Collagen proportions change based on location in the IVD, with the NP containing only type II collagen while both types I and type II are found in the AF.

The NP is the central proteoglycan and collagen Type II rich core distributes intradiscal pressure arising from applied compressive loads. The NP is comprised of a large aggregating proteoglycan, which contains a protein core. Up to 100 highly sulfated glycosaminoglycans (GAG) chains, principally chondroitin and keratin sulfate, are covalently attached to a core protein. The chains are long, linear carbohydrate polymers that have a net negative charge under physiological conditions. This negative charge attracts positively charged sodium ions which in return lures water molecules via osmosis. Research studies have proven a negative correlation between age and degeneration to the amount of water and GAGs present within the NP. As GAGs begin to diminish over time, it is replaced with fibrocartilage. The presence of fibrocartilage found in IVDs as GAGs diminish is a protective adaptation to physiological conditions.

The AF consists of circumferential exterior collagen sheets and is an essential constituent of the intervertebral joint. The AF is comprised of approximately 15-25 collagen sheets (lamellae), per IVD, which encompasses the NP. These lamellae are comprised of type I collagen fibers that are aligned in alternating patterns of ± 30 degrees to the horizontal axis of the spine. The AF can be divided into three layers: peripheral, intermediate, and inner regions. The annulus fibers are found to be thicker and more numerous anteriorly than posteriorly while the posterior fibers illustrate a more parallel
alignment. The bundles of fibrous cartilage (multi-layers) run obliquely from one vertebral body to the next to anchor the IVD to the cartilaginous endplates.

The hyaline cartilaginous endplates allow for nutrient supply to the IVD. Since IVD’s are avascular structures, they are dependent on the endplates for nutrient diffusion. By the age of eight, the cartilaginous endplates lose their blood supply, leaving holes from blood vessels that allow for the diffusion of metabolites. These holes which are essential for the supply of nutrients also cause structural deficiencies (weakness) to the endplate structure.

**Clinical Significance of Intervertebral Disc Diseases**

In a clinical setting, two relevant pathologies directly affect the IVD: IVD herniation (IVDH) and IVD degeneration (IVDD). An IVDH is referred to as a “herniated disc”, “bulging disc”, or “slipped disc”. IVDH as a consequence of traumatic (quick jolting motion), typically results in painful circumstances for the patient via nerve root impingement. As an IVDH begins to form, the AF begins to degrade and rupture progress radially through its layers. This results in a loss of IVD height, a decrease in IDP, and in many cases contributes significantly to LBP. This defect provides a pathway for the NP to bulge or migrate beyond the confines of the AF layers that can apply pressure to a nearby nerve within the spinal canal, “pinched nerve.” The applied pressure leads to irritation of the nerve which depending on the level of intensity may lead to a radicular pair. Radicular pain is a pain that radiates to the extremities of the body, i.e. arm and legs, which travels directly along the course of a spinal nerve root. A “pinched nerve” may lead to symptoms of dull/sharp pain in the lower back, muscle spasms/cramping, sciatica, leg weakness,
and/or loss of leg function. Although it is important to express, not all patients who develop an IVD herniation experience symptoms of pain.

Figure 2: Stages of Disc Herniation (IVDH)

There are four stages of IVD herniation as seen in Figure 2: Stage 1) Annular tear: IVD is intact, but a small tear in the outer layer (without herniation), Stage 2) Prolapse: the form of position of the IVD changes with some slight impingement into the spinal canal (i.e. bulge or protrusion), Stage 3) Extrusion: the gelatinous core of the NP breaks through the collagen sheets of the AF but remains within the IVD, Stage 4) Sequestration/Sequestered Disc: the NP breaks through the AF and lies outside the IVD in the spinal canal. The most frequently affected IVDs via IVDH are IVDs with the most substantial range of motion and/or axial loading forces: L3-4, L4-5, L5-S1.

IVDD is a prevalent musculoskeletal disease seen through the aging population. IVDD occurs via a multifactorial process that results in biochemical degradation of NP tissue, a decrease in IVD height, a decrease in IDP, and loss of mechanical function. IVDD is linked to multiple factors in the initiation and progression of the disease: aging, poor
nutrient supply, loading conditions, and hereditary factors.\textsuperscript{32–38} Consistent IDP changes of the NP result in assuage of the posterior AF. This is identified as part of the aging process after 45 years of age, thus resulting in total annular lamellae disorganization by the age of 80.\textsuperscript{39,40} A research study showed through magnetic resonance imaging (MRI) 20\% of patients tested over 45 years of age have an IVD bulge or IVDD, and nearly 60\% of patients over 65 years of age had IVDD.\textsuperscript{41}

IVDD is a loss of both hydraulic and viscoelastic properties of the IVD through 1) a decrease in the water content and, therefore, a reduction of the preloading effect of the NP, 2) a decrease in the elastic collagen tissue in the annulus with replacement of large fibrous inelastic bands, and 3) cartilage degeneration in the endplates.\textsuperscript{29} IVDD is characterized by changes identified in the cellular microenvironment, loss of proteoglycan, loss of IVD height, tears of AF tissue, spinal stenosis, herniated discs, neoinnervation, hypermobility, and inflammation.\textsuperscript{42–45}

Degeneration is diagnosed on a grading scale of 0-4 of the IVD (\textbf{Figure 3}) and can be referred to the degeneration of the facet joint: Grade 0) no changes in the physiological IVD, Grade 1) joint space narrowing, Grade 2) narrowing in addition to sclerosis or hypertrophy, Grade 3) severe osteoarthritis in addition to beginning narrowing, sclerosis, and osteophytes and Grade 4) advanced osteoarthritis in conjunction with hypertrophy, narrowing, sclerosis, and osteophytes.\textsuperscript{46}
The level of degeneration has a correlating effect on the amount of IDP observed by the IVD. The mean IDP showed a statistically significant difference between each grade of degeneration, with IDP reduced with increasing levels of degeneration. The IDP in the L4-L5 IVD measured in a patient with a grade 0 degeneration presented a mean of 0.091 [MPa] while a patient with a grade 4 degeneration displayed a mean IDP of 0.010 [MPa].

**Current Treatments of Intervertebral Disc Diseases**

Treatment plans for LBP are dependent on the patient’s history and the type/severity of pain. Until recently, clinicians believed pain due to herniation would resolve on its own without surgical intervention (i.e. spontaneous resorption of the herniation). However, despite a temporary reduction in pain, a recent study showed that when herniation’s are not treated, a resurgence of the pain does in fact occur. The study investigated the course of LBP in a general population of 2000 people aged 30-50 years over a five-year period. The study found that for more than 33% of people who experienced
LBP, the pain lasted for >30 days, and out of these 33% of patients, only 9% were pain-free five years later.49

The treatment method utilized by a physician is often dependent on the type of injury of the patient, with IVDD and IVDH requiring different treatment approaches. IVDH treatments typically begin with bed rest, NSAIDs, and pain medications. Sometimes physical therapy (PT) may also be prescribed to improve the patients’ symptoms. However, if symptoms persist, epidural injections of corticosteroids to reduce the nerve irritation and facilitate healing may be assessed. If a patient is not responding to 6-12 weeks of conservative conventional treatments, surgical intervention is often considered. For patients suffering from LBP, a microdiscectomy, and/or laminectomy may be performed. Within the United States, (U.S.) lumbar discectomy is the most frequently completed procedure to repair an IVDH, with over 300,000 operations performed annually,50 with an associated direct cost of approximately $3,445 per case.51

The surgical approach to a discectomy procedure, “minimal or aggressive,” is based on the surgeons’ personal preference. Both techniques have favorable and unfavorable results, pros and cons. The objective of the surgical procedure, to be considered a complete success, provides the maintenance of the patients’ IVD height and experiences low reoccurrences of IVDH. By maintaining the patient’s IVD height, this provides better patient outcomes. A “minimal” discectomy maintains the IVD height but is at an increased risk of reherniation. While an “aggressive” discectomy reduces the reoccurrence of herniation’s, it is linked with IVD height collapse.52-54 In both surgical approaches, there is the partial removal of the protruding herniated NP tissue and decompression of the neural
elements. During the procedure, a laminotomy/laminectomy, a small piece of the vertebrae removed, may need to be performed to provide access to the herniated fragments. This laminotomy permits a more advantageous visualization for the surgeon of the herniated fragments of the IVD. Consequently, by removing the herniated IVD fragments via discectomy, an open pathway (defect) remains through the layers AF, which provides a path of least resistance for recurrent herniation’s to occur. Regardless of surgical approach, an average IVD height loss of 25% has been reported after a discectomy, which is associated with increased back pain and disability.\textsuperscript{50} Various studies have shown that reoperation rates range from 9-25% at 4 and 10 years post-operatively,\textsuperscript{55–59} which results in direct costs ranging from $2.5k for conservative management to 35k for surgical re-intervention, respectively per case.\textsuperscript{60,61} It is imperative to express, current discectomy procedures are not directed to treat the damaged IVD, but rather to alleviate patients’ symptoms that may result in further aggravation of existing damage.\textsuperscript{10,62,63} Since discectomies are still the most performed spinal surgical procedure worldwide and mainly affects the employed population, the resulting socio-economic consequences are significant.\textsuperscript{64}

Alternatively, IVDD is often managed with over-the-counter pain medications (i.e. NSAIDS) and applying ice/heat to the affected area. More aggressive treatments include physical therapy (PT), chiropractic manipulation, electrical stimulation, massage, and last surgery. With surgical interventions, surgical treatments of IVDD are focused on treating symptoms experienced by the patient rather than restoring native structure and function.
Currently, two IVDD surgical procedures are performed: spinal fusion and total disc replacement (TDR). Both procedures have an objective to preserve the IVD height as seen in Figure 4. The traditional procedure, often considered as the “gold standard” surgical treatment, for an IVDD, is the generation of a bony fusion between two vertebral bodies to reduce spinal instability and minimize pain. Shortcomings of a fusion procedure include adjacent segment degeneration and other complications; thus, it is typically employed as a last resort option. Spinal fusions are approached from either the posterior or anterior position. While historically the posterior approach has been used, the anterior approach allows the surgeon to leave an IVD space that more likely will result in the patient having greater pain relief. The cost of spinal fusion typically ranges between $80k to $150k per case; a significantly greater cost as compared to a discectomy procedure.

Figure 4: IVDD End-Stage Devices Used for Surgical Repair. Left) Lumbar Fusion Device. Right) Lumbar Artificial Disc Replacement
The more contemporary surgical procedure, TDR, consists of the removal of the damaged IVD, but maintenance of motion is achieved using a prosthetic implant. However, during a TDR only one of the three joints of the vertebra is replaced, thus limiting the range of motion. Long-term clinical data is still needed to validate the efficacy of TDR. Due to adjacent segment disorders are seen with spinal fusions and lifetime of TDR devices revision surgery is an important factor that must be addressed with these clinical procedures. Clinical outcomes have shown that patients undergoing revision fusion surgery exhibited significantly higher rates of unfavorable discharge, prolonged length of stay at the hospital, high-end hospital charges, neurologic complications, pulmonary embolism, wound infections, wound complications, and gastrointestinal and respiratory complications.

**Repair of the IVD**

With the short-term lifespan of mechanically assisted devices and limitations of patient’s mobility, repair and/or regeneration of the IVD is needed to restore it to its healthy native state. A functional AF must be capable of withstanding the mechanical static and dynamic tensile, burst, and impact loading saw in a healthy IVD. To accomplish this ideology, it is advantageous and obligatory to have an intact AF to allow for native physiological and functional capabilities.

Current surgical procedures for IVDH and IVDD are palliative and suffer from drawbacks. Depending on the amount of herniated material removed, patients undergoing discectomy can be at an increased risk for re-herniation. Treatments for IVDD, as stated
above, are end-stage surgical interventions that could benefit from an early stage solution, such as nucleus arthroplasty. Current research is being conducted on developing an artificial NP, which would be used for replacing the degenerated native material; however, the defect in the AF would remain after implantation and migration, respectively. Therefore, in both clinical scenarios, it would be advantageous and obligatory to have an intact AF to prevent re-herniation. Accompanying techniques that treat the damaged AF are now increasingly recognized as mandatory to prevent re-herniation, to increase the potential of NP repair, and to confine NP replacement therapies that target early-stage IVDD and IVDH. These shortcomings and drawbacks of current surgical procedures constitute a need in which the tissue engineering discipline may be applied to repair the native IVD.

**Annulus Fibrosus Structure**

The AF is comprised of circumferential exterior sheets (lamellae) comprised of collagen type I and is an essential constituent of the intervertebral joint. The AF is divided into three layers: peripheral, intermediate, and inner segments. The annulus fibers are found to be thicker and more numerous anteriorly than posteriorly while the posterior fibers illustrate a more parallel alignment. The bundles of fibrous cartilage (multi-layers) run obliquely from one vertebral body to the next to anchor the IVD to the cartilaginous endplates. The formation of the AF begins at the mesoderm germ layer, one of the three primary germ layers in the early stages of embryonic development. The middle embryological germ layer, mesoderm, forms the mesenchyme that during embryogenesis develops the AF.
The composition of the AF is comprised of four main elements: water, collagen, proteoglycans, and non-collagenous proteins. The physiological classification by percentage of these elements is as follows: water (65-90%), collagen (50-70% dry weight), proteoglycans (10-20% dry weight), and non-collagenous proteins (i.e. elastin) (5-25%).\textsuperscript{73–75} Within the internal portions of the AF, the layers are less hydrated than the NP and more widely dispersed in comparison to the exterior region of the AF.\textsuperscript{76} The interior AF also consists of a more substantial amount of GAGs in comparison to the exterior region of the AF: aggrecan and versican.\textsuperscript{77–81} Small proteoglycans are found in greater amounts in the internal region: biglycan, decorin, fibromodulin, and lumican.\textsuperscript{82–84} The distribution of type I and II collagen also fluctuate based on location within the AF. The exterior lamellae consist purely of type I collagen, but as you move interiorly toward to the NP, overall type II collagen percentage increases as type I collagen percentage decreases. The internal AF is mainly composed of type II collagen, although other collagen types such as III, V, VI, IX, and XI were detected within the inner AF.\textsuperscript{85}

The highly organized structure of the AF results in a complex anisotropic behavior, with tensile, compressive, and shear properties differing in the axial, circumferential, and radial directions.\textsuperscript{74,86,87} The AF is comprised of collagen sheets (lamellae) with approximately 15-25 lamellae, per IVD,\textsuperscript{27} which the fibers are aligned in alternating patterns of $\pm 30^\circ$ to the horizontal axis of the spine, as seen in Figure 5.\textsuperscript{28}
Figure 5: Annulus Fibrosus Lamellae. Arranged in alternating patterns between subsequent layers.\textsuperscript{88}

The composition of the lamellae is filled with proteoglycans and interspersed with elastin fibers.\textsuperscript{89} In the exterior AF, long elastic fibers are present within the lamellae, running parallel to each other in the same direction as the collagen sheets. However, within the internal AF, the elastic fibers are present between adjacent lamellae as well as an increased regular organization within the lamellae.\textsuperscript{90} The spaces located between the separate layers of the AF are called interlamellar septae, and they contain proteoglycan aggregates, and a complex structure of linking elements creating interlamellar cohesion.\textsuperscript{73,91,92} The ends of the collagen sheets run obliquely from one intervertebral body to the next to attach/secure the IVD to the endplates.
Mechanical Properties of Native Annulus Fibrosus

Mechanically, the interior AF region is subjected to higher IDP generated from the NP as opposed to higher tensile forces exerted on the exterior region. Together these fiber networks couple adjacent lamellae together allowing them to work co-operatively during dynamic loading and prevent separation of lamellae during torsional compressive loading.

The posterolateral location of the AF experiences the highest frequency of layer interruption from various mechanical forces. This region is also where the highest stresses are observed during loading, and where annular tears, fissures, protrusions, extrusion, and/or sequestrations are known to develop. Table 1 illustrates the mechanical characteristics of the native AF.
Table 1: Mechanical Characteristics of the Native AF Tissue

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions L4/L5 IVD</td>
<td>IVD Width: 30.4 ± 4.5 mm\textsuperscript{100}</td>
</tr>
<tr>
<td></td>
<td>NP Width: 19.3 ± 2.9 mm\textsuperscript{100}</td>
</tr>
<tr>
<td></td>
<td>Anterior AF: 20.5% (total IVD)\textsuperscript{100}</td>
</tr>
<tr>
<td></td>
<td>Posterior AF: 15.6% (total IVD)\textsuperscript{100}</td>
</tr>
<tr>
<td>Burst Strength</td>
<td>Slit (6 mm vertical): 0.93 [MPa]\textsuperscript{16}</td>
</tr>
<tr>
<td></td>
<td>Circular Opening (3 mm): 0.64 [MPa]\textsuperscript{16}</td>
</tr>
<tr>
<td>Ultimate Tensile Strength</td>
<td>Anterior: 1.7 ± 0.8 [MPa]\textsuperscript{101}</td>
</tr>
<tr>
<td></td>
<td>Posterior: 3.8 ± 1.9 [MPa]\textsuperscript{101}</td>
</tr>
<tr>
<td>Elastic Modulus</td>
<td>Anterior: 7.2 ± 3.1 [MPa]\textsuperscript{101}</td>
</tr>
<tr>
<td></td>
<td>Posterior: 27.2 ± 10.2 [MPa]\textsuperscript{101}</td>
</tr>
<tr>
<td></td>
<td>Overall 12-24 [MPa]\textsuperscript{102}</td>
</tr>
<tr>
<td>Tensile Fatigue</td>
<td>1043 cycles at 2.3 [MPa]\textsuperscript{101}</td>
</tr>
<tr>
<td></td>
<td>10,000 cycles at 1.7 [MPa]\textsuperscript{101}</td>
</tr>
</tbody>
</table>

**Healing Potential of the Annulus Fibrosus**

To unlock the potential for AF regeneration, the native healing potential needs to be addressed. The intrinsic capacity of the AF to cope with damage or degenerative pathological changes have been studied in several animal trials\textsuperscript{103–112} Key and Ford studied
the healing capacity of three different types of posterior annulus lesions in a canine model: square annular window, transverse incision, and puncture with 20 gauge needle (0.91 mm). During post-operative procedures, the lesions were initially filled with extravasated blood, fibrin, bone, and cartilage debris. These were gradually replaced by a thin layer of fibrous tissue at later time points (up to 22 weeks). While the larger damaging lesions (window and incision lesion groups) developed slowly towards IVD protrusion, the lesions that were generated by needle puncture revealed nothing irregular and the site of puncture was unidentifiable after 22 weeks. This study was contradicted however using rabbit IVDs as an organ culture model. The needle puncture showed immediate and progressive mechanical and biological consequences that could lead to degenerative modeling if longer time points were observed.

Smith identified the healing process of the AF occurs in three different phases. In the 1st phase, the outer AF begins to heal. This is caused by a proliferative reaction in the fibrous tissue spreading from the lateral parts of the wound to the medial parts. During the 2nd phase changes occur in the inner annular fibers. The lateral parts of the interior AF layers gradually heal by a slow appositional spread in the medial direction. The process normally begins after a few weeks but can last up to one-year post-operatively. During the 3rd phase, an increase in the number of collagenous fibers found within the NP tissue, which has remained in the AF wound tract, become increasingly dense.

Although many hypotheses have been formed on what leads to the limited intrinsic healing capability of the AF, many contributing factors remain unknown. One of those reasons may be the fact that exterior repairs are not matched, or insulated to the demands
of the progressive recruitment of fibers to a tensile force.\textsuperscript{114,115} Nevertheless, no matter the underlying reason of why the limited intrinsic healing capability of the native AF negatively correlates with the success rates of discectomies and NP replacement therapies.\textsuperscript{71}

\textbf{Annulus Fibrosus Closure Techniques}

\textbf{Commercially Available Products}

With tissue engineering, medical devices still in the primitive stage, mechanical closure of the AF is the most effective closure technique currently available on the market. It has been proposed that improved annular closure procedures may reduce IVD re-herniation and reduce the need for spinal fusion. The most straight-forward solution is per operative suturing of the AF defect. However, suturing does not account for missing AF tissue and may not be strong enough to resist changes of the IDP generated by the NP tissue under different loading regimes. Efficacy and efficiency of suturing the AF have been studied by Ahlgren et al. in an animal study of an ovine model. It was reported that sutured IVDs showed a tendency towards stronger healing, but the results were not statistically significant.\textsuperscript{103} As the investigation of an AF closure device continues, Johannes Bron states, the clinical durability the eventual arbiter of technological value.\textsuperscript{71}

A diversity of medical devices has been designed or are currently being studied to preserve, repair, and reinforce the AF. The following devices are currently marketed for the proposed use in AF closure after a discectomy to reduce the risk of re-herniation. Anulex Technologies, Inc. has developed two devices: Xclose Tissue Repair System and Inclose Surgical Mesh System. Intrinsic Therapeutics developed the Barricaid Annular
Reconstruction Device. Lastly, Magellan Spine Technologies, Inc. has developed the Disc Annular Repair Technology (DART) System. Table 2 illustrates a comparison of AF repair devices currently available on the market.

Table 2: Comparison of AF Repair Devices Currently Available on the Market

<table>
<thead>
<tr>
<th>Competitors</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Barricaid Annulus Closure Device</strong></td>
<td>Polyethylene Terephthalate and Titanium Bone Anchor</td>
</tr>
<tr>
<td><strong>Xclose Suturing System</strong></td>
<td>Polyethylene Terephthalate</td>
</tr>
<tr>
<td><strong>Inclose Surgical Mesh</strong></td>
<td>Polyethylene Terephthalate</td>
</tr>
<tr>
<td><strong>Suture</strong></td>
<td>Nylon, Polyester, PVDF, and Polypropylene.</td>
</tr>
<tr>
<td><strong>Disc Annular Repair Technology (DART) System</strong></td>
<td>Polyetheretherketone (PEEK)</td>
</tr>
</tbody>
</table>

The Xclose Tissue Repair System received a 510(k) clearance on August 7th, 2006. The Xclose system consists of non-absorbable, synthetic, poly(ethylene terephthalate) surgical sutures modified with toggle anchors. The FDA labeled indications state the system is used for soft tissue approximation in general and orthopedic surgery procedures. The use of an AF repair was later deemed as outside the scope of the 510(k) clearance. The FDA required the manufacturer to submit a premarket approval application (PMA), in conjunction with clinical data from an investigational device exemption (IDE) study. Xclose and Inclose implants are now commercially available for annuloplasty and can be seen as modified sutures with anchors.9,11
The Inclose Surgical Mesh System received FDA 510(k) clearance on August 18, 2005. The surgical mesh is comprised of polyethylene terephthalate (PET) monofilament expandable braided material that is preloaded on a disposable delivery tool inserted through the defect of the tissue and affixed to surrounding soft tissue with the anchor bands (non-absorbable sutures). It is proposed as an alternative procedure for annular repair, following a discectomy, to re-approximate the compromised tissue of the AF.

These devices, however, do not address the underlying issue of using sutures to close the AF. Also, sutures and these devices are fully directed to the containment of the NP, but they do not compensate for the loss of annulus material nor reverse the biomechanical changes that have occurred in the damaged AF.

The Barricaid Device received the CE Mark approval for marketing in Europe in April 2009. It has thus far yet to receive FDA 510(k) clearance for marketing in the U.S. The Barricaid consists of a polytetrafluoroethylene (ePTFE) woven mesh supported by a titanium bone anchor. The function of the device is to form a mechanical barrier that closes the annular defect. The device is intended for use in individuals with primary IVDH with large annular defects. It is a commercially available implant used in adjunction to discectomies that fully bridge the defect in the AF.12 According to the manufacturers’ website, “the Barricaid enables surgeons to directly reconstruct the annulus in the region of the herniation.”

The DART System received CE Mark approval for marketing in Europe in April 2009. It has yet to receive FDA 510(k) clearance for marketing in the U.S. The device consists of a polyetheretherketone (PEEK) implant that provides closure of the AF. When
implanted, it is placed near the central axis of rotation along the posterior edge of the vertebral body. It is secured in place by the apophyseal ring along the vertebral body load column.

**Developing Scaffolds for Annulus Fibrosus Tissue Engineering**

Ideal AF devices used for tissue engineering is to achieve both immediate mechanical stability and to allow regeneration of native tissue over time. Currently, there is a missing link between addressing AF closure techniques and regenerative strategies. Closure techniques primarily focus on restoration of the mechanical integrity of the AF, while regenerative therapies target the engineering of healthy and functional AF tissue. Furthermore, closure techniques offer the clear implantation and fixation strategy; however, regenerative therapies lack the strategies for implantation and fixation that limits their clinical application.\(^7\)

To develop a suitable scaffold for tissue engineering, certain general principles have to be addressed including, biocompatibility, biodegradability, and method of delivery.\(^1\) On the microscale specific requirements for AF scaffolds include: fill and/or repair the AF defect to contain the NP, allow fixation to the surrounding structures (i.e. endplates and/or surrounding AF tissue), allow AF cells (AFCs) (or stem cells) to survive (differentiate/proliferate), synthesize and secrete the native ECM, have the characteristic anisotropic behavior to maintain/restore the mechanical properties of a spinal motion segment, and not irritate or adhere to the perineurium of the spinal cord.\(^7\) Table 3 highlights the difficulties and challenges in tissue engineering seen within the cells, scaffolds, anatomy, and physiology.
Table 3: Difficulties and Challenges in Tissue Engineering of the AF Reported by Sharifi et. al.\textsuperscript{3}

| Sources of Cells | • Limited Sources of human cells due to the unavailability of healthy tissue  
|                 | • Biopsies taken from healthy AF do not contain sufficient cells  
|                 | • Risk of damage to AF during the biopsy  
|                 | • Difficulty distinguishing inner and outer AFCs  
|                 | • Difficulties in cell culturing. Loss of cell phenotype in 2D cell culture and the requirement for specific media and culture conditions  
|                 | • Lack of suitable cell markers for AFCs  
|                 | • Poor survival of transplanted cells  
| Tissue-Engineering Scaffolds | • Requirement of anisotropic physical and mechanical characteristics mimicking the healthy AF  
|                           | • Requirements change with the extent of IVD degeneration  
|                           | • Limited integration with native AF tissue  
|                           | • Confirmation to the site of implantation that hinders implantation and restricts implant geometry  

Overall formation of a scaffold is summarized into two classifications: single composition (oriented or non-oriented to mimic the organized native lamellae) or gradient composition to simulate internal and external layers of the AF. A gradient structure would have an external layer enriched in collagen type I, and an internal layer comprised of more collagen type II to mimic the native structure of the AF. Several other novel sutures, sealants, and barrier techniques are currently being developed, resulting in an increasing attention at scientific workshops and conferences.8–15
Potential Cell Sources for Annulus Fibrosus Scaffolds

In the current research, as seen in Table 4, the cells used in conjunction with the mechanical scaffolds include both stem cells and native AFCs. Within the native human AF, mature subjects have a cell density of $\sim 9 \times 10^6$ cells/cm$^3$, which is two times greater than the NP. A recent study showed that cells derived from the human AF were able to differentiate into the chondrogenic and adipogenic lineages. This suggests that cells in the AF could be skeletal progenitor cells that could be recruited under pathologic conditions such as herniation. Otherwise, progenitor cells from surrounding tissue might perhaps be capable of migrating into the IVD. However, isolation of the cells retrieved from a human discectomy procedure typically does not allow for division between internal and external AFCs.

Table 4: Cell Types Used for AF Repair in Current Research for AF Repair

<table>
<thead>
<tr>
<th>Type of Cells</th>
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</thead>
<tbody>
<tr>
<td>Porcine Annulus Fibrosus Cells</td>
</tr>
<tr>
<td>Mesenchymal Stromal Cells (MSCs)</td>
</tr>
<tr>
<td>Human Annulus Fibrosus Cells</td>
</tr>
<tr>
<td>Bovine Annulus Fibrosus Cells</td>
</tr>
<tr>
<td>Porcine chondrocytes</td>
</tr>
<tr>
<td>Rabbit Bone Marrow-Derived MSCs (BMSCs)</td>
</tr>
<tr>
<td>Rabbit Chondrocytes</td>
</tr>
</tbody>
</table>
Investigated Scaffold Development

Abundant varieties of scaffolds have been investigated for AF tissue engineering in academic laboratories. With tissue engineering being a new scope of the study and associated high development costs, AF scaffolds are being developed mostly in the academic field. Researchers continue to try and create a suitable AF replacement. Table 5 lists current materials being used with the hope of mimicking either the native AF structure and/or function.

Table 5: List of Scaffolds Investigated for AF Repair

<table>
<thead>
<tr>
<th>Type of Scaffolds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodegradable Glue (HDI-TMC1-PEG200-TMC1-HDI tissue glue)</td>
</tr>
<tr>
<td>Alginate-based collagen shape-memory composite scaffolds</td>
</tr>
<tr>
<td>Poly(trimethylene carbonate) (PTMC) scaffolds with an elastic poly(ester-urethane) (PU) membrane</td>
</tr>
<tr>
<td>Electrospun biologic laminates</td>
</tr>
<tr>
<td>Biodegradable shape-memory polymer network prepared by photo-crosslinking poly(D,L-lactide-CO-trimethylene carbonate) dimethacrylate macromers</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Expandable scaffold-free neo-construct</td>
</tr>
<tr>
<td>Bioactive poly(L-lactide) (PLLA/TGF) electrospun scaffold</td>
</tr>
<tr>
<td>Biphasic whole IVD using silk protein for the AF and fibrin/hyaluronic acid gel for the NP</td>
</tr>
<tr>
<td>Biphasic IVD by electrospinning using PCL as the AF and agarose as the NP</td>
</tr>
<tr>
<td>Biphasic whole IVD using collagen 1 for the AF and alginate for the NP</td>
</tr>
<tr>
<td>Bone marrow-derived MSC sheets and silk scaffolds</td>
</tr>
<tr>
<td>Biphasic scaffold using demineralized bone matrix gelatin (BMG) as the AF and elastic material based on poly(polycaprolactone triol malate) (PPCLM) as the NP</td>
</tr>
<tr>
<td>Porous silk scaffolds</td>
</tr>
<tr>
<td>PDLLA (poly(D,L-lactide)/Bioglass composite foam scaffold</td>
</tr>
<tr>
<td>Alginate/chitosan hybrid fiber scaffold</td>
</tr>
<tr>
<td>Oriented electrospun nanofibrous scaffolds</td>
</tr>
<tr>
<td>Biodegradable poly(1,8 octanediol malate) (POM) scaffold</td>
</tr>
<tr>
<td>Polyglycolic acid (PGA) and calcium alginate mesh coated with 1.5% w/v polylactic acid</td>
</tr>
<tr>
<td>Atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold)</td>
</tr>
</tbody>
</table>

In 2015, Vergroesen et al. published a research study on a biodegradable glue for annulus closure. The aim of the study was to investigate the feasibility and biomechanical
properties of biodegradable glue for AF repair, using non-degenerated caprine IVDs. The glue consisted of a biodegradable HDI-TMC1-PEG200-TMC1-HDI tissue glue. The ultimate strength and endurance tests were performed using native and punctured IVDs as positive vs. negative controls. Results demonstrated that the biodegradable glue was able to provide a reduction in the risk of herniation; however, the majority of the IVDs (n=9/11) failed by nucleus protrusion. Before protrusion of the AF occurred, the glue demonstrated a trend toward the partial restoration of the native AF ultimate strength. Results of the study showed: 1) the glue partially restores annulus integrity after a puncture, 2) increases the amount of force required to cause nucleus protrusion through the AF puncture occurs, and 3) limits the number of herniations during ambulatory loading and ultimate strength tests. The use of biodegradable glue for AF repair provides a low-cost method, but the efficacy would need to be evaluated in long-term studies.127

In 2015, Guillaume et al. published a research study on enhancing AF cell migration in shape-memory alginate collagen composite scaffolds through in vitro and ex vivo assessment for IVD repair. For this study, collagen was incorporated into alginate-based shape-memory scaffolds. Addressed were the advantages of using alginate or collagen independently, in producing mechanical stability, injectability, and biological activity. In vitro studies demonstrated that performed alginate scaffolds promoted AF cell penetration and preserved the natural cell phenotype. Also, porcine AF and MSC-seeded scaffolds, with the incorporation of collagen, showed to have a beneficial effect on cell proliferation and ECM deposition. The matrix secretion was amplified by the local release of TGF-β3 growth factor compared to those of non-supplemented media. During ex vivo experiments,
collagen-enriched scaffolds maintained MSC viability and allowed for attachment of endogenous AFCs from the surrounding tissue and showed infiltration into the 3D constructs compared to alginate only scaffolds.128

In 2014, Sharifi et al. published a research study on the treatment of the degenerated IVD through closure, repair, and regeneration of the AF. With cell-based therapies to regenerate degenerated IVDs, mesenchymal stromal cells (MSCs) are more readily available in contrast to differentiated autologous or allogenic cells. One of the difficulties in cell therapy is most transplanted cells will not survive the ischemic conditions immediately after implantation. It was previously demonstrated with transplanted MSCs, 80-90% died within five days after implantation in an osteochondral defect via rabbits, thus using MSCs for IVD treatment seems imprudent.129 Further addressed were the advantages and disadvantages of cell types for direct transplantation into the degenerated IVD. Autologous NP or AFCs do not initiate an immune response, although they are not available in sufficient amounts, and healthy state, their phenotype change upon expansion in monolayer culture, and additional surgery are required to obtain NP or AFCs with the risk of causing further IVDD upon harvesting. Allogeneic NP or AFCs do not initiate an immune response, and healthy cells are available; although, there is a limited availability of allogeneic human NP or AFCs, and there is a risk of IVDD upon harvesting. Autologous stem cells elicit no immune response and are available in sufficient amounts; however, there presents a lack of definitive phenotype markers for NP or AFCs. Allogenic stem cells do not elicit an immune response and have “off the shelf” availability; although, they also
lack definitive phenotype markers for NP or AFCs. Lastly, chondrocytes are available in sufficient amounts, but they have a different phenotype compared to NP or AFCs.\(^3\)

In 2014, Pirvu, et al. published a study that included a combined biomaterial and cellular approach for AF rupture repair. The study utilized poly(trimethylene carbonate) (PTMC) scaffolds seeded with bone marrow-derived MSCs in a combination with an elastic poly(ester-urethane) (PU) membrane. The mechanical properties of the PU membrane showed an ultimate strength of 53.0 +/- 2.0 MPa, and a yield strength of 4.9 +/- 1.4 MPa. The surfaces and cross section showed the absence of large pores and minor amounts of non-connected micro-porosity. Through testing, it was demonstrated the PTMC implant combined with the sutured PU membrane restored IVD height of annulotomized IVDs and prevented herniation of NP tissue into the AF defect. In comparison, press-fitted implants without closure were not able to withstand dynamic loading and were pressed out of the defect immediately after starting loading cycles. The PU membrane sutured onto the AF tissue was used to cover the scaffold and seal the defect. After 14 days of repetitive dynamic load, the sutured PU membrane retained the PTMC scaffold within the AF defect, and no herniation of the NP was observed. Through cellular studies, MSCs showed the potential of positively modulating cell phenotype of native IVD tissue by up-regulating anabolic mechanisms and downregulating catabolic mechanisms in host IVD cells. MSCs were implanted into the AF defect by first embedding in fibrin hydrogel. After 14 days of culture, \textit{in situ}, with repetitive dynamic load, the implanted MSCs remained within the PTMC scaffolds. This suggests that implanted MSCs can adapt their phenotype during culture within IVDs.\(^{130}\)
In 2013, Guillaume et al. published a research study on shape-memory porous alginate scaffolds for regeneration of the annulus fibrosus and the effect of TGF-β3 supplementation and oxygen culture conditions. The porous shape-memory alginate scaffolds were structurally based on covalently cross-linked alginate. Manual manipulation of the scaffolds exhibited shape-memory capability suitable for delivery in AF defects of the IVD through minimally invasive approaches. The scaffold was developed as a support template for AF cell proliferation and tissue formation. In vitro testing demonstrated cytocompatibility when seeded with porcine AFCs and supported cell penetration, proliferation, and ECM deposition when cultured in IVD like micro-environmental conditions (low oxygen and low glucose concentrations) with TGF-β3 media supplementation. With TGF-β3, after three weeks of culture sulfated GAG (sGAG) and collagen type I were detected throughout the entire porous network of the alginate scaffold. Without TGF-β3, cells agglomerated in small clusters with limited proliferation which resulted in poor ECM deposition. It was also stated that cells did not appear to attach directly to the alginate structure itself. Guillaume believed this to be due to the low cell adhesion properties of alginate, and to mimic the natural orientation of collagen fibers in engineering scaffolds, various fabrication methods and specific architectures have been investigated previously. These structures could potentially be incorporated into the alginate solution before cross-linking to create a composite alginate-based porous scaffold with aligned fibers.\textsuperscript{131}

In 2013, Driscoll et al. published a research study on biaxial mechanics and inter-lamellar shearing of stem cell-seeded electrospun angle-ply laminates for AF tissue
engineering. The study focused on electrospun biologic laminates reaching biaxial properties of the native tissue. It was found that the properties of the opposing (± 30°) constructs were significantly higher than the aligned constructs despite showing similar fiber stretch ratios. This could indicate that a lamellar structure with native tissue fiber orientation can provide higher stiffness than a single aligned fiber population, due to biaxial boundary conditions. It is stated that the use of biaxial testing is necessary for an appreciation of this difference since previous uniaxial tensile testing showed single lamellar 0° constructs to be about twice as stiff as opposing constructs. This provides further evidence that a lamellar structure with two alternating or angle-ply fiber populations is an important design criterion for both native tissue and a mechanically functional engineered AF. The limitation of the electrospun angle-ply laminates was found to be the strain levels. Native AF function under large strains without failing and typical have highly nonlinear mechanics with large toe regions. The electrospun biologic laminates displayed a nonlinear response to strain, although at a lesser extent and with a smaller toe region than the native tissue.  

In 2013, Sharifi et. al. published a research study on an AF closure device based on a biodegradable shape-memory polymer network. The biodegradable elastic polymer network was prepared by photo-crosslinking poly(D,L-lactide-CO-trimethylene carbonate) dimethacrylate macromers. The mechanical properties of the scaffold were reported in the range of native human AF tissue for tensile strength, elastic modulus, and elongation at break. The cell culture of human AFCs (HAFCs) showed good cell adhesion and proliferation. Cell culturing studies were evaluated on network specimens that were either
pre-coated or not-coated with fibronectin. Fibronectin was utilized because it is previously reported as an ECM protein that improves cellular adhesion on a polymer surface. The feasibility of the shape-memory device was evaluated using a cadaveric canine model. Upon insertion into the IVD, the device had begun to deploy. It was reported that the network samples had a shape-recovery rate of 9.3% per minute when heated at a rate of 2°C per min. After overnight incubation at 37°C, the IVD was cross-sectioned. It was found that the implant recovery to its permanent shape was not complete. This was believed to be due to the shape-recovery force of 1 MPa not being larger than the force exerted by the surrounding AF tissue on the implant. Also, through manually applied flexion and extension forces, and axial rotations the implants dislodged from their original positions. Thus, it is necessary for an improvement in the design of the implant to increase the mechanical properties needed to withstand native forces.133

In 2013, Cho et al. published a research study on the construction of a tissue-engineered AF. In this study, they characterized an expandable scaffold-free neoconstruct using autologous AFCs. The construct was prepared from pellet cultures, derived from monolayer cultures of AFCs from mature pigs until the cells had reestablished a proteoglycan-rich cell-associated matrix. The second step consisted of the removal of the cells embedded in their own ECM products as a membrane from culture plates and subsequent maintenance as a detached explant tissue. This method consisted of using only the matrix produced by the cells themselves and does not require the use of exogenous matrices. The collagen content and biomechanical properties after three weeks of culture were similar to that found in mature porcine AF tissue. The principal advantages of these
constructs observed were the artificial materials are not required to obtain a sizable 3D AF tissue construct, and the cell distribution was uniform. This contrast could provide applications for analysis of cell behavior as it provides a well-defined 3D environment for studies of biomechanical load or growth-factor stimulation.\textsuperscript{134}

In 2012, Vadala et al. published a research study on bioactive electrospun scaffolds for AF repair and regeneration. The scaffold was synthesized by electrospinning, with a direct incorporation of TGF-β1 into the polymeric solution. The scaffold was a poly(L-lactide) (PLLA/TGF) electrospun scaffold. The electrospun scaffold consisted of a non-woven porous mesh with randomly oriented fibers. The study demonstrated a sustained release of growth factors and inducement of anabolic stimulus on bovine AFCs while mimicking the ECM three-dimensional environment of the AF tissue.\textsuperscript{135}

In 2012, Chan et al. published a research study on IVD regeneration or repair with biomaterials and stem cell therapy, and if it is feasible or fiction. They describe how commercially available implants for closing the AF reinforces the complete posterior annulus and could prevent contralateral herniation. Although, these devices are unable to maintain the biological AF structure in the long-term or stop AF degeneration. Tissue engineering methods aim to close the injured AF to prevent IVDH as well as to stop the AF from further degeneration. They also identified the previously attempted engineering approaches, such as hydrogels, alginate, PDLLA/Bioglass, silk, and electrospinning discussed further in this paper. Additionally, a whole IVD tissue engineered total disc replacement was addressed: A biphasic whole IVD using silk protein for the AF and fibrin/hyaluronic acid gel for the NP using porcine AFCs and chondrocytes respectively.
Another approach described was a fabricated biphasic IVD by electrospinning using PCL as the AF and agarose as the NP. Lastly, the last approach addressed was a biphasic whole IVD using collagen 1 for the AF and alginate for the NP. An issue they address is the major challenge of not de-differentiating cells in culture after in vitro culture. With primary cells starting to dedifferentiate when they are cultured in vitro over long passaging (p>6), strategies are needed to control the state of the cells to ensure these cells are not de-differentiated over long expansion time and remain suitable for IVD tissue engineering.136

In 2012, See et al. published a research study on simulated IVD-like assembly using bone marrow-derived MSC sheets and silk scaffolds for AF regeneration. The structure used to form the IVD-like assembly was constructed of a cylindrical silicone NP substitute. The study addressed the potential to regenerate the AF by using rabbit bone marrow-derived MSCs (BMSCs) to form cell sheets and incorporating them into silk scaffolds. The study demonstrated that BMSC cell sheets can be successfully transplanted and adhere well when combined with silk scaffolds. The cells within the assembly remained viable and showed the presence of GAGs throughout the ECM. Through immunohistochemical (IHC) staining, collagen type I and II were present and the composition of type II increased from 8% to 70% over a four-week culture. This could prove this method has the potential to regenerate ECM similar to that of the internal AF. One shortfall of the study was no specific markers or genes too are known to differentiate the IVD cells from other lineage-specific cells. However, due to the lack of specific markers, studies have only reported that the internal and external AF have a gene expression profile and ECM that resembles those synthesized by articular chondrocytes and fibroblasts, respectively.137
In 2008, Wan et al. published a research study on a biphasic scaffold for AF tissue regeneration. In this study, a biphasic scaffold was used to simulate the annulus both structurally and elastically. They describe the generation of a cylindrically shaped composite implant designed to emulate the AF. The outer phase of the scaffold consisted of demineralized bone matrix gelatin (BMG). The inner phase of the scaffold was constructed of an elastic material based on poly(polycaprolactone triol malate) (PPCLM). The BMG phase was used to increase the mechanical strength and to simulate the ligamentous structure of the outer AF. The PPCLM phase was seeded with rabbit chondrocytes that showed proliferation and ability to maintain their phenotype with a four-week culture.¹²³

In 2008, Chang et al. published a research study on enhancing AF tissue formation in porous silk scaffolds. This study was an extension to their previous publication, “Porous Silk Scaffolds to be used for Tissue Engineering.” The aim of this study was to determine whether dynamic culture and/or scaffold pore size would influence AF tissue formation and distribution in silk scaffolds in vitro. It was shown that scaffolds with an average 600 μm pore size appeared to be the best model to mimic the native AF. Scaffolds were seeded and grown in either static culture or spinner flasks (90 rpm). After two weeks, scaffolds in the dynamic culture showed greater than 3-fold increases in collagen accumulation per cell compared to static cultures, and a 1.5-fold increase per cell in proteoglycan accumulation. The tissue generated under dynamic conditions also appeared to be more cellular as the DNA content was significantly greater compared to static cultures. However, in both the static culture and spinner flasks the scaffolds had a similar pattern of tissue distribution as
more tissue was present on the outer surface, with little tissue formation on the interior of the scaffold.\textsuperscript{138}

In 2008, Helen et al. published a research study on cell viability, proliferation, and ECM production of HAFCs cultured within PDLLA/Bioglass composite foam scaffold \textit{in vitro}. The scaffolds were comprised of poly(D, L-lactide) (PDLLA)/Bioglass. The PDLLA composite foams were combined with varying percentages (0, 5, and 30 wt.\%) of Bioglass through thermally induced phase separation (TIPS) and characterized by scanning electron microscopy (SEM). HAFCs were used to determine viability, cell attachment, proliferation, and proteoglycan/collagen production. Live/dead staining showed viable HAFCs were present on the top surface of the foams as well as penetrating into the internal pore structure. SEM observations revealed larger quantities of clusters of HAFCs were attached to the pore walls to the foams with 5 and 30 wt.\% Bioglass in comparison to the 0 wt.\% Bioglass. DMMB assays showed HAFCs cultured within the PDLLA/30BG foam had a greater ability to deposit collagen and proteoglycan after four weeks of culture compared to lower wt.\% Bioglass. IHC analysis of collagen production demonstrated that collagen produced in all cultures was predominantly Type I collagen, similar to collagen found in the external AF.\textsuperscript{122}

In 2007, Chang et al. published a research study on porous silk scaffolds to be used for tissue engineering. The biomaterial used was a protein polymer made by silkworms, silk fibroin. The idea that the use of a silk scaffold would potentially degrade at a sufficiently slow rate would allow for proper tissue development and could be covalently coupled to peptides to allow for the potential of enhancing cell attachment. The results of
the study demonstrated that AFCs adhered to porous silk scaffolds and synthesized collagen and proteoglycans after attachment: with a 10-fold increase in collagen deposition and a small increase in proteoglycan content. After 24 hours, 35% cells seeded into the scaffold had attached, but only a significant increase in cellularity was observed on day 56. However, with the coupling of RGD-peptides, there was no evidence of further cell attachment, collagen accumulation, or tissue formation. RGD decoration showed to have no effect on cell seeding efficiency or morphology. The silk fibroin scaffold was found to be a suitable scaffold material for AF tissue engineering; however, it was observed that tissue growth was limited and not uniformly distributed throughout the scaffold.118

In 2007, Shao et al. published a research study on developing an alginate/chitosan hybrid fiber scaffold for AFCs. The study utilized a fibrous scaffold made of alginate or alginate/chitosan created by wet-spinning and lyophilization. Demonstrated through testing, alginate/chitosan hybrid scaffolds exhibited a slower degradation rate than pure alginate fiber scaffolds while both scaffold types did not display any cytotoxicity to 3T3 fibroblasts and could maintain canine AFC growth. Cellular studies showed AFCs retained their spherical shape within the fibrous scaffold at the beginning of the culture period and formed into cell aggregates at later time points. ECM molecules, including collagen I and II, aggrecan deposition were also detected in the AFC clusters.117

In 2007, Nerurkar et al. published a research study on the mechanics of oriented electrospun nanofibrous scaffolds for AF tissue engineering. The study utilized electrospun poly-ε-caprolactone nanofibrous polymer scaffolds. Bovine AFCs were seeded onto the scaffolds, and biochemical analysis was performed on days 1, 14, 28. The model was used
in contrast against single lamellae of the native internal and external regions of the AF. Results demonstrated elongated cells aligned along the predominant fibers direction with proteoglycan (s-GAG) and collagen content increase over the four-week culture. Uniaxial tension mechanical testing was performed on various fiber angles of the scaffold: 0°, 15°, 30°, 45°, and 90°, and showed that moduli varied nonlinearly between the different fiber angles. A nonlinear decrease in modulus was seen with increasing fiber angles.120

In 2007, Wan et al. published a research study on a novel biodegradable poly(1,8 octanediol malate) for AF regeneration. The scaffold was formed using malic acid-based polyester poly(1,8 octanediol malate) (POM), which was synthesized by direct polycondensation. Tensile strength, compressive stress, and compressive Young’s modulus of POM demonstrated an increase with the extension of the polymerization time of POM. However, degradation rates and elongations of POM decreased with extensive polymerization of POM. Rat AFCs were seeded onto the scaffold and showed that AFCs did not attach readily to the polymer after polymerization times less than two days. Rat AFCs were then cultured in monolayers for 1, 2, and 4 days. As time points increased, the proliferation of cells with stellate and elongated morphology was seen, with an increase in gene expression for aggrecan and type II collagen only seen on day 4.121

In 2007, Wan et al. published a research study on a biphasic scaffold for AF tissue regeneration. They created a biphasic elastic scaffold comprised of an inner and outer phase sections. The outer phase was a ring-shaped demineralized BMG extracted from cortical bone. The BMG was studied due to its ECM composition of mainly type I collagen, its natural biomechanical properties, and because it contains insoluble bone morphogenetic
proteins, non-collagenous proteins, and intrinsic growth factors. The inner phase was a biobiomaterial poly(polycaprolactone triol malate) (PPCLM) orientated in concentric sheets and seeded with chondrocytes. Mechanical testing was conducted on the scaffold to test for the incorporation of BGM into the PPCLM scaffold. Through testing, it was found that mechanical properties and degradation of PPCLM could be adjusted by controlling the post-polymerization time of the pre-polymer. Mechanical testing results demonstrated good biocompatibility in foreign body response in vivo assay, enhanced compressive strength and 50-fold greater tensile stress. Rabbit chondrocytes were seeded onto the scaffold and showed proliferation and penetration into the inner phase after four weeks. Production of type II collagen and aggregan was detected in both phases of the scaffold.

In 2006, Helen et al. published a research study on the potential use of PDLLA/45S5 (PDLLA-poly(D-L-lactide))/Bioglass composite films for the culture of AFCs in vitro. The study evaluated the attachment and matrix production of bovine AFCs to four different substrates. They chose to combine bioactive glass with degradable polymers due to the ion dissolution products of the bioactive ceramic counteract the acidic degradation products of the polymer to reduce the inflammatory response. Through SEM analysis it was shown that cells had attached and spread on all films after three days and had proliferated to form a confluent monolayer of cells by day 7. sGAG production was measured and shown that PDLLA/30BG had enhanced sGAG production after three weeks; although, after four weeks in cultures sGAG production decreased. The results of the study showed that PDLLA/Bioglass provided an appropriate substrate for AFCs and
that these films promote the production of an ECM containing abundant sGAG and collagenous proteins.\textsuperscript{139}

In 2004, Mizuno et al. published a research study on tissue-engineered composites of the AF and NP for IVD replacement. The study used a polyglycolic acid (PGA) and calcium alginate mesh coated with 1.5\% w/v polylactic acid solution. AF and NP ovine cells were seeded onto PGA and alginate scaffolds to create a tissue with AF and NP components. Based on histologic and biochemical characteristics of the tissue-engineered IVDs, they presented similar to those of native IVDs based on morphology and quantities of collagen and proteoglycans produced. DNA content remained constant during the first eight weeks but increased greatly between 12-16 weeks \textit{in vivo}. Amounts of hydroxyproline and GAG increased with time with GAG reaching native tissue levels after 16 weeks.\textsuperscript{119}

In 2002 and 2003, Sato et al. published research studies on an atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) and the regeneration of the IVD with an allograft of cultured AFCs. During manufacturing, it was found the diameter of the pores could be controlled by changing the concentration of collagen solution and ammonia gas. The first study demonstrated stability, cytocompatibility, uniform seeding of cells, and accumulation of produced matrix components within the ACHMS-scaffold. The objective of the second study was to investigate the regeneration of IVDs after laser discectomy using their tissue engineering methods. They used AFCs isolated from Japanese white rabbits and seeded them onto the ACHMS-scaffold for one week. They then implanted the scaffold into the lacunas of an
IVDs of recipient rabbits that had the NP vaporized by a laser. Results demonstrated cells proliferated and retained spherical shape, higher increases in type II collagen and GAG content than compared to monolayer content, and a presence of an accumulation of cartilage-like matrix after insertion.124,140

**Tissue Engineering and Regenerative Medicine Approaches**

Tissue engineering evolved from the field of biomaterials and refers to the combination of scaffolds, cells, and suitable biochemical and physicochemical factors to form functional tissues to improve/replace biological functions. There is a need to create an effective AF repair device with could improve patient outcomes and reduce healthcare costs while preventing re-herniation and the need for spinal fusion for patients with IVDH and IVDD, respectively. Regeneration of the damaged AF is an appealing concept since it permits restoration of all functions of the AF; however, this concept is exceptionally complex to attain. It must be able to endure the direct mechanical strength applied and be a size sustainable to contain the NP.141 The progression in development needed for the success of regenerative strategies of the AF consists of three major components: cell therapy, gene therapy, and scaffolds.63 Proclaimed in a recent study, the ideal strategy for IVD regeneration is to restore the function and integrity of the IVD by using biomaterials, native matrices, growth factors, and cells that produce matrices; although, with the complex biological, mechanical environment of the IVD makes the synthesis of an artificial IVD a difficult task.85 However, while a difficult task, without a functional AF repair device to
resist expulsion of NP tissue and the IDP, generated within, IVD regeneration and prevention of IVDD and IVDH is deemed to fail.

During the early 2000’s there was an increase in research of NP replacements with the goal of restoration of the physiological IDP and IVD disc height. However, there was a lack of effective strategies that combated the damaged AF (incurred via implantation of such devices or the pathological processes). Current strategies for NP replacements attempt to restore native NP mechanical properties but do not address the issue of the inferior quality of the surrounding AF, which is not capable of withstanding IDP.\textsuperscript{71} Recently in research, IVD engineering strategies are increasingly focusing on the regeneration or repair of the AF in order to reduce the number of re-herniation’s, increase the potential of NP engineering strategies, and to mechanically assist NP replacement therapies.\textsuperscript{141,142}

An effective AF closure/repair device in conjunction with a less aggressive discectomy for IVDH and/or NP arthroplasty for IVDD may result in improving patient outcomes, decreased pain, and provide fewer revision surgeries via lower re-herniation and expulsion rates.\textsuperscript{5,6} In either case, an intact AF must be re-established to prevent implant expulsion or re-herniation, thus addressing the two major spinal pathologies directly associated with an IVD. Therefore, it would be advantageous to develop a biomimetic patch for biological augment AF repair to help mitigate this re-herniation.

The objectives of this research were to investigate the development of a biomimetic patch for biological augment AF repair through: (1) characterizing the micro-architecture of the multi-laminate angle-ply AF patch, (2) evaluating the mechanical properties of the
developed multi-laminate angle-ply AF patch, and (3) evaluating the cytocompatibility of
the multi-laminate angle-ply AF patch.

**Use of Stem Cells for Regenerative Medicine**

Stem cell properties are unlike any other cell in the body. While mature cells play
an important bodily function, stem cells are unspecialized. The stem cells foremost feature
is the ability to proliferate and increase in number indefinitely. These cells can develop into
many different cell lineages within the body during early and later stages of life. Therefore,
stem cells have great potential for the development of tissue engineered models. There are
a variety of stem cells, often based on where in the body or what stage in development they
come from. The main types of stem cells are: Adult Stem Cells (ASCs), Embryonic Stem
Cells (ESCs), Induced Pluripotent Stem Cells (iPSCs) (a type of derived adult stem cell),
and Amniotic Fluid Stem Cells (AFSCs). All stem cells, regardless of their source, present
three general properties: the capability of dividing and renewing themselves for long
periods, they are unspecialized, and they can give rise to specialized cell lineages. Each
variety of stem cells have its own strengths and weaknesses for the development towards
tissue engineering. Recently, research has shifted to stem cell-based approaches because
of their ability to replicate and differentiate into various applications.

**Types of Stem Cells**

**Embryonic Stem Cells**
ESCs are derived from embryos. Contrary to public belief, most ESCs are derived from eggs which have been fertilized *in vitro* and donated for research purposes with the informed consent of the donors. ESCs are derived from the inner cell mass of the blastocyst, and able to differentiate into any type of cell or organ within the body.\(^{144}\) This includes the three primary germ layers: ectoderm, endoderm, and mesoderm. This allows the ESCs to develop into more than 200 cell types within the adult body as long as they are specified to do so.\(^{145}\) Their pluripotency distinguishes ESCs from ASCs found in native human tissue. While ESCs are fully pluripotent, ASCs are multipotent and can produce only a limited number of cell types. Additionally, ESCs can propagate themselves indefinitely. Thus, allowing ESCs to be employed as useful tools for both research and regenerative medicine.

However, there are ethical concerns surrounding the use of ESCs. This is because ESCs require the destruction of early human embryos, which is considered a “crime against humanity.” Alongside the ethical concern, there is also a technical issue with allogeneic stem cell transplantation. Since ESCs are generated from embryos that do not retain the same genome with the receiving patient. This may lead to a possible immune rejection after implantation. Also, once the cells are cultured, there is difficulty to control and regulate cell proliferation and differentiation to avoid spontaneous development of a teratoma from undifferentiation ESCs. Drawbacks of stem cell use for regeneration affected by tumorigenicity and immunocompatibility must yet to be overcome through laboratory research.

**Adult Stem Cells**
An ASC is thought to be an undifferentiated cell, found among differentiated cells in a tissue or organ. ASCs are found in a variety of locations within the body including, bone marrow, hair roots, epidermis, fat, and muscle. Within these locations, the ASCs are located within a stem cell niche, where they can be found in children, as well as adults. An ASC can renew itself and able to differentiate to yield some of the major specialized cell types found in different tissues and organs. The primary role of an ASC is to maintain and repair the tissue where they are located. Unlike ESCs, ASCs can be used in research and therapy non-controversially because the production of the ASCs does not require the destruction of an embryo. The clinical development for therapeutic applications of ASCs is also further along than the other two main stem cell approaches. One main concern with ASCs is that the number of cells found within each tissue is a limited, and once removed from the body the ability for the ASCs to proliferate is also very limited. ASCs can differentiate into more than one cell type but are often restricted to certain lineages. With different types of ASCs capable of transdifferentiation, the ability of a stem cell of one lineage to become another lineage, more than others. Current research being conducted attempts to discover a more efficient method to grow large quantities of ASCs in cell culture and manipulate them to generate specific cell types.
Normal differentiation pathways of ASCs include hematopoietic and stromal stem cells. Hematopoietic stem cells give rise to all types of blood cells. While stromal stem cells, also known as mesenchymal stem cells (MSCs), give rise to a variety of cell types including bone cells, cartilage cells, and fat cells. ASCs treatments have been used for many years to treat successfully leukemia and related bone/blood cancers through bone marrow transplants.
Induced Pluripotent Stem Cells

iPSCs are adult cells which have been genetically reprogrammed to an ESC-like state. This is caused by forcing the cells to express genes and factors important for maintaining the properties of ESCs. It has been shown that a handful of reprogramming factors are sufficient to reprogram adult differentiated cells into pluripotent stem cells. The iPSCs technology was discovered by Shinya Yamanaka’s lab in Kyoto, Japan in 2006. Their lab showed that the introduction of four specific genes encoding transcription factors could transform ASCs into a version of pluripotent stem cells, with the original set of reprogramming factors being genes Oct4, Sox2, cMyc, and Klf4. The development of these newer type of stem cells is able to rise into all three germ layers (ectoderm, endoderm, and
mesoderm), and differentiate into adult cells. As with ASCs, iPSCs are not considered controversial like ESCs. Since iPSCs are derived from adult tissues, they can be produced for patient-matched protocols. This regards to the ability of everyone being able to have their own pluripotent stem cell line. The possibilities of usage of autologous pluripotent stem cells are immeasurable. These supplies could be used to generate transplants without the risk of immune rejection seen in many other tissue engineering products.

Figure 8: Reprogramming of cells into iPSCs

Amniotic Derived Stem Cells

Amniotic-derived stems cells can be classified into two types: Amniotic fluid stem cells (AFSCs) and amniotic mesenchymal stem cells (AMSCs). AFSCs are typically
obtained during the second trimester by ultrasound-guided needle puncture. Conversely, AMSCs are typically obtained from the amniotic membrane surrounding the placenta following cesarean section (C-section). Previously, Topoluk et al. have shown that the yields from amniotic membrane tissue isolation are typically greater compared to stem cells isolated from amniotic fluid, adipose (fatty) tissue, or bone.149 Additionally, this research demonstrated the potential benefits of AMSCs compared to other stem cell types, including providing an enhanced chondroprotective effect in orthopaedic applications.150

**Concerns Perceived with Stem Cells**

To select appropriate stem cells for tissue regeneration it is important to consider a variety of factors: acquisition of adequate stem cells, ex vivo expansion efficiency, the optimal approach of administration, in vivo differentiation efficiency, and functional integration. It is challenging for the acquisition of ESCs due to ethical limitations, and ASCs are difficult to collect due to their unique locations and limited quantity spread throughout the body. Ex vivo expansion and purification are required to facilitate the utilization. Thus, to determine the best administration approach for the various candidate stem cells, it is necessary to assess through testing and compare all delivery methods during *in vitro* and *in vivo* models.

iPSCs may pose a significant risk which could limit their use in human’s contingent on the methods used to develop the iPSCs. For example, if a virus is used to genomically alter the cells, cancer-causing genes “oncogenes” expression may be triggered. In February 2008, scientists announced the discovery of a technique which could remove oncogenes
after the induction of pluripotency, thereby increasing the potential use of iPSCs and decreasing the risk.\textsuperscript{151} Later in 2009, it was demonstrated that the generation of iPSCs was possible without any genetic alteration of the adult cell.\textsuperscript{152} Unfortunately, iPSCs derivation is typically a slow and inefficient process, taking 3-4 weeks for human cells to proliferate with weak efficiencies around 0.01-0.1 \%.\textsuperscript{146}
CHAPTER II

‘All of the research contained within this chapter has been published as Borem, Ryan, Characterization of a Multi-Laminate Angle-Ply AF Patch for Annulus Fibrosus Repair, Clemson Master’s Thesis, December 2015’

FABRICATION AND CHARACTERIZATION OF A MULTI-LAMINATE ANGLE-PLY AF PATCH

MATERIALS & METHODS

Specimen Procurement, Dissection, And Treatment

Porcine hearts were obtained at the time of slaughter from a local abattoir. The parietal layer of the pericardium was separated from the heart, and excess adipose fatty tissue and connective tissue were removed by manual separation using tweezers and scalpel blades. The parietal pericardium layer was chosen due to its natural structure. The parietal pericardium is fused to and inseparable from the fibrous pericardium (most superficial layer of the pericardium). It consists of dense and loose connective tissue containing collagen type I. The dense connective tissue is divided into regular and irregular sections. Dense regular connective tissue contains collagen fibers bundled in a linear/parallel orientation. The presence of these collagen fibers was utilized during the fabrication of the scaffold. The decellularization procedure was adapted and modified from methods described by Teeder et al without elastase treatment.
Fabrication of Biological Collagen Angle-Ply Scaffold

Multi-laminar angle-ply collagen patches were designed to mimic the native architecture of the AF. Angle-ply patches consisted of multiple layers of pericardium tissue adhered together via a sewing machine, backing material, and suture thread (Rachel Reference if previously reported). Single layers of pericardium were placed on a light box to determine the preferred direction of the collagen fibers. The layers were then stacked one on top of the other at ± 30° alternations to create the native angle-ply formation (Figure 5). For sewing, the layers were placed on top of a water-soluble backing material (solvy-ultra). The backing tissue was necessary during sewing to provide even positioning, and to permit the needle to puncture all the layers of the pericardium. Dimensions of 8 x 8 mm² were sewn into the layers to create the multi-laminar angle-ply patch. The backing material was then removed manually by hand/tweezers and stored in ddH₂O for 4 hours to ensure complete degradation from the patch. The dimensions of a three-layer patch are: length (8 mm), height (8 mm), and width/thickness (0.8 mm).

Isolation and Expansion of Bovine AF Cells

Three bovine tails from young calves were obtained at the time of slaughter from a local abattoir. The caudal discs were separated using mechanical shears, and the NP was removed first through a 6mm biopsy punch to ensure the harvest of only the AF. The AF tissue was then removed and minced using two scalpels blades. The tissue was then transferred to a 50 ml conical tube with 25 ml of collagenase solution (1% Ab/Am, 0.2% Collagenase Type I (125 units/mg dry weight) and DMEM). Following tissue digestion
overnight (~18 hours) at 37°C, the solution was centrifuged at 1000 rpms for 5 minutes. The supernatant was removed, and the cells were re-suspended in cell culture media (1% Ab/Am, 10% FBS and DMEM). Passage 2 (P2) bovine AF cells were used in the current study.

**Cellular Analysis**

Cellular studies were conducted on the multi-laminar angle-ply AF patch to ensure cell viability on all layers of the patch. Previous laboratory testing showed that bovine AFCs were viable on the surfaces of the patches through day 15 studies; however, cell viability between the upper and lower layers was unattainable. Multiple variations of tissue disruption modifications were used with the hypothesis of degrading the tissue matrix to provide more nutrients to the inner layers. For all studies, patches were prepared by re-sterilization and neutralization before cell seeding. Sterilization occurred by placing the patches in a specimen cup with 100 ml of 0.1% Peracetic Acid on a shaker at room temperature for two hours with a speed of 150 rpms. The patches were then washed in 100 ml of sterile PBS for 1 hour (3x) on a shaker at room temperature for one hour with a speed of 150 rpms. The patches were then neutralized in 100 ml of sterile 50% fetal bovine serum (FBS)/48 % Dulbecco’s Modified Eagle Medium (DMEM) + 2% antibiotic/antimitotic (Ab/Am) overnight on a shaker at room temperature with a speed of 150 rpms. The tissue disruption performed and discussed herein was sonication of the tissue. Pericardium tissue was placed in a Sonicator for 20 minutes prior to patch fabrication. Sonication affected the tissue by pressure created through a probe which rapidly expands and contracts at high
frequencies. The high-frequency oscillation is due to the piezoelectric effect, with the rapid oscillation of the current causing tiny shock waves. 

**AF Cell Seeding onto Scaffold**

Following sterilization and neutralization, patches were divided into 2 separate groups: control and sonicated. 5 x 10^6 cultured bovine AF cells at passage 2 (P2) were placed in a predetermined amount of cell culture media. Previous studies not shown herein determined the optimized volume amount to prevent run-off of the patch. 6 x 10^5 cells were seeded per patch (16 x 10^3 cells per mm^2): 2 x 10^5 cells were seeded in 40 μl of CCM onto the top the patches, 2 x 10^5 cells seeded in 75 μl of CCM were injected in to the middle layers using an 18-gauge needle. The patches were then incubated at 37°C for four hours for cell attachment. After incubation, the patches were flipped using sterile tweezers, and the same process was completed for seeding 2 x 10^5 cells in 40 μl of CCM onto the bottom of the patch.

**Staining Histology**

Representative samples of single layer decellularized pericardium were aligned in alternating degrees to illustrate the articulation of the alternation of fibers within pericardium tissue. This was performed with the intent to mimic the native angle-ply foundation of the human AF. Patches were fabricated as stated above, in ± 30° and + 30° alternations. The samples were then analyzed via histological analysis. Representative samples were placed in histological cassettes with gauze to hold the sample in place and to
prevent swelling between the layers. The samples were then fixed in neutral buffered formalin for 24 hours, embedded with paraffin wax, and sectioned at 5 μm thickness. Hematoxylin and Eosin (H&E) staining was performed to visualize cellular material and tissue extracellular matrix. Masson’s trichrome staining was used to define the composition of the pericardium tissue, and to verify the angle-ply alternation between the layers. A Zeiss Axio Vert.A1 camera microscope with AxioVision SE64 Rel. 4.9.1 software was using for imaging and reviewing of the histological slides.

**Biomechanical Evaluation of Multi-Laminate AF Patches**

**Ultimate Tensile Strength**

Mechanical tensile testing was performed on decellularized single and multi-laminar angle-ply AF patches to ensure comparable tensile ultimate tensile strength (UTS) to native human AF tissue. This is deemed crucial to ensure the patch does not disrupt the function of the native tissue and is able to withstand the max tensional force experienced during extension and flexion. The representative samples for the decellularized single layer pericardium were tested in a fiber preferred (tension applied parallel to collagen fibers; n=5) and cross-fiber direction (tension applied perpendicular to collagen fibers; n=5). Representative samples of three-layered multi-laminar angle-ply AF patches were also tested (tension applied at ± 30° alternating angles of patch aligned to the vertical axis; n=5). The tensile testing protocol was adapted from methods describing single human AF lamellae tensile testing by Green et. al.¹⁰¹
Testing was performed on a mechanical testing system (MTS) Synergie-100 fitted with a 100 N load cell. Samples were preheated to 37°C in a water bath for 30 minutes prior to testing and sample length, width, and thickness was determined using digital calipers. Samples were placed in tensile grips lined with fine-grit sandpaper to the ends of each sample before insertion into the MTS clamps to prevent slippage. Once samples were secured, preconditioning (5 cycles to 10% strain at 10 mm/min), and testing to failure at a rate of 240 mm/min was performed. Stress-strain data was recorded, plotted, and collected for statistical analysis. The tensile elastic modulus was determined from the linear region of the graph. The UTS was determined by dividing the max peak load by the cross-sectional area of the sample.

**Patch Impact Resistance**

Impact strength was determined in accordance with ASTM D1709 with minor modification. Representative samples of multi-laminar angle-ply AF patches of 1, 2, 3 and 6 layers were tested (n=4 per group). The impact mechanical test was performed to obtain the minimum number of layers required to withstand the changes of IDP of the IVD. Understanding the impact force is crucial due to high rapid changes in IDP which could result in herniations of weakening or damaged areas of the IVD. The impact force was determined using a custom design sled fixture consisting of four rails attached to a base platform, with a free-moving apex platform. The testing apparatus for the tissue consisted of two wooden blocks bolted together with a 6.25 mm diameter hole drilled through the center of both blocks. On the inner surface of each block, a neoprene square glued down
with epoxy along with a layer of sandpaper to prevent the samples from slipping during testing. The representative samples were centered over the hole in the block and bolted together. A 6 mm steel ball and rod were slid through the top block and placed in contact with the tissue. The remaining rod extruded from the wooden block towards the apex of the apparatus. Concisely, various weights ranging from 0.18-0.58 kg were dropped from a consistent height of 10 in (0.254m) towards the base of the platform along the four rails. The impact between the weight of the free-moving apex platform and rod were used to measure the amount of force the patch could withstand.

Using the application of conservation of energy of a falling objectEQ 1, the impact velocity and kinetic energy were calculated. Thus, combined with the distance traveled, impact force [N] was calculated. In conjunction with the impact force data, the contact radius between the ball and patch was needed to convert to pressure. The law of conservation of energy and the following formulas were used to calculate the contact radius:
This formula represents the calculated contact surface [m] of the ball with the pericardium. F is the load [N] of the force exerted on the patch at the time of impact. R₁ and R₂ were the radius [m] of the ball (3 mm) and the patch (∞) respectively, where the patch was considered a sphere with an infinitely large radius. E₁ and E₂ were the elastic modulus [Pa] of the ball (200 GPa) and the patch (16.4 MPa, determined from tensile testing), respectively. Poisson’s ratios, v₁ and v₂, of the ball (0.27) and the pericardium (0.3), respectively. Potential Energy (PE) and kinetic energy (KE) were used based on the conservation of energy calculation to determine the impact force of a falling object. Impact force was then converted to pressure [Pa] to relate to IDP changes (EQ. 3). The data was collected and recorded for statistical analysis.
Tensile Fatigue Testing

Mechanical tensile fatigue testing was performed on three layered multi-laminar angle-ply patches to test the ability of the angle-ply patch to withstand 10,000 cycles of the UTS of the native AF tissue. The testing setup was in reference to previous fatigue testing of Green et. al. Testing was conducted on a Bose ElectroForce 3200 series, model: 3220, WinTest 7 (software), with a 100 lb. load cell. Representative samples were preheated to 37°C in a water bath for 30 minutes prior to testing and sample length, width, and thickness was determined using digital calipers. The samples were then attached by two U-shaped clamps with fine-grit sandpaper to prevent slippage of the material. The clamps were submerged in a bath chamber filled with sterile PBS solution and a protease inhibitor. Once the samples were in place, the sample was aligned and brought taught to have a minimum of 0.5 N load on the tissue prior to testing. Once the sample was secured, preconditioning (5 cycles at 10% strain of the gauge length) was performed. Following preconditioning, the base plate was adjusted to apply a minimum of 0.5 N load to the sample. The axial tensile force was then applied on the patch (tension applied with alternating angles of ±30° oriented in the vertical axis; n=16) at a pre-determined load [N] based on the representative percentage of UTS. Testing frequency was set at 0.5 Hz, and samples were run until structural failure or completion of 10,000 cycles. One cycle consisted of raising to the predetermined testing load with the sample in tension and lowering back down to the original testing load. The displacement, load control value, total time, cycles performed, and where the sample broke were recorded for statistical data analysis.
Statistical Analysis

Statistical analysis of the data was performed using Microsoft Excel or statistical analysis software (SAS Enterprise Edition 3.4, Version 9.4 or above). Results are represented as mean ± standard error of the mean. Data was analyzed using a one-way ANOVA or by a two-tailed Student’s t-test of unequal variance with significance defined as (p < 0.05).

RESULTS

Pericardium Sheet Fiber Alignment and AF Patch Assembly

Our group has previously confirmed the decellularization of porcine pericardium via immunohistochemistry, agarose gel electrophoresis and Nanodrop analysis demonstrating the complete removal of alpha-Gal (porcine antigenic epitope), and a 95.3% decrease in DNA as compared to the fresh porcine pericardium, respectively. This decellularization method was utilized before construction of AF patches.

Macroscopically, parietal pericardium sheets demonstrated a fiber preferred direction that was evident when the tissue was placed over a lightbox (Figure 10A). Histology of individual decellularized pericardium sheets confirmed the composition of the tissue (Figure 11A-B): dense regular collagen connective tissue (dense collagen type I fibers bundled and aligned in a parallel orientation, left) fused with dense irregular collagen connective tissue (dense arrangement of thick collagen type I fibers embedded in an amorphous ground substance, right). The average thickness of single-ply sheets of the decellularized pericardium was 0.026 ± 0.002 mm.
Figure 10: Fabrication of the AFRP through the alternation of individual sheets with collagen fibers oriented at ± 30° to a common horizontal axis. A) Representative image of a single piece of the porcine pericardium. Arrows indicate collagen fiber preferred direction within each pericardium layer, and boxes (dotted outline) represent regions cut out for use in AF patches. B) Representative image of a 3-layer AF patch (5 [L] x 5 [W] x 0.8 [T] mm) following patch fabrication methods. C) Schematic representation depicting the fiber alignment in individual decellularized porcine pericardium sheets, subsequent stacking, and suturing of sheets together.

Multi-laminate patches comprised of 2, 3, 4, and 6 layers were successfully assembled having an average thickness of 0.05 ± 0.004 mm, 0.08 ± 0.006 mm, 0.11 mm ± 0.009 mm, and 0.16 ± 0.02 mm, respectively. The average length and width of multi-laminate patches for mechanical testing and cellular studies were 12 x 12 mm and 7 x 7 mm, respectively. Predetermined by testing protocols, different patch dimensions were constructed to fit into different test apparatus. Patch sizes ranging from 4 x 4 mm to 30 x
30 mm were produced using our assembly method. Patches were assembled to mimic the human AF architecture with the multi-laminate sheets of porcine parietal pericardium aligned sequentially in alternating ± 30° to a common horizontal axis using a lightbox and secured by suture (Figure 11C).

**Confirmation of Angle-Ply Architecture**

Composition and microstructure of the AF dictate its mechanical function. Based on the organization of collagen fibers between subsequent lamellae dictates the amount of intradiscal pressure the AF is capable of resisting. Therefore, to develop a biomimetic biomaterial mimicking the native AF, the resulting AF patch must be capable of presenting fiber alignment similar to the native tissue. To evaluate the capability of aligning porcine pericardium in alternating fiber alignment, polarized light microscopy and histology were used to confirm the orientation of the fiber preferred direction within each layer of the multi-laminate AF patches. Figure 11C illustrates the proof of concept demonstrating the successful construction of a 3-layered angle-ply patch with aligned collagen fibers orientations with the collagen fiber preferred direction running parallel to the viewer. While the proof of concept demonstrating the alternations of individual layers was demonstrated with the middle layer collagen fibers running parallel to the viewer, and the outer layer(s) collagen fibers aligned coming towards the viewer. (Figure 11D).
Figure 11: Histological analysis of decellularized porcine pericardium stained with Masson’s trichrome (blue = collagen, red = cytoplasm), (arrow = fiber direction). A&B) Representative single layer pericardium illustrating dense regular connective tissue (X) and dense irregular connective tissue regions (■), (magnification: 100X and 200X, respectively). C&D) Histological image depicting the ability to alternate the fiber preferred orientation in additional pericardium sheets. Decellularized pericardial sheets were aligned based on alternating the angle of the fiber-preferred direction between each sequential layer (arrows indicate collagen fiber preferred direction within each pericardium layer), (magnification: 100X). [C] All three layers orientated with the collagen fiber preferred direction running parallel to the viewer, while [D] illustrates the alternation of fiber preferred direction with the middle layer.
collagen fibers running parallel to the viewer, and the outer layer(s) collagen fibers aligned coming towards the viewer.

**Ultimate Tensile Strength**

To determine the ultimate tensile strength (UTS) and elastic modulus (EM) of the AF patch and to understand how fiber alignment impacts these properties, static tensile testing was performed on 3-layered multi-laminate AF patches, as well as, single layer sheets of pericardium. Single layer pericardium was tested in the fiber preferred (tensile load applied parallel to collagen fiber) and cross-fiber (tensile load applied perpendicular to collagen fiber) directions. Average UTS of a 3-layer AF patch, single layer fiber preferred, and single layer cross-fiber testing was $5.9 \pm 0.3$ MPa, $5.6 \pm 1.1$ MPa, and $2.9 \pm 0.2$ MPa, respectively (Figure 12). A statistical difference ($p < 0.05$) was found between the UTS of three-layered patches and single layer tissue oriented in the cross-fiber direction.
Figure 12: Tensile UTS of decellularized single layer sheets and 3-layered AF patches were tested, and values were compared to human AF (Dotted horizontal line indicates measured human AF UTS reported in the literature). The representative graph illustrates the average UTS for a 3 layered AF patch and single layer sheets aligned in fiber preferred and cross fiber directions. * indicates significant difference (p < 0.05).

EM was calculated from the linear region of the stress vs. strain graphs. Average EM of a 3-layer AF patch, single layer fiber preferred, and single layer cross fiber was 16.4 ± 3.5 MPa, 62.0 ± 13.6 MPa, and 23.6 ± 6.0 MPa, respectively (Figure 13). A statistical
difference (p < 0.05) was found between the EM of 3-layered patches and single layer tissue oriented in the fiber preferred direction.

Figure 13: Tensile EM of decellularized single layer sheets and 3-layered AF patches were tested, and values were compared to human AF lamellae (Dotted horizontal line indicates measured human AF tensile modulus range reported in the literature). The representative graph illustrates the average tensile EM calculated from the linear region of the graph for decellularized 3 layered patch and single layer sheets aligned in fiber preferred and cross fiber directions. * indicates significant difference (p < 0.05).
**AF Patch Resistance to Instantaneous Changes of Intradiscal Pressure**

One of the primary mechanical functions of the AF is to resist the IDP generated by the NP. The IDP can change (increase) abruptly due to daily activities causing NP to herniate from native AF. Thus, the ability of the AF patch to resist bursting open from a sudden increase in impact force/pressure was evaluated. The mechanical evaluation of instantaneous change of IDP for 1-layer, 2-layer, 3-layer, and 6-layer multi-laminar patches demonstrated an average impact resistance of $< 6.81$ MPa, $6.8 \pm 0.0$ MPa, $7.99 \pm 0.22$ MPa, and $9.94 \pm 0.98$ MPa, respectively (Figure 14). Single layer pericardium sheet impact resistance occurred at $< 6.81$ MPa, the lowest measurable amount of the custom designed testing apparatus. A linear increase in average impact pressure was observed with increasing layers included in the patch. The statistical difference ($p < 0.05$) was observed between 2-layer and 6-layer patches, and between 3-layer and 6-layer patches.
Figure 14: Impact resistance testing indicates multi-laminate AF patches can withstand the instantaneous application of intradiscal pressure commonly experienced in the IVD. Representative graph of the average maximum calculated impact pressure withstood by a 2-, 3-, and 6-layer patches. (Dotted horizontal line indicates highest measured *in vivo* human IDP reported in the literature [2.3 MPa observed while bent over with a round back while lifting 20 kg.]) * indicates significant difference (p < 0.05).

The previous testing conducted in our lab determined the quasi-static burst strength of AF multi-laminate patches. The burst strength testing protocol was adapted from the ASTM
D3786/D3786M: Bursting Strength of Textile Fabrics Method. The mechanical evaluation of the burst strength was performed for 1-layer, 2-layer, 3-layer, and 6-layer multi-laminar patches demonstrated an average impact resistance of at 4.26 ± 0.40 MPa, 7.53 ± 0.52 MPa, 10.75 ± 1.47 MPa, and 18.49 ± 0.76 MPa for, respectively (Figure 15). Statistical difference (p < 0.05) was observed between all testing groups.
Figure 15: Burst resistance testing indicates multi-laminate AF patches can withstand the quasi-static application of pressure commonly experienced in the IVD. Representative graph of the average maximum calculated burst pressure for 1-, 2-, 3-, and 6-layer patches. (Dotted horizontal line indicates highest measured *in vivo* human IDP reported in the literature [2.3 MPa observed while bent over with a round back while lifting 20 kg.]) * indicates significant difference (p < 0.05). Picture reproduced with permission of R. McGuire.\(^{157}\)
**Dynamic Tensile Fatigue Testing**

One of the two primary spinal motions in the lumbar region is flexion. The bending motion associated with flexion is resisted by the posterior annulus and posterior longitudinal ligament. The ability of the AF patch to resist varying stress amplitudes during tensile fatigue loading was evaluated. Dynamic tensile fatigue testing of 3-layered multi-laminate AF patches was performed to predict the relative lifespan/endurance of the patch. Representative samples were tested to a mechanical run out of 10,000 cycles over a range of stress amplitudes observed during flexion to develop an S-N curve. Testing was conducted at stress amplitudes ranging between 0.95-2.66 MPa (25-70% UTS of the native human AF). AF patches withstood at minimum 1155 ± 75 cycles and achieved an endurance limit of 10,000 cycles at stress amplitudes of 2.66 MPa and 0.95 MPa, respectively. The S-N curve (**Figure 16**) illustrates the recorded data for AF patch overlaid on values reported for human AF tested under the same conditions. Supplemental data of Detailed applied load and cycles to failure of cyclic tensile fatigue experiments are listed in (**TABLE 6**).
Figure 16: Tensile fatigue endurance of 3-layer AF patch approximates that of the native human AF. S-N Curve illustrating the fatigue strength of our 3-layered multi-laminate AF patch (triangles), in comparison of native human AF (diamonds). A negative correlation between the level of applied stress and the number of loading cycles to failure was observed. Open diamonds, triangles, and arrows indicate specimens with no mechanical failure observed (mechanical run out to 10,000 cycles).
Table 6: Detailed applied load and cycles to failure of cyclic tensile fatigue experiments with native AF (left) vs. 3-layered multi-laminate AF patch (right) based on representative UTS (Ultimate Tensile Strength) percentages. Tests were halted following a mechanical run out to 10,000 cycles.

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<th>Cycles to failure</th>
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Cellular Analysis

Cellular studies were conducted on 3-layered multi-laminate angle-ply AF patches to ensure cell viability on all layers of the patch. Previous laboratory testing showed that bovine AFCs were viable on the surfaces of the patches through day 15 studies, however, following histological analysis cell viability between the upper and lower layers were
previously unidentifiable. To provide the greatest chance of probability of cell attachment, a volume optimization protocol was used to determine the total amount of volume (µl of cell culture media containing AF cells) an AF patch can hold to prevent run-off. It was determined a 7 x 7 x 0.8 mm³ AF patch can hold 45 µl on the top and bottom layers, and 75 µl between the layers before run-off occurred.

Tissue disruption modification (sonication) was used with the hypothesis of disrupting the tissue extracellular matrix to provide more nutrient transfer to the inner layers. Histological analysis of representative control and sonicated 3-layered multi-laminate AF patches of days 3 and 6, respectively, illustrated cell attachment and infiltration (Figure 17). Histological evaluation illustrates AF cell attachment on all layers of the multi-laminate AF patch for all samples and time points investigated.
Figure 17: Multi-laminate AF patches support AF cell viability, and infiltration demonstrated through stained H&E (purple = cellular nuclei, pink = ECM) samples. A-B) Representative histological cross-sectional images depicting the presence of AF cells within the tissue of a 3–layer multi-laminate AF patch at days 3 and 6 stained with H&E (magnification: 100X). C-D) Representative cross-sectional histological images depicting the presence of AF cells within the tissue of a 3–layer multi-laminate sonicated AF patch at days 3 and 6 stained with H&E (magnification: 200X and 50X, respectively).
Representative sections of day 6 AF patches for sonicated and controlled tissues were magnified to perceive clearly the cells infiltrated into the tissue (Figure 18). The width of the representative tissue layers was measured and recorded at 538 µm and 483 µm (Table 7). Average measured cell penetration depths for sonicated and control sample AF patches, measured using ImageJ software, were 278 µm and 52 µm, respectively. The furthest depth of cell infiltration was 503 µm (93.7% overall depth relative to tissue width) and 327 µm (67.7% overall depth relative to tissue width). A statistical difference (p < 0.05) was observed between the average cell infiltrations of sonicated and control patches on day 6.

Figure 18: Increased magnification of a multi-laminate AF patch section, one of the three layers, depicting infiltration of cells into the tissue layers for day 6 sonicated and controlled AF patches. (Left) Sonicated tissue treated for 20 minutes at time point day 6. (Right) Control tissue at time point day 6. (Left and Right original magnification: 200X).
Table 7: Measurement and analysis were conducted to illustrate cell infiltration. To determine the depth of penetration by the AF cells H&E histological staining and ImageJ software were used. Representative sections of a 3-layered multi-laminate AF patch were magnified (Figure 18) to perceive clearly the cells for sonicated and control tissue samples. The width of the representative tissue layers was measured, and the average/deepest depth of cell infiltration was analyzed for sonicated and control tissue samples, respectively. For average and deepest cell infiltration, the percentage of overall depth relative to tissue width is displayed in parenthesis. * indicates significant difference (p < 0.05).

<table>
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<td>(67.7%)</td>
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Effective biological AF repair is an unmet clinical need for several applications: to prevent recurrent IVDHs, to preserve the IVD biological structure and functionality, and/or to support NP arthroplasty devices for patients with early-stage interventions targeting IVDD. An ideal AF repair device must mimic the biological, structural, and functional characteristics while promoting tissue regeneration. The native AF is comprised of highly organized lamellae of aligned type I collagen fibers. When entirely intact, the AF confines the NP to the center of the IVD and provides resistance to IDP generated by the NP. When a defect is present, the associated underlying pathology and surgical repair typically contribute to long-term pain and long-term effects on adjacent IVDs (adjacent segment degeneration).

Until recently, clinicians believed pain associated with IVDH would resolve on its own without surgical intervention (i.e. spontaneous resorption of the herniation). However, despite a temporary reduction in pain, a recent study indicated when herniations were left untreated, a resurgence of pain occurs. For patients presenting with radicular symptoms or low back pain that is unresponsive to conservative therapy, and demonstrate through magnetic resonance imaging (MRI) or computed tomography (CT) confirmation of an NP herniation or extrusion, a discectomy procedure is often warranted. Annually, there are over 500,000 lumbar discectomies performed in the United States. While this procedure yields satisfactory clinical outcomes, an open pathway (defect) remains which provides a path of least resistance for recurrent IVDHs to manifest. It has been shown, the rate of re-herniation is greater in patients with an AF defect larger than 6 mm. The wide variety...
of approaches and techniques used to perform a discectomy can be debated with respect to
their advantages and disadvantages. While a less aggressive discectomy approach
(removing the protruding NP fragment without an extensive intradiscal compression)
results in improved patient outcomes, it does present a greater risk for re-herniation as
compared to an aggressive approach (removal of more than the protruding NP tissue [disc
decompression] in attempts to decrease the likelihood that re-herniation may occur).54
Therefore, an effective AF closure/repair device in conjunction with a less aggressive
discectomy may result in improved patient outcomes, decreased pain, and fewer revision
surgeries via lower re-herniation rates.5,6 Additionally, with the application of an AF
closure device, it could lead to a reduction of chemical pain mediators escaping through
the open AF following a conventional discectomy, thus reducing the effects of irritation of
the nerve root ganglion, epidural fibrosis, or the potential for ingrowth of nerve fibers that
are mediators of postoperative discogenic pain.160–162

The discectomy procedure itself is not benign to the disc – it is an ablative, not a
restorative procedure.158 Suturing is a simple and appealing repair method to be used for
AF closure due to its frequent use during surgery; however, suturing does not account for
missing AF tissue and may not be strong enough to resist changes in intradiscal pressure
(IDP).5,6,16 Therefore, there has been an increase in tissue engineering and regenerative
therapeutic being investigated over the last decade aiming to structurally and biologically
repair the AF.

A need exists to develop an AF closure/repair device that mimic the architecture
(multi-laminate lamellae of aligned collagen oriented in an angle-ply fashion), mechanical
properties (ultimate tensile strength and modulus), cytocompatibility (ability to promote cell viability and regeneration), and function (ability to resist tensile loading, intradiscal pressures, and fatigue) of the native human AF. The objective herein was to confirm our ability to generate multi-laminate patches comprised of decellularized porcine pericardium with a repeatable angle-ply architecture, to mechanically evaluate their strength and durability, and assess their cytocompatibility. It is hypothesized that the AF patches composed of decellularized porcine pericardium would mimic the biological, structural, and functional characteristics of the native AF, and therefore, be an ideal surrogate for use in its repair.

**Patch Formation**

The development of the biomimetic AF patch that mimics the native human AF architecture was vital. The alternation of collagen fibers between the native lamellae is the underlying foundation of what provides the AF strength when distributing the tensile bending and torsional forces throughout the functional spinal unit.\(^{163}\) The parietal pericardium layer from porcine hearts was chosen due to its natural underlying structure. The parietal pericardium is fused to and inseparable from the fibrous pericardium (most superficial layer of the pericardium). It consists of dense and loose connective tissue comprised of collagen type I,\(^ {153}\) mimicking the native composition of the AF. The dense connective tissue is divided into regular and irregular sections. Dense regular connective tissue contains collagen type I fibers bundled in a linear/parallel orientation. The presence of these collagen fibers was utilized during the fabrication of the scaffold.
By manually alternating the fiber preferred direction of the dense regular fiber architecture of the decellularized porcine pericardium over a light box, a repeatable method to overlay consecutive sheets of pericardium achieving an angle-ply orientation was developed. Following the alignment of subsequent layers, the patches were secured together using suture. The simplicity of this technique combined with the need for differently sized patch dimensions for testing protocols, patient IVD height, and varying patient AF defect sizes allowed for the construction of patches ranging in size from 4 x 4 mm to 30 x 30 mm using our assembly method. The mean disc height reported for the lumbar segment of the spine was 11.6 ± 1.8 mm for the L3/4 disc, 11.3 ± 2.1 mm for the L4/5, and 10.7 ± 2.1 mm for the L5/S1 level.\textsuperscript{164} While not surprisingly, the size of an AF defect exhibits an enormous effect on the re-herniation rate. In patients with massive AF defects (> ~6-6.5 mm), the re-herniation rate was 27% compared to patients with slit defects who experienced re-herniation rates of only 1%.\textsuperscript{10} Thus, based on these results, it would indicate that if larger defects are effectively reduced in size, re-herniation rates could fall dramatically in this “at-risk” group of patients.\textsuperscript{158} Taken together, we were able to produce patch sizes that are of clinical utility. In addition, the development method employed to make these patches can be easily scalable for batch manufacturing procedures compared to other formation methods of AF scaffolds which require more complex manufacturing techniques.\textsuperscript{165}

**Confirmation of Angle-Ply Architecture**

Verifying the proof of concept creating an angle-ply AF patch architecture was demonstrated through histological images of single sheets and three-layer stacking.
Masson’s trichrome staining and polarized light microscopy were used to view the fiber preferred direction histologically. Polarized light microscopy was used to refract the light based on collagen fiber orientation within the individual layer of pericardium. Since collagen fibers are an anisotropic material, it expresses birefringent under polarized light. With this optical property, the fiber preferred direction of the porcine pericardium was clearly visible to correspond to the fibers seen macroscopically. When stacked in multiple layers, the AF patch demonstrated the proof of concept for the alternation of fibers with the collagen fibers visible in the individual layers aligned coming towards the viewer, and the alternating layers oriented away from the viewer.

**Mechanical Testing of AF Patch**

Of the available repair methods for AF regeneration, decellularization of an extracellular matrix derived scaffold is an attractive option due to its ability to promote the expression of M2 macrophages (immune cells with the ability to promote tissue homeostasis through cell proliferation and tissue repair)\(^{166}\) and its ability to preserve the tissues microenvironment.\(^{167}\) In addition, these attributes promote cell survival, proliferation, morphogenesis, differentiation, and constructive tissue remodeling.\(^{168,169}\) However, the decellularization process of a biological tissue can have potentially significant effects on the biomechanical properties of the resulting scaffold. Thus, it is critical to evaluate how closely the mechanical characteristics of the resultant AF patch compare to the values of the native human AF. Although the mechanical advantage of closing annular defects to retain NP material seems intuitive, only recently have AF closure
devices been examined for their ability to withstand in situ IDP or flexibility testing.\textsuperscript{16} For this reason, it was paramount to evaluate the mechanical characteristics of our multi-laminate angle-ply AF patches with respect to tensile strength, impact resistance, and tensile fatigue.

**UTS Testing**

To characterize the maximum tensional stress our AF patch is capable of resisting, representative samples were placed under a static tensile load to develop a stress-strain relationship. These values were then compared to reported human AF tissue to approximate the maximum tensile loads endured by the AF during flexion/bending. The AF patch illustrated mechanical properties of UTS and elastic modulus (EM), which fell within the reported values of the native human AF (3.8 ± 1.9 MPa, and 12-24MPa, respectively).\textsuperscript{101,102} The biological function of a collagen-based material lies predominantly in its mechanical properties. The viscoelastic biological tissue (combined with strong underlying cross-linking fibers)\textsuperscript{170} demonstrated both viscous and elastic properties when undergoing deformation. This particular behavior results from the anisotropic microstructure of the AF tissue composed of oriented collagen fibers embedded in the ECM.

The measured UTS represents the maximum stress that the material could withstand before failing and is correlated with the highest stress value on the stress-strain graph. Collagen fibers have the optical property of birefringence that defines the direction they can bear a load in a tissue, and the strength of birefringence has been correlated with the UTS.\textsuperscript{171} In a stress-strain relationship, the EM is calculated from the linear portion of the
graph up to the yield point of the material. As a material stiffness increases, it demonstrates a higher EM. The low EM of the AF patch demonstrates its flexibility and ability to change shape while maintaining a relatively high UTS similar to the native AF tissue.

The UTS and EM of the single layer fiber preferred direction were shown to be greater than the single layer cross-fiber direction. This is attributable to the mechanical function of the collagen fibers that work to resist tension, and while applying a load perpendicular to or at an angle from the primary axis of the fiber preferred direction would essentially cause the interstitial matrix to endure most of the stress, not the fibers themselves. The difference seen between the fiber preferred and cross-fiber directions is also consistent with acellular scaffolds and continuous fibers in composite materials consisting of predominantly collagen fibrils. These acellular scaffolds and continuous fibers demonstrated the mechanical properties of the fiber preferred orientation and resulted in higher tensile strength and stiffness (elastic modulus) compared to that of the cross-fiber preferred direction.172,173

Analysis of tissue behavior increases in complexity as the hierarchical tissue structures become more complicated. The AF patch (aligned with ± 30° alternations between subsequent layers) demonstrated a higher UTS compared to single layer tissue aligned in the fiber preferred direction and cross-fiber direction. This is consistent with mechanical testing of angle-ply laminates which found that opposing (± 30°) constructs were stronger than the fiber preferred aligned constructs.132 While another study showed that during uniaxial tensile loading on various fiber angles, the elastic moduli varied with a nonlinear decrease seen in modulus with increasing fiber angles.120
Even though axial rotation and lateral bending in the lumbar discs are relatively limited, the range of motion of the IVD in flexion-extension can be as high as 24 degrees.174 Because the center of rotation of the disc is typically anterior of the posterior AF, the strain range of the posterior AF during these high ranges of motion can vary greatly. Maximal physiologic strains observed along the AF superficial tissue are about 4% when the disc is in compression or torsion, and ~6% when the disc is in flexion or extension but never exceeding 10%.175 Thus, designing a device that can maintain its integrity while sealing defects that also go through this strain range is extremely challenging.158 The amount of strain experienced to reach the UTS of our AF patch, relative to the native AF tissue UTS, was $5.7 \pm 0.43$ mm. Thus, demonstrating the ability of our AF patch to undergoing the native strain range, while maintaining its tensile strength integrity.

**Fatigue Testing**

To assess the capability of the AF patch withstanding dynamic loading, the AF patch underwent tensile fatigue testing for ranges of 25-70% UTS. Upon characterizing the UTS of the AF patch, it was necessary to address the levels of stress amplitude commonly experienced by the AF during flexion. The representative testing range, of 25-70%, was performed based on the lower range limit determined from the resulting endurance limit of the AF patch. While the upper range limit was chosen to be 70% (due to the fact, when placed *in vivo*, the IVD is unlikely subjected to stresses higher than 70% UTS due to the prevention of injury by ligaments of the neural arch during extension and lateral bending, and through soft tissues during flexion).176 Comparative analysis of the AF patch and native
human AF, illustrated by Figure 16, demonstrates the AF patches display of a similar S-N curve profile relating the level of applied stress to the number of loading cycles withstood prior to failure.

Adapted from a previously published testing protocol for AF tensile fatigue testing, \(^{101}\) 10,000 cycles were chosen for the mechanical run out of our AF patch. This was not to represent a true fatigue limit, but an estimation of how a biological tissue will last in a theoretical environment absent of intrinsic healing. While the AF is constantly subjected to cyclic tensile loading, typically the higher maximum tensile stresses only occur during a limited range of motions, such as bending forward at the waist (without bending your knees), sitting down in a low chair, or tying your shoes. When damage to the AF does occur, the intrinsic healing process begins. The AF is identified to heal in three phases: 1\(^{st}\) phase is when the outer AF begins to heal (day 0 – few weeks), the 2\(^{nd}\) phase is when the interior AF layers gradually heal (few weeks – one-year), and 3\(^{rd}\) phase is when there is an increase of collagenous fibers within the NP.\(^{112}\) Based on the tensile fatigue results, the AF patch has the capability to resist failure for between 1.0-2.7 years (conservatively assuming achievement of maximal flexion approximately 10-25 times per day), which is beyond the reported time required for native AF healing (which would eventually help off-load the mechanical patch responsibilities).\(^{112}\) Therefore, it is reasonable to assume that when placed under tensile loading the AF patch is capable of meeting the mechanical demands experienced during flexion for the posterolateral AF.
Impact Testing

Impact mechanical testing was performed to obtain the minimum number of layers required to withstand the changes of IDP generated by the NP of an IVD. Currently, IDP measurement is the only direct way to determine the loading conditions within the human spine.48 The posterior region of the AF is inherently weaker due to its more parallel alignment to the horizontal axis of the spine compared to other regions.29 Combined with instantaneous changes of IDP within preceding IVDH defects and/or discectomy procedure sites provide a weakened region in the AF lamellae that have been linked to the rupture of the AF; therefore, it was crucial to characterize the impact resistance for the AF patch.177 The AF patch must be capable of enduring the maximum impact force generated by the NP since the vacant area beneath the patch will represent the entire thickness of the AF in the repaired region.

Previously reported by our group, the quasi-static burst strength of the AF patch was calculated to determine the approximate amount of layers required to withstand typical IDP values generated by the NP.157 While herniations typically occur from a gradual degeneration of the disc (weakening of the posterior AF), the rate of IDP change varies based on AF tissue degradation and movement of the patient. Currently, no published studies specify the speed at which a herniation occurs in nondegenerated IVDs. Using calculations based on the reported IDP ranges seen in the native human AF, (0.1-2.3 MPa),177–179 combined with the idea that an instant change of force can lead to an increase in stress concentrations, the AF patches were tested at varying forces with a constant velocity of 2.23 m/sec. This velocity is consistent with compression testing conducted to
measure the failure mechanisms of the impact loading on the spine,\textsuperscript{180,181} and represent greater speeds that are commonly tested for physiological responses to impact loading.\textsuperscript{182–184} The impact resistance measured of AF patches indicated its ability to withstand instantaneous changes of IDP observed within the human IVD. A linear increase in average impact resistance was observed with increasing the number of pericardium layers within the AF patch. While theoretically, the number of layers required to withstand instantaneous changes in IDP commonly observed was less than three layers (optimized value chosen for the AF patch), previous results from the quasi-static burst strength testing illustrated a need for the minimum of 3-layers. Additionally, this impact mechanical testing does not account for repetitive cyclic increases in IDP which would be seen \textit{in vivo}. Future studies will be conducted to measure the efficacy of the patch to resist cyclic IDP and containment of the NP material through dynamic compression fatigue resistance testing, following attachment to cadaveric IVDs.

\textbf{Cytocompatibility Of Cell Seeding on AF Patch}

Aside from the AF patch having mechanical characteristics that closely approximate the human AF, it is also made of a biologic material that may lead to the promotion of tissue regeneration. This is something that cannot be said about synthetic AF repair techniques/materials. The regenerative potential the biological AF patch possesses is an advantage in the way it will aid in self-repair and integration. Following attachment of the AF patch to the native IVD, the entire mechanical load is supported by the biomaterial scaffold and attachment mechanisms. Thus, the patch must maintain sufficient
mechanical strength to support physiological loading. With the potential increase of integration between the AF patch and native AF tissue over time, the applied loads on the patch will decrease; thus, assisting in the durability of the AF patch and attachment mechanism. It is reported that AF regeneration could potentially be enhanced by the supplementation of cells to accelerate the healing process of large annular defects. While this is not essential as a mechanical surrogate, the AF patch should be capable of, at the minimum, supporting cell viability of cells within the patch as well as potentially promote the migration of neighboring cells into the patch.

Tissue engineering scaffolds are developed to influence the physical, chemical, and biological environment surrounding a cell population. The use of cells affects the scaffolds ability to regenerate the ECM by maintaining the long-term homeostatic balance of matrix turnover. Therefore, cytocompatibility studies were conducted to evaluate the AF patch ability to allow for cell infiltration of viable AF cells. Bovine AF cells were used in the current study to test for cytocompatibility of the AF patch with cells representing an end-stage phenotype found in the native AF. While bovine AF cells were suitable for studying the effects of cell behavior on the AF patch, there is a need for an alternative cell source if it is to be used in a clinical setting. Allogenic AF cell isolation requires the patient to be subjected to an invasive surgical procedure, and xenogeneic AF cells are not a viable option. Therefore, the use of stem cells integrated within the AF patch during implantation needs to be investigated to identify potential improvement for the regeneration capabilities of the patch. This will be addressed in future studies.
The previous testing conducted in our lab demonstrated the viability of AF cells on the outer layers of the AF patch; however, histologically cells were not observed within the inner layers.\textsuperscript{157} It was assumed that the cells seeded on the inner layers may have undergone premature cell death and detached before histological analysis was conducted due to the dense ECM preventing the nutrient transfer. However, the current study presumed that a non-optimized cell seeding process could have contributed to the failure of cells attaching to the inner layers. Therefore, a cell suspension volume optimization protocol was developed to ensure cell seeding accuracy. Additionally, ultrasonication studies were conducted with the intent of disrupting the ECM of the AF patch layers to improve nutrient transfer and cell migration within the inner layers of the AF patch.

The results indicated the AF patch does allow for the attachment and infiltration of AF cells in all three layers for an extended period and is capable of supporting cell viability. This was true for both the control (non-sonicated) samples and sonicated samples. While the volume optimization protocol ensured cell attachment on the inner layers of the AF patch was feasible without tissue disruption, sonicated samples showed a statistical difference in the distance cells were capable of infiltrating into the tissue; however, sonication treatment of the AF patches could also potentially weaken mechanical properties.

**Conclusions & Recommendations for Future Studies**

Coupled with our previously reported findings that illustrate the simplicity and scalability of our patch formation method concurrent with its ability to support AF cell
attachment, these mechanical testing data suggest our AF patch is an ideal surrogate to replace and regenerate the native human AF tissue. Overall results show similar mechanical properties to native human AF tissue through the ultimate tensile strength and elastic moduli, the ability to resist instantaneous changes of IDP, and strength to withstand tensile fatigue mimicking the intact (15-25 layer) native human AF. Thus, our device could improve patient outcomes and lower health care costs for those with IVDH by allowing for the utilization of less aggressive discectomy, minimizing the reoccurrence of IVDH, and may allow the use of early-stage interventions including nucleus arthroplasty for those with IVDD. Future studies should include burst fatigue strength, animal studies, and further cellular characterization studies (stem cell seeding, GAG content, and cross-linking).
CHAPTER III

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THE FABRICATION AND CHARACTERIZATION OF A MULTI-LAMINATE, ANGLE-PLY COLLAGEN PATCH FOR ANNULUS FIBROSUS REPAIR

INTRODUCTION

One major limitation of intervertebral disc (IVD) repair is that no ideal biomaterial has been developed which effectively mimics the angle-ply collagen architecture and mechanical properties of the native *annulus fibrosus* (*AF*). Furthermore, it would be beneficial to devise a simple, scalable process by which to manufacture a biomimetic biomaterial that could function as a mechanical repair patch to be secured over a large defect in the outer *AF* that will support *AF* tissue regeneration. Such a biomaterial would; 1) enable the employment of early-stage interventional strategies to treat IVD degeneration (i.e. *nucleus pulposus* (*NP*) arthroplasty), 2) prevent IVD re-herniation in patients with large *AF* defects, and 3) serve as a platform to develop full-thickness *AF* and whole IVD tissue engineering strategies. Due to the innate collagen fiber alignment and mechanical
strength of pericardium, a procedure was developed to assemble multi-laminate angle-ply AF patches derived from the decellularized pericardial tissue. Patches were subsequently assessed histologically to confirm angle-ply microarchitecture and mechanically assessed for biaxial burst strength and tensile properties. Additionally, patch cytocompatibility was evaluated the following of seeding with bovine AF cells. This study demonstrated the effective removal of porcine cell remnants from pericardium and the ability to reliably produce multi-laminate patches with angle-ply architecture using a simple assembly technique. Resultant patches demonstrated their inherent ability to resist biaxial burst pressures reminiscent of intradiscal pressures (IDP) commonly borne by the AF and exhibited tensile strength and modulus values reported for native human AF. Furthermore, the biomaterial supported AF cell viability, infiltration, and proliferation.

The intervertebral discs (IVDs) of the spine support complex loads and motions during activities of daily living. This function is imparted by the structure of the IVD which is comprised of two distinct anatomical regions; 1) the centrally located aggrecan- and collagen type II-rich core (known as the nucleus pulposus (NP)) which is sequestered by 2) 12-25 concentric multi-laminate lamellae composed of type I collagen (known as the annulus fibrosus (AF)) \(^{23,187}\). Evaluation of the microarchitecture of the AF reveals an alternating angle-ply microarchitecture in which the collagen fiber preferred direction within each subsequent lamella is oriented at \(\pm 28-43^\circ\) to the transverse (horizontal) axis of the spine thus providing an optimal structure for resisting tensile loading and intradiscal pressures (IDP) generated by the hydrophilic NP \(^{187}\). During IVD degeneration and herniation, the competency of the AF is often compromised resulting in fissures and
extrusion of the NP, which can impinge on or chemically irritate adjacent nerve roots contributing to discogenic low back pain and muscle weakness \(^{188,189}\). The AF has limited capacity for self-repair and thus in the aforementioned clinical scenarios, restoring AF integrity is critical \(^{71}\). It has been demonstrated that patients with AF tears measuring greater than 6mm are at increased risk for re-herniation \(^{10}\). Additionally, the use of NP implants as early-stage interventions to mitigate the progression of IVD degeneration, require an intact AF to prevent implant migration \(^{71}\). To further illustrate the criticality of AF repair, the AO Foundation (a preeminent musculoskeletal research institution) launched a collaborative research program in 2011 aimed at developing engineering solutions to combat AF rupture.

To date, few synthetic AF repair devices have been marketed, and while development of suturing techniques, adhesives, and various natural and synthetic biomaterials continues, none have yet illustrated comparable structural and mechanical characteristics of the native AF concomitant with the ability to support tissue regeneration \(^{41,50,190,191}\). Herein, we hypothesized that pericardium, the connective tissue that surrounds the heart, would serve as an optimal material with which to construct a multi-laminate angle-ply patch to repair the outer AF. This tissue is a durable, thin sheet of collagen fiber-reinforced matrix that has been extensively used in the manufacture of medical devices including bioprosthetic heart valves and tendon grafts. The pericardium itself is comprised of both dense regular and irregular connective tissue (predominantly type I collagen) that is organized into fibrils, fibers, fiber bundles and laminates \(^{192}\). The fibrous pericardium is the outermost layer of the pericardium containing aligned type I collagen fibers, which is
fused to an adjacent layer of parietal pericardium containing a multi-directional network of fine collagen fibers and elastin. Despite the presence of this multi-directional network; a discrete fiber preferred or predominant fiber directionality is evident within the fibrous pericardium. Thus, we hypothesized that multiple sheets of pericardium could be oriented relative to each other and overlaid such that the preferred fiber alignment could be tailored within each layer of the AF patch in order to achieve varying angle-ply orientations and mechanical properties similar to the human AF. Thus, the objectives of the studies described herein were to 1) confirm the complete decellularization of porcine pericardium, 2) demonstrate a simple and scalable method by which to create a multi-laminate angle-ply AF repair patch, and 3) assess its basic mechanical characteristics and cytocompatibility relative to the native AF and its cells, respectively. Quantitative results were expressed as a mean ± standard error and were statistically compared via one-way analysis of variance with significance denoted as p<0.05.

METHODS & RESULTS

Porcine pericardium was obtained from a local abattoir and transported to the lab within three hours of harvest. Tissue was cleaned of extraneous fat and subjected to a decellularization process previously described by Tedder et al., with modification. Briefly, the pericardium was submerged in distilled water for 24 hours at 4°C to lyse porcine cells via hypotonic shock. Tissue specimens (3 pieces ~2x5cm each) were then transferred to 100ml decellularization (decell) solution (pH 7.8) containing 50mM Tris, 0.15%(v/v) Triton X-100, 0.25%(w/v) deoxycholic acid, 0.1%(w/v)
ethylenediaminetetraacetic acid and 0.02% (w/v) sodium azide while maintained at room temperature under constant agitation (150RPM) for 3 days. Decell solution was changed on day 3 and the process was continued for a total of 6 days prior to sequential washes in 70% ethanol and distilled water (two washes each for 10 minutes while agitating at room temperature). Tissues were placed in a solution (pH 7.5) of DNase/RNase (720U/ml each) containing 5mM magnesium chloride at 37°C for 24 hours at 150RPM. Efficacy of decellularization was assessed via routine, paraffin-embedded histology (5μm sections) followed by hematoxylin & eosin (H&E) staining for the evaluation of cell nuclei as well as agarose gel electrophoresis and Nanodrop spectrophotometry for residual porcine DNA which was isolated from tissue via a Qiagen DNeasy Blood and Tissue kit according to manufacturer’s instructions. Additionally, immunohistochemistry (IHC) for the porcine antigenic epitope alpha-gal (biotinylated GSL1-isolectin B4; 2.5μg/ml; VectorLabs) was performed. Histology results indicated the complete absence of intact porcine cell nuclei in decellularized samples with some evidence of tissue swelling and minor matrix disruption indicated by an increase in overall tissue thickness to ~250μm as compared to fresh pericardium (~150μm) (Figure 19A&B). Furthermore, staining with 4’, 6-diamidino-2-phenylindole (DAPI) for cell nuclei was absent (data not showed). Additionally, IHC for alpha-gal, the xenogenic epitope responsible for acute rejection of porcine-derived materials in humans was below the detection limit of the antibody in the decellularized pericardium (Figure 19C&D). One percent agarose gels stained with ethidium bromide demonstrated the absence of residual DNA greater than 300 base pairs (bp), concomitant with a significant (p<0.05) 95% reduction in double-stranded DNA
content in decellularized pericardium compared to fresh tissue as determined by spectrophotometry (96.2±13.4 and 2051±112.7ng/mg dry weight, respectively) (Figure 19E&F). Removal of xenogeneic DNA is required when developing biomaterials for implantation into humans so as not to elicit an immune reaction. Our results are in alignment with Gilbert et al. and Crapo et al. and who provide initial benchmarks defining minimal criteria for effective tissue decellularization. Furthermore, herein we corroborate the effectiveness of the decellularization technique developed by Tedder et al. illustrating the repeatability of the procedure.

Figure 19: Representative histological images of A) fresh and B) decellularized porcine pericardium stained with H&E, respectively (pink = ECM, arrowheads =
Representative histological images of C) fresh porcine pericardium demonstrating positive (brown) IHC staining for alpha-gal epitope and D) decellularized pericardium illustrating a lack of positive staining (blue = matrix, black = cell nuclei), (total magnification: 200X). (Inserts = negative IHC controls). E) Ethidium bromide-stained agarose gels for DNA isolated from fresh (lanes 1-5) and decellularized (lanes 6-10) pericardium (white bands = presence of DNA). A 300-24000bp DNA standard ladder (lanes 11-12) is shown for comparison. F) DNA quantification of fresh and decellularized pericardium performed with Nanodrop spectrophotometry. G) Diagrammatic representation of multi-laminate AF patch formation in which at least three ply’s of decellularized pericardium were stacked (red tubes = aligned collagen type I fibers in the fibrous pericardium layer of each ply) oriented at ±30° to each other. H) Schematic representation depicting histology sectioning of AF patches using an oblique cut (dotted red line) across multiple layers performed to microscopically visualize collagen fiber alignment in fibrous pericardium surfaces stacked directly adjacent to one another demonstrating a ±30° “chevron” (*) pattern. Alternatively, when fibrous and parietal pericardium surfaces were directly adjacent to each other, a “half chevron” (#) was achieved. I) Chevron and J) half chevron patterns (dashed white outlines), respectively were observed within each patch via polarized light microscopy confirming the presence of oriented collagen fiber alignment, (total magnification: 100x). K) Macroscopic image of a 6-ply AF patch sewn with suture (black outline).
Solid lines connecting different study groups on the graph indicate a significant difference (p<0.05).

Next, in order to create multi-laminate AF patches (Figure 19G), decellularized pericardium sheets were gently dried with tissue paper and sections of the tissue with a clearly defined collagen fiber preferred/aligned direction were identified in the fibrous pericardium and cut out into squares. The fiber aligned direction of each square was then oriented ±30° (verified via a protractor) relative to a stationary grid containing a common horizontal axis. Once aligned, sections were stacked, and the multi-laminate sheets were placed upon a dissolvable embroidery backing material (Sulky Fabri-Solvy; 100% polyvinyl alcohol), which allowed for easy positioning within a sewing machine (Brother JX2517) and enabled sewing needle penetration through all pericardium layers. A square pattern was sewn around the periphery of the sheets using a thread diameter equivalent to a 2-0 suture followed by removal of excess tissue and backing material. The patches, along with the backing were soaked in saline for 30 minutes to ensure that the backing had completely dissolved. To illustrate that patches could be made with adjacent ply’s having an aligned collagen fiber-preferred direction oriented at ±30° relative to each other, polarized light microscopy was performed in conjunction with employing a red fluorescence wavelength filter to visualize multi-laminate patches that had been sectioned obliquely (Figure 19H) across the sample in order to observe multiple layers. Histological inspection of patch sections illustrated the presence of “chevrons” (Figure 19H*) indicating the location at which the collagen-preferred directions in immediately adjacent layers of fibrous pericardium intersect at opposing ±30° angles (Figure 19I&J). While one
of the major hurdles of AF regeneration is to promote the development of a highly oriented multi-laminate structure, one must also consider that any biomaterial developed to assist the repair/regenerative process must also have the appropriate dimensions in order to adequately cover large (~6-6.5mm) defects results from NP herniation while allowing for attachment to lumbar IVDs. Considering the dimensional constraints of lumbar IVD heights, AF patches as small as 4x4mm and upward of 15x15mm were made using this technique and thus were able to meet the aforementioned critical design requirements for use in either the anterior or posterior AF due to their inherent micro-architecture and customizable dimensions. It should be noted that mechanical evaluations of AF patches described below were carried out on 10x10mm multi-laminate patches (Figure 19K) consisting of one to six sheets (or ply’s). The approximate thickness of every single sheet of the decellularized pericardium was 0.25mm whereas six-ply patches were approximately 1.60mm in total thickness.

The native AF is subject to complex stretching and loading including biaxial strains and circumferentially directed tensile hoop stresses developed due to spinal motions and intradiscal pressure (IDP) generated by the NP. Thus, it is critical to assess the inherent strength of the AF patches to resist biaxial burst. Briefly, single layers of the pericardium and AF patches of 2-, 3-, and 6-ply (n=6 each) were assembled and subjected to biaxial ball burst directed radially (perpendicular to the axis of the patch fibers) generate/simulating a potential large AF herniation or expulsion of an NP replacement out through the patch thickness. Briefly, patches were placed in a custom designed stationary test fixture, which centered and secured the AF patch between two restraining blocks lined with coarse grit
sandpaper and contained a 6.25mm diameter thru-hole. The fixture and patches were secured to the base of an Instron mechanical test frame fitted with a 1000N load cell. A 6mm diameter stainless steel ball was welded to a push rod, which was secured to the test frame crosshead. The crosshead was lowered such that the rod and ball were directed into the thru-hole of the stationary test fixture until contact was made with the secured AF patches (indicated by the generation of a 0.1N preload). Testing was performed at a rate of 300mm/min until patch rupture. The resultant ball-burst pressure at failure was calculated given the maximum force at rupture and its relationship with ball-burst pressure according to established equations (Equations 1-3) given the geometric constraints of our test set-up.

**Equation 1:** \[ P = \frac{F}{A} = \frac{F}{(2\pi d^2(1-\cos(\varphi)))} \]

**Equation 2:** \[ \varphi = \pi - \left(\frac{\pi}{2} - \tan^{-1}\left(\frac{d}{a}\right)\right) - \tan^{-1}\frac{a}{b} \]

**Equation 3:** \[ f = \sqrt{(b^2 + a^2 - d^2)} \]

Where \( P \) is the ball burst pressure, \( F \) is the maximum recorded burst force, \( A \) is the contact area between the patch and the surface of the steel ball, \( \varphi \) is the contact angle between the AF patch material and ball, \( d \) is the radius of the steel ball, \( f \) is the magnitude of the vector representing the stretching material which is geometrically determined from: \( a \); the distance between the central axis of the ball and tissue clamp set-up (3.25mm), \( b \); the position of the steel ball and push rod relative to its starting point (3mm) which maintains the tangential relationship between the patch material and surface of the ball as described by. Burst strength results illustrated a positive correlation between the number of layers
used in patch construction and biaxial ball burst strength (Figure 20A). AF patches of 1-, 2-, 3-, and 6-ply exhibited average biaxial burst strengths of 1.28±0.12MPa, 2.25±0.16MPa, 2.92±0.46MPa and 5.53±0.23MPa, respectively. All values were significantly different from each other (p<0.05) except between 2- and 3-ply patches. The three-ply patch material inherently exhibited biaxial burst strengths exceeding the highest reported in vivo IDP value of 2.3MPa measured in human lumbar IVDs when lifting a 20kg load with flexed back.

Figure 20: A) Graph of the average maximum biaxial burst strength of 1-, 2-, 3-, and 6-ply AF patches. B) Histological image of a 3-ply patch (with layers separated for clarity) illustrating the presence of AF cells on the surface and penetrating (insert,
total magnification: 200x) the $AF$ patch after 6 days of in vitro culture (purple = nuclei, pink = ECM) (total magnification: 50x). C) Graph illustrating bovine $AF$ cell DNA content within 3-ply $AF$ patches following 6 and 15 days of culture, respectively, as compared to a DNA standard curve generated from a known number of viable $AF$ cells. D) Graph illustrating percent lactate dehydrogenase produced by bovine $AF$ cell seeded 3-ply patches immediately following seeding (Day 0) and after 6 and 15 days of culture relative to a positive cell death control (i.e. $AF$ cell seeded 3-ply patches subjected to snap freezing with liquid nitrogen to induce 100% cell death). Solid lines connecting different study groups on graphs indicate a significant difference (p<0.05).

Uniaxial tensile testing was performed on 3-ply $AF$ patches (n=6) according to methods described by Green et al. who assessed the circumferential tensile properties of human $AF^{101}$. Briefly, patches were affixed between two tensile grips such that the fiber alignment of the patches was oriented ±30° to the axis of applied tension. Additionally, herein single layer sheets of the pericardium (n=6) were tested in the fiber-preferred (tensile loading applied in the direction of collagen fiber alignment) and cross-fiber (tensile load applied perpendicular to collagen fiber alignment) directions. The testing protocol consisted of applying 5 preconditioning cycles to 10% strain followed by testing to failure at a rate of 240mm/min. Modulus values were determined from the linear region of the resultant stress-strain curves. Stress-strain curves demonstrated a non-linear profile as is reminiscent of a viscoelastic material (data not shown). The average ultimate tensile strength (UTS) of the 3-ply $AF$ patch, single-ply decellularized pericardium in the fiber-
preferred and cross-fiber directions were 5.9±0.3MPa, 5.6±1.1MPa, and 2.9±0.2MPa, respectively. The UTS of the single-ply pericardium tested in the cross-fiber direction was significantly different (p<0.05) from the single-ply sampled tested in the fiber-preferred direction as well as the multi-laminate AF patch. Average linear circumferential modulus of the 3-ply AF patch, single-ply decellularized pericardium tested in the fiber-preferred and cross-fiber directions were 16.4±3.5MPa, 62.0±13.6MPa, and 23.6±6.0MPa, respectively. Overall, the UTS and modulus values of the 3-ply AF patches mirror the values reported for posterolateral human AF tissue (3.8±1.9MPa, and 12-24MPa, respectively)\textsuperscript{101,102,197,198}. Furthermore, the average linear modulus of a single sheet of decellularized pericardium in the fiber preferred direction matched values reported for single AF lamellae from the outer AF (64.8 MPa)\textsuperscript{198}.

Cytocompatibility of multi-laminate AF patches was assessed after seeding patches with bovine caudal IVD AF cells. Briefly, cow tails were collected within two hours of slaughter and caudal IVDs were isolated via blunt dissection. AF tissue was minced into 2-4mm\textsuperscript{2} pieces and digested in DMEM containing 0.2% collagenase (Type I–125U/mg) and 1% antibiotic/antimycotic (Ab/Am; 10,000 I.U. penicillin, 10,000 µg/ml streptomycin, 25µg/ml amphotericin) for 18 hours at 37°C. Prior to seeding, multi-laminate AF patches were sterilized using 0.1% peracetic acid in phosphate buffered saline solution (pH 7.5) for 2 hours at room temperature prior to thorough rinsing in sterile saline and overnight neutralization in a solution of 48% FBS, 50% DMEM and 2% Ab/Am. AF cells were seeded drop-wise in 75µl of media containing 1x10\textsuperscript{5} cells (passage 4) on to the surface of 10x10 mm patches. Cells were allowed to attach for 3 hours prior to being flipped and
seeded on the opposite surface. Additionally, following surface seeding, patches were injected with $1 \times 10^5$ cells between the layers using a 20G syringe. Cells were allowed to culture under standard conditions for up to 15 days. Histological analysis of cell-seeded patches ($n=3$/time-point) was completed on paraffin embedded, 5μm sections stained with H&E, which were imaged on a Zeiss AxioVert A1 microscope with Axiovision software. DNA content and cell death on the patches was assessed via Picogreen ($n=3$ patches/time-point) and lactate dehydrogenase (LDH; $n=3$ patches/time-point) assays, respectively according to the manufacturer’s instructions. To determine the number of cells attached to the multi-laminate AF patches, a standard curve was developed from known numbers of bovine AF cells seeded in well plates subjected to Picogreen analysis. Additionally, LDH values were expressed as a percentage of a positive cell death control developed by snap freezing AF cell seeded patches 3 days prior to LDH analysis on the culture media. Histological results confirmed AF cell attachment to the surfaces of the multi-laminate patches forming a monolayer. Furthermore, there was evidence of cellular infiltration into the lamellae of the patches as well (Figure 20B). DNA content of multi-laminate patches significantly ($p<0.05$) increased between day 6 and day 15 (0.264±0.081 and 0.625±0.090μg DNA/ml, respectively) suggesting AF cell proliferation over time in culture (Figure 20C). Interpolation from a standard curve developed from known numbers of bovine AF cells demonstrated greater than $3 \times 10^5$ cells on each patch by day 15. LDH content of culture media surrounding patches immediately following seeding (day 0) and after 6 and 15 days of culture was 10.31±0.48%, 28.72±1.22%, and 29.94±0.90%, respectively as compared to positive controls at each respective time-point (Figure 20D).
While LDH values at day 6 and 15 were both significantly different (p<0.05) compared to day 0, there was no difference between days 6 and 15 indicating that there was no increase in cell death with increasing time in culture, therefore, illustrating cytocompatibility of multi-laminate AF patches.

**DISCUSSION & CONCLUSION**

In conclusion, this is the first study to report the development of an angle-ply multi-laminate AF repair patch using a simple and scalable process resulting in a biomaterial that demonstrates structural and mechanical properties comparable to that of native human AF tissue. Furthermore, this multi-laminate biomaterial supports the viability and proliferation of AF cells thus illustrating its regenerative potential. Taken together, the potential clinical value of this multi-laminate AF patch for patients undergoing surgical procedures for IVD degeneration and herniation is immense. Ongoing investigations of this biomaterial include 1) assessing the fatigue properties (tensile and torsional), 2) developing a surgical fixation technique to secure the patch in place, 3) evaluating matrix deposition and cell phenotype following seeding with an alternative cell source (i.e. human amniotic mesenchymal stem cells) and 4) further developing a multi-laminate implant to fill defects throughout the depth of the AF which could be accomplished by combining the patch with a mechanically competent and cytocompatible material.
ANGLE-PLY BIOMATERIAL SCAFFOLD FOR ANNULUS FIBROSUS REPAIR REPLICATES NATIVE TISSUE MECHANICAL PROPERTIES, RESTORES SPINAL KINEMATICS, AND SUPPORTS CELL VIABILITY

ABSTRACT

Annulus fibrosus (AF) damage commonly occurs due to intervertebral disc (IVD) degeneration/herniation. The dynamic mechanical role of the AF is essential for proper IVD function and thus it is imperative that biomaterials developed to repair the AF withstand the mechanical rigors of the native tissue. Furthermore, these biomaterials must resist accelerated degradation within the proteolytic environment of degenerate IVDs while supporting integration with host tissue. We have previously reported a novel approach for developing collagen-based, multi-laminate AF repair patches (AFRPs) that mimic the angle-ply architecture and basic tensile properties of the human AF. Herein, we further evaluate AFRPs for their: tensile fatigue and impact burst strength, IVD attachment
strength, and contribution to functional spinal unit (FSU) kinematics following IVD repair. Additionally, AFRP resistance to collagenase degradation and cytocompatibility were assessed following chemical crosslinking. In summary, AFRPs demonstrated enhanced durability at high applied stress amplitudes compared to human AF and withstood radially-directed biaxial stresses commonly borne by the native tissue prior to failure/detachment from IVDs. Moreover, FSUs repaired with AFRPs and nucleus pulposus (NP) surrogates had their axial kinematic parameters restored to intact levels. Finally, carbodiimide crosslinked AFRPs resisted accelerated collagenase digestion without detrimentally effecting AFRP tensile properties or cytocompatibility. Taken together, AFRPs demonstrate the mechanical robustness and enzymatic stability required for implantation into the damaged/degenerate IVD while supporting AF cell infiltration and viability.

**INTRODUCTION**

Intervertebral discs (IVD) support axial compressive loading of the spine while allowing for flexibility and a defined range of motion during activities of daily living. IVD’s are comprised of two distinct regions: the central gelatinous core is known as the nucleus pulposus (NP) which is circumferentially constrained by the annulus fibrosus (AF). The NP is a highly-hydrated tissue composed primarily of collagen type II and aggrecan, which provides load support due to its low permeability and the generation of intradiscal pressure (IDP). The AF is a highly organized lamellar structure consisting of 15-25 sheets of collagen type I with fibers aligned in alternating orientations of ± 28-43° to the transverse axis of the spine yielding an ‘angle-ply’ architecture. The AF functions to
circumferentially confine the NP and resist tensile strains experienced during rotational and bending spinal motions.93,94

Annually, over 5.7 million Americans are diagnosed with IVD disorders including IVD degeneration (IVDD) and herniation (IVDH) which ultimately compromise the structural integrity of the AF.2 This results in a loss of IVD height, impaired IVD mechanical function, patient pain, and disability.32–38 Accordingly, discogenic low back pain (LBP) affects approximately 80% of the adult population during their lifetime resulting in a diminished quality of life,2,17–19 and estimated health care expenditures exceeding $85.9 billion.3,199 Surgical treatments for late-stage IVDD include spinal fusion and total disc replacement, however, these methods suffer from significant drawbacks.65 Newer technologies, including NP replacement (NPR), are being developed as interventional strategies to mitigate IVDD progression.200,201 Such devices have not yet realized clinical utility, due in part to the lack of a mechanically robust AF repair method. Additionally, the nearly 500,000 patients undergoing discectomies annually to remove protruding/herniated NP tissue may benefit from a biomaterial that can restore AF integrity following the procedure. Studies demonstrate that conservative discectomies (i.e. those removing minimal NP material) often result in maintenance of IVD height, biomechanics, and improved patient outcomes; however, these patients are at increased risk for re-herniation and incur significant reoperation costs.6,55,59,159,161,162,202–205 These detrimental consequences may be moderated via utilization of an AF repair method.

Accordingly, there has been a recent increase in the development of AF repair strategies ranging from simple mechanical closures to biomaterial scaffolds. While many
biomaterials developed for AF repair have been assessed for their ability to promote tissue regeneration in vitro,\textsuperscript{120,130,138,206,207} few have undergone thorough testing to evaluate their mechanical competency required for implantation into the spine.\textsuperscript{102,198,207,208} Moreover, even fewer have been assessed for their contribution to restoring functional spinal unit (FSU) kinematics following injury and repair; arguably one of the most important functional outcomes of any motion preserving/sparing spinal implant. Finally, biomaterials to be implanted into a damaged IVD must demonstrate resistance to accelerated degradation as investigations have illustrated increased concentrations of destructive proteases which could jeopardize their mechanical integrity.\textsuperscript{23,209,210} This is of particular importance for biomaterials composed of extracellular matrix (ECM) components, which often have to be chemically crosslinked to impart resistance to accelerated enzymatic degradation, yet should demonstrate cytocompatibility. Taken together, a critical need exists to create an effective AF repair biomaterial, which demonstrates the ability to survive in the mechanical, and biochemical environment of the damaged IVD and which will allow for eventual integration or regeneration of healthy AF tissue. The development of such a biomaterial may reduce the rate of IVD re-herniation, improve patient outcomes, and delay the need for spinal fusion procedures.\textsuperscript{211}

We have previously reported the development of a novel collagen sheet-based annulus fibrosus repair patch (AFRP) biomaterial derived from decellularized porcine pericardium, which has been assembled using a simple, scalable, and repeatable process. The resulting AFRPs have been shown to mimic the multi-laminate angle-ply (i.e. layered) architecture and basic tensile mechanical properties of the human AF.\textsuperscript{212} Herein we aimed
to further mechanically evaluate this biomaterial for its tensile fatigue strength, resistance to impact loading, attachment strength to IVDs, and its ability to assist in restoring axial kinematics following repair of injured FSUs. Additionally, we have assessed the ability of various crosslinking chemistries to render AFRPs resistant to accelerated protease degradation and evaluated their effects on AFRP tensile properties. Finally, considering the long-term goal of using this biomaterial in conjunction with autologous or allogenic cells to regenerate healthy AF tissue, we evaluated the ability of the AFRP to support AF cell viability and infiltration.

**MATERIALS & METHODS**

**Fabrication of Annulus Fibrosus Repair Patches (AFRPs)**

Multi-laminate angle-ply AFRPs were developed and assembled from decellularized porcine pericardium as previously described by McGuire et al.\textsuperscript{212} AFRPs were maintained in a phosphate buffered saline storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing.

**Preparation of Functional Spinal Units**

Bovine tails from 2-3-year-old calves were obtained from a local abattoir and transported on wet ice to the lab within an hour. Excess tissue surrounding the vertebral bodies and intervertebral discs were removed via dissection and functional spinal units (FSUs: vertebrae-IVD-vertebrae) were isolated via shears. Three FSUs were harvested from three caudal levels (cc1-2 to cc3-4: IVDs closest to the rear end) and were potted
using wood screws and urethane potting resin to prevent slippage of the samples during testing. In general, bovine IVDs have been shown to have similar swelling pressure, geometry and resting stress compared to human lumbar IVDs.213 Prior to testing, FSUs were wrapped in gauze saturated with storage solution and stored at -20°C. Samples were thawed within the sealed zip-lock bag, which was submerged for four hours in PBS at ambient temperature thus not allowing tissue swelling.

**Biomechanical Evaluations of Non-Crosslinked Multi-Laminate AFRPs**

**Biaxial Impact Burst Strength of AFRPs**

Biaxial impact burst strength testing was modeled after ASTM D1709: “Standard Test Methods for Impact Resistance of Plastic Film by the Free-Falling Dart Method” with modification. Representative samples of AFRPs (2-, 3-, and 6-ply; n=5/group; AFRP dimensions: 2-ply; 12 mm (L) x 12 mm (W) x 0.5mm (T), 3-ply; 12 mm (L) x 12 mm (W) x 0.75mm (T), 6-ply: 12 mm (L) x 12 mm (W) x 1.5mm (T)) were tested using a custom designed free-fall impact testing drop-tower (Figure 21). The base platform of the drop-tower consisted of a tissue holding clamp and four vertical rails, which guided a free-falling platform. The tissue holding apparatus consisted of two stacked blocks lined with coarse-grit sandpaper each having an aligned thru-hole of 6.25mm diameter. AFRPs was sandwiched between the two blocks centered over the two thru-holes. Subsequently, a 6mm steel ball attached to a 3-inch pushrod was placed in contact with the AFRP via the thru-hole in the superior block. Various weights ranging from 0.18-0.58kg were stacked on the free-fall platform, which was then dropped from a constant height of 0.254m. Impact
energy (E) was calculated using the equation for kinetic energy, \( E = \frac{1}{2} m v^2 \), where \( m \) = mass and \( v \) = velocity. The resultant ball-burst pressure was calculated given the maximum force at rupture and its relationship with ball-burst pressure and geometric constraints according to established procedures.\(^{195,196,212}\) AFRPs were kept moist throughout testing via saline spray.

Figure 21: A) Representative image of a custom designed free-fall impact testing drop-tower consisting of a base platform with a four vertical rail system to guide a free-falling platform with a predetermined attached weight. The tissue holding apparatus consists of two stacked blocks with an aligned thru-hole of 6.25mm diameter. AFRP samples were sandwiched between two blocks centered over the two thru-holes. 1) a 6mm steel ball and attached 3-inch pushrod placed in contact with an AFRP. 2) As the top platform falls, an increase of stress is applied across the AFRP until 3) AFRP failure. B) Representative image of a functional spinal unit (vertebrae-
IVD-vertebrae) with a 6mm ball and push rod fed through a predrilled 6.25mm pathway through the IVD. An axial force is being applied radially into an AFRP until failure of AFRP attachment mechanism. *Red arrow indicates the direction of the applied force.*

**Tensile Fatigue Strength of AFRPs**

Tensile testing was performed using the methods described by Green et al. with minor modification.\(^{214}\) Testing was performed using a Bose ElectroForce 3200 series (model 3220), fitted with a 100-lb. load cell in a test chamber filled with PBS and protease inhibitor solution at ambient temperature. Three-ply AFRPs (n=16; AFRP dimensions: 7 mm (L) x 7 mm (W) x 0.75mm (T)) were preconditioned using 5 cycles of 10% strain applied at a rate of 10mm/min prior to initiation of fatigue testing. Varying stress amplitudes ranging from 0.95 – 2.66MPa (i.e. stress values typically observed in the lumbar spine during activities of daily living\(^{214}\)) were applied at a frequency of 0.5Hz; the number of cycles to failure at each applied stress for each sample was recorded and plotted to develop an S-N curve. Testing was stopped if failure did not occur prior to achieving 10,000 loading cycles, which was defined as a run-out. Displacement, load, total testing time, cycle count, and location of material failure were recorded for all samples.

**Strength of Non-Crosslinked AFRP Attachment to IVDs**

The strength of attachment of the AFRP to the IVDs was performed using a ball-burst test modeled after ASTM D3786/D3786M: “Standard Test Method for Bursting Strength of Textile Fabrics – Diaphragm Bursting Strength Tester Method” with
modification. Laterally directed thru-holes were drilled through the center of bovine tail IVDs using a 6.25mm drill bit to simulate a large herniation or implantation pathway for a pre-formed NPR. AFRP’s (n=6; AFRP dimensions: 7 mm (L) x 7 mm (W) x 0.75mm (T)) were then secured to the FSUs over the center of the defect. Attachment included that application of a topical adhesive (Dermabond Advanced) to temporarily secure the AFRP while it was being sutured (4-0 FiberWire; Arthrex) in place at the four corners of the AFRP with the suture inserted 1-2mm from the edge of the AFRP with throws directed perpendicular to the surface of the AF tissue. Testing was performed on an Instron mechanical testing system with a 1000N load cell. A 3-inch long pushrod with a 6mm steel ball was placed in the Instron’s upper grip and force was directed radially through the IVD and perpendicular to AFRP (i.e. load was applied perpendicular to collagen fiber alignment in the AFRP similar to radial forces applied to the native intact AF by the NP) at a constant rate of 300mm/min. Displacement and load were recorded until failure of the attachment occurred, and the resultant ball-burst pressure was calculated given the maximum force at rupture and its relationship with ball-burst pressure according to established equations.\(^{195,196,212}\) AFRPs were kept moist throughout testing via saline spray.

**FSU Kinematic Testing Following Repair with Non-Crosslinked AFRPs And NPRs**

Axial kinematic testing was performed on a Bose ElectroForce 3200 series, model: 3220, fitted with a 100-lb. load cell in a test chamber filled with PBS and protease inhibitor solution at ambient temperature. The loading methodology and analysis were adapted from previous reports.\(^{206,208}\) Briefly, each FSU (n=5) was tested sequentially as follows: Intact,
Annulotomy, Discectomy, and Repair (Figure 22A). An annulotomy was performed by perforation of the IVD using a 6mm biopsy punch (7mm depth) and the subsequent removal of the AF tissue. Aggressive discectomy was performed by removing $1.0 \pm 0.2 \text{mL}$ of NP tissue through the previously formed aperture remaining after annulotomy. The repair group consisted of replacing the excised tissue with an equal volume of a biologic NPR (termed ABNP - developed and described previously by our lab\textsuperscript{201}) prior to closure with the AFRP (n=5; AFRP dimensions: 7 mm (L) x 7 mm (W) x 0.75mm (T)) which was secured initially with topical adhesive prior to suturing. Suturing of the AFRP consisted of four corners suturing with sutures being passed through the AFRP 1-2mm from the edge of the AFRP with throws directed in alignment with the $\pm 30^\circ$ collagen fibrils in the AFRP such that the fibers were captured within the knot. Prior to test initiation, samples were preloaded to a mean stress of $-0.125 \text{MPa}$; a physiologically relevant stress magnitude borne by the IVD \textit{in vivo}. Between each test, IVD samples were equilibrated in the saline bath for approximately 5 minutes before test initiation. FSUs were then subjected to three consecutive loading regimes: 1) a 1-hour creep period at $-0.5 \text{MPa}$, 2) immediately followed by 35 cycles of tension-compression between 0.25 MPa and $-0.5 \text{MPa}$ at 0.1Hz, and 3) ending with a constant slow-ramp to $-0.5 \text{MPa}$ at a rate of 1 N/s. The testing rate of 0.1Hz has been shown to represent normal physiological spinal frequency (Figure 22B).\textsuperscript{206,215}
Figure 22: Study design for *in vitro* kinematic testing using AFRP and ABNP repair biomaterials. A) Representative images of assigned testing groups and schematic of preparation for kinematic testing of bovine caudal functional spinal units (FSUs). B) Loading scheme for FSU testing depicting creep, axial cyclic tension-compression, and slow constant-rate ramp tests. C) Representative axial force-displacement curve from axial tension-compression loading and its post-test analysis. D) Representative creep displacement curves from a single FSU.
The force-displacement curve (Figure 22C) for the unloading phase of the 35th cycle was used to analyze the cyclic loading data as previously described to ensure dynamic equilibrium was achieved. Tensile and compressive stiffness were calculated via linear fit of the force-displacement curve from 60-100% of the respective peak loads. The axial range of motion (RoM) was defined as the total peak-to-peak displacement of the IVD. Neutral zone (NZ) length was determined by fitting a third-order polynomial to the data and finding the maxima and minima with the correlating range between the peaks. Creep data (Figure 22D) was analyzed using a non-linear constitutive model fit to the data using R statistical software as described previously. Step displacement was defined as the initial deformation that occurred until the target load was achieved, while the creep displacement was the displacement that occurred after the step displacement. A four-parameter rheological model equation: 

\[
\frac{d(t)}{L_o} = \frac{1}{\Psi_1} \left( 1 - e^{-\frac{t}{\eta_1}} \right) + \frac{1}{\Psi_2} \left( 1 - e^{-\frac{t}{\eta_2}} \right)
\]

was fit to the creep displacement data, where \( \Psi_i \) represents the elastic response and \( \eta_i \) represents the viscous response. Short-term and long-term creep time constants (\( \tau_1 \) and \( \tau_2 \), respectively) were derived from constitutive model coefficients using the equation: \( \tau_i = \frac{\Psi_i}{\eta_i} \). The constant-rate slow-ramp compression stiffness was determined using a linear fit of the slow-ramp load-displacement response.

**Compression to Failure of FSUs Repaired with Non-Crosslinked AFRPs And NPRs**

Following kinematic testing of FSUs, samples were transferred to an Instron mechanical testing system fitted with a 10kN load cell. Samples were compressed to failure
at a rate of 300mm/min until FSU failure (i.e. contact of the adjacent vertebral endplates and/or AFRP failure / NPR herniation). The sample load and the initial cross-sectional area of the IVDs were determined to calculate the engineering compressive stress applied to the IVD. Digital video was used to record mode and time of failure.

**AFRP Crosslinking Studies**

**Crosslinking Procedures**

Crosslinking of AFRPs using various concentrations of either carbodiimide- or glutaraldehyde-based chemistries were evaluated. Carbodiimide-based crosslinking was carried out using either 6mM or 30mM 1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide HCL (EDC) with 1.2mM or 6mM N-hydroxysuccinimide (NHS), respectively while buffered in 50mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.5) at ambient temperature. Glutaraldehyde (GLUT) treated AFRPs were prepared using 0.2% or 0.6% GLUT in 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) at ambient temperature. Following 24 hours of crosslinking, all AFRPs were rinsed and placed in a storage solution for a maximum of 3 days at 4°C prior to testing.

**Collagen Stability of Crosslinked AFRPs**

Thermal denaturation temperature (T_d) was used as a measure of collagen stability of non-crosslinked and crosslinked (6mM and 30mM EDC, 0.2% and 0.6% GLUT) AFRPs in addition to native bovine AF tissue for comparison (n=3 non-crosslinked AFRPs and fresh AF) (n=4 /crosslinked group). Briefly, a differential scanning calorimeter (DSC:
Model Q1000) was used to determine $T_d$ which was defined as the maximum value of the endothermic peak (thermal transition midpoint). Heating of each sample was performed from 20 to 120°C at a rate of 10°C/minute in accordance with the previous literature.\textsuperscript{216,217}

### Crosslinked AFRP Resistance to Accelerated Collagenase Degradation

The resistance to accelerated collagenase degradation was assessed for non-crosslinked and crosslinked (6mM and 30mM EDC, 0.2\% and 0.6\% GLUT) AFRPs (n=5/group) in addition to native bovine AF tissue (n=3/group per time point). Samples were rinsed in TRIS buffer, blotted dry, frozen at -80°C, lyophilized, weighed, and rehydrated in TRIS buffer. Samples were then incubated in 2mL of 33.6 U/mL collagenase type I at 37°C for up to 7 days. Sample mass loss was determined by comparing the dry mass of each sample before and after enzymatic digestion. At Day 7, samples were further incubated for an additional 7 days in an increased concentration of collagenase, 336 U/mL, to ensure full tissue degradation.

### Tensile Properties of Crosslinked AFRPs

To define the effect of crosslinking on AFRPs, tensile testing was performed using an MTS mechanical system using methods previously described by Green et al. with minor modification.\textsuperscript{214} Crosslinked AFRPs (n=5/group; AFRP dimensions: 14 mm (L) x 14 mm (W) x 0.75mm (T)) were affixed between two tensile grips such that the fiber alignment of the AFRPs was oriented ±30° to the axis of applied tension. AFRPs were preconditioned for 5 cycles to 10\% strain at 10mm/min and tested to failure at 240mm/min to determine
elastic modulus (EM: linear region of the stress-strain curve), ultimate tensile strength (UTS: maximum peak load divided by the initial cross-sectional area of the sample) and maximum tensile strain at failure (TSF: % strain at AFRP failure).

Cell Seeding Studies on Crosslinked AFRPs

Isolation and Expansion of Bovine AF Cells

Primary bovine AF cells (bAFCs) were enzymatically isolated from freshly isolated bovine tail caudal IVDs (n=4 IVDs from different donors). Briefly, the NP was removed first via an 8mm biopsy punch to ensure the harvest of only AF tissue. The AF tissue was then removed, minced via scalpel, and transferred to a 50mL conical tube with 25mL of collagenase solution (1% Ab/Am, 0.2% Collagenase Type I (125 units/mg) and Dulbecco’s Modified Eagle Medium (DMEM). Following overnight (~18 hours) digestion at 37°C, and the digested tissue was filtered through a 100µm cell strainer and the filtered cell suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and the cells were re-suspended in cell culture media (CCM), pooled and expanded. CCM consisted of DMEM (with L-glutamine, 1g/l glucose and sodium pyruvate) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Ab/Am). Passage 3 (P3) bAFCs were used for all studies.

Preparation and Seeding of Crosslinked AFRPs

Prior to cell seeding, all AFRPs (n=5/group; AFRP dimensions: 7 mm (L) x 7 mm (W) x 0.75mm (T)) were sterilized in 0.1% neutral buffered (pH 7.4) peracetic acid for two
hours on an orbital shaker at 150 rpm, rinsed in sterile PBS (3x: 1 hour/wash), and neutralized (50% FBS/ 48% DMEM + 2% Ab/Am) while agitated on an orbital shaker at 150 rpm at ambient temperature. AFRPs were then seeded on the surface of each ply with bAFCs (9x10^6 cells/cm^3) via dropwise addition. To cell seed the surface of the interior ply, a 21G needle was used to puncture through the edge of the AFRP immediately adjacent to the suture line and cells were injected. Cell-seeded AFRPs were cultured under standard culture conditions at 37°C for up to 14 days.

**Cytocompatibility of Crosslinked AFRPs**

Cytotoxicity of crosslinked AFRPs was assessed following bAFC seeding via a lactate dehydrogenase assay (LDH; n=3/time-point/group) performed on the CCM. Positive death controls consisted of AFRPs seeded with bAFCs, which were allowed to culture in parallel with the experimental groups. Subsequently, three days prior to LDH analysis on crosslinked AFRPs, the corresponding positive death controls were snap frozen and placed back in culture for the remainder of three days so LDH could accumulate. LDH values are expressed as a percentage of the positive (100%) cell death control.

**Cell Infiltration into Crosslinked AFRPs**

To determine the effect of AFRP crosslinking on bAFC infiltration depth, histological analysis was used to quantify cell infiltration and distribution on 3-ply AFRPs (n=5/group; AFRP dimensions: 7 mm (L) x 7 mm (W) x 0.75mm (T)). Following seeding and culture, AFRPs were fixed in 10% neutral buffered formalin for 24 hours prior to
paraffin embedding and sectioning to 5µm followed by staining with Hematoxylin and Eosin (H&E). A Zeiss Axio Vert.A1 microscope with AxioVision SE64 Rel. 4.9.1 software was used for imaging slides. Additionally, ImageJ 1.50b software was used to quantitatively define cellular infiltration depth via imaging three random locations per AFRP (n=3/group) at 200x total magnification and averaging the bAFC infiltration depth of the 20 deepest cell nuclei from the outer edge of the respective ply.

**Sonication of Crosslinked AFRPs**

To determine if sonication would assist in improving bAFC infiltration depth into crosslinked AFRPs, a Branson ultrasonic cleaner (model 2800; 40kHz) was used to physically disrupt 6mM EDC crosslinked AFRPs for 1-, 5-, and 10-minutes, respectively.

**Tensile Properties of Crosslinked & Sonicated AFRPs**

To define the effect of sonication on mechanical properties of 6mM EDC crosslinked AFRPs (n=5/group), tensile testing was performed as outlined above in section 2.5.4.

**Cell Infiltration into Crosslinked & Sonicated AFRPs**

To define the effect of sonication on cell infiltration within 6mM EDC crosslinked AFRPs (n=5/per group), histological analysis was performed as outlined above in section 2.6.4.
Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism 7 software. Results are represented as mean ± standard error of the mean (SEM) and were statistically compared via a one-way ANOVA followed by a Tukey’s (Biaxial Impact Burst Strength, Tensile Fatigue Strength, IVD Attachment Strength, and Compression to Failure) or Dunnet’s (Collagen Stability, Resistance to Collagenase Degradation, Cytocompatibility-LDH Assay, Cell Infiltration, and Tensile Properties of Crosslinked and Sonicated AFRPs) post-hoc statistical analyses. FSU kinematic data were evaluated with a one-way repeated measures ANOVA followed by Dunnet’s post-hoc analysis. Significance was defined as (p<0.05).

RESULTS

Non-Crosslinked AFRPS Withstand Physiologically Relevant Impact Stresses

Multi-laminate AFRPs underwent biaxial impact burst strength to evaluate its resistance to failure during sudden changes in IDP. Mean burst strength of 2-, 3-, and 6-ply AFRPs was 0.93 ± 0.0 MPa, 1.51 ± 0.06 MPa, and 3.42 ± 0.09 MPa, respectively (Figure 23A). Additionally, the average kinetic energy absorbed by 2-, 3-, and 6-ply AFRPs was 0.32 ± 0.00 J, 0.51 ± 0.02 J, and 1.16 ± 0.03 J, respectively. Expectedly, burst strength increased with increasing number of plies; the burst strength of 6-ply AFRPs was statistically greater than 2- (p=0.0003) and 3-ply (p=0.0006) AFRPs.
Figure 23: Biomechanical evaluations of non-crosslinked multi-laminate AFRPs subjected to biaxial impact burst and tensile fatigue. A) Representative graph of the average maximum calculated impact burst strength withstood by 2-, 3-, and 6-ply AFRPs. Solid lines connecting different groups on the graph indicate a significant difference ($p<0.05$). B) S-N curve illustrating the fatigue strength of 3-ply AFRPs, in comparison of native human AF (Dotted horizontal line indicates 70% UTS [46] of measured human AF. Open diamonds and triangles indicate specimens with no mechanical failure observed (i.e. run-out to 10,000 cycles).

**Non-Crosslinked AFRPS Fatigue Strength is Comparable to Human AF Tissue**

Tensile fatigue failure was evaluated to determine the endurance limit of AFRPs by measuring the cycles to failure across three different applied stress amplitude ranges (low: 1.5-2 MPa, moderate: 2-2.5 MPa, and high: 2.5-3 MPa) which represent 25-80% of the UTS range of native posterolateral human AF (Figure 23B).\textsuperscript{214} The average number of cycles to failure when exposed to low and moderate stress amplitude ranges for AFRPs was $6932 \pm 946$ and $4068 \pm 1055$ cycles, respectively which were not statistically different from the number of cycles to failure reported for native human AF tissue ($7959 \pm 2041$ and $2536 \pm 1076$ cycles, respectively). However, at higher applied stress amplitudes, the number of cycles endured prior to failure by AFRPs ($1155 \pm 75$ cycles) was significantly greater ($p=0.0443$) compared to native human AF tissue ($447.5 \pm 252.5$ cycles).
Attachment of Non-Crosslinked AFRPS To IVDS Can Resist Physiologically Relevant Pressures

The attachment strength of 3-ply AFRPs following suturing to bovine tail caudal IVDs was determined using a modified ball-burst test. The average biaxial attachment strength of 3-ply AFRPs was $1.45 \pm 0.08$ MPa (Figure 24). Macroscopic evaluation of AFRPs following testing illustrated three primary failure modes: AFRP rupture (1 of 6 AFRPs), failure by suture break (4 of 6 AFRPs), and a combination of suture breakage and AFRP rupture (1 of 6 AFRPs) (Figure 24G-I). Together, these results illustrated that the 4-0 suture used to attach the AFRP commonly failed prior to the AFRP itself. The UTS of a single loop of suture was also determined to be $21.24 \pm 1.87$ N via suture pull-out strength testing from a 3-ply; no AFRP failure occurred during these tests again confirming that the suture was primary failure location.
Figure 24: Strength of non-crosslinked AFRP attachment to IVDs. A-C) Representative images of one test specimen illustrating the eventual burst of a 6mm stainless steel ball through an AFRP (Arrow represents the direction of force applied by the ball and rod against the AFRP). D-F) Evaluation of failure mechanism prior to removal of the AFRP (Scale bar = 10 mm). G-I) AFRPs illustrating representative failure modes including; G) AFRP rupture, H) failure by suture break, and I) combined failure modes of suture break and AFRP rupture.
Non-Crosslinked AFRPS Contribute to The Restoration of FSU Kinematics

Following injury (i.e. aggressive annulotomy and discectomy) of bovine caudal IVDs, changes were noted in each mechanical testing parameter evaluated. Results were subsequently normalized to the corresponding intact control; these ratios are shown in Figure 25. In summary, implantation of both the ABNP and the AFRP demonstrated their ability to restore most mechanical parameters to intact values (Tables 8 and 9). A representative axial force-displacement curve from the cyclic tension-compression testing is shown in Figure 22C. Cyclic testing demonstrated a significant increase (p=0.0475) in the range of motion (RoM) between the discectomy (4.62 ± 0.18 mm) and intact groups (4.18 ± 0.17 mm); however, the RoM was restored to intact levels following implantation of the AFRP and ABNP (4.42 ± 0.2 mm; p > 0.05) as was indicated by a lack of statistical difference comparing between the two groups. Furthermore, trends towards achieving restoration to intact values of compressive and tensile stiffness values and neutral zone length (NZ) (intact: 1120.9 ± 48.6 MPa, 319.6 ± 31.0 MPa, and 1.63 ± 0.1 mm, respectively) were observed in the group repaired with AFRP and ABNP (1093.2 ± 44.0 MPa, 315.79 ± 20.6 MPa, and 1.74 ± 0.1 mm, respectively). Slow ramp compressive stiffness of the intact FSUs (527.2 ± 20.9 MPa) demonstrated a significant decrease (p=0.0001) following discectomy (325.0 ± 12.9 MPa); however, the compressive stiffness following annulotomy and repair (412.13 ± 46.7 MPa and 350.7 ± 52.7 MPa, respectively) were not statistically different compared to the intact condition thus indicating restoration.
Figure 25: Kinematic testing results normalized to intact controls. Comparison between groups for A) compressive stiffness and tensile stiffness, B) slow constant-rate slow ramp stiffness C) axial range of motion, D) neutral zone length, and creep parameters E) elastic damping coefficients and F) viscous damping coefficients (i.e.
normalized to the intact IVD (dotted black line at y=1) for each test). Axial biomechanical parameters were partially or completely restored to Intact levels for the AFRP + ABNP repair groups. # and & on the graph indicates a significant difference (p<0.05) compared to the intact or discectomy groups, respectively.

Table 8: Summary of RoM and slow ramp compression results from kinematic testing.

<table>
<thead>
<tr>
<th>Cyclic Loading</th>
<th>Slow Ramp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Compressive Stiffness [N/mm]</td>
<td>Tensile Stiffness [N/mm]</td>
</tr>
<tr>
<td>Intact</td>
<td>1120.95 ± 48.64</td>
</tr>
<tr>
<td>Annulotomy</td>
<td>1009.17 ± 79.4</td>
</tr>
<tr>
<td>Discectomy</td>
<td>1021.77 ± 101.0</td>
</tr>
<tr>
<td>Repair</td>
<td>1093.23 ± 43.96</td>
</tr>
</tbody>
</table>

* (bold) indicates significant difference (p<0.05) from intact controls.
Statistical analysis was performed using a one-way repeated measures ANOVA.

Table 9: Summary of creep results from kinematic testing.

<table>
<thead>
<tr>
<th>Creep Period</th>
<th>Creep Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Step Disp. [mm]</td>
<td>Creep Disp. [mm]</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>1.22 ± 0.3</td>
</tr>
<tr>
<td>Annulotomy</td>
<td>2.25 ± 0.2</td>
</tr>
<tr>
<td>Discectomy</td>
<td>2.89 ± 0.5</td>
</tr>
<tr>
<td>Repair</td>
<td>^1.61 ± 0.4</td>
</tr>
</tbody>
</table>

* (bold) indicates significant difference (p<0.05) to intact controls.
^ (bold) indicates significant difference (p<0.05) between repair and discectomy groups.
Statistical analysis was performed using a one-way repeated measures ANOVA.

A representative creep rate curve was determined from the average curve fit parameters of the test groups developed from a four-parameter exponential model as shown in (Figure 22D). The overall step displacement followed a graded response with the largest
step displacement observed in the discectomy group (2.89 ± 0.46 mm). This large step displacement was associated with the subsequent statistically smaller (p=0.0040) creep displacement (1.04 ± 0.1 mm) compared to intact values. Following repair, step and creep displacement values (1.61 ± 0.4 mm and 1.5 ± 0.1 mm, respectively) demonstrated a restoration to intact values (1.22 ± 0.3 mm and 1.45 ± 0.1 mm, respectively; p>0.05) as was indicated by a lack of statistical difference comparing between the two groups. Early viscoelastic creep response coefficients, $\Psi_1$ and $\eta_1$, were significantly lower for both the discectomy ($\Psi_1$: 663.9 ± 65.9; p=0.0137 and $\eta_1$: 6.8 ± 0.9; p=0.0343, respectively) and repair ($\Psi_1$: 445.2 ± 55.3; p=0.0070 and $\eta_1$: 4.9 ± 0.9 p=0.0087, respectively) groups compared to the intact testing condition. Viscoelastic coefficients reflecting late creep response, $\Psi_2$ and $\eta_2$, demonstrated significant increases in the annulotomy ($\Psi_2$: 215.6 ± 15.4; p=0.0011 and $\eta_2$: 72.3 ± 5.3; p=0.0108, respectively) and discectomy ($\Psi_2$: 254.2 ± 21.9; p=0.0002 and $\eta_2$: 73.2 ± 5.2; p=0.0056, respectively) groups compared to intact controls. The $\Psi_2$ of the repaired group ($\Psi_2$:197.1 ± 12.7; p=0.0108) was significantly different compared to intact values whereas the long-term creep parameter $\eta_2$ was not statistically different from intact which demonstrated restoration in the repair group ($\eta_2$: 47.9 ± 2.86).

**Non-Crosslinked AFRPS Prevent NPR Herniation from IVDS**

Compressive failure strength of the repaired FSUs was assessed under axial compression testing concomitant with visually assessing for AFRP attachment failure and/or ABNP herniation. Testing to failure in all cases was stopped once an average
compressive stress of 4.74 ± 0.43 MPa and compressive displacement of 3.0 ± 0.21 mm was achieved. At this point, video recordings illustrated that the vertebral endplates contacted each other, and the axial load was no longer applied. Macroscopic and video analysis illustrated that although outward bulging of the AFRP was noted, no failure was observed in that the AFRP did not rupture, nor did herniation/extrusion of ABNP occur.

**Collagen Stability of AFRPS is Enhanced by Crosslinking**

DSC analysis illustrated Td values of 73.61 ± 1.93 °C, 67.22 ± 0.14 °C, and 64.86 ± 0.29 °C for the native bovine AF, fresh and decellularized porcine pericardium, respectively. Td values increased for AFRPs crosslinked with 6mM and 30mM EDC and 0.2% and 0.6% GLUT: 78.66 ± 0.31 °C, 86.0 ± 0.37 °C, 87.5 ± 0.26 °C and 90.28 ± 2.0 °C, respectively. Compared to native AF and pericardial tissue, these increases were significant (p=0.0001 for all groups) for the EDC and GLUT treated AFRPs (Appendix B).

**Crosslinked AFRPS Resist Accelerated Collagenase Degradation**

AFRPs were subjected to accelerated collagenase digestion to assess AF resistance to the degradative environment associated IVDD (Figure 26). Following 1 day in digestion solution, non-crosslinked controls lost 46.3 ± 7.1% of their dry mass, while EDC and GLUT crosslinked AFRP groups experienced statistically lower (p=0.0001 for all groups) mass loss (6mM EDC: 2.0 ± 1.0%, 30 mM EDC: 3.8 ± 1.0%, 0.2% GLUT: 1.3 ± 1.0%, and 0.6% GLUT: 2.0 ± 1.7%, respectively). Subsequently, by day 7 a statistical increase in mass loss for non-crosslinked controls (80.8 ± 4.5% average mass loss) occurred.
compared to day 1 values. However, the mass loss did not significantly progress in the EDC or GLUT treated AFRPs compared to their respective day 1 values. After subjecting AFRPs to an enzyme solution containing a 10-fold increase in collagenase, day 14 samples demonstrated a significant increase in collagenase-induced mean mass loss for EDC and GLUT samples (6mM EDC: 10.0 ± 2.3%; p=0.029, 30 mM EDC: 9.5 ± 1.9%; p=0.008, 0.2% GLUT: 7.0 ± 0.8%; p=0.0097, and 0.6% GLUT: 6.8 ± 1.0% p=0.0447, respectively), but these values were significantly lower (p=0.0001) than non-crosslinked controls (100 ± 0.0% average mass loss). Additionally, a significant difference in mass loss comparing between EDC and GLUT treated AFRPs was not evident by day 14, indicating both crosslinking methods imparted similar levels of collagen protection (Appendix C).
Figure 26: Representative images of non-crosslinked (NC), native bovine AF, and crosslinked sample groups illustrating their degradation over time, up to 14 days, simulating an enhanced enzymatic environment. NC AFRPs and native bovine AF experienced 100% mass degradation following day 14, while crosslinked AFRPs
experienced a smaller degree of degradation, which corresponds to the quantitative analysis of percent mass loss.

**AFRP Tensile Properties Are Not Altered by Crosslinking**

Tensile testing of crosslinked 3-ply AFRPs demonstrated that cross-linking itself had minimal effects on AFRP tensile mechanical properties. EM for non-crosslinked controls (18.73 ± 1.56 MPa) was not statistically different from EDC (6mM EDC: 16.05 ± 2.15 MPa, 30mM EDC: 15.63 ± 2.4 MPa) or GLUT (0.2% GLUT: 14.73 ± 2.37 MPa, and 0.6% GLUT: 17.06 ± 1.11 MPa) crosslinked groups (Figure 27A-C). Additionally, UTS of non-crosslinked controls (7.09 ± 0.48 MPa) was not statistically different from 6- and 30-mM EDC or 0.2% and 0.6% GLUT (7.83 ± 0.43 MPa, 5.71 ± 1.02 MPa, 5.85 ± 0.93 MPa, and 6.65 ± 0.55 MPa, respectively). However, tensile strain at failure (TSF) of these groups demonstrated an increasing trend, 77.71 ± 6.9%, 92.69 ± 15.81%, 101 ± 14.9%, and 92.96 ± 11.87%, respectively versus non-crosslinked controls (70.74 ± 3.21%). Additionally, all groups retained comparable mechanical properties to values reported for native human AF (EM: 19.8 MPa, UTS: 5.9 MPa, and TSF: ~65%, respectively).102,207,214
Figure 27: Tensile testing of crosslinked AFRPs compared to native human AF lamellae. A-C) Representative graphs illustrating mean elastic modulus (EM), ultimate tensile strength (UTS), and tensile strain at failure (TSF) for crosslinked AFRPs. (Dotted horizontal line indicates human AF tensile properties reported in the literature).102,214,218

**Crosslinked AFRPS Support AF Cell Viability**

LDH content of culture media from non-crosslinked AFRPs after 3, 6, and 12 days of culture was 21.5 ± 1.2%, 39.6 ± 3.7%, and 35.0 ± 1.1%, respectively as compared to positive death control values at each respective time point (Figure 28B). Both 6mM and 30mM EDC treated AFRPs demonstrated low levels of LDH (6mM: 26.4 ± 1.3%, 41.3 ± 1.7%, and 34.1 ± 1.4%; 30mM: 39.2 ± 2.1%, 36.5 ± 2.3%, and 37.7 ± 1.7%, respectively) which were not significantly different from non-crosslinked controls. Additionally, non-crosslinked and EDC treated AFRPs illustrated no statistical increase in cytotoxicity between days 3, 6, and 12 within each group. Thus, indicating there was no increase in cell death with increasing time in culture. However, both 0.2% and 0.6% GLUT treated AFRPs demonstrated significantly higher (p=0.0001 for all groups) cytotoxicity across each respective time point (0.2% GLUT: 159.4 ± 5.5%, 97.9 ± 2.6%, and 74.7 ± 9.3%; 0.6% GLUT: 104.5 ± 8.1%, 152.8 ± 8.2%, and 84.6 ± 3.0%, respectively) compared to non-crosslinked controls. Furthermore, high levels of cytotoxicity for GLUT samples were further validated with the relative absence of cells within histological samples (Figure 28C) obtained at each respective time point.
Figure 28: Cytotoxicity of crosslinked AFRPS. A) Representative image of bovine AF cells (bAFCs) (P2) (100x) harvested and isolated from skeletally mature bovine caudal IVDs (Insert: Representative image of AF cell fibroblast-like phenotype (400x)). B) Graph illustrating percent lactate dehydrogenase (LDH) produced by bAFC seeded 3-ply AFRPs following seeding and culture of 3, 6, and 12 days relative to a positive cell death control (i.e. bAFC seeded 3-ply AFRPs subjected to snap freezing with liquid nitrogen to induce 100% cell death). Solid lines with (#) connecting different study groups on graphs indicate a significant difference (p<0.05) from non-crosslinked controls. C) Representative H&E images (200x) of 0.2% and 0.6% GLUT
crosslinked AFRPs illustrating minimal to no cell presence at respective time points. *(Black arrowhead indicates cell nuclei).*

**AF Cell Infiltration is Limited Within Crosslinked AFRPs**

Cellular migration within tissue engineering scaffolds is required for tissue ingrowth and regeneration. Therefore, 3-ply AFRPs were evaluated histologically for average bAFC infiltration depth (defined as the average infiltration depth of bAFCs from the seeded ply surface) *(Figure 29).* Representative sections of non-crosslinked AFRPs demonstrated an average bAFC infiltration depth of 105 ± 4.8 µm (71.1 ± 3.2% overall relative tissue thickness [RTT] by day 12), and a maximum bAFC infiltration depth (defined as the farthest bAFC distance from the seeded ply surface) of 141 µm (95.6% overall RTT). However, following EDC crosslinking bAFCs were found forming a monolayer on the outer seeded edge surfaces of the AFRP plies with minimal infiltration observed. At day 12, 6mM EDC crosslinked AFRPs illustrated a bAFC infiltration depth of 3.62 ± 0.2 µm (1.4 ± 0.1% overall RTT) and a maximal infiltration depth of 4.7 µm (1.7% overall RTT) which was statistically lower *(p=0.0058)* compared to non-crosslinked controls. Similarly, 30mM EDC crosslinked AFRPs demonstrated an average bAFC infiltration depth of 14.9 ± 2.9 µm (6.8 ± 1.3% overall RTT) and a maximum infiltration depth of 35.7 µm (16.4% overall RTT), which was statistically greater *(p=0.0089)* compared to 6mM EDC crosslinked AFRPs, however infiltration depth was statistically lower *(p=0.0001)* compared to non-crosslinked controls.
Figure 29: Cell infiltration in crosslinked AFRPS. Representative H&E images (200x) of non-crosslinked, 6mM EDC, and 30mM EDC AFRPs following cell seeding. Crosslinked samples illustrate minimal cell infiltration into AFRPs in contrast to non-crosslinked samples at respective time points (Insert: Magnified image highlighting representative region of interest outlined by dotted-line). *(Black arrowhead indicates cell nuclei).*
Sonication of Crosslinked AFRPS Enhances AF Cell Infiltration and Does Not Detrimentally Alter Tensile Properties

To increase cellular infiltration into the optimized 6mM EDC crosslinked AFRPs, 3-ply samples were sonicated for various times. Sonication has been demonstrated to disrupt fibrous tissue architecture and promote cell infiltration. Representative histological sections subjected to 1-, 5-, and 10-minute sonication periods illustrated an increased average bAFC infiltration depth of 149 ± 31.6 µm (27.4 ± 5.8% overall relative tissue thickness), 234.7 ± 37.8 µm (50.6 ± 8.2% overall RTT), and 98.0 ± 19.3 µm (33.4 ± 6.6% overall RTT), respectively by day 14 (Figure 30). The maximum bAFC infiltration depth of 1-, 5-, and 10-minute sonicated crosslinked AFRPs was 333.9 µm (72.0% overall relative tissue thickness), 416.3 µm (89.7% overall RTT), and 272.9µm (93% overall RTT), respectively. Overall, histological analysis following 7 and 14 days of cell culture illustrated AFRPs subjected to 5- and 10-minute sonication periods demonstrated improved cell presence on the middle ply of the AFRPs and enhanced bAFC infiltration within all plies. All sonicated testing groups demonstrated a significant increase (1- vs. 5-minute: p=0.0058; 1- vs 10-minute: p=0.0009) in maximum cell infiltration depth compared to non-sonicated controls. Additionally, AFRPs sonicated for 5-minutes demonstrated a significant increase (p=0.0001) in maximum bAFC infiltration depth compared to a 1-minute sonication period.
Figure 30: Cell infiltration of bAFCs into crosslinked and sonicated AFRPS. A) Representative H&E images (200x) of 6mM EDC crosslinked AFRPs following 1-, 5-, and 10-minutes of sonication at the respective time points. (Insert: Magnified image highlighting representative region of interest outlined by dotted-line). (Black arrowhead indicates cell nuclei). B-C) Representative graphs of the average cell infiltration depth. Solid connecting lines indicate significant difference (p<0.05).
Based on histological results of improved bAFC infiltration, tensile testing of 6mM EDC crosslinked 3-ply AFRPs was performed following 5- and 10-minute sonication periods to evaluate the effect of sonication times on AFRP tensile mechanical properties. Following sonication, the EM for 5- and 10-minute sonicated 6mM EDC treated AFRPs (17.36 ± 1.88 MPa and 11.42 ± 1.78 MPa, respectively) were not statistically different from non-sonicated 6mM EDC treated AFRPs (16.05 ± 2.15 MPa) (Figure 31). However, the EM of 10-minute sonicated AFRPs was significantly lower (p=0.0083) than AFRPs subjected to 5-minute sonication. Additionally, 5- and 10-minute sonication periods did not significantly alter the UTS of 6mM EDC treated AFRPs (6.07 ± 0.95 MPa and 6.38 ± 1.19 MPa, respectively) compared to non-sonicated 6mM EDC treated AFRPs (7.83 ± 0.43 MPa). However, tensile strain at failure (TSF) tended to be lower (not statistically) for the 6mM EDC treated AFRPs exposed to 5- and 10-minute sonication periods (63.31 ± 1.77% and 74.17 ± 11.57%, respectively) versus non-sonicated 6mM EDC treated AFRPs (77.71 ± 6.9%).
DISCUSSION

To date, few biomaterials developed for AF repair have been extensively evaluated for their mechanical characteristics and ability to restore FSU kinematics. Likipanichkul et al. describe the mechanical evaluation of a cytocompatible fibrin-based injectable hydrogel for inner AF repair which demonstrated resistance to expulsion following dynamic compression and restoration of compressive stiffness following implantation into bovine FSUs.\textsuperscript{206} More recently, the fibrin-based hydrogel was combined with a synthetic NP scaffold and a polyurethane membrane sutured to the outer AF to repair FSUs. Biomechanical evaluations of the repaired FSUs commonly demonstrated NP scaffold herniation/extrusion from the IVDs when physiological loading was applied.\textsuperscript{198} In this investigation, we have demonstrated an in-depth mechanical characterization of a collagen-based, multi-laminate angle-ply AF repair patch (AFRP) biomaterial that illustrated its ability to mimic the static and dynamic biomechanical characteristics of the native human AF. Additionally, a second major finding of this study demonstrated that the use of the
AFRP could prevent herniation of an NPR and aids in the restoration of the axial kinematics of FSUs following aggressive discectomy and application of physiologic loading. A third major finding of this study confirmed that chemical crosslinking of AFRPs rendered them resistant to accelerated collagenase degradation without detrimentally impacting AFRP tensile mechanical properties or AF cell viability.

The AF is composed of an angle-ply architecture which exhibits unique material properties which are dependent on orientation (axial, circumferential, or radial), hierarchical structure (single lamellae or multi-laminate), and anatomic location (anterior, posterior, inner, or outer AF). The primary role of the AF is to mechanically confine the NP by providing resistance to IDP through the generation of tensile hoop stresses, while resisting tensile stretching and torsional rotations. Human lumbar IVDs can experience axial compressive loads ranging from 340-1200N during activities of daily living and up to 2350N during strenuous activities which can result in the generation of IDPs ranging from 0.1-2.3 MPa that must be endured by the AF. The tensile material properties of the AF, including its UTS (3.8 MPa), EM (12-24 MPa), and TSF (~65% in fully flexed position) allow for it to bear such stresses, in addition to allowing the AF to withstand varying applied strain rates arising from complex intradiscal deformations and strain patterns. Considering this, an effective AF repair device must recapitulate these material parameters to function and survive within the mechanical environment of the IVD.

Accordingly, AFRPs were evaluated for several mechanical characteristics. First, we evaluated the ability of AFRPs to absorb energy and resist impact loading as sudden variations in applied force with respect to time (i.e. ‘jerk’ or ‘force-jerk’ loading) can occur.
when missing a step for falling on your buttocks,223–225 which results in increased IDPs,226,227 and can lead to AF rupture.177 The AFRP demonstrated the ability to withstand up to 3 g-forces (equivalent to a rollercoaster ride) prior to failure. Second, considering that lumbar IVDs undergo millions of loading cycles over the life of the patient, AFRPs were evaluated for their tensile fatigue strength. Previous reports by others have demonstrated that human lumbar AF promptly fails following the application of cyclic tensile stress amplitudes ranging from 2.5-3 MPa, whereas application of moderate amplitudes (reminiscent of those experienced during activities of daily living) result in years of functionality.214 Under similar test conditions, the AFRP illustrated an S-N curve profile comparable to full-thickness human lumbar AF when subjected to low-stress amplitudes (1.5-2 MPa); however, AFRPs demonstrated enhanced durability compared to native AF tissue tested at moderate and high-stress amplitude ranges (2-3 MPa). Finally, the attachment strength of the AFRP to IVDs was investigated as many clinical studies have demonstrated this to be an ‘Achilles heel’ of current AF repair devices causing them to fail at stresses approaching 1.5 MPa.6,16,103,202,228–230 Herein, we utilized a simple suturing technique using 4-0 FiberWire suture. Burst strength testing of the AFRP attachment to the IVD demonstrates that the suturing technique employed can withstand physiologically relevant stresses without failure. Although suturing in the confined space of the posterolateral spine using minimally invasive spine surgery techniques can be challenging, nervous tissue can be gently retracted, and suture can be placed in the four corners of the AFRP prior to introducing it down a tubular retractor.
The primary function of the IVD is to allow for flexibility of the spine and thus together, the healthy NP and AF help maintain normal spinal kinematics. Two primary kinematic parameters are often used to define spinal motion: 1) NZ (i.e. the absolute measure of joint laxity around the neutral position demonstrating minimal internal resistance), and 2) RoM (i.e. the entire range of the physiological spinal segment motion). Reported NZ and RoM values for healthy human lumbar spine segments are 10.4° and 7.6°, 1.5° and 3.8°, 1.6° and 6.6°, and 0.7° and 2.4° in flexion, extension, lateral bending, and torsion, respectively.\textsuperscript{231} During IVDD these parameters often change.\textsuperscript{231,232} The primary goal of NPR and AF repair devices is to restore normal spinal kinematics to healthy (intact) values. In general, when used together our ABNP and AFRP were able to restore axial FSU kinematics following repair.

Creep displacement of the IVD is thought to be dominated by the NP in the short-term, and by the AF in the long-term.\textsuperscript{208} This was supported by the testing herein of annulotomized FSUs which resulted in significant changes in the long-term creep parameters. In addition, FSUs that subsequently underwent aggressive nucleotomy demonstrated persistent long-term changes in creep parameters in addition to imparting detrimental effects in the short-term creep parameters. Repair of the IVD with the ABNP and AFRP helped restore these values. However, the short-term creep response remained significantly lower in the repaired group as compared to intact, likely due to the swollen and porous nature of the ABNP leading to increasing fluid diffusivity and a reduced capacity to resist compressive loading. Cyclic testing data demonstrated statistical increases in RoM following discectomy and subsequent restoration towards intact values.
following repair with the AFRP and ABNP biomaterials. There was no significant difference observed in the constant slow ramp compressive stiffness comparing intact and repaired FSUs, however, the compressive stiffness of injured IVDs was significantly lower compared to the intact condition.

Subsequent to kinematic testing, compression to failure testing was used to evaluate AFRP repair strength under extreme axial loading conditions.\textsuperscript{220,221} The strength of attachment of the AFRP exceeded values obtained during biaxial ball-burst testing. It was also noted that no ABNP material extruded but that the vertebral end-plates eventually contacted each other. This is likely explained by two potential phenomena; 1) the nucleotomy space may have not been completely filled with ABNP and/or 2) when the ABNP was compressed at supraphysiological loads (>2.5 MPa) the water within the ABNP was driven out effectively depressurizing the material thus allowing the end-plates to make contact.

Disc degeneration is best defined as an aberrant cell-mediated process that results in ECM degradation eventuating in the structural demise of the IVD. Increasing grades of IVDD have been positively correlated with increasing concentrations of proteases. More specifically, mean concentrations of MMP-1 (collagenases type I) have been shown to increase with increasing grades of IVDD (grade III: 133pg/ml, grade IV: 279pg/ml, grade V: 319pg/ml).\textsuperscript{210} Therefore, any ECM-based biomaterial placed in this caustic environment must resist accelerated degeneration so that it can maintain its mechanical integrity and function. To prevent collagen degradation, AFRPs were crosslinked using well-established chemistries and were evaluated for accelerated mass loss following incubation in
collagenase concentrations that were 1000 – 10,000 times the concentrations typically found in the degenerative IVD. Minimal degradation was observed in EDC and GLUT crosslinked AFRPs that may be advantageous allowing for the prolonged mechanical function of the biomaterial and eventual tissue remodeling and regeneration. The tensile mechanical properties of AFRPs did not change significantly following crosslinking; this was likely due to the testing rate used to assess the AFRPs. Jang et al. have previously demonstrated that when pericardium tissue is tested under high tensile strain rates (>100mm/min; similar to testing rates of native AF tissue) the viscoelastic nature of the tissue predominates the effect of different crosslinking chemistries on its mechanical properties.233

Tissue remodeling and regeneration are in large part influenced by cytotoxicity of the biomaterial and the ability of cells to infiltrate into the AFRP. Previous studies by others have demonstrated the cytocompatibility of EDC when used as a collagen crosslinker.36 Conversely, while GLUT crosslinking has been shown to effectively stabilize tissue engineered scaffolds, it significantly reduces cell viability likely due to the toxicity of unreacted side-groups.234,235 Herein, we examined the effects of EDC and GLUT crosslinking chemistries on AF cell cytotoxicity and infiltration into the AFRP. Non-statistical differences were observed comparing between non-crosslinked and EDC treated AFRPs demonstrating their cytocompatibility. However, AF cell infiltration was significantly hampered as has been previously observed by others,234 likely due to the high density of ‘zero-length’ crosslinks created between collagen molecules. Thus, ultrasonication; a procedure previously demonstrated to increase porosity and thus cell
infiltration depth, was used to mechanically disrupt the AFRPs. Subsequent mechanical testing of AFRPs demonstrated that the tensile properties of the biomaterial were not detrimentally altered following 5-minute sonication periods, yet bAFC infiltration depth was statistically increased. Together, these results suggest the AFRP is cytocompatible and will allow endogenous or exogenous cells to infiltrate and potentially remodel the AFRP.

LIMITATIONS

As with any study, the authors acknowledge some limitations within this study. First, fatigue testing was only run to a maximum of 10,000 cycles despite the spine undergoing millions of cycles over the lifetime of an individual. However, there are only a limited number of daily activities (i.e. those involving bending at the waist) which would create the stress amplitudes evaluated herein. Therefore, the AF may only be subjected to these stresses a few times per day and thus achieving 10,000 cycles of fatigue would represent years to a decade of in vivo use. The authors also recognize that the full-thickness human AF constitutes a gradient of ECM components and material properties while the AFRP described herein anatomically represents only the outer AF. Despite this, our results demonstrate that the 3-ply AFRP has the tensile strength typically observed for a full-thickness segment of human posterolateral AF. Moreover, the authors recognize that the inclusion of an additional testing group (i.e. repaired annulotomy) within the kinematic study may have more clearly delineated the exact contribution of the AFRP and ABNP to FSU kinematics; however, we did not want to induce excessive damage to the native AF via repeated attachment and detachment of the AFRP following annulotomy and
discectomy. It should also be noted that the AFRPs were sterilized using 0.1% peracetic acid, however further study is warranted to evaluate other commonly used medical device sterilization techniques and their effect on AFRP mechanical properties and cytocompatibility. Finally, the authors also recognize that the laboratory-based mechanical testing performed herein do not reflect the potential changes in AFRP or suture strength or accumulation of damage over time when implanted *in vivo*.

**CONCLUSION**

In conclusion, the AFRP has demonstrated its mechanical appropriateness for implantation into the mechanically demanding environment of the spine while illustrating its biologic functionality supporting IVD cell activity. Utilization of this biomaterial may allow for the implementation of interventional strategies thereby potentially improving clinical outcomes for a significant number of patients suffering from IVD degeneration and/or herniation. On-going work includes evaluating the ability of the AFRP and ABNP to support stem cell differentiation and tissue regeneration *in vitro* and their ability to mitigate IVD degeneration in a large animal model.
CHAPTER V

‘All of the research contained within this chapter is currently in revision and was submitted for peer-review as Ryan Borem, Allison Madeline, Joshua Walters, Henry Mayo, Sanjitpal Gill, Jeremy Mercuri, Multi-Laminate Annulus Fibrosus Repair Scaffold with An Interlamellar Matrix Enhances Impact Resistance, Prevents Herniation, And Assists In Restoring Spinal Kinematics, Journal of the Mechanical Behavior of Biomedical Materials, September 2018, Preprint: https://doi.org/10.1101/418103’

MULTI-LAMINATE ANNULUS FIBROSUS REPAIR SCAFFOLD WITH AN INTERLAMELLAR MATRIX ENHANCES IMPACT RESISTANCE, PREVENTS HERNIATION, AND ASSISTS IN RESTORING SPINAL KINEMATICS

ABSTRACT

Focal defects in the annulus fibrosus (AF) of the intervertebral disc (IVD) from herniation or surgical injury have detrimental impacts on IVD mechanical function. Thus, biomaterial-based repair strategies, which can restore the mechanical integrity of the AF and support long-term tissue regeneration are needed. Accordingly, a collagen-based multi-laminate scaffold with an underlying “angle-ply” architecture has been previously reported demonstrating similar mechanical properties to native AF tissue. The objectives of this
work were to: 1) enhance the biomaterials impact strength and 2) assess its ability to spinal
kinematic restoration and prevent IVD herniation. First, AFRP’s were enriched with a
glycosaminoglycan-based (GAG) interlamellar matrix (ILM) and then tested for its
radially-directed impact resistance under physiological stresses. Subsequent kinematic
testing was conducted using a characterized GAG-enriched AFRP as an AF focal defect
closure device. In summary, AFRPs demonstrated 1) incorporation of a GAG-based ILM
significantly increased radial impact strength, 2) restoration of axial FSU kinematics and
3) ability to prevent herniation of native IVD tissues. Together, these results suggest that
the AFRP demonstrates the mechanical robustness and material properties to restore an
IVD’s physiological mechanical function through the adequate closure of an AF focal
defect.

INTRODUCTION

Intervertebral discs (IVDs) consist of a gelatinous core known as the nucleus
pulposus (NP), which is circumferentially constrained by a fiber-reinforced, multi-laminate
structure known as the annulus fibrosus (AF). The NP’s hydrophilic extracellular matrix
(ECM) is composed of aggrecan and collagen type II, while the AF is composed of 15-25
concentric lamellae of collagen type I with fibers aligned in a ± 28-43° “angle-ply”
microarchitecture. Between each AF lamellae, non-fibrillar ground substance (e.g.,
glycosaminoglycan; GAG), elastin, collagens type I and VI, and cells form an inter-
lamellar matrix (ILM). The AF’s highly organized, fiber-reinforced structure resists
tension and torsion during spinal bending and twisting, but also bears hoop stresses
developed from pressurization of the NP during compressive loading of the IVD.\textsuperscript{219–221,238} Furthermore, the ILM reinforces the AF against these radially-directed intradiscal pressures (IDPs) and prevents de-lamination of adjacent lamellae.\textsuperscript{239} Thus, the ECM composition and organization of these IVD tissues allow for stable, multi-axial motion of the spine through their mechanical interplay.

Physiologically, human lumbar IVDs experience axial compressive loads that generate IDPs ranging from 0.1-2.3 MPa.\textsuperscript{179} IDPs fluctuate depending on body position and can increase abruptly due to activities such as weight lifting, jumping/landing, and Valsalva maneuvers, resulting in radially-directed impact loading on the AF.\textsuperscript{223,225–227} Abrupt spinal loading, in the presence or absence of IVD degeneration (IVDD), can induce damage resulting in IVD herniation (IVDH) of the NP through the development of focal defects in the AF (Figure 32).\textsuperscript{177} Patients with IVDH often undergo discectomy to remove the herniated IVD fragments; however, these remnant focal defects are not surgically repaired. Instead, the outer AF is left to heal on its own through a process that generates fibrotic scarring.\textsuperscript{240} This scarring does not restore the microarchitecture or mechanical integrity of the AF which is evidenced by re-herniation rates of 9-25\% at four and ten years follow-up in discectomy patients.\textsuperscript{55,59,230} Furthermore, surgical intervention can result in focal AF injury. More specifically, patients with early- to moderate IVDD (an aberrant cell-mediated inflammatory process initiating in the NP) may benefit from early-stage interventions including NP replacement (NPR). However, pre-formed NPRs require injury to the AF to implant the device in the center of the IVD (Figure 32).\textsuperscript{241} Thus, following implantation the AF needs to be closed to prevent subsequent herniation. Taken together,
a need exists to develop a mechanically competent AF repair biomaterial, which allows for the immediate closure of focal AF defects and mimics the native structure and function of the outer AF to guide regeneration of native tissue.  

Figure 32: Representative image depicting the disc degenerative cascade. Increase in inflammation and proteases lead to increases in disc degeneration. IVDs suffering from moderate disc degeneration and herniation which are surgically repaired via discectomy result in a remnant annular defect (yellow circle). This allows for a pathway of least resistance for NP tissue to extrude/herniate; thus, leading to spinal instability.

In response to this need, there have been several attempts towards developing an outer AF closure system, including sutures (e.g., X-Close; Annulex)\textsuperscript{5,242}, adhesives (e.g., cyanoacrylates, glutaraldehyde-albumin, fibrin glues)\textsuperscript{243,244}, or obstructive devices (e.g., Barricaid)\textsuperscript{245,246}. However, these approaches do not possess the same composition, microarchitecture, or mechanical properties of the native AF, which has been shown to
play a crucial role in AF function. Thus, over the last decade increasing advancements in creating tissue-engineered scaffolds for the repair and regeneration of AF focal defects have been investigated. Many groups have focused on developing void-filling biomaterials that can replace missing AF tissue throughout the full-thickness (≈5-6mm depth) of the AF. These include genipin-crosslinked fibrin hydrogels, riboflavin cross-linked collagen gels, disc-like angle ply structures (DAPS), and synthetic poly(trimethylene carbonate) scaffolds. These innovative biomaterials have previously demonstrated the ability to support cell adhesion, proliferation, and ECM production, and are currently being evaluated for their mechanical competency and ability to assist in the restoration of functional spinal unit (FSU) kinematics. Of significant note, full-thickness AF repair biomaterials undergoing kinematic testing were sequestered within the IVD using an auxiliary synthetic membrane or ‘patch’ secured to the outer AF. Taken together, this suggests that a combinational approach may be required for successful IVD repair by utilizing an outer AF closure that can 1) resist impact loading, 2) aid in restoring FSU kinematics, and 3) secure a full-thickness AF repair biomaterial.

Thus, our lab has developed a biological scaffold, which is a biomimetic outer annulus fibrosus repair patch (AFRP) comprised of multiple sheets of type I collagen. AFRP’s are fabricated from decellularized porcine pericardium with its innate collagen fibers aligned with a ±30° “angle-ply” microarchitecture mimicking the native AF. Additionally, the AFRPs mechanical properties have been previously characterized demonstrating similarity to human AF; however, its ability to withstand high-impact loading warranted enhancement. The AF’s ability to resist radially-directed impact is
thought to be supported in part by the energy dissipative function of GAG found within the ILM. (Nerurkar et al., 2011; Nerurkar et al., 2011; Perie et al., 2006) Thus, we hypothesized the impact resistance of the AFRP would be increased via the inclusion of a GAG-based hydrogel ILM. Additionally, when using a combinational approach for AF repair, which includes both an outer AF closure device and a full-thickness AF repair biomaterial, it is essential to understand the effects on FSU kinematics. Thus, the objectives of this research were to: 1) characterize the radially-directed impact resistance of the AFRP following incorporation of a GAG-based ILM, 2) determine the effects of an AFRP and AF void-filler towards restoring injured FSU axial and torsional kinematics, and 3) evaluate the AFRP’s ability to provide adequate closure of an annular focal defect by preventing native IVD tissue herniation.

**MATERIALS AND METHODS**

**Fabrication of Annulus Fibrosus Repair Patches (AFRPs)**

Multi-laminate “angle-ply” AFRPs were developed and assembled from decellularized porcine pericardium to form tri-layer scaffolds and crosslinked in 6mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with 1.2mM N-hydroxysuccinimide (NHS) as previously described. 212,255 were maintained in a phosphate buffered saline (PBS) storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing.
HA-Gel Preparation and AFRP Enrichment

Hyaluronic acid (HA)-gel was prepared by reconstituting 1% sodium hyaluronate (a water-soluble salt form of HA) in PBS. AFRPs were then enriched by injecting the HA-gel between the AFRP lamellae (~62.5ug/mg) to form a GAG-based inter-lamellar layer (Figure 33A&B).

Figure 33: Glycosaminoglycan (GAG) enrichment of the AFRP. A) Representative schematic illustrating the proteoglycan-rich interlamellar matrix between native AF lamellae. B) Injection of hyaluronic acid (HA- non-sulfated GAG) between the AFRP lamellae. C) Representative image depicting GAG-enriched AFRP [* indicates AFRP
Histological Staining for Confirmation of Inter-Lamellar GAG

AFRPs were fixed in 10% neutral-buffered formalin for 24 hours before undergoing successive washes in graded ethanol, xylene, and paraffin followed by embedding and sectioning to 5µm thickness. Slides were stained with Alcian Blue (1% Alcian Blue in 3% aqueous acetic acid; pH 2.5) and counterstained with 0.1% aqueous Nuclear Fast Red for visualization of glycosaminoglycan deposition. Histological images were then captured on a Zeiss Axio Vert.A1 microscope with AxioVision software (SE64 Rel. 4.9.1 SP08-2013).

Impact Resistance Testing

Radial impact strength testing was performed according to methods previously described. Briefly, representative samples of crosslinked and crosslinked + HA-gel enriched AFRPs (3-ply; n=15/group; AFRP dimensions: 12 mm (L) x 12 mm (W) x 0.75mm (T)) were tested using a custom designed free-fall impact testing drop-tower. The base platform of the drop-tower consisted of a tissue-holding clamp and four vertical rails, which guided a free-falling platform. The tissue holding apparatus consisted of two stacked blocks lined with coarse-grit sandpaper each having an aligned thru-hole of 6.25mmØ. AFRPs was interposed between the two blocks centered over the two thru-holes. Subsequently, a 6mmØ steel ball attached to a 3-inch pushrod was placed in contact with the AFRP via the thru-hole in the superior block. Various weights ranging from 0.32-
0.65kg were stacked on the free-fall platform, which was then dropped from a constant height of 0.254m. Impact energy (E) was calculated using the equation for kinetic energy,

\[ E = \frac{1}{2} \times m \times v^2 \]

where \( m \) = mass and \( v \) = velocity. The resultant ball-burst pressure was calculated given the maximum force at rupture and its relationship with ball-burst pressure and geometric constraints according to established procedures.

**Preparation of Functional Spinal Units**

Bovine caudal tails, from 2-3-year-old calves, were obtained from a local abattoir and transported on wet ice to the lab within an hour. Excess tissue and ligaments surrounding the vertebral bodies and intervertebral discs were removed via dissection, and functional spinal units (FSUs: vertebrae-IVD-vertebrae) with posterior elements attached were isolated via a bandsaw. FSUs were harvested from two caudal levels (cc1-2 to cc2-3) and were potted using 3mm steel rods and urethane potting resin to prevent slippage of the samples during testing. Prior to testing, FSUs were wrapped in gauze saturated with storage solution and stored at -80°C. Samples were thawed within the sealed zip-lock bag, which was submerged for four hours in PBS at ambient temperature; thus, to prevent excessive tissue swelling.

**Biomechanical Evaluation of Functional Spinal Unit Axial and Torsional**

**Kinematics**

FSU’s (n=5/group) underwent kinematic testing according to methods previously described by our group, with modification. Briefly, FSU’s (n=5/group) were tested
sequentially in the following groups: Intact, Annulotomy with an AFRP only, and Repair with HA-gel enriched AFRP + AF tissue plug (Figure 34A). To initiate injury of the FSU, an annulotomy was performed by perforation of the IVD using a 6mmØ biopsy punch (7mm depth) and subsequent removal of the AF tissue. The annulotomy testing group included the closure of the outer annular focal defect with an AFRP (AFRP dimensions: 7mm (L) x 7mm (W) x 0.75mm (T)), which was secured via a 4-0 FiberWire suture. The defect of the annulus was left unobstructed to allow for NP swelling and redistribution similar to a herniation. Suturing of the AFRP consisted of suturing at the four corners with sutures being passed through the AFRP 1-2mm from the edge of the patch. Suture throws were made in alignment with the ±30° collagen fibrils of the AFRP. The AFRP + AF tissue plug group consisted of the restoration of the full-thickness annular defect using the previously incised native AF tissue recovered during the annulotomy, and the tissue was secured in place with an AFRP as described above.

FSU’s first underwent creep loading on a Bose ElectroForce (model: 3220, TA Instruments) equipped with a 100-lb. load cell and a test chamber filled with 1xPBS/protease inhibitor at 25°C. Samples were loaded to a mean amplitude level of -0.125 MPa and then underwent a 1-hr. creep period at -0.50 MPa. Samples were then transferred to a servohydraulic test frame (model: 8874, Instron) fitted with a 20kN load cell, and subjected to 35 cycles of axial compression (-0.50 MPa) and tension (0.25 MPa) at 0.1 Hz. Compression was then maintained at -0.50 MPa as samples underwent 35 torsional cycles to ±3°. Finally, samples underwent a slow-rate compressive ramp (1 N/s) from a mean amplitude level of -0.125 MPa to -0.50 MPa (Figure 34B). Tensile and
Compressive stiffness was determined using a linear fit of the loading force-displacement curve from 60-100% of the 35th cycle (Figure 34C). The axial range of motion was defined as the total peak-to-peak displacement of the IVD. Axial neutral zone (NZ) length was determined by fitting a third-order polynomial to the data and finding the maxima and minima with the correlating range between the peaks. A non-linear constitutive model was fit to the creep data (Figure 34D) using GraphPad Prism 7 software to yield elastic ($\Psi$) and viscous ($\eta$) damping coefficients for the short-term ($\eta_1$ and $\Psi_1$) and long-term ($\eta_2$ and $\Psi_2$), as described previously. Torsional stiffness was calculated from a linear fit of the loading torque-rotation curve of the 35th cycle. Torque range and axial range of motion (RoM) was calculated as the peak-to-peak torque and displacement, respectively. Changes in torque hysteresis were calculated by measuring the height and width of the curve along the geometric axes as shown in Figure 34E&F. The constant-rate slow-ramp compression stiffness was determined using a linear fit of the slow-ramp load-displacement response.
Figure 34: Study design for *in situ* kinematic testing of bovine IVD functional spinal units (FSUs) using GAG-enriched AFRPs. A) Representative images of the progression of testing groups (plus a pictorial image of annular injury prior to repair) and preparation for kinematic testing of FSUs. B) Loading scheme for FSU testing depicting creep loading, axial cyclic tension-compression loading, torsional loading,
and slow constant-rate ramp loading. C-E.1) Representative graphs depicting the axial, creep, and torsional response of an IVD and its associated parameter measures. E.2) Graph depicting the torsional hysteresis height and width; parameters were calculated based on the dimensions of their respective geometric axes (orange solid line = height; green dotted line = width).

**Compression to Failure of Repaired FSUs**

Following kinematic testing of FSUs, repaired samples were loaded to a mean amplitude level (-0.125 MPa) and then compressed at a rate of 300 mm/min (5mm/sec) until FSU failure (i.e., contact of the adjacent vertebral endplates and/or AFRP failure/AF Plug herniation). Ultimate compressive strength was calculated from the sample load and the initial cross-sectional area of the IVDs. Digital video was used to record mode and time of failure.

**Statistical Analysis**

Statistical analysis was performed on raw data using GraphPad Prism 7 software. Results are represented as mean ± standard error of the mean (SEM), and significance was defined as (p≤0.05). Impact resistance data was evaluated using a one-way ANOVA with Dunnett’s post-hoc analysis. Kinematic data were evaluated using a one-way repeated measures ANOVA followed by Dunnett’s post-hoc analysis.
RESULTS

Confirmation of GAG-Enrichment of AFRPS

HA-gel enriched AFRPs were histologically evaluated to confirm the presence of GAG between the AFRP lamellae following injection. Positive Alcian blue staining of the crosslinked + HA-gel AFRPs demonstrated positive staining within the inter-lamellar region confirming the presence of the GAG (Figure 33C).

Impact Testing

Multi-laminate AFRPs underwent radially-directed impact loading to evaluate its resistance to failure during the application of sudden changes in IDP (Figure 35A&B). Mean impact strength of non-crosslinked, crosslinked, and crosslinked + HA-gel AFRPs was 1.51 ± 0.06 MPa, 2.04 ± 0.03 MPa, 2.57 ± 0.02 MPa, respectively (Figure 35C). Additionally, the average kinetic energy absorbed by non-crosslinked, crosslinked, and crosslinked + HA-gel AFRPs was 0.51 ± 0.02 J, 0.69 ± 0.01 J, 0.87 ± 0.01 J, respectively (Figure 35D). Expectedly, impact strength was statistically increased following crosslinking (p<0.001) and crosslinking + HA-gel (p<0.001) compared to non-crosslinked AFRPs. Furthermore, impact energy was statistically increased in crosslinked AFRPs (p<0.001) and crosslinked + HA-gel AFRPs (p<0.001) compared to non-crosslinked AFRPs.
Figure 35: Biomechanical evaluations of non-crosslinked, crosslinked, and crosslinked + HA-gel AFRPs subjected to radial impact burst strength. A&B) Representative schematics illustrating the radial loading axis the NP exerts on the AF through increases in intradiscal pressures (IDPs). C) Graph depicting the average radial impact strength withstood by AFRP testing groups. Dotted line indicates reported human values of IDPs 0.1-2.3 MPa. D) Graph depicting the average radial impact kinetic energy absorbed by the AFRP testing groups.
**Impact on Axial Spinal Kinematics**

Axial and torsional FSU kinematics were evaluated to determine the impact of AF focal defect repair (created via annulotomy) compared to a combinational approach using a full-thickness AF tissue plug (excised intact AF tissue) and an outer closure device. Of note, completion of kinematic testing with the AF tissue plug could not be successfully achieved without sequestering it within the IVD with an AFRP. This was due to the pressurization of the native NP, which caused the AF tissue plug to extrude/herniate prior to mechanical loading (Figure 36).
Figure 36: Pressurization of the IVD prevented repair with the native AF Plug only without an AFRP. A) Annular defect following annulotomy procedure (purple dotted circle). B&C) Top and Side views of an AF Plug placed into FSU illustrating the immediate extrusion out of the defect following implantation (AF Plug centered in a red dotted circle).

**Axial Cyclic Loading**

Axial cyclic kinematic testing of FSU’s with an annulotomized defect or repaired with the AFRP scaffold and AF tissue plug did not significantly alter axial compressive stiffness, slow ramp compressive stiffness, or tensile stiffness kinematic parameters.
compared to intact controls (Figure 37A&B). Conversely, annulotomy of FSUs resulted in a statistical increase compared to intact controls for axial range of motion (AFRP: 4.03 ± 0.27 mm, Intact: 3.45 ± 0.29 mm; p=0.045) and neutral zone (AFRP: 1.55 ± 0.11 mm, Intact: 1.28 ± 0.13 mm; p=0.037) (Figure 37C&D). However, incorporation of the native AF tissue plug, sequestered by an outer AFRP closure, restored these parameters to intact values (axial range of motion: 3.67 ± 0.29 mm; p=0.476 and neutral zone: 1.31 ± 0.11 mm; p=0.908).
Figure 37: Axial cyclic tension-compression kinematic testing results of bovine IVD FSUs. Graphs depicting A) axial and slow ramp compressive stiffness, B) tensile stiffness, C) axial range of motion, D) axial neutral zone. Solid lines connecting groups indicates significant difference (p<0.05) compared to uninjured controls.

Creep Loading

Creep loading of FSU’s with an annulotomized defect or repaired with the AFRP scaffold and AF tissue plug did not significantly alter the step displacement or short-term elastic damping coefficient (Figure 38). However, annulotomy of FSUs resulted in a statistical increase compared to intact controls in creep displacement (AFRP: 1.29 ± 0.07 mm, Intact: 1.56 ± 0.07 mm; p=0.03), long-term elastic damping coefficient (AFRP: Ψ₂: 201.30 ± 14.97 N/mm, Intact: 140.10 ± 3.53 N/mm; p=0.022), short-term and long-term viscous damping coefficients (AFRP: η₁: 7532.40 ± 1251.02 Ns/mm and η₂: 46.59 x 10⁴ ± 3.29 x 10⁴ Ns/mm, respectively, Intact: η₁: 4.73 x 10³ ± 1.07 x 10³ Ns/mm and η₂: 3.70 x 10⁵ ± 0.11 x 10⁵ Ns/mm, respectively; p₁=0.003 and p₂=0.024). However, IVD repair incorporating the native AF tissue plug, sequestered by an outer AFRP closure, restored these parameters to intact values for creep displacement (1.74 ± 0.06 mm; p=0.134), long-term elastic damping coefficient (Ψ₂: 144.48 ± 7.50 N/mm; p=0.751), short- and long-term viscous damping coefficient (η₁: 5839.60 ± 1327.04 Ns/mm and η₂: 31.33 x 10⁴ ± 1.92 x 10⁴ Ns/mm, respectively; p₁=0.538 and p₂=0.058).
Figure 38: Creep kinematic testing results of bovine IVD FSUs. Graphs depicting A) step displacement, B) creep displacement, C&D) elastic & viscous damping coefficients. Solid lines connecting groups indicates significant difference (p<0.05) compared to uninjured controls.

Torsional Cyclic Loading

Focal injury to the AF resulted in significant changes in FSU torsional kinematics, which were not restored by with repair of the IVD (Figure 39). Torsional rotation of FSU’s
with an annulotomized defect or repaired with the AFRP scaffold and AF tissue plug demonstrated a significant decrease in torsional stiffness (0.48 ± 0.02 Nm/°; p=0.001 and 0.53 ± 0.03 Nm/°: p=0.024, respectively) (Figure 39A), torque range (3.45 ± 0.12 Nm: p=0.0002 and 3.68 ± 0.17 Nm: p=0.022, respectively) (Figure 39B), and torque hysteresis height (0.28 ± 0.03 Nm: p=0.028 and 0.30 ± 0.04 Nm: p=0.023, respectively) (Figure 39C) compared to intact FSUs (0.66 ± 0.02 Nm/°, 4.60 ± 0.10 Nm, 0.42 ± 0.05 Nm, respectively).
Figure 39: Torsional kinematic testing results of bovine IVD FSUs. Graphs depicting A) torsional stiffness, B) torque range, C) torque hysteresis width (NZ), D) torque hysteresis height. Solid lines connecting groups indicates significant difference (p<0.05) compared to uninjured controls.
**Compression to Failure**

Axial compressive testing of FSU’s with an annulotomized defect or repaired with the AFRP scaffold and AF tissue plug were assessed for compressive failure strength concomitant with visual assessment. Testing demonstrated no failure in the AFRP attachment nor herniation of native IVD tissues. Testing was stopped once video recordings illustrated that vertebral endplates were no longer applying a linear displacement, and an axial load was no longer applied. Furthermore, no statistical differences were observed between the repair groups ultimate compressive strength (Annulotomy: 5.28 ± 0.62 MPa; AF Plug: 3.84 ± 0.48 MPa; p=0.101) or compressive displacement (Annulotomy: 2.58 ± 0.25 mm; AF Plug: 2.96 ± 0.61 mm; p=0.619).

**DISCUSSION**

Focal injuries of the AF, produced by NP herniation or surgical trauma to enable implantation of an NPR, ultimately results in mechanical dysfunction of the IVD. Thus, repair of AF defects using mechanically competent implants that can withstand abrupt spinal loading and assist in restoring IVD kinematics are required. Herein, we characterized a multi-laminate AFRP scaffold containing a GAG-based ILM for its ability to resist radially-directed impact loading and its contribution to FSU kinematics when used in conjunction with a full-thickness AF tissue plug. The primary findings from these studies include: 1) incorporation of an ILM significantly increased the impact strength of the AFRP, 2) the AFRP enabled the restoration of axial FSU kinematics, and 3) the AFRP prevented herniation of native NP and a full-thickness AF tissue plug under applied...
physiological loading. Together, these results suggest that the AFRP can be used as a mechanical closure system to sequester NP and AF tissues and/or repair biomaterials within the IVD to assist in restoring its native mechanical function.

The first major finding from this study was that the inclusion of an ILM composed of an HA-hydrogel between the layers of the AFRP scaffold resulted in a significant increase in its radial impact strength. To the best of the author's knowledge, this is one of the first attempts to incorporate a GAG-based ILM within a biologic annulus repair scaffold to better recapitulate the anatomy and physiology of the native AF. GAGs, including chondroitin sulfate and HA, make up approximately 10-20% of the overall dry weight of the AF. Moreover, these molecules have been shown to play a functional role in contributing to the overall compressive properties of the IVD (e.g., compressive equilibrium modulus and peak stress) and helps the AF to resist IDP and prevent delamination and shearing of the AF lamellae. Herein, we demonstrate that the inclusion of a GAG-gel between the layers of the AFRP can play a significant role in dissipating radial-directed impact loads. This is somewhat intuitive given they biochemical properties of GAGs and the load-bearing role they play in many tissues including heart valves and cartilage. In fact, our results suggest that incorporation of a GAG-gel ILM increases the AFRPs radial impact resistance from 3 g’s (non-crosslinked AFRPs) to a conservative 10 g’s of gravitational force (equivalent to the force exerted when falling on your buttocks backward into low office chair, and 3x the force experienced on an aggressive roller coaster), both of which are beyond the magnitudes expected for normal activities of daily living following IVD repair.
The second major finding from these studies demonstrated that repair of AF focal defects in injured FSUs required a combinational approach, using both an outer AF closure and a full-thickness repair, to restore axial kinematics to intact levels. Moreover, when only an IVD which is annulotomized is repaired with only an outer closure, FSU kinematics were detrimentally affected; however, it was visually confirmed these changes were attributed to the migration of the native NP tissue into the void space of the focal AF defect and were not directly attributed to the outer AFRP closure itself. Furthermore, this migrated NP tissue had to be forcibly pushed back into the center of the IVD to replace the full-thickness AF tissue plug for subsequent testing. This pressurization phenomenon made it impossible for completing the full-thickness repair without the use of an outer AF closure patch which has been observed in other studies. 130,198

Notably, torsional kinematic parameters were not restored with either repair methodology. This could be contributed to the lack of integration with the surrounding annulus tissue. While the native full-thickness AF tissue plug occupied the structural void, lack of adhesion to surrounding tissue resulted in a lack of continuity. Therefore, it is presumed the use of a biocompatible adhesive could restore the continuity between the full-thickness AF tissue plug and the adjacent native annular tissue. However, previous investigations of AF repair biomaterials using adhesives have demonstrated only partial restoration of torsional kinematic parameters toward intact levels. 198,265 Thus, the recovery of mechanical torsional properties may also be dependent on the restoration of circumferential residual stresses and pre-strains found within the AF. 266,267 Accordingly, future investigations of AF repair biomaterials should take into consideration the
restoration of native residual stresses and pre-strains to further assist in restoring torsional kinematic parameters.

The third major finding was that repair of the outer AF with the AFRP scaffold prevented herniation of both native NP and a full-thickness AF tissue plug repair. This is significant given the proposed role of this biological scaffold is to sequester either native tissue or implants which restore the native function of the IVD. In general, IVD function is dependent on the interaction of the NP and AF. More specifically, under compressive loading, the NP becomes pressurized and exerts radially-directed compressive forces on the AF that in turn help to reinforce the AF and prevents inward buckling of the lamellae. Thus, the AFRP must be able to withstand these forces generated by the native tissue or by implant surrogates. Previous studies of AF repair biomaterials (with and without synthetic membranes) have demonstrated that under axial compressive loading these biomaterials experience the inclination to herniate at or below physiological stresses. Additionally, while other studies using similar AF repair biomaterials have demonstrated the ability to resist expulsion from the AF focal defect, a substantial portion of native NP tissue is often removed which in turn may presumably reduce the IVD pressurization. Therefore, these AF repair biomaterials may not be exposed to the full magnitude of radially-directed loading following the removal of native NP tissue. Therefore, under a more rigorous testing scenario (full pressurization of native IVD tissue), we have shown that the AFRP can resist the coordinate pressurization and radially-directed translation of native NP and a full-thickness AF tissue plug under physiological relative loads. Additionally, while macroscopic and video analysis of the AFRP illustrated minimal
outward bulging during axial compressive loading, the AFRP showed no failure at the patch/suture interface nor did herniation/extrusion of the native NP or full-thickness AF tissue plug occurs.

CONCLUSIONS

In summary, AFRP’s demonstrated its ability to resist physiological impact loading IDPs, prevent herniation of a full thickness AF void-filler, and assisted in the restoration of spinal kinematic parameters. Moreover, kinematic testing results expressed herein demonstrated the necessity of both 1) a full thickness AF void-filler and 2) an outer annular closure biomaterial to restore spinal kinematic parameters to intact values. Therefore, to restore physiological functionality, surgical repair of IVD disorders should include both a full thickness annular replacement to fill the mechanical void and an AFRP biomaterial to reinforce the immediate closure of the AF.

LIMITATIONS

As with any study, the authors acknowledge some limitations within this study. Concerning impact testing, the authors recognize that the ILM is composed of a variety of components, all of which play a crucial role in the maintenance of physiological function of the native AF. Although, the purpose of this study was to improve the impact resistance of AFRPs, and the proteoglycan-component of the ILM demonstrates a viscous response that was hypothesized to serve as a shock absorber to dissipate the force applied during impact loading. An additional limitation acknowledged by the authors, regarding impact
testing, is that AFRPs were not evaluated over multiple cycles for their impact fatigue strength. However, the magnitudes of impact pressures used during testing are considered infrequent (e.g., falling on your buttocks, riding a rollercoaster, and car accidents), and not expected to be a common occurrence for people who recently undergone IVD repair. Additionally, for this study, AFRPs impact strength was evaluated for its ability to resist the complete absorption of the impact energy using a 6mmØ steel-ball to represent a large annular defect \(^{230}\), however, this is thought to serve as a “worse-case” scenario, as the inclusion of a full-thickness AF repair biomaterial will also absorb IDP exerted by the NP and dissipate the energy applied to the AFRP. Concerning kinematic testing, one limitation was FSUs were not re-equilibrated for 8 hours submerged in saline between testing cycles, which has been recently shown by others to effect FSUs kinematics.\(^{269}\) Lastly, the authors acknowledge that laboratory-based mechanical testing performed herein may not directly reflect the potential changes of accumulation in damage over time when implanted \textit{in vivo}. 
AMNION- AND ADIPOSE-DERIVED MESENCHYMAL STROMAL CELLS RESPOND TO INFLAMMATION DIFFERENTLY: POTENTIAL IMPLICATIONS FOR THEIR USE IN INTERVERTEBRAL DISC THERAPY

ABSTRACT

Background: Intervertebral disc (IVD) degeneration is a progressive condition marked by proteolytic tissue destruction which is mediated by chronic inflammation. The well-established therapeutic effector functions (e.g., anti-inflammatory cytokine production and differentiation capacity) of mesenchymal stromal cells (MSCs) makes them an attractive therapy for patients diagnosed with IVD degeneration. While several sources of MSCs exist, controversy surrounding which one may be optimal for use in the inflamed environment of the IVD remains. Adipose (AD)- and amnion (AM)-derived MSCs have several advantages compared to other sources including higher cell yields and lower donor
site morbidity during harvest. However, no study to date has directly compared the impact of degenerate IVD inflammation on the effector function of these two MSC populations.

**Purpose**: To compare the effect of inflammation on the proliferation, cytokine production profile and differentiation capacity of human AD- and AM-MSCs.

**Study Design**: Controlled laboratory study.

**Methods**: Human AD- and AM-MSCs were cultured in media with or without supplementation of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) at concentrations reportedly produced by degenerate IVD cells. MSC proliferation was evaluated via cell counting. MSC production of pro- and anti-inflammatory cytokine was quantified following 24- and 48-hours of culture via cytokine array and enzyme-linked immunosorbent assay (ELISA). Additionally, the osteogenic and chondrogenic potential of AD- and AM-MSCs was characterized via histology and biochemical analysis of extracellular matrix (ECM) production following 28 days of culture.

**Results**: Under inflammatory culture conditions, AD-MSCs proliferated more than AM-MSCs resulting in higher cell numbers (p<0.048) compared to AM-MSCs. Additionally, AD-MSCs produced higher concentrations of MCP-1 (1154.20 ± 52.57 pg/ml; p=0.074), PGE₂ (1593.80 ± 46.51 pg/ml; p=0.030), and IL-1β (185.40 ± 7.63 pg/ml; p=0.010) compared to AM-MSCs (MCP-1: 1018.30 ± 22.34 pg/ml, PGE₂: 1291.40 ± 78.47 pg/ml, IL-1β: 144.10 ± 4.57 pg/ml). Conversely, AM-MSCs produced significantly more IL-10 (14.47 ± 2.39 pg/ml; p=0.004) compared to AD-MSCs (0.00 ± 0.00 pg/ml). AD-MSCs also produced more mineralized ECM (3.34 ± 0.05 RAU; p<0.001) under inflammatory osteogenic conditions compared to AM-MSCs (1.09 ± 0.06 RAU). However, under
inflammatory chondrogenic conditions AM-MSCs produced larger chondrogenic pellets (5.67 ± 0.26 mm²) with greater percent area staining positively for glycosaminoglycan (GAG: 82.03 ± 3.26%) and higher GAG content (8.34 ± 0.88 μg/pellet) compared to AD-MSCs (pellet area: 2.76 ± 0.18 mm², percent area positive for GAG: 34.75 ± 2.49%, GAG content: 5.90 ± 0.57 μg/pellet).

Conclusions: A different effector response was observed comparing between AD- and AM-MSC when cultured under identical inflammatory conditions. AD-MSCs produced more pro-inflammatory cytokines and demonstrated enhanced osteogenesis compared to AM-MSCs, which produced more anti-inflammatories and demonstrated enhanced chondrogenesis.

Clinical Relevance: In the context of the degenerate IVD, AD-MSCs may be a more appropriate cell source for inducing bone formation and thus may be advantageous for interbody fusion as compared to AM-MSCs. Conversely, AM-MSCs appear to be more optimal for promoting an anti-inflammatory environment and supporting cartilaginous IVD tissue regeneration.

Key Terms: Mesenchymal stromal cell; intervertebral disc degeneration; amnion; adipose; inflammation; differentiation; cytokine

What Is Known About the Subject: In general, it is known that MSCs can produce both pro- and anti-inflammatory cytokines depending on the environmental context. Additionally, it is known that the presence of inflammation can detrimentally impact the ability of MSCs to regenerate tissue. Furthermore, researchers have been investigating the ability of individual MSC types to regenerate IVD tissue when used in the presence or
absence of biomaterials under idealized (e.g., non-inflammatory) in vitro culture conditions. Currently, controversy exists as to which MSC type/source may be most appropriate for therapeutic use in the degenerate IVD, and to the best of the authors knowledge no controlled comparative analyses been performed to determine the effect of degenerate IVD-like inflammation on MSC effector function (e.g., cytokine production and differentiation capacity) to help identify an optimal MSC source for intradiscal administration.

**What This Study Adds to Existing Knowledge:** This is the first study to compare the effect of inflammation on the pro- and anti-inflammatory cytokine production and differentiation capacity of AD- and AM-MSCs. The study also provides fundamental insights into critical differences in these MSCs effector response to the same inflammatory conditions which may have clinical implications for their administration within degenerate IVD. This study also provides the basis for further investigations into the mechanisms which may underlie the observed differences. The findings herein will also have implications in the broader context of musculoskeletal tissue engineering and regenerative medicine research for bone, cartilage, and IVD tissue.

**INTRODUCTION**

Intervertebral disc (IVD) degeneration imparts significant socioeconomic burden resulting in annual direct costs estimated to exceed $100B in the U.S. This pathology is mediated in part by increased levels of pro-inflammatory cytokines including interleukin-
1 beta (IL-1β) and tumor necrosis factor – alpha (TNF-α). Elevated levels of these cytokines stimulate resident IVD cells to produce extracellular matrix (ECM) degrading proteases that break down tissue. IVD tissues are unable to regenerate as they have limited blood supply and relatively low numbers of active resident cells. Current treatments for IVD degeneration are not curative; they primarily attempt to alleviate pain and inflammation. Ultimately, this pathology becomes debilitating and thus warrant spinal fusion or arthroplasty. Due to the shortcomings associated with current surgical treatments, the orthopaedic community has turned their attention to the therapeutic potential of mesenchymal stromal cells (MSCs).

Populations of MSCs are found throughout adult tissues; however, their numbers are limited. Thus, MSCs are often harvested and expanded in vitro prior to administration for therapeutic purposes. The most common tissue sources of MSCs utilized include bone marrow (BM), adipose (AD), and more recently amniotic membrane (AM). It has been established through laboratory studies that MSCs possess several effector functions which make them excellent candidates as biologic therapies for IVD degeneration. MSCs secrete soluble chemicals (i.e., cytokines, chemokines, and growth factors) including prostaglandin E2 (PGE2), transforming growth factor-beta (TGF-β), and TNF-α stimulated gene/protein 6 (TSG-6) that function as anti-inflammatories. Additionally, soluble signals released by MSCs have been shown to stimulate resident tissue cells to produce new ECM. Moreover, MSCs have been shown to differentiate into several musculoskeletal cell types and generate new IVD ECM in vitro. These findings have prompted the initiation of several clinical trials evaluating the safety and efficacy of
intradiscal administration of MSCs to attenuate the progression of IVD degeneration. These trials have yielded promising results including improved pain and functional outcomes in patients with Pfirrmann grade II-V IVD degeneration.\textsuperscript{279,280} However, identification of an MSC source which demonstrates optimal outcomes within the complex pathological environment of the IVD has yet to be defined.\textsuperscript{277,278,280} Therefore, controlled studies which expand our understanding of the extent to which the effector functions of various MSCs differ when utilized within a specified pathological musculoskeletal niche are warranted.

Towards this goal, research groups have performed comparative analyses on the differentiation capacity of several MSC sources using established \textit{in vitro} differentiation protocols.\textsuperscript{149,281,282} More specifically, Topoluk et al. aimed to identify an optimal MSC source for bone and cartilage regeneration by comparing the osteogenic and chondrogenic differentiation capacity of AM- and AD-MSCs.\textsuperscript{149} These two MSC sources were chosen for study as they have higher yields and impart less donor site morbidity at harvest compared to BM-MSCs. The authors demonstrated that AM-MSCs exhibited enhanced gene and ECM markers for bone and cartilage formation compared to AD-MSCs.\textsuperscript{149} More recently, the same authors also demonstrated that AM-MSCs are more effective at chondroprotection and at skewing pro-inflammatory M1 macrophages towards a pro-regenerative M2 phenotype compared to AD-MSCs.\textsuperscript{150} However, considering these MSCs could be administered into inflamed joints and IVD tissues, it is imperative to better understand the influence of inflammation on the therapeutic effector functions of AM- and AD-MSCs. Having this information will help further identify which of these MSC types
may be more optimal (e.g., produce more anti-inflammatory cytokines and/or ECM) for use as pro-regenerative therapeutic agents in IVD degeneration. Thus, the objective herein was to determine and compare the effect of inflammation on AM- and AD-MSC proliferation and effector function (e.g., production of soluble cytokines and differentiation capacity). To achieve this objective, AM- and AD-MSCs were cultured under identical conditions in the presence or absence of IL-1β and TNF-α. The concentration of the proinflammatory cytokines used was chosen to mimic levels produced within degenerate (e.g., Pfirrmann Grade IV-V) human IVD tissues. Subsequently, MSC proliferation, production of soluble inflammatory mediators, osteogenesis, and chondrogenesis was determined, compared, and considered in the context of the degenerate IVD microenvironment.

**MATERIALS & METHODS**

**MSC Expansion**

Human AD-MSCs were purchased from Invitrogen. Human AM-MSCs were isolated via informed consent under an IRB approved protocol (PRO00031185) according to previously published methods. All MSCs were expanded under standard culture conditions (37°C with 5% CO₂) with media changes occurring every three days until passage 3 (P3). Culture medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), and 1% antibiotic/antimitotic (Ab/Am).
**IVD Degeneration-Mimetic Inflammatory (INF) Conditions**

INF media conditions included the study’s respective control media supplemented with human recombinant IL-1β (500pg/ml; PeproTech) and TNF-α (400pg/ml; PeproTech).

**Impact of Inflammation of MSC Proliferation and Cytokine Production**

**Non-Inflammatory and Inflammatory Culture Conditions**

Non-inflammatory (control) conditions included culturing MSCs in DMEM containing 2% FBS and 1% Ab. INF conditions described above. Cells were seeded at 1x10^5 into tissue culture-treated 12-well plates (n=3/condition/cell-type) and cultured for 12- and 48-hours.

**MSC Proliferation**

Proliferation of MSCs was evaluated by determining the total cell count per well (n=3 wells/treatment/time-point) following culture at respective 12- and 48-hr time-points. Following the removal of time-point media, cells were trypsinized (0.25% Trypsin, 0.1% EDTA), centrifuged for 5 minutes at 1000 rpm, resuspended in culture media, and counted using a TC20 Automated Cell Counter (Bio-Rad).
Cytokine Array and ELISA Analysis of Cell Culture Media for MSC Cytokine Production Studies

Media samples (n=3/condition/cell-type/time-point) were analyzed for several inflammation mediating cytokines including interleukins-1 beta (IL-1β), -1 alpha (IL-1α), -6 (IL-6), -8 (IL-8), -4 (IL-4), -10 (IL-10), and monocyte chemotactic protein-1 (MCP-1) using a quantitative glass slide array (RayBiotech) according to manufacturer’s instructions. Prostaglandin E2 (PGE2) was analyzed from media samples (n=3/condition/cell-type/time-point) via enzyme-linked immunosorbent assay (ELISA; Abcam) according to the manufacturer’s instructions. All detected cytokine values were calculated as the difference in cytokine concentration (pg/ml) produced by MSCs relative to media only controls.

Impact of Inflammation on MSC Osteogenesis and Chondrogenesis

Non-Inflammatory and Inflammatory Osteogenic Culture Conditions

To induce osteogenic differentiation, cells were seeded at 1.0 x10^5 into tissue culture-treated 12-well plates (n=3/condition/cell-type) and cultured in monolayer in differentiation media (StemPro Osteogenic Media; ThermoFisher Scientific) for up to 28 days (control). INF conditions described above. Differentiation was assessed histologically and semi-quantitatively via Alizarin Red staining for ECM mineralization.
**Analysis of ECM Mineralization**

Osteogenic differentiation was assessed histologically via Alizarin Red staining. Briefly, MSC-seeded well plates were rinsed with sterile DPBS (3X) and fixed in 4% paraformaldehyde solution for 15 minutes at room temperature (RT) before incubation in 1mL/well of 40mM Alizarin Red S for visualization of calcium deposition. Wells were rinsed with ddH₂O (5X) to remove excess stain, and histological images were captured. Quantification of staining was performed via a colorimetric analysis of the stain. Briefly, wells were treated with 10% acetic acid and incubated for 30 minutes at room temperature. Wells were agitated using a cell scraper, and the fluid was transferred to 1.5mL microcentrifuge tubes. Microcentrifuge tubes were heated in a water bath to 85°C for 10 minutes followed by 5 minutes of incubation on ice. Samples were then centrifuged at 20,000g for 15 mins. The supernatant was collected, resuspended in 10% ammonium hydroxide to neutralize the acid, and the colorimetric absorbance was read at 405nm. Results are expressed as relative absorbance units (RAUs).

**Non-Inflammatory and Inflammatory Chondrogenic Culture Conditions**

To induce chondrogenic differentiation, MSCs were seeded in pellets (1.0 x 10^5 cells per pellet; n=3 pellets/condition/cell-type) and cultured in differentiation media (StemPro Chondrogenic Media; ThermoFisher Scientific) for up to 28 days (control). INF conditions described above. Differentiation was assessed histologically via Alcian Blue staining and quantitatively via pellet area analysis, the percent area of pellet stained positively for GAG, DNA, and GAG content analyses, respectively.
Analysis of Chondrogenic Pellet Area

Following 28 days of culture, MSC pellets were imaged using phase contrast in well plates. Cross-sectional area \([\text{mm}^2]\) of pellets \((n=3/\text{condition/cell-type})\) were calculated from pellet diameters obtained using NIH ImageJ software.

Analysis of Chondrogenic Pellet Glycosaminoglycan Staining

Chondrogenic MSC pellets \((n=3/\text{condition/cell-type})\) were fixed in 10\% non-buffered formalin for 24 hours before undergoing dehydration in graded ethanol and xylene prior to paraffin embedding and sectioning to 5\(\mu\text{m}\) thickness. Slides were stained with Alcian Blue (1\% Alcian Blue in 3\% aqueous acetic acid; pH 2.5) and counterstained with 0.1\% aqueous Nuclear Fast Red for visualization of glycosaminoglycan deposition and cell nuclei, respectively. Histological images were captured, and the percentage of the total cell pellet area stained positively for GAG was quantified via color threshold analysis using NIH ImageJ software.

Analysis of Chondrogenic Pellet DNA and Glycosaminoglycan Content

Chondrogenic MSC pellets \((n=3/\text{condition/cell-type})\) were analyzed for DNA and GAG content using PicoGreen and DMMB assays, respectively. Briefly, cell pellets were digested in PBE buffer (pH 7.5) containing 5 mM L-Cysteine, 100 mM dibasic phosphate buffer, and 5 mM EDTA and 125 \(\mu\text{g/mL}\) papain at 65\(^\circ\text{C}\) for 24 hours. Fifty microliters of each sample were assayed in with 200ul of DMMB reagent (40 mM sodium chloride, 40 mM glycine 46 \(\mu\text{M}\) DMMB; pH: 3.0) in a 96-well plate. Absorbance was read at a
wavelength of 525nm and GAG content was determined from a standard curve containing known concentrations of chondroitin-6-sulfate. DNA content was assessed via PicoGreen according to manufacturer’s instructions from papain digests that had been frozen overnight at -20°C. Briefly, digestate was thawed and diluted in Tris-EDTA buffer prior to combining 100μl of the sample with 100μl of PicoGreen reagent in a black-walled 96-well plate. Fluorescence was detected using an excitation wavelength of 480nm and an emission wavelength of 520nm. DNA content was determined from a standard curve developed from known concentrations of DNA supplied by the manufacturer.

**Microscopic Imaging**

All images were captured on a Zeiss Axio Vert.A1 microscope with AxioVision software (SE64 Rel. 4.9.1 SP08-2013).

**Statistical Analysis**

Statistical analysis of the data was performed using GraphPad Prism 7 software. Quantitative results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed via Student’s t-tests of equal variance comparing between control and inflammatory study groups at respective time-points. Significance was defined as p≤0.050, and significant trends were defined as p≤0.080.
RESULTS

Impact of Inflammation on Proliferation of AD- and AM-MSCs

To determine the effect of inflammation on AD- and AM-MSC proliferation, cell number was quantified for each MSC type at 12- and 48-hours in control and inflammatory media. In general, inflammation promoted an increase in cell number between 12- and 48-hours in both AD- and AM-MSCs compared to control conditions; however, this was found to be significant only in AD-MSC cultures. The number of AD-MSCs significantly increased over time in inflammatory media (12-hours: 113,000 ± 4,163 cells, 48-hours: 221,000 ± 8,021 cells; p<0.001), but not in control media (12-hour: 119,350 ± 22,650 cells, 48-hour: 173,333 ± 20,305 cells). The number of AM-MSCs also increased over time in inflammatory (12-hour: 87,250 ± 8,151 cells, 48-hour: 109,667 ± 5,696 cells) and control media (12-hour: 101,250 ± 2,250 cells, 48-hour: 114,500 ± 12,500 cells), however these increases were not significant.

Comparing the two MSC types, inflammation resulted in a significantly higher number of AD-MSCs at both 12- (p=0.048) and 48- (p<0.001) hours compared to AM-MSCs.

Impact of Inflammation on Cytokine Production by AD- and AM-MSCs

To determine the effect of inflammation on AD- and AM-MSC production of soluble inflammatory mediators, the cytokine content of media containing each MSC type was quantified via antibody array or ELISA at 12- and 48-hours in control and inflammatory media. Inflammation resulted in an increase in MSC production of both pro-
and anti-inflammatory cytokines (Figure 40). Compared to controls at 12- and 48-hours, respectively, AD-MSCs in inflammation demonstrated significant increases in the production of PGE$_2$ (12-hour: 1593.80 ± 46.51 pg/ml; p<0.001, 48-hour: 1118.30 ± 115.56; p=0.005), MCP-1 (12-hour: 1154.20 ± 52.57 pg/ml; p<0.001, 48-hour: 1301.70 ± 63.71; p<0.001), IL-1β (12-hour: 220.27 ± 32.48 pg/ml; p=0.013, 48-hour: 185.40 ± 7.63; p<0.001) and IL-8 (12-hour: 800.00 ± 0.00 pg/ml; p<0.001, 48-hour: 800.00 ± 0.00 pg/ml; p<0.001) (Table 10). Compared to controls at 12- and 48-hours, respectively, AM-MSCs in inflammation demonstrated significant increases in the production of PGE$_2$ (12-hour: 1291.40 ± 78.47 pg/ml; p=0.001, 48-hour: 1158.70 ± 138.99; p=0.008), MCP-1 (12-hour: 1018.30 ± 22.34 pg/ml; p<0.001, 48-hour: 1198.60 ± 32.20; p<0.001), IL-1β (12-hour: 226.73 ± 22.81 pg/ml; p=0.005, 48-hour: 144.10 ± 4.57; p<0.001), IL-6 (12-hour: 4000 ± 0.00 pg/ml; p<0.001, 48-hour: 4000.00 ± 0.00 pg/ml; p<0.001), IL-8 (12-hour: 800.00 ± 0.00 pg/ml; p<0.001, 48-hour: 800.00 ± 0.00 pg/ml; p<0.001), and IL-10 (12-hour: 5.37 ± 0.73 pg/ml; p=0.002, 48-hour: 14.47 ± 2.39 pg/ml; p=0.018) (Table 10).
Figure 40: Cytokine production profile of human MSCs in control and inflammatory culture conditions. Graphs depicting production of pro- and anti-inflammatory cytokines by AD- and AM-MSCs following 24- and 48-hours of culture in the absence (Control; gray bars) or presence (INF: pink bars) of IL-1β and TNF-α. Solid lines connecting bars indicate significant differences between cell types within the same culture condition (p≤0.050). Dotted lines connecting bars indicate a significant trend between cell types within the same culture condition (p≤0.080).
Table 10: Summary of cytokine production profile of human MSCs in control and inflammatory culture conditions. Table depicting pro- and anti-inflammatory cytokine concentrations produced by AD- and AM-MSCs following 24- and 48-hours of culture in the absence (Control) or presence (INF) of IL-1β and TNF-α. * indicates a significant difference between culture conditions within MSC type (p≤0.050).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>12hr</th>
<th>48hr</th>
<th>p-value</th>
<th>p-value</th>
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<tr>
<td></td>
<td>Control</td>
<td>INF</td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PGE2</td>
<td>0.00 ± 0.00</td>
<td>1593.80 ± 46.51*</td>
<td>&lt;0.001</td>
<td>23.00 ± 4.38</td>
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<tr>
<td>MCP-1</td>
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<td>334.27 ± 85.05</td>
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<td>IL-1α</td>
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<td>1.03 ± 1.03</td>
<td>0.495</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>IL-1β</td>
<td>0.00 ± 0.00</td>
<td>220.27 ± 32.48*</td>
<td>0.013</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.00 ± 0.00</td>
<td>1.13 ± 1.13</td>
<td>0.495</td>
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<tr>
<td>IL-6</td>
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<td>4000.00 ± 0.00</td>
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<tr>
<td>IL-8</td>
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<td>&lt;0.001</td>
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<tr>
<td>IL-10</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
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<th>Cytokine</th>
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<tr>
<td></td>
<td>Control</td>
<td>INF</td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PGE2</td>
<td>74.72 ± 9.17</td>
<td>1291.40 ± 78.47*</td>
<td>0.001</td>
<td>39.99 ± 4.71</td>
</tr>
<tr>
<td>MCP-1</td>
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<td>&lt;0.001</td>
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<td>IL-1α</td>
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<td>IL-1β</td>
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<td>0.005</td>
<td>4.30 ± 4.30</td>
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<td>IL-4</td>
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<td>IL-8</td>
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<td>IL-10</td>
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<td>5.37 ± 0.73</td>
<td>0.002</td>
<td>0.00 ± 0.00</td>
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</table>

* indicates significance (p≤0.050) compared between culture conditions at respective time point
Comparing the two MSC types, AD-MSCs produced significantly more PGE₂ and IL-1β at 12- and 48-hours, respectively compared to AM-MSCs in inflammatory media (Figure 40). Additionally, AD-MSCs trended towards producing statistically more MCP-1 (p=0.074) at 12-hours compared to AM-MSCs in inflammatory media. Conversely, AM-MSCs produced significantly more IL-10 at 12- (p=.002) and 48-hours (p=0.004) compared to AD-MSCs when cultured in inflammatory media (Figure 40). Of note, significant differences were also observed comparing the cytokine production of AD-MSCs and AM-MSCs in control media (Figure 40 & Table 10).

**Effect of Inflammation on Extracellular Matrix Mineralization by AD- and AM-MSCs**

To determine the effect of inflammation on the osteogenic potential of AD- and AM-MSCs, Alizarin Red staining for mineralized ECM was imaged and quantified following 28 days of culture in control or inflammatory differentiation media. In non-inflammatory conditions, histological imaging confirmed that AD- and AM-MSCs produced calcified ECM as evidenced by positive (red) alizarin staining on the bottom of all wells (Figure 41). Additionally, macroscopic images of wells illustrated the formation of white, calcified ECM nodules in AD- and AM-MSC cultures (Figure 41; inserts). Inflammation affected osteogenesis of both MSC types to different degrees. Inflammation resulted in a significant reduction in the number of calcified nodules (1.33 ± 0.04 per well; p=0.013) in AD-MSC cultures compared to control conditions (3.33 ± 0.33 per well). However, inflammation did not significantly affect nodule diameter (inflammation: 0.22 ±
0.33 mm, control: 0.18 ± 0.02 mm) or the amount of Alizarin Red staining (inflammation: 3.34 ± 0.05 RAU, control 3.46 ± 0.07 RAU) in AD-MSC cultures (Figure 41). Conversely, inflammation resulted in a significant increase the number of nodules (inflammation: 27.67 ± 0.23 per well, control: 9.33 ± 1.86 per well; p=0.009) in AM-MSC cultures; however, a significant reduction in nodule diameter (inflammation: 0.23 ± 0.02 mm, control: 0.36 ± 0.05 mm; p=0.002) and the amount of Alizarin Red staining (inflammation: 1.08 ± 0.06 RAU, control: 1.80 ± 0.03 RAU; p<0.001) was observed in AM-MSC cultures (Figure 41).

Figure 41: Osteogenic potential of human MSCs in control and inflammatory culture conditions. Representative alizarin red staining (red = mineralized ECM) in A) AD-
and B) AM-MSC osteogenic cultures in control (non-inflammatory) conditions. (inserts = macroscopic imaging of wells illustrating mineralization (white pellets). C) Graph depicting quantification of alizarin red staining of AD- and AM-MSCs in control conditions. Representative alizarin red staining (red = mineralized ECM) in D) AD- and E) AM-MSC osteogenic cultures in inflammatory conditions. (inserts = macroscopic imaging of wells illustrating mineralization (white pellets). F) Graph depicting quantification of alizarin red staining of AD- and AM-MSCs in inflammatory conditions. Scale bars = 100µm. Solid lines connecting bars indicate significant differences between MSC type within the same culture conditions (p≤0.050). * Indicates a significant difference between culture conditions within MSC type (p≤0.050).

Comparing the two MSC types, AD-MSC osteogenesis was impacted to a lesser degree by the presence of inflammation as compared to AM-MSCs. AD-MSCs demonstrated the formation of a thin white film of ECM coating the bottom of the wells resulting in significantly fewer nodules (p=0.001) compared to AM-MSC cultures in inflammatory differentiation media (Figure 41; inserts). However, AD-MSC cultures had significantly higher amounts of total alizarin red staining (p<0.001) compared to AM-MSC cultures in inflammatory differentiation media (Figure 41).
Effect of Inflammation on Glycosaminoglycan-Containing Extracellular Matrix Formation by AD- and AM-MSCS

To determine the effect of inflammation on the chondrogenic potential of AD- and AM-MSCs, Alcian Blue staining for glycosaminoglycan (GAG)-containing ECM and morphological analysis was performed on MSC pellets following 28 days of culture in control or inflammatory differentiation media. In non-inflammatory conditions, histological imaging confirmed that AD- and AM-MSCs produced ECM containing GAG as evidenced by positive (blue) staining within all pellets (Figure 42). Inflammation did affect chondrogenesis of both MSC types to different degrees. Inflammation resulted in ECM irregularities and voids within the AD-MSC pellets which appeared more fibrous (Figure 42) and demonstrated significant reductions in the percentage of pellet area staining positively for Alcian blue (inflammation: 34.75 ± 2.49%, control: 57.83 ± 2.10%; p<0.001) and overall pellet cross-sectional area (inflammation: 2.76 ± 0.18mm², control: 4.25 ± 0.22mm²; p=0.006) compared to control conditions (Figure 42). Conversely, AM-MSC pellets remained intact with no voids under inflammatory conditions (Figure 42) and did not demonstrate a significant reduction in pellet cross-sectional area (inflammation: 5.67 ± 0.26mm², control: 6.20 ± 0.61mm²) (Figure 42). However, the percentage of pellet area staining positively for Alcian blue (inflammation: 82.03 ± 3.26%, control: 92.86 ± 1.01; p=0.019) was significantly reduced.
Figure 42: Chondrogenic potential of human MSCs in control and inflammatory culture conditions. Representative Alcian blue staining (blue = GAG-containing ECM, purple = cell nuclei) in A) AD- and B) AM-MSC chondrogenic pellet cultures in control (non-inflammatory) conditions. Graphs depicting quantification of C) the percentage of pellet area stained positively for GAG and D) cross-sectional pellet area of AD- and AM-MSCs in control conditions. Representative Alcian blue staining in E) AD- and F) AM-MSC chondrogenic pellet cultures in inflammatory conditions. Graphs depicting quantification of G) the percentage of pellet area stained positively for GAG and H) cross-sectional pellet area of AD- and AM-MSCs in inflammatory conditions. Scale bars = 100µm. Solid lines connecting bars indicate significant differences between MSC type within the same culture conditions (p≤0.050). * Indicates a significant difference between culture conditions within MSC type.
Comparing the two MSC types, AM-MSC chondrogenesis was impacted to a lesser degree by the presence of inflammation as compared to AD-MSCs. AM-MSCs had significantly greater GAG staining (p<0.001) and cross-sectional pellet area (p=0.04) compared to AD-MSCs in inflammation.

**Effect of Inflammation on DNA and Glycosaminoglycan Quantification by AD- and AM-MSCS**

To further evaluate the impact of inflammation on AD- and AM-MSC chondrogenesis, DNA and GAG were quantified via PicoGreen and DMMB assays, respectively on MSC pellets following 28 days of culture in control or inflammatory differentiation media. Inflammation resulted in increased DNA and GAG content in both AD- and AM-MSC pellets (Figure 43). AD-MSC pellet DNA (inflammation: 0.14 ± 0.01 μg/pellet, control: 0.05 ± 0.01 μg/pellet; p=0.002) and GAG content (inflammation: 5.90 ± 0.57 μg/pellet, control: 2.63 ± 0.40 μg/pellet; p=0.009) significantly increased compared to control conditions (Figure 43). AM-MSC pellet DNA (inflammation: 0.18 ± 0.03 μg/pellet, control: 0.10 ± 0.001 μg/pellet; p=0.048) and GAG content (inflammation: 8.34 ± 0.88 μg/pellet, control: 6.95 ± 0.25 μg/pellet) increased, however the latter was not significant (Figure 43).
Figure 43: DNA and GAG quantification of human MSCs in control and inflammatory culture conditions. Graphs depicting quantification of A) DNA and B) GAG content per AD- and AM-MSC chondrogenic pellet culture in control (non-inflammatory) conditions. Graphs depicting quantification of C) DNA and D) GAG content per AD- and AM-MSC chondrogenic pellet culture in inflammatory conditions. Solid lines connecting bars indicate significant differences between MSC type within the same culture conditions (p≤0.050). * Indicates a significant difference.
between culture conditions within MSC type. Dotted lines connecting bars indicate a significant trend between cell types within the same culture condition (p≤0.080).

Comparing the two MSC types, AM-MSCs trended toward having significantly (p=0.081) higher GAG content per pellet compared to AD-MSCs in inflammation. This also held true when normalizing pellet GAG content to DNA content (AD-MSC: 41.46 ± 1.46 μg GAG/ μg DNA, AM-MSC: 47.99 ± 2.66 μg GAG/ μg DNA).

Table 11: Summary of osteogenesis and chondrogenesis differentiation quantification of human MSCs in control and inflammatory culture conditions. Table depicts the representative quantitative outcomes of the differentiation potential of AD- and AM-MSCs following 28 days of culture in the absence (Control) or presence (INF) of IL-1β and TNF-α. Osteogenesis outcomes include: Relative absorbance units (RAU) of Alizarin Red staining for mineralized extracellular matrix production. Chondrogenesis outcomes include: Normalized glycosaminoglycan (GAG) content to the respective pellet’s DNA content, the percentage of pellet positively stained for Alcian Blue indicating GAG presence, pellet cross-sectional area, and percent reduction of pellet cross-sectional area from its respective Day 0 measurements. * indicates a significant difference between culture conditions within MSC type (p≤0.050).
DISCUSSION

The number of clinical trials employing intradiscal administration of MSCs in patients with IVD degeneration is increasing. Several MSC types have been investigated in these studies; however, controversy exists as to which MSC type will provide optimal therapeutic benefit when administered into these inflammation incumbered environments. More specifically, Tong et al. recently stated that “direct comparison of the cells’ ability to attenuate local inflammation and to improve disc structure and biomechanical function is needed.” Thus, in this study, we compared the effect of degenerate IVD levels of inflammation on AD- and AM-MSC effector function (e.g., cytokine production and differentiation capacity). This was an initial attempt to identify which of the two MSC sources may be more therapeutically beneficial for mitigating IVD degeneration and/or promoting tissue regeneration. In general, our findings suggest that inflammation affects

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<td>Control</td>
<td>INF</td>
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<tr>
<td>Alizarin Red staining</td>
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<td>[µg GAG / µg DNA]</td>
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<td>Alcian Blue Staining</td>
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<tr>
<td>for GAG [% of Pellet</td>
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<tr>
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<td>57.83 ± 2.10</td>
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<tr>
<td>Area [mm²]</td>
<td>4.25 ± 0.22</td>
<td>2.76 ± 0.18*</td>
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<td>% Reduction of Pellet</td>
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<tr>
<td>Cross-Sectional Area</td>
<td>5.63 ± 10.62</td>
<td>38.59 ± 7.66*</td>
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* indicates significance (p ≤ 0.050) compared between culture conditions
cytokine production and the differentiation capacity of AD- and AM-MSCs differently. These differences may have significant clinical implications when considered in the context of the local environment of the degenerate IVD. More specifically, AD-MSC culture in inflammation resulted in 1) increased cell number, 2) production of significantly more pro- and less anti-inflammatory cytokines, 3) enhanced osteogenesis, and 4) diminished chondrogenesis compared to AM-MSCs under the same conditions.

The first significant difference comparing between the two MSC sources was that culture in inflammation resulted in a significant increase in AD-MSC numbers compared to AM-MSCs. In many tissues, inflammation has been shown to promote activation, migration, and proliferation of resident MSCs as a mechanism to increase cell numbers at a site of injury and prime them to promote tissue repair and regeneration.275,285 Thus, increases in MSC number is generally thought to be a beneficial response in normal wound healing conditions. However, in the context of the degenerate IVD, it has been hypothesized that an increase in MSC numbers could quickly deplete the already limited nutrient supply within the tissue and thus may not provide therapeutic benefit.286 Furthermore, it is essential to identify the species and amounts of soluble cytokines being released by these MSCs and to consider their effects on other local cells present in the degenerate IVD.

Thus, the second significant difference found comparing between the two MSC sources was that AD-MSCs produced more PGE₂ and MCP-1 at the early (12-hour) time-point compared to AM-MSCs. MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL2), is produced by many cell types to recruit macrophages selectively and other
inflammatory cells to sites of tissue injury, inflammation, or infection. PGE$_2$ produced by MSCs is thought to exert an anti-inflammatory effect by shifting infiltrating pro-inflammatory, M1-macrophages towards a pro-regenerative, M2 phenotype. Thus, enhanced recruitment of macrophages via increased expression of MCP-1 coupled with increased expression of PGE$_2$ could subsequently quell local tissue inflammation. However, in the context of the intact degenerate IVD relatively few blood vessels are found, and thus infiltration of exogenous macrophages is limited to only the periphery the IVD (e.g., cartilaginous end-plates). Therefore, increased concentrations of MCP-1 and PGE$_2$, as observed by AD-MSCs in the current study, may not be beneficial in the context of the degenerate IVD. More specifically, studies have demonstrated that PGE$_2$ has a negative impact on IVD cell health and ECM homeostasis even after short-term exposure. Moreover, increased MCP-1 expression by cells in the IVD correlate positively with increasing histological grades of IVD degeneration. However, additional studies investigating the impact of elevated levels MCP-1 on IVD cell-mediated ECM homeostasis in a three-dimensional (e.g., IVD tissue mimetic) inflammatory environment are warranted.

Another significant difference identified when comparing the cytokine production of AD- and AM-MSCs in the presence of inflammation was that AD-MSCs produced significantly more IL-1$\beta$ and significantly less IL-10 at the later time-point (48-hour) compared to AM-MSCs. IL-1$\beta$ is a pro-inflammatory cytokine, which along with TNF-$\alpha$, plays a key role in the progression of IVD degeneration. Increased concentrations of IL-1$\beta$ have been shown to cause IVD cells to increase production of tissue degrading
proteases, pro-inflammatory and chemotactic cytokines, concomitant with inhibiting ECM biosynthesis.\textsuperscript{271,272} Thus, the increased production of IL-1\(\beta\) by AD-MSCs observed herein suggests that this MSC type could exacerbate the pro-inflammatory environment more than AM-MSCs. Conversely, IL-10 is a potent anti-inflammatory and immune suppressant which plays a prominent autoregulatory role in the production of pro-inflammatory cytokines by macrophages.\textsuperscript{291} In the context of the degenerate IVD, IL-10 is found in increased levels compared to non-degenerate tissues,\textsuperscript{283} suggesting an endogenous attempt to quell inflammation. Moreover, administration of exogenous IL-10 to degenerate IVD cell cultures has been shown to decrease transcription of TNF-\(\alpha\) and IL-1\(\beta\).\textsuperscript{292} Thus, the observed increase in the production of IL-10 by AM-MSCs in the inflammatory conditions studied herein could serve as an exogenous source of anti-inflammatories which were not found to be produced by AD-MSCs. Of note, the decreased production of IL-1\(\beta\) observed in the AM-MSC group may be related to their increased production of IL-10 suggesting the possibility of a similar autoregulatory mechanism to that observed in macrophages, however further study of the specific mechanisms involved are warranted.

The fourth significant difference observed comparing between MSC types was that the presence of inflammation detrimentally impacted the osteogenic differentiation of AM-MSCs, but not AD-MSCs. Previous \textit{in vitro} studies have demonstrated that inflammatory mediators TNF-\(\alpha\) and IL-1\(\beta\) enhance osteogenesis of human MSCs in a dose-dependent manner,\textsuperscript{293} but only if the MSCs are osteogenically primed or are cultured in the presence of osteogenic signals.\textsuperscript{294} While we observed similar effects in AD-MSC cultures (as was indicated macroscopically by more uniform mineralization in the wells compared to their
non-inflammatory conditions), the converse was true for AM-MSCs. The difference could be explained in part by the short-term cytokine data which not only demonstrated elevated levels of IL-1β produced by AD-MSC cultures but also higher concentrations of PGE2. PGE2 has been shown to be a key cytokine necessary for promoting bone healing and regeneration.\textsuperscript{295,296} However, to further confirm this hypothesis cytokine analysis on the inflammatory differentiation media at early- and late time-points are warranted. From a clinical perspective, these results suggest that AD-MSCs may be more prone to forming bone in the context of the inflammatory environment of the degenerate IVD compared to AM-MSCs. Provided an osteoinductive cue (e.g., autologous bone graft), AD-MSCs may be more amenable to promoting fusion to combat the effects of severe degeneration as compared to AM-MSCs.

Another key difference observed comparing between MSC types was that the presence of inflammation detrimentally impacted chondrogenesis of AD-MSCs to a greater extent compared to AM-MSCs. It is well established that the presence of TNF-α and/or IL-1β impairs chondrogenesis of MSCs. This is mediated in part via increased translocation of nuclear factor kappa beta (NF-kβ) and inhibition of the transcriptional activator sex determining region Y-box 9 (Sox-9) and transforming growth factor beta (TGF-β) signaling which is required for chondrogenesis.\textsuperscript{297,298} In the present study, although chondrogenesis appeared to be hampered by inflammation in both MSC types compared to their respective non-inflammatory controls, AM-MSC cultures yielded larger, intact chondrogenic pellets, enhanced histological staining and quantification of GAG compared to AD-MSCs. Similar results were observed by others when comparing the effects of IL-
1β (10ng/ml), TNF-α (50ng/ml), or human osteoarthritic synovial fluid on AD- and BM-MSC chondrogenesis.\textsuperscript{299} In the previous studies, it was found that although AD-MSCs pellets demonstrated histological irregularities including; voids in the pellet, altered cell nucleus morphology, smaller pellet size, and reduced GAG staining compared to non-inflammatory controls, they appeared to fare better than BM-MSCs cultures.\textsuperscript{299} The reasons for the observed differences between these studies are still unclear and require further investigation. However, it could be hypothesized that differences in MSC cytokine receptor expression and thus sensitivity to inflammation may play a role. Regarding the clinical application of MSCs for IVD degeneration, our results suggest that AM-MSCs may have an enhanced ability to produce ECM containing GAG, as is typically found within the nucleus pulposus (NP)-region of the IVD, compared to AD-MSCs. Thus, AM-MSCs may have a higher propensity to regenerate IVD tissue in mild- to moderately degenerate IVDs.

As with any study, limitations were noted. First, the effect of other degenerate IVD environmental stimuli including other inflammatory cytokines, oxygen tension, pH, limited nutrient supply and osmolarity, which have been shown to impact MSC effector function, was not investigated.\textsuperscript{300} However, our objective was to elucidate the effects of the primary inflammatory mediators involved in IVD degeneration without overcomplicating the study system. Secondly, we did not specifically investigate the effect of inflammation on MSC differentiation toward IVD cell-specific phenotypes (e.g., nucleus pulposus and annulus fibrosus cells). However, our understanding of distinguishing phenotypic markers for IVD cells continues to be investigated,\textsuperscript{301–303} and repeatable differentiation protocols to achieve these phenotypes are still being defined,\textsuperscript{304} which will enable further investigation.
Moreover, we chose to evaluate bone and cartilage formation as these particular phenotypes are relevant in the context of the degenerate IVD. For example, the ectopic bone formation has been observed following leakage of intradiscally injected MSCs from degenerate IVDs, and products containing MSCs have been investigated to improve IVD fusions. Additionally, although the relative quantities are different, the primary ECM components that comprise the nucleus pulposus region of the IVD are similar to that of articular cartilage. Another limitation to the study was that the differential response of the MSC types to the same inflammatory conditions could have been due to differences in MSC age and donor variability which was not controlled for in this study. However, it was not feasible to obtain amnion and adipose tissue samples from the same donors, and moreover, the younger age of amnion-derived MSCs may represent an actual clinical advantage of over other adult sources.

**CONCLUSION**

Taken together, our findings suggest that AM-MSCs may be more amenable to promote IVD tissue regeneration as compared to AD-MSCs in the context of the inflammatory environment found in the degenerate IVD. Conversely, AD-MSCs may be more prone to form bone and thus promote IVD fusion. The studies herein also provide the impetus for further investigation into the mechanisms underlying the observed differences between AD- and AM-MSC efficacy as therapeutics for IVD degeneration.
CHAPTER VII

BIOMIMETIC ANNULUS FIBROSUS REPAIR SCAFFOLDS DEMONSTRATE SIMILAR BIAXially DEPENDENT STRETCH RATIO AND STRAIN RATE MECHANICAL PROPERTIES COMPARED TO THE NATIVE ANNULUS FIBROSUS

INTRODUCTION

The annulus fibrosus (AF) is a dense fibrocartilage tissue that undergoes multi-directional tensile strain to allow for spinal movement and withstand compression of the spine. The mechanical properties of the AF have been extensively researched in the uniaxial tension direction and more recently the material properties of the AF have been subjected to multi-directional (biaxial) tensile loading conditions. While the AF does experience tensile loading, most tensile stresses are applied biaxially as the annular tissue is constrained by the adjacent vertebral bodies. Furthermore, the biaxial properties of other tissues throughout the human body have been examined (mitral valve, pericardium, lung, and arteries),\textsuperscript{310–314} while only seven studies, to the author’s knowledge, have examined the AF’s biaxial tensile response. Bruehlmann et al. examined the intercellular mechanics of the AF using the outer layers of the bovine AF tissue, but did not record the mechanical forces or calculate material properties.\textsuperscript{315} Bass et al. examined the biaxial mechanical properties of “thick” cube samples of the human AF and found that biaxial tension resulted in higher stresses than uniaxial; however, the constitutive model which was used fixed the
circumferential direction strain and therefore may not directly represent physiological loading.\textsuperscript{316} Gregory et al. evaluated the influence of strain rate and found that porcine AF tissue is strain rate independent at physiological loading rates.\textsuperscript{317} Additionally, Gregory et al. examined the differences between uniaxial and biaxial properties of porcine AF tissue and observed a higher linear-region modulus with biaxial loading that may be explained by boundary constraints using elasticity theory.\textsuperscript{102,318} O’Connell et al. examined the biaxial properties of human AF tissue through the use of different stretch ratios at a set strain rate to develop and validate a constitutive model of the material properties of the AF.\textsuperscript{102} Additionally, this model was used to simulate shear and uniaxial tensile behavior to investigate the effect of degeneration demonstrating the fiber-matrix interactions play an important contribution to the stress and was detrimentally affected through degeneration. Driscoll et al. evaluated the dependence of biaxial mechanical properties on lamellar orientation (parallel, perpendicular, and angle-ply alignment of collagen fibers) and inter-lamellar shearing using bilayer nanofibrous scaffolds seeded with bovine mesenchymal stromal cells (MSCs).\textsuperscript{132} Results from this particular study reaffirmed the need of an angle-ply architecture, replicating native AF structure, is needed to achieve the native AF biaxial properties. Lastly, Gooyers et al. examined the effects of porcine AF tissue on cyclic biaxial loading to measure effects of cycle-varying changes in peak stress and if the location of the AF tissue (posterior or anterior) influenced the material properties.\textsuperscript{319}

However, to date, no mechanical studies have been conducted to determine the biaxial material properties (elastic modulus and peak stress) of bovine annulus fibrosus
tissue. Given that the bovine’s caudal IVD is similar in size, geometry, matrix components, and resting stress, it is often been regarded as a surrogate for human IVD tissue testing. Therefore, to address this our lab has developed a physio-mimetic AF repair patch (AFRP) which is a novel, extracellular matrix-based implant with an underlying angle-ply architecture. The AFRP has previously shown to exhibit similar tensile mechanical properties to human AF tissue, and one of the primary advantages of the AFRP scaffold possesses, compared to others, is its biomimetic structure and inherent mechanical strength. Physiologically, the native AF tissue undergoes multidirectional tensile strains (biaxial loading) during spinal motion. Therefore, the examination of the AFRP under physiological biaxial stresses and various strain rates is vital. With this in mind, to date, only a few studies have assessed the biaxial mechanical properties of AF repair devices despite being the most common loading modality borne by the AF.\textsuperscript{102,132,318,320} Thus, the objectives of this study were to 1) evaluate the biaxial material properties of the native bovine AF tissue and 2) assess the \textit{in-situ} mechanical properties of AFRPs in comparison with the native bovine AF.

\textbf{MATERIALS AND METHODS}

\textbf{Fabrication and Sterilization of AFRPs}

Multi-laminate “angle-ply” AFRPs (n=5) were developed and assembled from decellularized porcine pericardium to form tri-layer scaffolds [10 mm (L) x 10 mm (W) x 0.75mm (T)] and crosslinked in 6mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with 1.2mM N-hydroxysuccinimide (NHS) as previously
described. AFRPs were maintained in a phosphate buffered saline (PBS) storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing.

**Native Annulus Fibrosus Tissue Isolation**

Native bovine AF samples (L x W x H: 7 x 10 x 0.75mm$^3$) were isolated from anterior/lateral sections of the caudal IVD (cc2-3 and cc3-4) within the skeletally mature bovine tails (n=5) obtained from a local abattoir. AF samples were maintained in a PBS storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing.

**Biaxial Mechanical Response of AFRPs**

Biaxial testing methods were adapted and modified from literature. Native AF samples and acellular AFRPs (n=5/group) were examined for their biaxial tensile mechanical properties in the circumferential and axial (X:Y) directions under increasing rates of strain at three stretch ratios (20%:20%-equiaxial, 20%:10%-circumferentially dominant, 10%:20%-axially dominant). Briefly, AFRP scaffolds were mounted, with rakes as seen in Figure 44A&B, in a loading apparatus on a CellScale BioTester such that tension could be applied in the circumferential and axial directions. PBS was used to keep each tissue scaffold hydrated during preparation and testing. AFRPs were preconditioned three times to 10% in the axial and circumferential directions (equiaxial) at 1%/s. AFRPs were then tested to each of the three stretch ratios listed above at increasing strain rates of 1%/s, 4%/s, and 10%/s with a five-minute rest between stretch ratios to allow for tissue equilibrium for a total of nine variations (Figure 44D). During each test, tensile force and
rake displacement was recorded using BioTester software and used to calculate each sample’s corresponding elastic modulus (EM) and maximum peak stress. Force was normalized within AFRPs by obtaining the amount of stress from its cross-sectional area. Stretch ratio for the rake-to-rake displacement was calculated as the current length of the scaffold divided by the original length of the tissue sample. EM was determined as the linear region of the stress-strain curve. Maximum peak stress was calculated based on the amount of stress applied at the peak loading curve. Heat mapping was used to determine the distribution of force and displacement of the samples at the peak of the loading curve (Appendix G).
Figure 44: Biaxial testing overview and mechanical loading conditions. A) Representative images of the CellScale BioTester biaxial testing mainframe with B)
AFRP attached by four sets of tines. C) Representative image depicting the angle-ply architecture of the AFRP during fabrication. D) Representative loading schematic beginning with pre-conditioning and illustrating the separate stretch ratios and increasing strain rates: 1%, 4%, and 10%. The stretch ratio is displayed as the tension applied to the circumferential (X) and axial directions (Y) (i.e. 2:1 ratio = 20% strain in circumferential (X) and 10% strain in the axial (Y) directions).

**Statistical Analysis**

Statistical analysis was performed on raw data using GraphPad Prism7 software. Results are represented as mean ± standard error of the mean (SEM), and significance was defined as (p≤0.05). Biaxial studies were statistically compared between native AF and non-seeded AFRP samples using a student’s t-test.

**RESULTS**

**Biaxial Mechanical Response of Annulus Fibrosus Repair Patches Compared to Native AF**

Biaxial mechanical testing at various stretch ratios was performed to assess the mechanical competency of AFRPs compared to physiological loading conditions of native AF tissue. Overall, the biaxial loading of AFRPs demonstrated similar elastic modulus and peak stress mechanical properties to native AF tissue (Figure 45 and Table 12). Loading conditions in the circumferential and axial test orientations illustrated a nonlinear stress-strain response for all tissue samples. However, once the tissue was deformed beyond its
toe region of the stress-strain curve, all tissue samples demonstrated a linear elastic region. Linear curve fitting showed all samples had a mean $R^2$ value of $0.99\pm0.02$. Strain rate significantly altered the elastic modulus and peak stress of bovine native AF tissue within stretch ratios; thus, demonstrating the native bovine AF tissue’s strain rate dependency. Similar trends in strain rate dependency were also observed in AFRPs.
Figure 45: Biaxial testing of AFRPs mimic mechanical properties of native bovine annulus fibrosus tissue for varying stretch ratios and increasing strain rates. A) Circumferential response and B) axial response of elastic modulus derived from 20% strain in the circumferential direction and 20% in the axial direction. C) Circumferential response and D) axial response of elastic modulus derived from 20% strain in the circumferential direction and 10% in the axial direction. E) Circumferential response and F) axial response of elastic modulus derived from 10% strain in the circumferential direction and 20% in the axial direction. Bold * illustrates significant differences (p<0.05) compared to native bovine AF tissue.
Table 12: Biaxial testing of AFRPs mimic native bovine annulus fibrosus tissue. Top, the elastic modulus of AF tissue and AFRP’s demonstrate similar biaxial mechanical properties across stretch ratios and increasing strain rates. Bottom, peak stresses of AF tissue and AFRP’s demonstrate similar biaxial mechanical properties across stretch ratios and increasing strain rates. Grey boxes with bold text and * illustrate significant differences (p<0.05) compared to native bovine AF tissue.

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<td>0.74 ± 0.10</td>
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<td>0.39 ± 0.07</td>
</tr>
<tr>
<td>1:2 1%</td>
<td>0.43 ± 0.04</td>
<td>0.32 ± 0.08</td>
<td>0.43 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.50 ± 0.03</td>
<td>0.33 ± 0.09*</td>
<td>0.49 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>0.52 ± 0.03</td>
<td>0.32 ± 0.09*</td>
<td>0.50 ± 0.06</td>
</tr>
</tbody>
</table>
DISCUSSION

Ideal repair of focal injuries of the AF requires mechanically competent implants which must display similar mechanical properties to native AF tissue. Herein, we characterized multi-laminate AFRP scaffolds for its ability to mimic native AF biaxial mechanical loading properties. The primary findings from these studies include: AFRPs exhibit similar elastic modulus and peak stress mechanical properties compared to native AF tissue and provide the mechanical competency needed for immediate implantation without cell-mediated \textit{in vitro} ECM maturation.

The major finding from this study demonstrated the ability of AFRPs to mimic the biaxial mechanical properties of native AF tissue under various stretch ratios and strain rates without cell-mediated \textit{in vitro} ECM maturation. To the best of the author’s knowledge, this is the first attempt to characterize bovine AF tissue for their biaxial elastic modulus and peak stress material properties. The native AF tissue is subjected to multi-directional loading, and therefore biaxial testing of AF repair biomaterials may provide a more accurate understanding of how AF repair biomaterials will perform \textit{in vivo}. Furthermore, the use of only uniaxial testing may be limited since the unconstrained edges may prevent fiber stretching.\textsuperscript{102} First, testing of native bovine AF tissue demonstrated consistent maximum stresses (0.49-0.74 MPa) compared to previous findings of human AF tissue by Bass et al. (0.56 MPa) and porcine AF tissue Gregory et al. (0.69 MPa).\textsuperscript{316,318} Peak stresses experienced by AFRP samples tended to be slightly higher (1.09 MPa) than that of the bovine AF tissue, although this was not statistically different. Additionally, the resilience to permanent deformation (elastic modulus) of AFRPs mimicked the material
properties of bovine AF tissue in circumferentially dominant stretch ratios. This was expected due to the angle-ply architecture of both tissues with the 30° alignment of collagen fibers aligned from the horizontal (circumferential) axis. Moreover, previous studies have demonstrated the necessity for this circumferentially oriented alignment to functionally restore the native AF tissue mechanical properties.\textsuperscript{120,132,322}

Conversely, changes observed between the AFRP and bovine AF tissue in mechanical properties in the axially dominant stretch ratio are thought to be contributed to the interlamellar matrix (ILM) components. The ILM is composed of multiple components including elastic fibers, cross-bridges, and proteoglycans.\textsuperscript{237} These components are suggested to play a vital role in the mechanical integrity of the AF, particularly in the axial response under biaxial loading. While collagen fibers within the intra-lamellar lamellae of the AF predominately work to resist tensile deformation, the ILM components can reorientate under different loading modalities.\textsuperscript{256} Therefore, current studies are being conducted for the inclusion of an ILM within the AFRP layers which may assist in the biaxial strength of axial dominant material properties.\textsuperscript{323}

\textbf{CONCLUSIONS}

In summary, AFRP scaffolds demonstrated similar biaxial mechanical properties to native AF tissue and its mechanical competency to withstand native mechanical loading modalities. Findings within this study illustrate the potential of the AFRP to withstand the mechanically demanding environment of the IVD. Therefore, future utilization of this AF
focal defect repair biomaterial may improve clinical outcomes for patients impacted from IVD degeneration and/or herniation.

LIMITATIONS

A limitation of this study would be the use of 20% strain during biaxial mechanical testing. Physiologically, the native human AF typically undergoes 4-6% to a 10% maximum strain, and only on rare occasions does the AF experience 20% strain. However, the purpose of the current study was to determine the material properties of both the native AF and AFRP scaffolds in worst-case scenarios. Additionally, preliminary uniaxial testing demonstrated that 20% strain in the axial and circumferential directions of both tissue samples demonstrated minimal to none detrimental impacts on tissue recovery.
CHAPTER VIII

CELL SEEDING OF AN ANGLE-PLY SCAFFOLD FOR ANNULUS FIBROSUS FOCAL DEFECT REPAIR PROMOTES STEM CELL DIFFERENTIATION AND DEMONSTRATES ENHANCED BIAXIAL MECHANICAL PROPERTIES THROUGH TISSUE REMODELING

INTRODUCTION

The annulus fibrosus (AF) is a multi-laminate angle-ply structure that undergoes multidirectional tensile strain to allow for spinal movement and withstand compression of the spine. Focal injuries that occur to the AF have shown to cause detrimental changes in spinal kinematics in addition to increasing the risk of further damage to the surrounding tissue through intervertebral disc (IVD) herniations (IVDHs) and degeneration (IVDD).

Clinically, therapeutic injections of MSC’s for spinal applications are currently being utilized, but the efficacy of different cell sources is still currently being investigated \textit{in vitro}.\textsuperscript{149,324} While the concept of MSC therapy is well understood in the basic science of healing and regeneration, little is known about individual MSC populations in terms of their inclination to promote the repair and/or regeneration of specific IVD tissues. Furthermore, the injection of MSCs into this inflammatory environment may cause the cells to release additional pro-inflammatory cytokines, thus, resulting in further damage to the tissue. To counter this, MSCs may be seeded on biomimetic biomaterial scaffolds, which would allow for an underlying “healthy” matrix for the MSCs to survive. Moreover,
implantation of this scaffold could provide the MSCs a starting point to promote tissue regeneration. However, no previous studies of AF repair biomaterials have been assessed for the differentiation potential and extracellular matrix (ECM) production of MSCs for long-term remodeling.

Previously, Nerurkar et al. developed an electrospun bi-layer tissue construct to assess this approach.\textsuperscript{325} Therein, a single layer of the electrospun scaffold required two weeks of cell-mediated \textit{in vitro} maturation prior to capably stacking of additional layers. Furthermore, an additional 6-10 weeks of cell-mediated \textit{in vitro} maturation were needed to obtain the uniaxial mechanical properties similar to native AF tissue. Thus, limiting its potential as an implant to provide immediate structural and mechanical closure of AF focal defects. Furthermore, while the electrospinning process allows for the alignment of collagen fibers to mimic the “angle-ply” architecture of the native AF, it also demonstrates inherent disadvantages: 1) organic solvents can be cell toxic, 2) process is dependent on many variables which are often un-repeatable, and 3) have demonstrated problems in producing 3D structures as well as sufficient pore size for biomedical applications.\textsuperscript{326}

Therefore, to address this, our lab has developed a biomimetic AF repair patch (AFRP) which is a novel, ECM-based scaffold with an underlying angle-ply architecture which has previously shown to exhibit similar tensile and biaxial mechanical properties to human AF tissue. One of the primary advantages of the AFRP scaffold possesses, compared to others, is its biomimetic structure and inherent mechanical strength. Physiologically, the native AF tissue undergoes multidirectional tensile strains (biaxial loading) during spinal motion, and therefore, the examination of the AFRP under
physiological biaxial stresses and various strain rates is vital. To date, few studies have assessed the biaxial mechanical properties of AF repair devices despite being the most common loading modality borne by the AF. Moreover, while AFRP scaffolds have demonstrated the innate mechanical competency to withstand the physical demands of the IVD, it is hypothesized the addition of MSCs could promote tissue regeneration/integration of the scaffold with the surrounding annular tissue through remodeling; thus, impacting its mechanical properties. Therefore, it is important to assess the change in the AFRPs mechanical properties with respect to MSC-directed collagen turnover.

Thus, the objectives of this study were to 1) determine the differentiation potential of human adipose-derived MSCs (AD-MSCs) under standard culture conditions using real-time reverse transcription PCR (RT-PCR) for putative AF profiles and 2) evaluate tissue remodeling of long-term culture of seeded AFRPs through changes in biaxial mechanical properties and histological collagen composition. It is hypothesized that the AFRP’s underlying aligned collagen microarchitecture will assist in the differentiation of MSCs into an AF-phenotype cell as shown by the gene expression of MSCs seeded AFRPs. Additionally, it is hypothesized that the long-term culturing of MSCs onto AFRPs will lead to remodeling of the scaffold and provide an increase in the AFRPs mechanical properties.

**MATERIALS AND METHODS**

**Fabrication and Sterilization of AFRPs**

Multi-laminate “angle-ply” AFRPs (n=5/group/time-point) were developed and assembled from decellularized porcine pericardium to form tri-layer scaffolds [10 mm (L)]
x 10 mm (W) x 0.75 mm (T)] and crosslinked in 6 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with 1.2 mM N-hydroxysuccinimide (NHS) as previously described. AFRPs were maintained in a phosphate buffered saline (PBS) storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing. Prior to cell seeding, AFRPs were sterilized in 0.1% neutral buffered (pH 7.4) peracetic acid for two hours on an orbital shaker at 150 rpm. AFRPs were then rinsed in sterile PBS (3x: 1 hour/wash), and neutralized (50% FBS/48% DMEM + 2% Ab/Am) while agitated on an orbital shaker at 150 rpm at ambient temperature.

**Cell Seeding and Culture Conditions**

Primary AF cells were harvest according to previous literature, and human AD-MSCs were commercially purchased. For all studies, AFCs and AD-MSCs were seeded at passage 3 (P3) (1x10⁶) onto the surface of each ply via dropwise using a pipette, and the interior ply of the AFRP was seeded using a 21G needle by puncturing through the edge of the AFRP immediately adjacent to the suture line to inject the cells. Cell-seeded AFRPs were cultured under standard static (no mechanical stimulus) culture conditions, which consisted of 3 mL of DMEM (with L-glutamine, 1 g/l glucose and sodium pyruvate) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Ab/Am) at 37°C.

**Biaxial Mechanical Response of Annulus Fibrosus Repair Patches**

Biaxial testing methods were adapted and modified from literature. Seeded and acellular AFRP scaffolds cultured concurrently (n=5/group/time-point) were examined
following 28 and 56 days (4 and 8 weeks) in static culture for their biaxial tensile mechanical properties in the circumferential and axial (X:Y) directions under increasing rates of strain at three stretch ratios (20%:20%-equiaxial, 20%:10%-circumferentially dominant, 10%:20%-axially dominant). Briefly, AFRP scaffolds were mounted, with rakes as seen in Figure 46, in a loading apparatus on a CellScale BioTester such that tension could be applied in the circumferential and axial directions. PBS was used to keep each tissue scaffold hydrated during preparation and testing. AFRPs were preconditioned three times to 10% in the axial and circumferential directions (equiaxial) at 1%/s. AFRPs were then tested to each of the three stretch ratios listed above at increasing strain rates of 1%/s, 4%/s, and 10%/s with a five-minute rest between stretch ratios to allow for tissue equilibrium for a total of nine variations (Figure 46). During each test, tensile force and rake displacement was recorded using BioTester software and used to calculate each sample’s corresponding elastic modulus (EM) and maximum peak stress Force was normalized within AFRPs by obtaining the amount of stress from its cross-sectional area. Stretch ratio for the rake-to-rake displacement was calculated as the current length of the scaffold divided by the original length of the tissue sample. EM was determined as the linear region of the stress-strain curve. Maximum peak stress was calculated based on the amount of stress applied at the peak loading curve.
Figure 46: Biaxial testing overview and mechanical loading conditions. A) Representative images of the CellScale BioTester biaxial testing mainframe with B)
AFRP attached by four sets of tines. C) Representative image depicting the angle-ply architecture of the AFRP during fabrication. D) Representative loading schematic beginning with pre-conditioning and illustrating the separate stretch ratios and increasing strain rates: 1%, 4%, and 10%. The stretch ratio is displayed as the tension applied to the circumferential (X) and axial directions (Y) (i.e. 2:1 ratio = 20% strain in circumferential (X) and 10% strain in the axial (Y) directions).

**Study Design Layout**

AFRPs were seeded with hADSCs. First, the relative gene expression of these cells will be evaluated using RT-PCR at Days 3, 8, and 15. Secondly, AFRPs were assessed for their long-term cytocompatibility, changes in biaxial mechanics, and changes in collagen composition following 28 and 56 days in culture.

**Gene Expression of Human AD-MSC’S on AFRP Scaffolds**

**Cell Seeding and Culture Conditions**

AFRPs were evaluated for the potential to differentiate human AD-MSCs into an AFC-phenotype using AF gene markers. AFRPs (n=3/time-point) were cultured under standard culture conditions described above for 3, 8, and 15 days.

**Human AD-MSC Gene Expression on AFRP Scaffolds**

Differentiation of AD-MSCs seeded onto AFRPs (n=3/time-point) were evaluated using reverse transcription polymerase chain reaction (RT-PCR) for putative AF profiles (Collagen 1, Collagen 5, Collagen 12, Decorin, and P4HA1) (Table 13). Briefly, RNA
was isolated via Trizol from AD-MSC seeded AFRPs. RNA was reverse transcribed via a RETROscript kit and resultant cDNA was amplified using validated primers and a Quantitect SYBR green master mix on a Roto-Gene Q. Expression was quantified using the $2^{-\Delta\Delta C_t}$ method with normalization to an internal control 18s ribosomal RNA and normalized to Day 3 seeded AFRP controls. Additionally, external controls day 0 AD-MSCs (i.e. RNA isolated from AD-MSCs immediately prior to seeding on AFRPs) and human AF RNA (purchased from ScienCell) were evaluated (Supplemental).

Table 13: Representative table depicting the putative gene markers for Annulus Fibrosus cells to be used for gene expression analysis using RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temp [°C]</th>
<th>G-C Content</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
</table>
| Collagen I A1 | Forward 5'-CCA GAA GAA CTG GTA CAT CAG CAA-3'  
Reverse 5'-CGC CAT ACT CGA ACT GGA ATC-3' | 53.0 | 45.8% | 24 |
| Collagen V A1 | Forward 5'-TGA GTT GTG GAG CTG ACT CTA ATC-3'  
Reverse 5'-TAA CAG AAG CAT AGC ACC TTT CAG-3' | 53.0 | 45.8% | 24 |
| Collagen X H A1 | Forward 5'-TGA CAA CCC TTT CCG ACA CA-3'  
Reverse 5'-CTC CTC ACG AAT CTA AAA TTT GC-3' | 53.0 | 50.0% | 20 |
| P4HA1 | Forward 5'-CCC ATT TTG ACT TTG CAC GG-3'  
Reverse 5'-CCC CAG CTC TTT GAA AGC ATC-3' | 53.0 | 50.0% | 20 |
| 18s | Forward 5'-GTA ACC CGT TGA ACC CCA TT-3'  
Reverse 5'-CCA TCC AAT CGG TAG TAG CG-3' | 53.0 | 50.0% | 20 |
| Decorin | Forward 5'-CTC TGC TGT TGA CAA TGG CTC TCT-3'  
Reverse 5'-TGG ATG GCT GTA TCT CCC AGT ACT-3' | 57.0 | 50.0% | 24 |
**Long-Term Remodeling of AFRPs**

**Cell Seeding and Culture Conditions**

AFRPs seeded with AD-MSCs and AFCs (n=5/group/time-point) were cultured under standard culture conditions described above for up to 56 days.

**Cytocompatibility of AFRPS**

Long-term cell cytocompatibility of AFRPs was assessed via alamarBlue. Seeded and acellular (control) AFRPs were assessed longitudinally bi-weekly for cellular metabolic activity. Briefly, 300uL of the alamarBlue solution was added to each well and incubated for 3 hours at 37°C protected from light. Relative fluorescence units (RFUs) was determined using an excitation wavelength of 570nm and the fluorescence emission was read at 585nm. Following 56 days of culture, separate AFC and AD-MSC seeded AFRPs were evaluated using a live/dead assay (n=2/group) according to the manufacturer’s instructions (Biotium). AFRPs were imaged using a fluorescent microscope to obtain images of both live (green) and dead (red) staining for each scaffold. Qualitative analysis was performed by overlaying the live/dead images.

**Changes in Biaxial Mechanical Properties**

Seeded and acellular AFRP scaffolds cultured concurrently in static culture (n=5/group/time-point) were examined following 28 and 56 days (4 and 8 weeks) for their biaxial tensile mechanical properties, as described above, followed by histological analysis.
Histological Staining for Collagen Production

AFRPs were fixed in 10% neutral-buffered formalin for 24 hours before undergoing successive washes in graded ethanol, xylene, and paraffin followed by embedding and sectioning to 5µm thickness. Slides were stained using Herovici’s Stain Kit (American MasterTech) to identify collagen production (i.e., immature/young collagen) from native collagen (i.e., Type 1/mature collagen). Staining colors depict: Young collagen and reticulum are stained blue, mature collagen is stained purplish-red, cytoplasm is stained yellow, and nuclei are stained black. Histological images were then captured on a Zeiss Axio Vert.A1 microscope with AxioVision software (SE64 Rel. 4.9.1 SP08-2013).

Statistical Analysis

Statistical analysis was performed on raw data using GraphPad Prism7 software. Results are represented as mean ± standard error of the mean (SEM), and significance was defined as (p≤0.05). Gene expression studies were statistically compared to Day 3 seeded AFRPs using a Student's t-test between the groups ΔCt values. Long-term culture biaxial studies were statistically compared to concurrently cultured non-seeded control AFRPs and native AF-phenotype (AFCs) seeded AFRPs via a one-way ANOVA followed by a Dunnett’s post-hoc analysis and t-test between non-seeded controls and testing specimen.
RESULTS

Gene Expression

Transcript levels of AF differentiation markers (Col I, Col V, Col XII, P4HA1, and Decorin) were analyzed using RT-PCR, normalized to internal control 18s, and compared to gene expression levels of Day 3 scaffolds (Table 14). Results herein are described as relative fold change ± SD, and statistical changes were evaluated on the ΔCt values. At day 8 and 15 there was a statistical increase in the relative fold change of Col I (5.122±0.555; p=0.002 and 5.242±0.451; p=0.001) compared to Day 3 controls (Figure 47A). Additionally, a statistical increase was observed in the relative fold change of Col XII at Day 12 (2.935±1.016; p=0.05) compared to Day 3 controls (Figure 47B). The transcript levels of P4HA1 were statistical up-regulated at day 8 (1.753±0.502; p=0.049) and a similar trend continued at day 15 compared to day 3 controls (Figure 47D). No statistical changes were observed in gene markers Col V or Decorin at either time-points.
Figure 47: Representative images of the relative fold change ± CI ($2^{-\Delta\Delta Ct\pm2SD}$) of Day 8 and 15 AFRPs compared to Day 3 controls for AF putative gene profiles: A) Collagen I, B) Collagen 12, C) Collagen 5, D) P4HA1, and E) Decorin. The dotted orange line indicates Day 3 control values.
Table 14: Gene expression values (fold change) for select AF putative gene markers of Day 3, 8, and 15 AFRPs. Fold change was calculated as $2^{-\Delta\Delta Ct}$. 95% confidence interval is calculated as $2^{-\Delta\Delta Ct \pm 2SD}$. Statistical difference was calculated using a paired student’s t-test performed at the $\Delta Ct$ level (Ct target – Ct internal control [18s]). Statistical significance ($p \leq 0.05$) is denoted with a bold * and red text. Significant trend ($p \leq 0.07$) is denoted with a bold ^.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Fold Change</th>
<th>95% CI ($2^{-\Delta\Delta Ct \pm 2SD}$)</th>
<th>Average $\Delta Ct \pm SD$</th>
<th>p-value (vs. Day 3 Non-INF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col 1</td>
<td>3</td>
<td>1.000</td>
<td>(0.473, 2.114)</td>
<td>3.523 ± 0.382</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5.122</td>
<td>(2.373, 11.053)</td>
<td>1.167 ± 0.403</td>
<td><strong>0.002</strong>*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5.242</td>
<td>(2.805, 9.796)</td>
<td>1.133 ± 0.240</td>
<td><strong>0.001</strong>*</td>
</tr>
<tr>
<td>Col 5</td>
<td>3</td>
<td>1.000</td>
<td>(0.635, 1.575)</td>
<td>9.593 ± 0.232</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.301</td>
<td>(0.713, 2.374)</td>
<td>9.213 ± 0.367</td>
<td>0.204</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.376</td>
<td>(0.478, 3.956)</td>
<td>9.133 ± 0.726</td>
<td>0.355</td>
</tr>
<tr>
<td>Col 12</td>
<td>3</td>
<td>1.000</td>
<td>(0.745, 1.343)</td>
<td>9.957 ± 0.150</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.329</td>
<td>(0.544, 9.975)</td>
<td>8.737 ± 1.038</td>
<td>0.114</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.935</td>
<td>(0.717, 12.006)</td>
<td><strong>8.403 ± 1.005</strong></td>
<td><strong>0.057</strong>^</td>
</tr>
<tr>
<td>P4HA1</td>
<td>3</td>
<td>1.000</td>
<td>(0.852, 1.174)</td>
<td>9.740 ± 0.082</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.753</td>
<td>(0.873, 3.518)</td>
<td><strong>8.930 ± 0.496</strong></td>
<td><strong>0.049</strong>*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.498</td>
<td>(0.683, 3.289)</td>
<td>9.157 ± 0.561</td>
<td>0.149</td>
</tr>
<tr>
<td>Decorin</td>
<td>3</td>
<td>1.000</td>
<td>(0.590, 1.695)</td>
<td>5.750 ± 0.269</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.030</td>
<td>(0.663, 1.602)</td>
<td>5.707 ± 0.170</td>
<td>0.825</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1.077</td>
<td>(0.449, 2.582)</td>
<td>5.643 ± 0.571</td>
<td>0.784</td>
</tr>
</tbody>
</table>
Long-Term Remodeling of AFRPS

Cytocompatibility of AFRPS

AFRPs seeded with AD-MSCs and AFCs (Figure 48A&B, respectively) were evaluated for their cellular metabolic activity via alamarBlue assay across increasing time-points to assess long-term cytocompatibility of AFRPs. Both cell types demonstrated significantly higher metabolic activity across all time-points compared to acellular AFRPs (p<0.001) (Figure 48C). Increasing time in culture demonstrated a statistical increase in metabolic activity of AD-MSC seeded AFRPs at Day 28 (7641.23±117.90 RFU; p=0.002) and Day 56 (11358.87±94.15 RFU; p<0.001) compared to values recorded on Day 0 (6906.13±111.16 RFU). AFC-seeded AFRPs demonstrated similar metabolic activity at Day 0 (10211.23±221.20 RFU) and Day 28 (10043.57±96.12 RFU), but with additional time in culture, AFC-seeded AFRPs demonstrated a statistical increase in metabolic activity at Day 56 (11624.60±219.57 RFU; p<0.001) compared to values recorded on Day 0. At the final time-point (Day 56), representative seeded-AFRP scaffolds were evaluated for cell viability via Live/Dead staining. Qualitative analysis demonstrated high quantities of live (green) cells and minimal to none dead (red) cells (Figure 48D).
Figure 48: Cytocompatibility of AFRPs over increasing time in culture. Representative images of passage 3 A) hADSCs and B) AFCs prior to seeding on AFRP scaffolds. C) Relative fluorescence of alamarBlue assay demonstrates the cellular metabolic activity over increasing time in culture. D) Representative images of Live/Dead staining of human AD-MSC seeded and AFC-seeded AFRPs following 56 days (8 weeks) in culture. Bold * indicates significant difference (p≤0.05) from Day
1. Bold # indicates significant difference (p≤0.05) of acellular and seeded AFRPs. **Green** staining illustrates live cells and **red** staining indicates dead cells.

**Tissue Remodeling Through Changes in Biaxial Mechanical Properties**

AFRPs seeded with AD-MSCs and AFCs were first evaluated for tissue remodeling (e.g. deposition and synthesis of extracellular matrix (ECM)) through the assessment of changes in biaxial mechanical properties with increasing time in culture. Relative changes in mechanical properties between acellular and seeded AFRPs under a normal physiological strain rate (4%/s) are shown in **Figure 49**.

AFRPs seeded with AD-MSCs demonstrated statistical increases in elastic modulus and peak stress for the circumferential response of 2:2 and 2:1 stretch ratios (**Table 15**). Elastic modulus and peak stress in the circumferential response continued to statistically increase following 8 weeks (56 days) of culture compared to acellular AFRPs cultured concurrently. However, no changes were observed in the circumferential response of the 1:2 stretch ratio nor any stretch ratios’ axial response.
Figure 49: Representative line graphs illustrating the changes in circumferential and axial biaxial material properties over increasing time in the culture of AFRPs under A-B) equiaxial (2:2) stretching and C-D) circumferentially dominant (2:1) stretching at a physiological 4% strain rate. Bold * indicates significant difference from acellular AFRPs. The dotted line and text on x-axis depict the native bovine AF tissue mechanical properties under the same loading conditions.
Table 15: Biaxial testing of AD-MSC seeded AFRPs following 4 and 8 weeks of culture. Top, elastic modulus of acellular and human AD-MSC seeded AFRP’s across varying stretch ratios and strain rates. Bottom, peak stresses of acellular and human AD-MSC seeded AFRP’s across varying stretch ratios and increasing strain rates. Bold text and ^ illustrates significant differences (p<0.05) compared to native bovine AF tissue.

<table>
<thead>
<tr>
<th>Biaxial Loading</th>
<th>Circumferential Response</th>
<th>Axial Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elastin Modulus (MPa)</td>
<td></td>
</tr>
<tr>
<td>Strain Ratio</td>
<td>Testing Rate</td>
<td>Acellular AFRP</td>
</tr>
<tr>
<td>(C:A) 1%</td>
<td>9.80 ± 0.79</td>
<td>10.81 ± 1.07</td>
</tr>
<tr>
<td>2:2 4%</td>
<td>9.55 ± 0.74</td>
<td>10.80 ± 1.21^</td>
</tr>
<tr>
<td></td>
<td>9.39 ± 0.76</td>
<td>10.44 ± 1.28^</td>
</tr>
<tr>
<td>2:1 1%</td>
<td>5.76 ± 0.79</td>
<td>6.74 ± 1.16^</td>
</tr>
<tr>
<td></td>
<td>5.77 ± 0.79</td>
<td>6.73 ± 1.13^</td>
</tr>
<tr>
<td></td>
<td>5.74 ± 0.79</td>
<td>6.60 ± 1.14^</td>
</tr>
<tr>
<td>1:2 4%</td>
<td>5.66 ± 0.13</td>
<td>3.41 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>3.65 ± 0.14</td>
<td>3.46 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>3.71 ± 0.19</td>
<td>3.62 ± 0.71</td>
</tr>
</tbody>
</table>

AFRPs seeded with AFCs demonstrated statistical increases in elastic modulus and peak stress for the circumferential response of 2:2, 2:1, and 1:2 stretch ratios following 8 weeks (56 days) of culture compared to acellular AFRPs cultured concurrently (Table 16).
Additionally, statistical increases were observed in the elastic modulus and peak stress for the axial response of 2:2, 2:1, and 1:2 stretch ratios following 4 weeks (day 28). However, the statistical changes which were observed in the 2:2 and 1:2 stretch ratios at week 4 were statistically lower at week 8. This loss of elastic modulus and peak stress was not significantly different from acellular AFRPs. Furthermore, the elastic modulus and peak stress of the 2:1 stretch ratio at week 8 maintained the mechanical properties seen at week 4 and remained statistically higher compared to acellular AFRPs.
Table 16: Biaxial testing of AFC-seeded AFRPs following 4 and 8 weeks of culture. Top, the elastic modulus of acellular and AFC-seeded AFRP’s across varying stretch ratios and strain rates. Bottom, peak stresses of acellular and AFC-seeded AFRP’s across varying stretch ratios and increasing strain rates. Bold text and ^ illustrates significant differences (p<0.05) compared to native bovine AF tissue.

Histological Staining for Collagen Production

Following mechanical testing, AFRPs were histologically evaluated using Herovici’s stain for the deposition of new “immature” collagen (blue stain) compared to the “mature” Type I collagen scaffold (reddish-purple stain). Minimal tissue disruption was
observed for all samples within all testing groups as they were evaluated following biaxial loading. Acellular AFRPs illustrated predominantly type I collagen staining with minimal to none staining of immature collagen the or nuclei (black) (Figure 50, left). AFRPs seeded with AD-MSCs illustrated the distribution of human AD-MSC nuclei along the seeded exterior and interior layers of the AFRP. Additionally, the deposition of a thick immature collagen band (Figure 50, middle) was observed along the seeded edges of the AFRP and between the AFRP layers at both 4 and 8 weeks. Similar distribution of AFC nuclei and immature collagen deposition were observed around the periphery of the seeded edges and the inner layer of the AFRP at the week 4 time-point. Conversely, at the week 8 time-point, AFC nuclei were observed infiltrating further within the AFRP layers. Additionally, the underlying collagen Type I matrix was observed to being remodeled and replaced with the deposition of immature collagen within the AFRP layers (Figure 50, right).
Figure 50: Representative histological images of left, acellular; middle, AD-MSC seeded; right, AFC-seeded AFRPs following 4 and 8 weeks in culture. AFRPs are stained with Herovici’s stain illustrating the deposition of new immature collagen (blue stain with arrowhead) between the AFRP layers and within AFRP layers compared to the scaffold’s mature collagen (purple #). (nuclei = black).
DISCUSSION

Ideal repair of focal injuries of the AF requires mechanically competent implants which must display similar mechanical properties to native AF tissue while allowing for the potential of tissue regeneration. Herein, we characterized a multi-laminate AFRP scaffold for its ability to support the differentiation of human AD-MSCs toward putative AF gene markers and promote stem cell-mediated tissue remodeling. The primary findings from these studies include: 1) the underlying microarchitecture and biochemical composition of AFRPs promotes differentiation of human AD-MSCs towards an AF cell phenotype and 2) long-term culture of seeded AFRPs lead to increases in biaxial mechanical properties and allows for tissue remodeling through new collagen deposition.

The first major finding from this study was that the AFRPs underlying microarchitecture and biochemical composition promotes differentiation of human AD-MSCs towards an AF cell phenotype in the absence of media growth factor supplementation or mechanical stimulus. Previous studies of gene expression in biological-based engineered AF repair biomaterials are limited due to the research of the AF’s role in IVD pathologies only recently being investigated. Most in vitro investigations into MSC treatments for IVD pathologies have focused on NP cell differentiation, and very few studies have focused on the differentiation of MSCs into AF cells. Additionally, it is currently unclear in the literature whether NP and AF cells are from distinct developmental lineages or if the local microenvironment determines the functionality and morphology of the different phenotypes. However, what is known are that matrix components of the IVD tissues, with the outer AF shown to be a more fibrous structure and illustrate greater
amounts of Collagen I compared to the inner AF and NP regions. Previously, a variety of expression studies have evaluated putative marker genes of AF cells based on different tissue origins. More recently, van den Akker et al. used healthy, non-degenerate, IVDs to identify potential distinct AF markers by comparing the differences of expression between NP and AF cells. This study established a set of AF gene expression markers which were distinctly different from NP cells: COL1A1, COL5A1, COL12A1, SFRP-2, ADAMTS-17, and P4HA1. Both COL1A1 and COL12A1 are mature collagen markers, while COL5A1 and P4HA1 are early collagen makers have been shown to demonstrate their involvement in collagen fiber triple-helix formation and matrix contraction. Of note, this study used TGF-β supplementation to induce the cells collagen fibrillogenesis, and moreover, collagen sheet formation only occurred in the presence of this supplemented growth factor. Thus, insinuating that the collagen maturation and fibrillogenesis are largely dependent on TGF-β signaling. However, previous literature has also indicated that the microarchitecture and biochemistry signaling of the underlying scaffold, of which the cells are seeded upon, also plays a large role in collagen production. Therefore, the AFRP’s biological collagen composition and fiber alignment provided a support structure for cells to differentiate in addition to its angle-ply architecture which served as a structural guide for a more homogenous cell distribution. This underlying structural and biochemical composition is contributed to the permitted differentiation of human AD-MSCs towards an AF cell phenotype through the up-regulation of AF putative markers: COL1A1, COL12A1, and P4HA1. Furthermore, this structural template guides cellular remodeling and can function as a future delivery vehicle of cells
Previous animal and human studies have demonstrated the positive effects of MSC delivery to degenerate IVDs. Animal studies using an intradiscal injection for MSC delivery has demonstrated that the cells have a fast clearance rate with the high quantities often leaving the site within the first 24-48 hours and most cells are non-detectable after 7-14 days.\textsuperscript{341} Although the cell presence of MSCs is limited, these injections have shown to improve IVDD symptoms including a reduction in pain, improvement of impaired movement, and restoration of ataxia (loss of full control of bodily movements).\textsuperscript{342} Moreover, the use of MSCs in human clinical trials via intradiscal injections have been investigated to determine the effects on patients with chronic low back pain. Two recent human trials have resulted in \textasciitilde 40\% of patients showed an improvement in pain relief, mobility, and strength.\textsuperscript{279,343} Furthermore, follow-up studies years afterward have shown no aberrant growths or abnormalities. Thus, these results give promise to the feasibility and safety of the use of MSC delivery to the IVD.\textsuperscript{344}

The second major finding was that long-term culture of AD-MSC and primary AFC seeded AFRPs allowed for tissue remodeling to occur as seen through increases in biaxial mechanical properties and the deposition of the new collagen matrix. This is significant given the proposed roles of this biological scaffold is to first provide mechanical closure of an AF focal defect, and second, allow for the regeneration of the damaged native AF tissue. To be able to provide adequate ECM production, which is needed to observe changes in mechanical properties, AFRPs must allow for biocompatibility, support cell growth, and provide uniformly distributed and interconnected pore structures to provide transport and cell migration of seeded cells.\textsuperscript{322} Additionally, a successful biomaterial
scaffold provides physical support for cell attachment and promotes cell proliferation to promote the deposition of desired ECM components. Tissue remodeling and regeneration are greatly influenced by the cytotoxicity of the biomaterial, and the ability of cells to infiltrate into the tissue. Previous studies in our lab have demonstrated the EDC crosslinked AFRPs cytocompatibility up to 15 days, however, this was the first attempt to determine the long-term viability of cells seeded on the AFRP. Herein, both AD-MSC and primary AFC cell types remained viable on the AFRP biomaterial throughout 8 weeks of culture. Furthermore, AFCs demonstrate the ability to infiltrate further within the AFRP layers compared to AD-MSCs. This infiltration allowed for the collagen turnover to occur within the AFRP layer, which conversely was taken place along the periphery of the AD-MSC seeded AFRPs. This cellular infiltration and collagen deposition/turnover potentially explain the differences that were observed within the changes of mechanical properties between the two cell types. Previous studies which focused on the infiltration depth of cells into AFRP layers were conducted using primary AFCs. These studies found that EDC crosslinking and sonication allowed for ~50% infiltration following 14 days in culture. The limited infiltration depths of AD-MSCs within this study is thought to be contributed to the difference in size between the AD-MSCs and AFCs. The pore size between EDC crosslinks could prevent the infiltration of the larger AD-MSCs and should be investigated in future studies.
CONCLUSIONS

In summary, AFRP scaffolds demonstrated similar biaxial mechanical properties to native AF tissue and its mechanical competency to withstand native mechanical loading modalities. Additionally, AFRPs showed their ability to promote differentiation of human AD-MSCs towards an AF cell phenotype in addition to allowing for long-term cell-mediated remodeling by AD-MSCs and primary AFCs. Findings within this study illustrate the potential of the AFRP to withstand the mechanically demanding environment of the IVD while illustrating its biologic functionality to support IVD cell activity. Therefore, future utilization of this AF focal defect repair biomaterial may provide a tissue regeneration approach to improve clinical outcomes for patients impacted from IVD degeneration and/or herniation.

LIMITATIONS

As with any study, the authors acknowledge some limitations within this study. First, gene expression results at days 8 and 15 were compared to hADSC seeded scaffolds at day 3, and a more direct control would be the examination of human AFC RNA. However, the RNA isolated from primary AFCs are mature phenotype cells and the gene transcript levels produced would better represent the gene expression of cells following long-term culturing of seeded AFRPs. Herein, the early changes in gene transcription levels were evaluated to determine the relative production of putative AF gene markers for the potential of AFRPs to promote an AFC phenotype with increasing time in culture. The second limitation of this study would be the use of 20% strain during biaxial mechanical
testing. Physiologically, the native human AF typically undergoes 4-6% to a 10% maximum strain, and only on rare occasions does the AF experience 20% strain. However, the purpose of the current study was to determine the material properties of both the native AF and AFRP scaffolds in worst-case scenarios. Additionally, preliminary uniaxial testing demonstrated that 20% strain in the axial and circumferential directions of both tissue samples demonstrated minimal to none detrimental impacts on tissue recovery.
CHAPTER IX

‘All of the research contained within this chapter is currently in revision and was submitted under peer-review as Ryan Borem, Joshua Walters, Allison Madeline, Lee Madeline, Jeremiah Easley, Sanjitpal Gill, Jeremy Mercuri, A Pilot Investigation of Chemonucleolysis-Induced Intervertebral Disc Degeneration in The Ovine Lumbar Spine, PLOS One, August 2018, Preprint: https://doi.org/10.1101/384743’

A PILOT INVESTIGATION OF CHEMONUCLEOLYSIS-INDUCED INTERVERTEBRAL DISC DEGENERATION IN THE OVINE LUMBAR SPINE

ABSTRACT

Intervertebral disc (IVD) degeneration (IVDD) initiates in the nucleus pulposus (NP) and is marked by elevated levels of pro-inflammatory cytokines and matrix-degrading proteases, leading to structural and functional disruption. IVDD therapeutics are currently being investigated; however, such approaches require validation using large animal models that recapitulate clinical, biochemical, and biomechanical hallmarks of the human pathology. Others have previously utilized intradiscal administration of chondroitinase-ABC (C-ABC) to initiate IVDD in the NP of sheep lumbar IVDs. While these studies examined changes in IVD height, hydration, and tissue micro-architecture, changes in biochemical content and mechanical properties were not assessed. Thus, the objective herein was to comprehensively characterize this ovine model IVDD for salient features reported in human degenerate IVDs by evaluating biochemical, biomechanical, and
histological changes. Briefly, C-ABC (1U) was administered via intradiscal injection into the L1/2, L2/3, and L3/4 IVDs, and degeneration was assessed at 6- and 10-weeks via longitudinal magnetic resonance (MR) imaging. After 6 weeks, degenerative samples showed significant reductions in IVD heights (p=0.048) and MR imaging index (p=0.048), which worsened at 10 weeks. Post-mortem degenerate and controls IVDs were evaluated for differences in interleukin-1β concentration, axial and torsional functional spinal unit kinematics, and histological microarchitecture. Degenerate IVDs demonstrated significantly elevated concentrations of interleukin-1β (p=0.002). Additionally, degenerative samples showed increased creep displacement (p=0.022) and compressive stiffness’s (p=0.007) concurrent with decreased long-term elastic (p=0.007) and viscous dampening coefficients (p=0.002). Histological analysis of degenerative IVDs showed changes in microarchitecture, including derangement of the nucleus pulposus and annulus fibrosus tissue as well as cartilaginous end-plate irregularities. This pilot study demonstrated that intradiscal injection of 1U C-ABC induces significant and progressive degeneration of sheep lumbar IVDs over the time course investigated. The changes observed in this pilot’s study small sample size resemble the hallmarks of moderate to severe IVD degeneration observed in humans. Further study is warranted on a larger sample size to further validate these findings.

INTRODUCTION

The intervertebral disc (IVD) is a fibro-cartilaginous structure adjoining vertebral bodies of the spine. The primary role of the IVD is to support and transmit axial loads while
allowing for spinal mobility and stability during activities of daily living. Each IVD comprises three morphologically distinct regions. The centralized core of the IVD is known as the nucleus pulposus (NP); a hydrophilic, matrix composed of aggrecan and collagen type II. The NP is circumferentially sequestered by the annulus fibrosus (AF); a ring-like structure consisting of 15-25 concentric layers of type I collagen whose fiber preferred direction is oriented at ± 28-43° to the transverse axis of the spine in alternating layers.\textsuperscript{187} This results in a fiber-reinforced composite structure with an ‘angle-ply’ microarchitecture. The third region of the IVD is known as the cartilaginous end-plate (CEP) which is a thin layer of hyaline cartilage that is located between the inferior and superior surfaces of the IVD and adjacent vertebral bodies. The CEP serves as a mechanical barrier and allows for nutrient transport between the IVD and adjacent vertebral bodies.\textsuperscript{345}

Approximately 1.5 to 4 million adults in the U.S. have IVD-related low back pain (LBP), leading to lost wages and reduced productivity exceeding $100 billion in the U.S. and $12 billion in the U.K. annually.\textsuperscript{64,346} The most common diagnosis for patients experiencing LBP is intervertebral disc degeneration (IVDD),\textsuperscript{347} which presents with several clinical characteristics. X-ray and magnetic resonance (MR) imaging often demonstrate significant reductions in overall IVD height, NP desiccation, intra-vertebral herniations (i.e. Schmorl nodes), changes in the vertebral body end-plates (i.e. Modic changes), and osteophyte formation.\textsuperscript{348} These changes are associated with IVDD, an aberrant, cell-mediated process originating in the NP due in part to genetic predispositions, mechanical overload, and limited nutrient supply.\textsuperscript{349} Once initiated, IVDD is marked by elevated concentrations of pro-inflammatory mediators that promote the production of
matrix-degrading enzymes by resident cells. In turn, matrix turnover will favor catabolism over anabolism, eventuating the desiccation and breakdown of the NP tissue; specifically, its proteoglycan constituents. Degraded NP matrix promotes inflammation, as evidenced by increased concentrations of pro-inflammatory cytokines including interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). This positive-feedback cycle coupling inflammation and matrix degradation may amplify small perturbations in IVD physiology, ultimately leading to altered IVD microarchitecture and reduced matrix mechanical properties. This begets altered spinal kinematics, and non-physiologic loading of the AF which can result in lamellar disorganization and damage leading to herniation.

The symptoms of IVDD are currently addressed by surgical discectomy to remove IVD tissue, joint fusion, or total disc replacement. These strategies suffer from significant limitations and may lead to degeneration of adjacent levels. To address these issues and restore IVD microarchitecture and function, regenerative medicine approaches are being investigated, including biologic administration and tissue engineering using novel biomaterial scaffolds and/or mesenchymal stem cells. Using such approaches, investigators have demonstrated the ability to promote IVD tissue regeneration in vitro, as well as the attenuation of degeneration in small animal models. However, clinical translation of these strategies requires evaluating their efficacy in large animal models that recapitulate the salient biochemical, mechanical, and clinical features of mild to moderate IVDD prior to human clinical trials.
Selecting the appropriate animal model to investigate IVDD and evaluate therapeutics has been controversial. Reitmaier et al reviewed large animal models used in IVD research and identified several shortcomings within the field, including minimal (e.g. based on available in vitro data) or no justification of animal model selection. Additionally, the authors suggested that logistical factors, such as behavioral differences, animal cost, availability, or researcher preference, may influence model selection in the absence of empirical differences. The authors concluded that the current limitations of data concerning large animal models minimized the impact of currently published literature, warranting further comprehensive characterization of in vivo models to determine their suitability for IVD research. Of those reviewed, caprine (goat) and ovine (sheep) models were found to be the most common quadrupeds used to study IVD pathology and repair.

In goats, Hoogendoorn and colleagues extensively characterized the ability of intradiscal injection of chondroitinase-ABC (C-ABC) to initiate and consistently produce progressive, degenerative changes in lumbar IVDs as evidenced by clinical imaging, biochemical, and histological analyses. These results were re-affirmed in a similar study by Gullbrand et al who administered 1U C-ABC which resulted in moderate IVDD in goats by 12 weeks. In contrast, equally comprehensive studies have not been performed in sheep, despite significant similarities to human lumbar IVDs with respect to geometry, range of motion, matrix composition, age-related changes, notochordal cell absence, and intradiscal pressures. To date, only two studies have utilized intradiscal C-ABC injection to induce IVDD in this sheep. Saski et. al
performed intradiscal injections of 1, 5, or 50 U C-ABC per IVD, which resulted in significant reductions in intravital lumbar intradiscal pressures and heights over 4 weeks across all concentrations.\(^3\)\(^7\) Ghosh et. al utilized 1U C-ABC to induce degeneration over a period of 12-weeks prior to administering progenitor cells to evaluate their efficacy to regenerate the IVD.\(^2\)\(^8\)\(^6\) However, the study was not specifically designed to characterize the degenerative model and only included imaging and histological outcome analyses.

Thus herein, a pilot study was completed using the ovine model to induce IVDD and to compare the resulting degeneration to that observed in human pathology. The pilot study was undertaken to further characterize changes observed in MR imaging, biochemical cytokines, mechanical properties, and histology in sheep lumbar IVDs following intradiscal injection of 1U C-ABC.

**MATERIALS & METHODS**

**Institutional Review Board (IRB) Approval**

The study was approved by the Institutional Animal Care and Use Committee of Colorado State University (IACUC Protocol: 16-6891A). IVDs from five lumbar levels of three skeletally mature female sheep (*Ovis Aries*, Rambouillet ewes; 65-71 kg, 3 years-of-age, K&S Livestock, Fort Collins, CO) were utilized in this study. Animals were group housed in an indoor/outdoor pen with access to a three-sided shelter and evaluated daily by a veterinarian for signs of pain, behavior changes, or gait abnormalities for the duration of the study.
Surgical Procedure and Intradiscal Administration of C-ABC

Peri-operatively, a transdermal fentanyl patch (150 mcg/hr/sheep) was applied to each animal for five days starting one day prior to surgery. Twenty-four hours prior to surgery, five doses of procaine penicillin G (3 million units) and phenylbutazone (1 gram) was administered to each sheep. The animals were induced with ketamine (2mg/kg IV) and midazolam (0.2mg/kg IV) then intubated and maintained on 1.5-3% isoflurane in 100% oxygen throughout the surgical procedure. Using standard surgical preparation and aseptic technique, the lumbar IVDs were located via fluoroscopy. IVDD was induced via percutaneous intradiscal injection of 1U of C-ABC (Amsbio, Cambridge, MA) in 200 µL of the vehicle solution (sterile 0.1% bovine serum albumin in 1x PBS - Fisher Scientific, Hampton, NH) into the L1/2, L2/3, L3/4 IVDs. To confirm accurate needle position prior to injection, needle visualization was achieved using lateral and anterior-posterior fluoroscopy. The L4/5 and L5/6 IVDs served as both vehicle and uninjured controls, respectively. Post-operatively, sheep were monitored until ambulatory, and then returned to standard housing conditions. Following the progression of IVDD, driven by C-ABC injections, animals were euthanized by intravenous barbiturate overdose (pentobarbitone sodium, 88mg/kg) in compliance with the 2013 American Veterinary Medical Association guidelines. Immediately following euthanasia, AF and NP tissues from selected IVDs were immediately excised and frozen at -80°C for biochemical analysis. Lumbar spines were then harvested en bloc and shipped overnight on wet ice to Clemson University for further analysis.
Figure 51: Animal study design overview and surgical approach. A) Animal study progressed over 10 weeks beginning with MR imaging and IVD degeneration (Degen) induction at week 0. At 6- and 10-weeks, IVDs were evaluated via MR imaging, kinematic testing, histological, and biochemical analysis. B) Table illustrating allocation of the treatment groups distributed across all sheep (n=3) for this pilot study. C) Table illustrating the sample distribution for outcome evaluation from each animal based on lumbar disc level. D) Representative lateral fluoroscopy image used.
to guide injections of 1U C-ABC in via 29-gauge needle. E) Representative T1-weighted MR image of an ovine vertebral body and adjacent IVD demonstrating IVD height (white solid arrows) and vertebral body height (red dotted arrows) measurements used for calculating DHI. F) Representative image of potted FSU undergoing kinematic creep loading in a saline bath. G) Representative histological image of an uninjured IVD stained with FAST: green/blue = NP, red = AF, and yellow = vertebral bone.

**Magnetic Resonance Imaging of IVDS**

Sagittal MR imaging was performed immediately prior to intradiscal injection of C-ABC (week 0 – baseline) and tracked longitudinally at 6- and 10-weeks post-injection to monitor IVDD of degenerate, vehicle, and uninjured IVDs. MR image scans were obtained using a 1.5 Tesla clinical imager (GE Signa). T2-weighted, T1-weighted, and Short-T1 Inversion Recovery (STIR) MR imaging sequences were performed on the explanted lumbar spines. Sagittal images were constructed using a T2-weighted fast spin echo sequence using a spine array coil (time to repetition: 2782 ms; time to echo: 101 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), a T1-weighted fast spin echo sequence using a spine array coil (time to repetition: 616 ms; time to echo: 18 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), and a STIR sequence using a spine array coil (time to repetition: 3500 ms; time to echo: 40 ms; voxel size: 1.56 mm x 1.56 mm x 3.0 mm, with a 0 gap and inversion time of 150).

Semi-quantitative MR image analysis was performed as described by Hoogendoorn et al.\textsuperscript{362} MR imaging index was calculated from T2-weighted images as the product of the
cross-sectional area and mean signal intensity of the encircled NP using IMPAX 6.6.1.4024 (AGFA HealthCare N.V., Mortsel, Belgium) software. Consistent mid-sagittal IVD imaging was confirmed by ensuring the full cross-sectional of the spinal cord was in view. MR imaging index is expressed as a percentage of week 0 (pre-C-ABC injection) values for normalization. It should be noted that normalization of signal intensity was not performed by comparing to the spinal cord, as has been described by others, because it is a mobile structure with too much Gibbs and pulsation artifact. Thus, normalization to normal (week 0) IVDs was deemed most appropriate. Two researchers (R.B. and J.W.) quantified the MR imaging index for each IVD. A board-certified neuroradiologist (L.M.) verified the quantitation and performed a qualitative analysis of MR images using the classification scale described by Pfirrmann et al.371

IVD height index (DHI) was measured from mid-sagittal, T1-weighted MR images in accordance with Hoogendoorn et al. with minor modifications.362 IVD and vertebral body heights were each quantified using three height measurements at ventral-dorsal quartiles using ImageJ (NIH, Bethesda, MD) (Fig 1C). DHI was calculated as each IVD’s mean disc height divided by the mean adjacent vertebrae height, with changes expressed as a percent of week 0 DHI. Measurements were performed by two researchers (R.B. and J.W.) to evaluate the interobserver reliability and blindly confirmed by a board-certified neuroradiologist (L.M.).
**Enzyme-Linked Immunosorbent Assay for Interleukin-1β**

Ten weeks post-injection (pilot), AF and NP tissues from degenerate and uninjured IVDs (n=3/group) were assessed for the pro-inflammatory cytokine IL-1β. Tissues were cryo-pulverized and homogenized on ice in lysis buffer (500uL/10mg of tissue; 100mM TRIS buffer (pH=7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5% Deoxycholic Acid, and 1x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). Homogenates were then agitated for 60 min at 4°C, and the supernatant was isolated via centrifugation (5 minutes at 10,000xg). Supernatants underwent Enzyme-linked immunosorbent assay (ELISA) for ovine IL-1β (AB Clonal Science, Inc., Woburn, MA) according to the manufacturer’s instructions, the results of which were normalized to the total protein quantified via Bicinchoninic Acid (BCA) assay (ThermoFisher Scientific, Waltham, MA).

**Functional Spinal Unit Axial and Torsional Kinematics**

Ten weeks post-injection, functional spinal units (FSU’s: vertebrae-IVD-vertebrae) of degenerate, vehicle, and uninjured (n=3/group) IVDs underwent biomechanical kinematic testing according to methods previously described by our group, with modification.321 Briefly, FSU’s (with intact posterior elements) were potted in urethane resin (Goldenwest Manufacturing, Grass Valley, CA) (Fig 1D) first underwent creep loading on a Bose ElectroForce (model: 3220, TA Instruments, New Castle, DE) equipped with a 100-lb. load cell and a test chamber filled with 1xPBS/protease inhibitor at 25°C. Samples were loaded to -0.125 MPa and then underwent a 1-hr. creep period at -0.50 MPa. Samples were then transferred to a servohydraulic test frame (model: 8874, Instron,
Norwood, MA) fitted with a 20kN load cell, and subjected to 35 cycles of axial compression (-0.50 MPa) and tension (0.25 MPa) at 0.1 Hz. Compression was then maintained at -0.50 MPa as samples underwent 35 torsion cycles to ±3°. Finally, samples underwent a slow-rate compressive ramp (1 N/s) from -0.125 MPa to -0.50 MPa. A non-linear constitutive model was fit to the creep data using GraphPad Prism 7 software (La Jolla, CA) to yield elastic (Ψ) and viscous (η) damping coefficients for the short-term (η₁ and Ψ₁) and long-term (η₂ and Ψ₂), as described previously. Tensile and compressive stiffness was determined using a linear fit of the loading force-displacement curve from 60-100% of the 35th cycle. Torsional stiffness was calculated from a linear fit of the loading torque-rotation curve of the 35th cycle. Torque range and axial range of motion (RoM) was calculated as the peak-to-peak torque and displacement, respectively. The constant-rate slow-ramp compression stiffness was determined using a linear fit of the slow-ramp load-displacement response.
Figure 52: Representative images of A) macroscopic view of lumbar spinal section *en bloc* with excessive tissue excised, B) the isolation of IVDs into functional spinal units (FSUs) with the adjacent vertebral bodies attached. C&D) illustrate schematics of the Bose ElectroForce 3200 and Instron 8874 testing frames used for kinematic evaluation.
Macroscopic Evaluations and Histology of IVDs

Following mechanical testing, samples were excised from their pots and fixed for 7 days in 10% neutral-buffered formalin, followed by decalcification in 12% formic acid. Samples were cut into 3-mm sagittal slices and images were captured for macroscopic evaluation by 3 blinded observers (R.B., J.W., and J.M.) using the Thompson grading scale (n=3/degen and uninjured IVDs; n=2/vehicle IVDs). These mid-sagittal slices were paraffin-embedded and 7µm sections were obtained; however, two of the three vehicle controls were lost during tissue processing. Staining was performed in accordance with methods form Leung et al. Briefly, sections were rehydrated via ethanol gradations and stained with 3% Alcian blue (pH 1.0) for 8 min, 0.1% safranin-O for 6 min, 0.25% tartrazine for 10 sec, 0.001% fast green for 4 min. Micrographs were acquired using an Axio Vert.A1 microscope with AxioVision SE64 Rel. 4.9.1 software (Zeiss, San Diego, CA), and composite images were stitched together using FIJI analysis software. Composite IVD micrographs (n = 3/group for degenerate and uninjured; n = 1 for vehicle) were scored by two blinded observers (R.B., J.M.) and an unblinded observer (J.W.) using the IVD degeneration scale described by Walter et al. Endplate Integrity, AF Morphology, AF/NP Demarcation, NP Matrix Homogeneity, and NP Matrix Stain Intensity were each assigned scores from 0 to 2, and these were summed to produce a semi-quantitative aggregate score.
Statistical Analysis

Results are represented as mean ± standard error of the mean (SEM) and significance was defined as (p≤0.05). Statistical analysis was performed using Prism 7 software. Comparisons were performed using a one-way ANOVA with Holm-Sidak method for multiple comparisons (MR imaging Index, DHI, ELISA) or Dunnett’s post-hoc (FSU kinematics and Thompson grading) analysis. Pfirrmann scoring was evaluated via a Kruskal-Wallis test with Dunn’s multiple comparisons. Assessment of the inter-observer reliability of macroscopic Thompson grading was evaluated via Fleiss’ kappa statistic. Inter-observer reliability of histological scores was evaluated using IBM Statistical Package for Social Sciences (SPSS 24.0; IBM, Armonk, NY). The Intra-class Correlation Coefficient (ICC) was calculated using a two-way random model for absolute agreement as described by McGraw and Wong. An ICC of 0.4-0.75 indicates good agreement, while >0.75 is considered excellent. Mean histological scores were compared between degenerate and uninjured groups via one-tailed Mann Whitney test.

RESULTS

All animals tolerated the intradiscal C-ABC injection procedure, recovered from anesthesia, and began weight bearing and eating within one hour postoperatively. At 6 weeks, one animal had difficulty rising from a recumbent position with severe hind end weakness and moderate pain response upon palpation of the lumbar region. MR imaging of the animal’s lumbar spine revealed signs of severe IVDD and a large IVD protrusion/herniation into the spinal canal causing cord compression at the L1/2 level. The
animal was euthanized, cultures and histopathology were obtained from the herniated IVD and the spine was harvested en bloc. No infection was found in the L1/2 IVD. Histopathology revealed reactive fibroplasia with mixed inflammation of lymphocytes, plasma cells, and macrophages. The remaining two degenerate IVDs (L2/3 and L3/4) were excluded from further analysis. The vehicle and uninjured IVDs (L4/5 and L5/6, respectively) were stored at -80°C, and later used for biochemical, biomechanical, and histological analysis. The remaining two animals recovered as expected without any signs of complication throughout the 10-week post-injection period.

**Intradiscal C-ABC Injection Results in Significantly Reduced MR Imaging Index and Increased Pfirrmann Grades of Degeneration in IVDs**

T2-weighted MR images illustrated a progressive darkening of the NP-region and adjacent vertebral endplates in degenerate IVDs (Fig 2A). Conversely, uninjured IVDs and those injected with vehicle demonstrated no such changes (Fig 2A). Of note, darkening in adjacent vertebral bodies adjacent to degenerate sheep IVDs were also observed on MR images indicative of endplate abnormalities (Fig 2A). Normalized MR imaging index of degenerate IVDs was significantly lower at week 6 (65.63±4.01%; p=0.024) and week 10 (57.10%; p=0.023) values. Moreover, these values were significantly lower compared to respective uninjured controls at week 6 (p=0.049) and continued to progress at week 10 (Fig 2B). No significant changes in MR imaging index were observed between uninjured and vehicle control IVDs. Degenerate IVDs also demonstrated a significant increase in Pfirrmann grade, indicating worsening degeneration at week 6 (2.11±0.261; p=0.014) and
week 10 (2.833±0.401; p<0.001) compared to respective week 0 values (Fig 2C). Uninjured and vehicle control IVDs both had average Pfirrmann scores of 1±0 at all time points investigated.
Figure 53: Longitudinal image tracking of sheep lumbar IVDs. A) Representative T2-weighted MR longitudinal images of a single sheep over the duration of the 10-week study. Graphs depicting quantitative MR image analysis for B) normalized MR image index, C) Pfirrmann grading, and D) normalized disc height index. (*) indicates a
significant difference (p<0.05) between groups within the same time-point. (#) indicates a significant difference (p<0.05) within the study group compared across time-points. Solid lines connecting groups indicate a statistical difference (p<0.05).

Intradiscal C-ABC Injection Results in Significant Reductions in IVD Height

Normalized DHI values of degenerate IVDs significantly decreased over time, reaching 84.91±2.98% (p=0.007) at week 6 and 79.23±0.74% (p<0.001) at week 10 (Fig 2D). These values were also significantly lower compared to uninjured IVD values at week 6 (94.99±2.00%; p=0.048) and week 10 (99.78±0.61%; p= 0.002), respectively. No significant changes in DHI were observed between uninjured and vehicle control IVDs.

Intradiscal C-ABC Injection Results in Significantly Increased Concentrations of IL-1β

ELISA analysis of degenerate IVDs demonstrated a significant (p=0.002) increase in pro-inflammatory IL-1β concentrations compared to uninjured IVDs (483.2±79.0 [pg/mL]/mg vs. 39.7±34.1 [pg/mL]/mg, respectively) (Fig 3). IL-1β was found to be increased in both the NP- and AF-regions of degenerate IVDs compared to uninjured IVDs; however, this was significant (p=0.019) only in the NP (Fig 3).
Figure 54: IL-1β quantification of sheep IVDs. Graphical and tabular results of normalized IL-1β concentrations from the NP and AF regions of degenerate and uninjured sheep IVDs. Solid lines connecting groups indicate a statistical difference (p<0.05). Dotted lines indicate a trend towards significance (p<0.085).

* indicates significant difference (p<0.05) compared to respective uninjured tissue
^ indicates significant trend (p<0.085) compared to respective uninjured tissue

<table>
<thead>
<tr>
<th>IVD Treatment</th>
<th>Normalized IL-1β Content [(pg/mL) / mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Annulus (AF)</td>
</tr>
<tr>
<td>Uninjured</td>
<td>75.5 ± 66.6</td>
</tr>
<tr>
<td>Degen</td>
<td>546.4 ± 149.4^</td>
</tr>
</tbody>
</table>
Intradiscal C-ABC Injection Results in Significant Alterations in Functional Spinal Unit Kinematics

Kinematic loading (Fig 4A) of FSUs containing degenerate IVDs demonstrated a reduced step displacement (0.11±0.02 mm) and significantly (p=0.022) increased creep displacement (0.50±0.05 mm) compared to FSUs with uninjured IVDs (0.16±0.016 and 0.30±0.02 mm, respectively) (Fig 4C). Additionally, long-term elastic (Ψ₂: 482.17 ± 69.89 N/mm) and viscous (η₂: 92.1 ± 2.78 x10⁴ Ns/mm) damping coefficients of FSUs with degenerate IVDs were significantly (p=0.0074 and p=0.0016, respectively) lower compared to FSUs with uninjured IVDs (Ψ₂: 862.26 ± 29.28 N/mm and η₂: 229.52 ± 17.86 x10⁴ Ns/mm, respectively) (Figs 4D and 4E).
Figure 55: Kinematic testing of IVD FSUs. A) Loading scheme for FSU testing depicting creep, axial cyclic tension-compression, axial torsion, and slow constant-rate ramp testing. B) Representative graph depicting the creep response of a sheep IVD and associated creep parameter measures. Graphs depicting C) step and creep...
displacement, D) elastic damping and E) viscous damping coefficients, F) slow ramp compressive stiffness, G) compressive stiffness, H) tensile stiffness, I) axial RoM, J) torque range and K) torsional stiffness values of uninjured, vehicle and degenerate (degen) sheep IVDs, respectively. Solid lines connecting groups indicates significant difference (p≤0.05) compared to uninjured controls.

Slow-ramp compressive loading demonstrated a significant increase (p=0.007) in compressive stiffness of FSUs containing degenerate IVDs compared to those with uninjured IVDs (1272.00±31.76 MPa and 959.4±52.87 MPa, respectively) (Fig 4F). A similar trend was observed for cyclic compressive stiffness of FSUs containing degenerate IVDs (Fig 4G). No significant changes were observed in the axial tensile stiffness or range of motion (Figs 4H and 4I). Torsional rotation of FSUs containing degenerate IVDs demonstrated a decrease in torque range (17.74±0.81 Nm vs. 18.83±0.82 Nm, respectively) and torsional stiffness (2.61±0.17 Nm/° vs. 2.75±0.08 Nm/°, respectively) compared to uninjured IVDs (Figs 4J and 4K). No significant changes were observed within the kinematic parameters between FSUs containing uninjured and vehicle control IVDs.

**Intradiscal C-ABC Injection Induced Significant Changes in IVD Morphology and Microarchitecture**

Macroscopic evaluation of IVDs using the Thompson scale (Fig 5) showed substantial agreement for inter-observer variability (κ grade: 0.75) and showed that the average Thompson grades for degenerate IVDs (3.89±0.20), were significantly greater
compared to uninjured (1.00±0.00; p<0.001) and vehicle controls (1.00±0.00; p<0.001), respectively.

**Figure 56: Mid-sagittal gross sections of ovine IVDs.** Representative IVDs utilized for Thompson grading depicting the macroscopic changes between A-C) 1U C-ABC injected IVDs (degen), D-F) uninjured IVDs, and G-H) vehicle IVDs.

Semi-quantitative histological scoring showed excellent agreement among observers (ICC: 0.975) and significant differences for uninjured and degenerate scores.
(Fig 6) Qualitatively, degenerate IVDs revealed significant alterations and disruption of tissue architecture compared to control IVDs (Figs 6A-F). Prominent intravertebral herniations were observed in all evaluated degenerate IVDs (Figs 6D-F), and these consistently exhibited several abnormalities compared to control IVDs: 1) failure and displacement of the CEP into the subchondral bone, 2) extrusion of marked amounts of NP into the vertebrae, 3) apparent remodeling around the herniated tissue, and 4) thickening of the vertebral bone. The AF of degenerate IVDs also showed a notable change in concavity, in contrast to the control IVDs that showed normal, convex AF orientation (Fig 6). Furthermore, gross morphological changes in degenerate IVDs seemed to have pronounced effects on AF structure. Specifically, loss of IVD height seemed to compress AF lamellae while CEP displacement resulted in reduced convexity. Higher magnifications revealed changes in distinct regions of the IVD (Fig 7). The ability of the CEP and NP to retain stain was severely diminished by C-ABC injection. Additionally, thinning and permeabilization of the CEP was seen through microscopic NP expulsion and reduction in CEP thickness. The vehicle control showed no qualitative histological differences compared to the uninjured samples and received a comparable aggregate score (0.6±0.4 for uninjured vs 0 for the vehicle).
Figure 57: Semi-quantitative scoring of ovine lumbar IVDs. Macroscopic images of (A-C) uninjured and (D-F) degenerative IVDs were used for scoring. G) Aggregate scores for uninjured and degenerative IVDs were calculated as the average of each observer and sample score within the respective treatment. Significance is indicated with ‘*’ (p≤0.05). Scale bar = 5 mm. FAST Staining: Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.
Figure 58: Qualitative histological comparison of anatomical regions of the IVD across study groups. Left column, representative histological images of AF lamellae from degenerate, uninjured and vehicle control IVDs depicting differences in lamellar thickness (red = AF lamellae). Center column, representative histological images of the NP region of degenerate, uninjured and vehicle control IVDs illustrating the difference in glycosaminoglycan staining intensity (blue/green = glycosaminoglycan). Right column, representative histological images of the cartilaginous end-plates of degenerate, uninjured and vehicle control IVDs illustrating differences in integrity and thickness. Scale bar = 400 µm. FAST Staining:
Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.

**DISCUSSION**

The present pilot study aimed to build upon the initial work of Saki et. al and Ghosh et. al who demonstrated that intradiscal administration of C-ABC resulted in mild to moderate IVDD as evidenced by reductions in IVD intradiscal pressure, height, hydration, and changes in microarchitecture. Two-hundred microliter intradiscal injections of 1U C-ABC were performed in the lumbar spine of sheep and degenerative changes were tracked for up to 10 weeks in alignment with previous studies by others. The primary findings of these studies herein suggest that intradiscal injection of 1U C-ABC in sheep resulted in moderate to severe IVD degeneration marked by progressive and significant alterations in IVD hydration, height, inflammation, kinematics, and IVD tissue morphology (including the formation of end-plate disruptions). The observed changes appeared to be potentially more severe as compared to previous reports, however, these changes were still reminiscent of that observed in human lumbar IVDD.

In the present pilot study, clinical imaging of sheep C-ABC injected IVDs demonstrated significant reductions in hydration and height, which worsened over time. This was evidenced by progressive decreases in both T2-weighted MR image signal intensity and IVD height measurements. No evidence of spontaneous regeneration was observed during the study time-course. Such observations are likely due to a loss of aggregating proteoglycan, leading reduced NP osmotic potential, lowered water content,
and the apparent increase in matrix permeability. Together, these alterations diminish the ability of the tissue to effectively support compressive loading leading to a reduction in IVD height and overall mechanical dysfunction. Similarly, imaging of degenerate human IVDs often display progressive darkening of the IVDs and loss of height with increasing grade of degeneration.

Trends in the Pfirrmann grading scores for degenerate IVDs reflected macroscopic deterioration, with some IVDs receiving scores of 3-4. This is indicative of demarcation between the NP and AF regions of the IVD in conjunction with reductions in MR image signal intensity and IVD height. From a clinical perspective, IVDs having a Pfirrmann grade 3 or lower remain functional and would be targeted for regenerative medicine approaches employing biomaterials, biologics, and cells. Notably, MR imaging corroborated the presence of subchondral end-plate irregularities adjacent to degenerate IVDs as evidenced by the darkening and obscuring of these structures. Likewise, patients with lumbar IVDD often exhibit similar findings which are referred to as Modic changes. Such changes represent a spectrum of biological alterations occurring within vertebral bodies which can include fatty marrow changes, endplate sclerosis, trabecular fracture, as well as edema and inflammation.

Biochemical analysis of degenerate sheep IVDs demonstrated an increased concentration of IL-1β compared to control samples in the pilot study, indicating elevated inflammation. These findings are likely related to the observed endplate changes discussed above. Degenerate human lumbar IVDs have been shown to contain increased concentrations of pro-inflammatory cytokines, including IL-1β, which are produced by
endogenous IVD cells and infiltrating inflammatory cells.\textsuperscript{271} The increased presence of this cytokine has been shown to result in the up-regulation of matrix-degrading proteases by NP and AF cells, which further compromise the extracellular matrix (ECM) and mechanical function of the IVD.\textsuperscript{385–387}

Cyclic axial and torsional kinematics of degenerate sheep IVDs were significantly altered compared to uninjured and vehicle control samples. Most notably, the creep displacement was significantly affected in conjunction with significant reductions in the long-term elastic and viscous dampening time constants. Together, this indicates that the ECM of the IVD has been disrupted and is no longer able to as effectively resist compressive creep loading as compared to controls. This is likely an effect of degradation of the ECM in conjunction with an increase in its permeability leading to reduced pressurization. Interestingly, the compressive stiffness of the degenerate sheep IVDs was significantly greater than uninjured controls. This has been observed by others and could be due to a reactive process involving the posterior elements in response to inflammation and overloading, and thus are attempting to stabilize the degenerate FSU’s.\textsuperscript{388} This could also explain why overall FSU range of motion was ultimately unaltered in the degenerate pilot study group. Similar phenomena have been observed in human degenerate lumbar IVDs in which spinal instability (i.e. increase in the neutral zone and overall range of motion) occurs in early- and mild degeneration,\textsuperscript{389,390} however, restoration or re-stabilization of axial spinal kinematics has been shown to occur as degeneration progresses.\textsuperscript{388}
Semi-quantitative and qualitative histological evaluations of degenerate sheep IVDs demonstrated significant alterations in the NP, AF, and endplate micro-architecture as compared to controls. This was demonstrated by decreases in proteoglycan staining intensity, alterations in lamellar architecture, and the formation of Schmorl nodes, respectively. Taken together, these histomorphological features are suggestive of moderate to severe IVDD. We hypothesize that C-ABC degraded NP proteoglycan and the cartilaginous endplates. In fact, cartilaginous endplate thickness was found to be irregular in both macroscopic (i.e. Thompson scoring images) and microscopic analysis. Loss of cartilaginous endplate integrity due to C-ABC treatment could account for the observed formation of Schmorl nodes and the resultant increase in inflammation. The cartilaginous endplate functions to prevent the NP from breaching the vertebral body which prohibits its contact with bone marrow and the immune system. This is critical as NP tissue has been shown to be immunogenic when it herniates extradiscally. It is plausible that because sheep lumbar IVDs have been shown to have higher intradiscal pressures compared to human IVDs, in combination with enzymatic disruption of the cartilaginous endplate, most likely resulted in NP tissue breaching into the vertebral body. Such breaches were not observed in vehicle control IVDs which suggests that enzymatic damage to the endplate, as opposed to an increase in intradiscal pressure due to injection volume, caused the observed Schmorl node formation. Additionally, the formation of Schmorl nodes could be due to alterations in the vertebral body bone as a result of inflammatory edema due to a breach of the cartilaginous end-plate and exposure of the NP, however, this was not directly assessed in the present study. In the context of lumbar IVDD in humans, the
presence of Schmorl nodes has been shown to be correlated with Modic changes and grade of degeneration in human IVDs.\textsuperscript{393,394}

As with any study, limitations were noted. First, the pilot study used a small sample size (n=3 sheep) to evaluate the effects of intradiscal injection of C-ABC on IVDD. Despite this fact, resultant degeneration was severe enough to cause statistical differences in many of the outcomes measured. Moreover, the promising results of the pilot study provide the foundation for the investigation of this ovine model using a larger sample size and additional outcome measures: including, changes in IVD GAG and collagen content, in addition to, quantifying several pro-inflammatory mediators and proteases. Second, while we used an established T2-weighted MR imaging modality to evaluate ECM degradation as a function of IVD hydration, it would be advantageous to include a T1rho relaxation parameter, which may correlate more strongly with changes in IVD GAG content and histology.\textsuperscript{395} Third, IVDs receiving a C-ABC injection and those serving as controls were not randomized to account for differences in spine level. However, data from each IVD was normalized to its respective week 0 (baseline) value to account for this.

**CONCLUSIONS**

In conclusion, we have begun the in-depth characterization of a chemonucleolysis-induced model of IVDD in sheep. Intradiscal administration of 1U C-ABC per lumbar IVD resulted in many of the salient hallmarks observed in the human pathology. Although further study is warranted using a larger sample size, the characterization of this ovine
model may represent a valuable tool in the future for evaluating strategies to attenuate IVDD and its resultant sequelae.
CHAPTER X

THE CHARACTERIZATION OF A CHEMONUCLEOLYSIS-INDUCED
INTERVERTEBRAL DISC DEGENERATION MODEL IN VIVO: THE OVINE
LUMBAR SPINE

INTRODUCTION

Herein, a larger sample size (n=9 sheep) of the pilot study (Chapter IX) was used to provide a more detailed evaluation of the chemonucleolysis-induced degeneration using an ovine lumbar spine compared to degeneration observed in human pathology. Analyses were conducted using methods which were enhanced during the pilot study which allowed for a more rigorous characterization of the ovine model. This model study further characterizes changes observed in MR imaging, mechanical properties, and histology in sheep lumbar IVDs following intradiscal injection of 1U C-ABC.

MATERIALS & METHODS

Institutional Review Board (IRB) Approval

The study was approved by the Institutional Animal Care and Use Committee of Colorado State University (IACUC Protocol: 16-6891A). IVDs from five lumbar levels of nine skeletally mature female sheep (Ovis Aries, Rambouillet ewes; 65-71 kg, 3 years-of-age, K&S Livestock, Fort Collins, CO) were utilized in this study. Animals were group housed in an indoor/outdoor pen with access to a three-sided shelter and evaluated daily by
a veterinarian for signs of pain, behavior changes, or gait abnormalities for the duration of the study.

**Surgical Procedure and Intradiscal Administration of C-ABC**

Peri-operatively, a transdermal fentanyl patch (150 mcg/hr/sheep) was applied to each animal for five days starting one day prior to surgery. Twenty-four hours prior to surgery, five doses of procaine penicillin G (3 million units) and phenylbutazone (1 gram) was administered to each sheep. The animals were induced with ketamine (2mg/kg IV) and midazolam (0.2mg/kg IV) then intubated and maintained on 1.5-3% isoflurane in 100% oxygen throughout the surgical procedure. Using standard surgical preparation and aseptic technique, the lumbar IVDs were located via fluoroscopy. IVDD was induced via percutaneous intradiscal injection of 1U of C-ABC (Amsbio, Cambridge, MA) in 200 µL of the vehicle solution (sterile 0.1% bovine serum albumin in 1x PBS - Fisher Scientific, Hampton, NH) into the L1/2, L2/3, L3/4 IVDs. To confirm accurate needle position prior to injection, needle visualization was achieved using lateral and anterior-posterior fluoroscopy. The L4/5 and L5/6 IVDs served as both vehicle and uninjured controls, respectively. Post-operatively, sheep were monitored until ambulatory, and then returned to standard housing conditions. Following the progression of IVDD, driven by C-ABC injections, animals were euthanized by intravenous barbiturate overdose (pentobarbitone sodium, 88mg/kg) in compliance with the 2013 American Veterinary Medical Association guidelines. Immediately following euthanasia, AF and NP tissues from selected IVDs were immediately excised and frozen at -80°C for biochemical analysis. Lumbar spines were
then harvested *en bloc* and shipped overnight on wet ice to Clemson University for further analysis.

**Magnetic Resonance Imaging of IVDs**

Sagittal MR imaging was performed immediately prior to intradiscal injection of C-ABC (week 0 – baseline) and tracked longitudinally at 6- and 17-weeks post-injection to monitor IVDD of degenerate, vehicle, and uninjured IVDs. MR image scans were obtained using a 1.5 Tesla clinical imager (GE Signa). T2-weighted, T1-weighted, and Short-T1 Inversion Recovery (STIR) MR imaging sequences were performed on the explanted lumbar spines. Sagittal images were constructed using a T2-weighted fast spin echo sequence using a spine array coil (time to repetition: 2782 ms; time to echo: 101 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), a T1-weighted fast spin echo sequence using a spine array coil (time to repetition: 616 ms; time to echo: 18 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), and a STIR sequence using a spine array coil (time to repetition: 3500 ms; time to echo: 40 ms; voxel size: 1.56 mm x 1.56 mm x 3.0 mm, with a 0 gap and inversion time of 150).

Semi-quantitative MR image analysis was performed as described by Hoogendoorn et al. MR imaging index was calculated from T2-weighted images as the product of the cross-sectional area and mean signal intensity of the encircled NP using IMPAX 6.6.1.4024 (AGFA HealthCare N.V., Mortsel, Belgium) software. Consistent mid-sagittal IVD imaging was confirmed by ensuring the full cross-sectional of the spinal cord was in view. MR imaging index is expressed as a percentage of week 0 (pre-C-ABC injection) values.
for normalization. It should be noted that normalization of signal intensity was not performed by comparing to the spinal cord, as has been described by others, because it is a mobile structure with too much Gibbs and pulsation artifact. Thus, normalization to normal (week 0) IVDs was deemed most appropriate. Two researchers (R.B. and J.W.) quantified the MR imaging index for each IVD. A board-certified neuroradiologist (L.M.) verified the quantitation and performed a qualitative analysis of MR images using the classification scale described by Pfirrmann et al.\textsuperscript{371}

**Radiographs of IVDs**

IVD height index (DHI) was measured from mid-sagittal, Radiographs (X-Rays) in accordance with Hoogendoorn et al. with minor modifications.\textsuperscript{362} IVD and vertebral body heights were each quantified using three height measurements at ventral-dorsal quartiles using ImageJ (NIH, Bethesda, MD) (\textbf{Fig 1C}). DHI was calculated as each IVD’s mean disc height divided by the mean adjacent vertebrae height, with changes expressed as a percent of week 0 DHI. Measurements were performed by two researchers (R.B. and J.W.) to evaluate the interobserver reliability and blindly confirmed by a board-certified neuroradiologist (L.M.).

**Functional Spinal Unit Axial and Torsional Kinematics**

Seventeen weeks post-injection, functional spinal units (FSU’s: vertebrae-IVD-vertebrae) of degenerate, vehicle, and uninjured (n=6/group) IVDs underwent biomechanical kinematic testing according to methods previously described by our group,
with modification. Briefly, FSU’s (with intact posterior elements) were potted in urethane resin (Goldenwest Manufacturing, Grass Valley, CA) first underwent creep loading on a Bose ElectroForce (model: 3220, TA Instruments, New Castle, DE) equipped with a 100-lb. load cell and a test chamber filled with 1xPBS/protease inhibitor at 25°C. Samples were loaded to -0.125 MPa and then underwent a 1-hr. creep period at -0.50 MPa. Samples were then transferred to a servohydraulic test frame (model: 8874, Instron, Norwood, MA) fitted with a 20kN load cell, and subjected to 35 cycles of axial compression (-0.50 MPa) and tension (0.25 MPa) at 0.1 Hz. Compression was then maintained at -0.50 MPa as samples underwent 35 torsion cycles to ±3°. Finally, samples underwent a slow-rate compressive ramp (1 N/s) from -0.125 MPa to -0.50 MPa. A non-linear constitutive model was fit to the creep data using GraphPad Prism 7 software (La Jolla, CA) to yield elastic (Ψ) and viscous (η) damping coefficients for the short-term (η₁ and Ψ₁) and long-term (η₂ and Ψ₂), as described previously. Tensile and compressive stiffness was determined using a linear fit of the loading force-displacement curve from 60-100% of the 35th cycle. Torsional stiffness was calculated from a linear fit of the loading torque-rotation curve of the 35th cycle. Torque range and axial range of motion (RoM) was calculated as the peak-to-peak torque and displacement, respectively. The constant-rate slow-ramp compression stiffness was determined using a linear fit of the slow-ramp load-displacement response.
Macroscopic Evaluations and Histology of IVDs

Separate samples (n=3/group) were fixed for 7 days in 10% neutral-buffered formalin, followed by decalcification in 12% formic acid. Samples were cut into 3-mm sagittal slices and images were captured for macroscopic evaluation by 3 blinded observers (R.B., J.W., and J.M.) using the Thompson grading scale (n=3/group) [Data not showed]. These mid-sagittal slices were paraffin-embedded and 7µm sections were obtained. Staining was performed in accordance with methods form Leung et al. Briefly, sections were rehydrated via ethanol gradations and stained with 3% Alcian blue (pH 1.0) for 8 min, 0.1% safranin-O for 6 min, 0.25% tartrazine for 10 sec, 0.001% fast green for 4 min. Micrographs were acquired using an Axio Vert.A1 microscope with AxioVision SE64 Rel. 4.9.1 software (Zeiss, San Diego, CA). Micrographs were then qualitatively evaluated for endplate Integrity, AF Morphology, AF/NP Demarcation, NP Matrix Homogeneity, and NP Matrix Stain Intensity.

Statistical Analysis

Results are represented as mean ± standard error of the mean (SEM) and significance was defined as (p≤0.05). Statistical analysis was performed using Prism 7 software. Comparisons were performed using a one-way ANOVA with Holm-Sidak method for multiple comparisons (MR imaging Index, DHI, ELISA) or Dunnett’s post-hoc (FSU kinematics and Thompson grading) analysis. Pfirrmann scoring was evaluated via a Kruskal-Wallis test with Dunn’s multiple comparisons.
RESULTS

All animals tolerated the intradiscal C-ABC injection procedure at 6 weeks, recovered from anesthesia, and began weight bearing and eating within one hour postoperatively. For the following 11 weeks, no animals showed no signs of complication including hind end weakness or pain response upon palpation of the lumbar region and recovered as expected. Tables 17&18 depicts the treatment and sample allocations for the model study.

Table 17: Representative table depicting the total sample sizes of each animal and their respective treatments per IVD level.

<table>
<thead>
<tr>
<th>Disc Level</th>
<th>C-ABC Degen</th>
<th>C-ABC Degen</th>
<th>C-ABC Degen</th>
<th>Vehicle</th>
<th>Uninjured</th>
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Table 18: Representative table depicting the sample allocation and sample size per outcome measure separated by disc level. Each outcome measure illustrates the distribution of different animal samples based on treatment.

<table>
<thead>
<tr>
<th>Disc Level</th>
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<th>L4/5</th>
<th>L5/6</th>
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<td><strong>Sample Size</strong></td>
<td><strong>Animal ID</strong></td>
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<td>F</td>
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<td>--</td>
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<td>--</td>
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</table>

**Intradiscal C-ABC Injection Results in Significantly Reduced MR Imaging Index of Degeneration in IVDs**

T2-weighted MR images illustrated a progressive darkening of the NP-region and adjacent vertebral endplates in degenerate IVDs. Conversely, uninjured IVDs and those injected with vehicle demonstrated no such changes. Of note, darkening in adjacent vertebral bodies adjacent to degenerate sheep IVDs were also observed on MR images indicative of endplate abnormalities. Normalized MR imaging index of degenerate IVDs has illustrated a significant trend at week 6 (75.52 ± 11.40%; p=0.075) and was significantly lower at week 17 (67.65 ± 16.63%; p=0.049) values (Fig.). Moreover, these values trended towards significantly lower compared to respective uninjured controls at week 6 (p=0.080) and were significantly lower at week 17 (p=0.033). No significant changes in MR imaging index were observed between uninjured and vehicle control IVDs. Degenerate IVDs also demonstrated an increase in Pfirrmann grade at the L1/2 IVD,
indicating worsening degeneration at week 17 (1.25±0.25) however this was not significantly different compared to week 0 values (Fig. ). Uninjured and vehicle control IVDs both had average Pfirrmann scores of 1±0 at all-time points investigated. No changes in Modic scores were observed across all groups.

![Representative T2-weighted MRI images tracking experimental groups across time-points (Left, week 0; Middle, week 6; Right, week 17) of one respective animal. Adjacent vertebral bodies are labeled on the left as L1, L2, etc. and IVD levels are labeled on the right as L1/2, L2/3, etc. Progressive darkening of the NP (central region of the IVD) can be seen across time-points. Development of Modic Type changes in adjacent vertebral bodies is seen at week 17.](image)

Figure 59: Representative T2-weighted MRI images tracking experimental groups across time-points (Left, week 0; Middle, week 6; Right, week 17) of one respective animal. Adjacent vertebral bodies are labeled on the left as L1, L2, etc. and IVD levels are labeled on the right as L1/2, L2/3, etc. Progressive darkening of the NP (central region of the IVD) can be seen across time-points. Development of Modic Type changes in adjacent vertebral bodies is seen at week 17.
Figure 60: Longitudinal image tracking of sheep lumbar model IVDs. A) Representative graphs depicting quantitative MR image analysis for normalized MRI Index for IVDs between treatment groups for A) all time-points B) within the week 6 time-point, and C) within the week 17 time-point. D-F) Representative graphs illustrating changes within treatment group across all time-points. Solid black lines connecting groups indicate a significant difference compared to week 0 values (p≤0.05).
Table 19: Representative tables depicting quantitative MR image analysis for A) Pfirrmann grading and B) Modic Type changes over the three time-points. *Bold indicates significant difference compared to week 0 values (p≤0.05).

<table>
<thead>
<tr>
<th>IVD Treatment</th>
<th>Pfirrmann Grade</th>
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<th>6 Week Post C-ABC Injury</th>
<th>17 Week Post C-ABC Injury</th>
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<tr>
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<td>1±0</td>
<td>1±0</td>
<td></td>
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<tr>
<td>Uninjured</td>
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<td>1±0</td>
<td>1±0</td>
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<table>
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<th>IVD Treatment</th>
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<th>6 Week Post C-ABC Injury</th>
<th>17 Week Post C-ABC Injury</th>
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<td>0±0</td>
<td>0±0</td>
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</tr>
<tr>
<td>Vehicle</td>
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<td>Uninjured</td>
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</tr>
</tbody>
</table>

**Intradiscal C-ABC Injection Results in Significant Reductions in IVD Height**

Normalized DHI values of degenerate IVDs significantly decreased over time, reaching 73.34±5.74% (p=0.031) at week 6 and 70.35±4.16% (p=0.009) at week 17. These values were also significantly lower compared to uninjured IVD values at week 6 (96.17±4.04%; p<0.001) and week 17 (90.40±1.34%; p<0.001), respectively. Vehicle IVDs also illustrated a significant decrease over time, reaching 83.84±3.37% (p=0.028) at week 6 and 81.01±0.99% (p<0.001) at week 17. These values were also significantly lower
compared to uninjured IVD values at week 6 (96.17±4.04%; p<0.001), but not at week 17. Of note, uninjured IVDs also illustrated a significant decrease in DHI at the week 17 (p=0.009) compared to week 0 values.

Figure 61: Representative radiograph images depicting the change of disc height across all time-points (Top, week 0; Middle, week 6; Bottom, week 17) in ovine model study. Lumbar IVD levels are labeled L_{1/2}, L_{2/3}, etc. adjacent to the IVD location.
Figure 62: Longitudinal image tracking of sheep lumbar model IVDs. A) Representative graphs depicting quantitative radiograph analysis for normalized disc height index (DHI) for IVDs between treatment groups for A) all time-points B) within the week 6 time-point, and C) within the week 17 time-point. D-F) Representative graphs illustrating changes within treatment group across all time-points. Solid black lines connecting groups indicate a significant difference compared to week 0 values (p≤0.05).
Intradiscal C-ABC Injection Results in Significant Alterations in Functional Spinal Unit Kinematics

Kinematic creep loading (Fig ) of FSUs containing degenerate IVDs demonstrated a significantly (p=0.004) increased creep displacement (0.49±0.04 mm) compared to FSUs with uninjured IVDs (0.33±0.03 mm, respectively) (Fig ). Additionally, long-term elastic ($\Psi_2: 537.40 \pm 36.05$ N/mm) and viscous ($\eta_2: 1.33 \pm 0.18 \times 10^6$ Ns/mm) damping coefficients of FSUs with degenerate IVDs were significantly (p=0.016 and p=0.016, respectively) lower compared to FSUs with uninjured IVDs ($\Psi_2: 766.75 \pm 69.98$ N/mm and $\eta_2: 2.07 \pm 0.19 \times 10^6$ Ns/mm, respectively) (Figs ). No significant changes were observed within the creep kinematic parameters between FSUs containing uninjured and vehicle control IVDs.

Axial cyclic kinematic loading of FSUs containing degenerate IVDs demonstrated a significant decrease in tensile stiffness (339.60±58.46 N/mm; p=0.034) and a significant increase in the axial range of motion (0.77±0.07 mm; p=0.007) compared to FSUs with uninjured IVDs (584.71±81.70 N/mm and 0.45±0.06 mm, respectively). Additionally, significant trends were observed in the loss of compressive stiffness for both FSUs with degenerate (1372.60±221.50 N/mm; p=0.065) and vehicle (1347.10±331.36 N/mm; p=0.055) IVDs compared to FSUs with uninjured IVDs (1933.70±155.52 N/mm). Torsional rotation of FSUs containing degenerate IVDs demonstrated a significant decrease in torque range (15.68±0.75 Nm; p = 0.001) and torsional stiffness (2.45±0.12 Nm/°, p=0.021) compared to uninjured IVDs (19.57±0.41 Nm and 2.78±0.03 Nm/°,
respectively). (Figs). No significant changes were observed within the torsional kinematic parameters between FSUs containing uninjured and vehicle control IVDs.

Figure 63: Kinematic testing of model IVD FSUs. A) Loading scheme for FSU testing depicting creep, axial cyclic tension-compression, axial torsion, and slow constant-
rate ramp testing. B) Representative graph depicting the creep response of a sheep IVD and associated creep parameter measures. Graphs depicting C) step and creep displacement, D) elastic damping and E) viscous damping coefficients, F) slow ramp compressive stiffness, G) compressive stiffness, H) tensile stiffness, I) axial RoM, J) torque range, and K) torsional stiffness values of uninjured, vehicle and degenerate (degen) sheep IVDs, respectively. Solid lines connecting groups indicates significant difference (p≤0.05) compared to uninjured controls.

Intradiscal C-ABC Injection Induced Significant Changes in IVD Morphology and Microarchitecture

Qualitative histological analysis illustrates significant differences from uninjured and degenerate IVDs. Qualitatively, degenerate IVDs revealed significant alterations and disruption of tissue architecture compared to control IVDs in the AF (Figure 64), NP (Figure 65), and CEP (Figure 66) regions of the IVD. Prominent intravertebral herniations (Schmorl’s nodes) were observed in all evaluated degenerate IVDs (Figure 67), and these consistently exhibited several abnormalities compared to control IVDs: 1) failure and displacement of the CEP into the subchondral bone, 2) extrusion of marked amounts of NP into the vertebrae, 3) apparent remodeling around the herniated tissue, and 4) thickening of the vertebral bone. The AF of degenerate IVDs also showed a notable change in concavity, in contrast to the control IVDs that showed normal, convex AF orientation. Furthermore, gross morphological changes in degenerate IVDs seemed to have pronounced effects on AF structure. Specifically, loss of IVD height seemed to compress AF lamellae
while CEP displacement resulted in reduced convexity. Higher magnifications revealed changes in distinct regions of the IVD. The ability of the CEP to retain stain was severely diminished by C-ABC injection. Additionally, thinning and permeabilization of the CEP was seen through microscopic NP expulsion and reduction in CEP thickness. The vehicle control showed no qualitative histological differences compared to the uninjured samples.

**Figure 64:** Qualitative histological comparison of model groups for the AF region of the IVD. Representative histological images of AF lamellae from A-C) uninjured control, D-F) vehicle control, and G-I) degenerate IVDs depicting differences in
lamellar thickness and organization (Red – AF Lamellae). Scale bar = 500 µm. *FAST Staining:* Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.

**Figure 65:** Qualitative histological comparison of model groups for the NP region of the IVD. Representative histological images of NP tissue from A-C) uninjured control, D-F) vehicle control, and G-I) degenerate IVDs depicting differences in illustrating the difference in glycosaminoglycan staining intensity (blue/green = glycosaminoglycan). Scale bar = 500 µm. *FAST Staining:* Greenish-blue =
glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.

Figure 66: Qualitative histological comparison of treatment groups for the CEP region of the IVD. Representative histological images of the cartilaginous end-plates from A-C) uninjured control, D-F) vehicle control, and G-I) degenerate IVDs depicting differences in illustrating the difference in illustrating differences in integrity and thickness. Scale bar = 500 µm. FAST Staining: Greenish-blue =
glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.

**Degen: Schmorl’s Nodes**

Figure 67: Qualitative histological comparison of Schmorl’s node formation in A-C) degenerate IVDs. Representative histological images of the surrounding cartilaginous end-plates, NP tissue, and bone depicting protrusion of NP tissue into the adjacent vertebral body bone. Scale bar = 500 µm. *FAST Staining*: Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.
DISCUSSION

The present study aimed to build upon the initial pilot study which demonstrated that intradiscal administration of C-ABC resulted in mild to moderate IVDD as evidenced by reductions in IVD height and hydration, changes in kinematic mechanical properties, and changes in microarchitecture.\textsuperscript{286,370} Two-hundred microliter intradiscal injections of 1U C-ABC were performed in the lumbar spine of sheep and degenerative changes were tracked for up to 17 weeks (model) in alignment with previous studies by others.\textsuperscript{286,363,370} The primary findings of these studies herein suggest that intradiscal injection of 1U C-ABC in sheep resulted in moderate to severe IVD degeneration marked by progressive and significant alterations in IVD hydration, height, inflammation, kinematics, and IVD tissue morphology (including the formation of end-plate disruptions). The observed changes appeared to be potentially more severe as compared to previous reports,\textsuperscript{286,361–363,370} however, these changes were still reminiscent of that observed in human lumbar IVDD.

Therefore, in the present studies, similar conclusions were drawn as from the pilot study. Clinical imaging of sheep C-ABC injected IVDs demonstrated significant reductions in hydration and height, which worsened over time. This was evidenced by progressive decreases in both T2-weighted MR image signal intensity and IVD height measurements. No evidence of spontaneous regeneration was observed during the study time-course. Such observations are likely due to a loss of aggregating proteoglycan, leading reduced NP osmotic potential, lowered water content, and the apparent increase in matrix permeability.\textsuperscript{379–382} Together, these alterations diminish the ability of the tissue to effectively support compressive loading leading to a reduction in IVD height and overall
mechanical dysfunction.\textsuperscript{348,352,383} Similarly, imaging of degenerate human IVDs often display progressive darkening of the IVDs and loss of height with increasing grade of degeneration.

Cyclic axial and torsional kinematics of degenerate sheep IVDs were significantly altered compared to uninjured and vehicle control samples. This is likely an effect of degradation of the ECM in conjunction with an increase in its permeability leading to reduced pressurization.

Qualitative histological evaluations of degenerate sheep IVDs demonstrated significant alterations in the NP, AF, and endplate micro-architecture as compared to controls. This was demonstrated by decreases in proteoglycan staining intensity, alterations in lamellar architecture, and the formation of Schmorl nodes, respectively. Taken together, these histomorphological features are suggestive of moderate to severe IVDD. We hypothesize that C-ABC degraded NP proteoglycan and the cartilaginous endplates. In fact, cartilaginous endplate thickness was found to be irregular in both macroscopic and microscopic analysis. Loss of cartilaginous endplate integrity due to C-ABC treatment could account for the observed formation of Schmorl nodes and the resultant increase in inflammation. The cartilaginous endplate functions to prevent the NP from breaching the vertebral body which prohibits its contact with bone marrow and the immune system. This is critical as NP tissue has been shown to be immunogenic when it herniates extradiscally.\textsuperscript{391} It is plausible that because sheep lumbar IVDs have been shown to have higher intradiscal pressures compared to human IVDs,\textsuperscript{358,392} in combination with enzymatic disruption of the cartilaginous endplate, most likely resulted in NP tissue
breaching into the vertebral body. Such breaches were not observed in vehicle control IVDs which suggests that enzymatic damage to the endplate, as opposed to an increase in intradiscal pressure due to injection volume, caused the observed Schmorl node formation. Additionally, the formation of Schmorl nodes could be due to alterations in the vertebral body bone as a result of inflammatory edema due to a breach of the cartilaginous end-plate and exposure of the NP, however, this was not directly assessed in the present study. In the context of lumbar IVDD in humans, the presence of Schmorl nodes has been shown to be correlated with Modic changes and grade of degeneration in human IVDs.

As with any study, limitations were noted. First, the model study used contained IVDs where were also treated with biomimetic implants at adjacent levels (n=18 IVDs total). Second, while we used an established T2-weighted MR imaging modality to evaluate ECM degradation as a function of IVD hydration, it would be advantageous to include a T1ρ relaxation parameter, which may correlate more strongly with changes in IVD GAG content and histology. Third, IVDs receiving a C-ABC injection and those serving as controls were not randomized to account for differences in spine level. However, data from each IVD was normalized to its respective week 0 (baseline) value to account for this.
CHAPTER XI

NOVEL, BIOMIMETIC ANNULUS FIBROSUS AND NUCLEUS PULPOSUS IMPLANTS FOR THE TREATMENT OF A CHEMONUCLEOLYSIS-INDUCED INTERVERTEBRAL DISC DEGENERATION: AN OVINE LUMBAR SPINE

INTRODUCTION

The model study (Chapter X) of the chemonucleolysis-induced degeneration of an ovine lumbar provided an in vivo platform to evaluate the efficacy of a novel, biomimetic IVD repair implants (acellular bovine nucleus pulposus: ABNP and annulus fibrosus repair patch: AFRP).\textsuperscript{201,212,255} Following 6 weeks of chemonucleolysis-induced degeneration, degenerate ovine lumbar IVDs were repaired with these biomimetic IVD implants and the efficacy of these implants was evaluated using the same outcome measures of the model study. The treated IVDs were evaluated for changes observed in MR imaging, mechanical properties, and histology following repair of IVDs injected with 1U C-ABC.

MATERIALS & METHODS

\textbf{Institutional Review Board (IRB) Approval}

The study was approved by the Institutional Animal Care and Use Committee of Colorado State University (IACUC Protocol: 16-6891A). IVDs from two lumbar levels of nine skeletally mature female sheep (\textit{Ovis Aries}, Rambouillet ewes; 65-71 kg, 3 years-of-age, K&S Livestock, Fort Collins, CO) were utilized in this study. Animals were group
housed in an indoor/outdoor pen with access to a three-sided shelter and evaluated daily by a veterinarian for signs of pain, behavior changes, or gait abnormalities for the duration of the study.

**Surgical Procedure and Intradiscal Administration of C-ABC**

Peri-operatively, a transdermal fentanyl patch (150 mcg/hr/sheep) was applied to each animal for five days starting one day prior to surgery. Twenty-four hours prior to surgery, five doses of procaine penicillin G (3 million units) and phenylbutazone (1 gram) was administered to each sheep. The animals were induced with ketamine (2mg/kg IV) and midazolam (0.2mg/kg IV) then intubated and maintained on 1.5-3% isoflurane in 100% oxygen throughout the surgical procedure. Using standard surgical preparation and aseptic technique, the lumbar IVDs were located via fluoroscopy. IVDD was induced via percutaneous intradiscal injection of 1U of C-ABC (Amsbio, Cambridge, MA) in 200 µL of the vehicle solution (sterile 0.1% bovine serum albumin in 1x PBS - Fisher Scientific, Hampton, NH) into the L1/2, L2/3, L3/4 IVDs (Fig). To confirm accurate needle position prior to injection, needle visualization was achieved using lateral and anterior-posterior fluoroscopy (Fig). The L4/5 and L5/6 IVDs served as both vehicle and uninjured controls, respectively. Post-operatively, sheep were monitored until ambulatory, and then returned to standard housing conditions. Following the progression of IVDD, driven by C-ABC injections, animals were euthanized by intravenous barbiturate overdose (pentobarbitone sodium, 88mg/kg) in compliance with the 2013 American Veterinary Medical Association guidelines. Immediately following euthanasia, AF and NP tissues from selected IVDs were
immediately excised and frozen at -80°C for biochemical analysis. Lumbar spines were then harvested en bloc and shipped overnight on wet ice to Clemson University for further analysis.

**Fabrication of Novel, Biomimetic Implants**

Multi-laminate “angle-ply” AFRPs were developed and assembled from decellularized porcine pericardium to form tri-layer scaffolds and crosslinked in 6mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with 6mM N-hydroxysuccinimide (NHS). These scaffolds were then maintained in a phosphate buffered saline (PBS) storage solution containing protease inhibitor at 4°C for up to two weeks prior to testing.

ABNPs were developed from decellularized NP tissue isolated from bovine coccygeal discs (CC1/2-CC3/4). Briefly, the NP was isolated using an 8mm biopsy punch and decellularized using a series of detergents, ultrasonication, and nucleases. ABNPs were then crosslinked in 30mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with 1.2mM N-hydroxysuccinimide (NHS) as previously described.
Implantation of Novel, Biomimetic Implants

Figure 68: Left) Representative image of the implantation of the ABNP into the lumbar IVD. Middle) Representative image of the implantation of the AFRP to secure the ABNP within the repaired IVD. Right) Representative macroscopic image depicting minimal to none fibrous encapsulation following 11 weeks in vivo.

Following discectomy for the removal of the degenerate NP tissue, AFRP and ABNP scaffolds were implanted. The scaffolds were divided into two groups: seeded and acellular. The acellular groups were implanted directly into the voids of the nucleotomized NP IVDs. The seeded AFRP and ABNP scaffolds were first seeded with $1 \times 10^6$ human adipose-derived stem cells (hADSCs), and cell viability was confirmed immediately prior to implantation.

Magnetic Resonance Imaging of IVDs

Sagittal MR imaging was performed immediately prior to intradiscal injection of C-ABC (week 0 – baseline) and tracked longitudinally at 6- and 17-weeks post-injection to monitor IVDD of treated, degenerate, vehicle, and uninjured IVDs. MR image scans were obtained using a 1.5 Tesla clinical imager (GE Signa). T2-weighted, T1-weighted, and Short-T1 Inversion Recovery (STIR) MR imaging sequences were performed on the
explanted lumbar spines. Sagittal images were constructed using a T2-weighted fast spin echo sequence using a spine array coil (time to repetition: 2782 ms; time to echo: 101 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), a T1-weighted fast spin echo sequence using a spine array coil (time to repetition: 616 ms; time to echo: 18 ms; voxel size: 0.78 mm x 0.78 mm x 3.0 mm, with a 0 gap), and a STIR sequence using a spine array coil (time to repetition: 3500 ms; time to echo: 40 ms; voxel size: 1.56 mm x 1.56 mm x 3.0 mm, with a 0 gap and inversion time of 150).

Semi-quantitative MR image analysis was performed as described by Hoogendoorn et al.\textsuperscript{362} MR imaging index was calculated from T2-weighted images as the product of the cross-sectional area and mean signal intensity of the encircled NP using IMPAX 6.6.1.4024 (AGFA HealthCare N.V., Mortsel, Belgium) software. Consistent mid-sagittal IVD imaging was confirmed by ensuring the full cross-sectional of the spinal cord was in view. MR imaging index is expressed as a percentage of week 0 (pre-C-ABC injection) values for normalization. It should be noted that normalization of signal intensity was not performed by comparing to the spinal cord, as has been described by others, because it is a mobile structure with too much Gibbs and pulsation artifact. Thus, normalization to normal (week 0) IVDs was deemed most appropriate. Two researchers (R.B. and J.W.) quantified the MR imaging index for each IVD. A board-certified neuroradiologist (L.M.) verified the quantitation and performed a qualitative analysis of MR images using the classification scale described by Pfirrmann et al.\textsuperscript{371}
Radiographs of IVDs

IVD height index (DHI) was measured from mid-sagittal, Radiographs (X-Rays) in accordance with Hoogendoorn et al. with minor modifications. IVD and vertebral body heights were each quantified using three height measurements at ventral-dorsal quartiles using ImageJ (NIH, Bethesda, MD) (Fig 1C). DHI was calculated as each IVD’s mean disc height divided by the mean adjacent vertebrae height, with changes expressed as a percent of week 0 DHI. Measurements were performed by two researchers (R.B. and J.W.) to evaluate the interobserver reliability and blindly confirmed by a board-certified neuroradiologist (L.M.).

Functional Spinal Unit Axial and Torsional Kinematics

Seventeen weeks post-injection, functional spinal units (FSU’s: vertebrae-IVD-vertebrae) of treated (n=6/group) IVDs underwent biomechanical kinematic testing according to methods previously described by our group, with modification. Briefly, FSU’s (with intact posterior elements) were potted in urethane resin (Goldenwest Manufacturing, Grass Valley, CA) first underwent creep loading on a Bose ElectroForce (model: 3220, TA Instruments, New Castle, DE) equipped with a 100-lb. load cell and a test chamber filled with 1xPBS/protease inhibitor at 25°C. Samples were loaded to -0.125 MPa and then underwent a 1-hr. creep period at -0.50 MPa. Samples were then transferred to a servohydraulic test frame (model: 8874, Instron, Norwood, MA) fitted with a 20kN load cell, and subjected to 35 cycles of axial compression (-0.50 MPa) and tension (0.25 MPa) at 0.1 Hz. Compression was then maintained at -0.50 MPa as samples underwent 35
torsion cycles to ±3°. Finally, samples underwent a slow-rate compressive ramp (1 N/s) from -0.125 MPa to -0.50 MPa. A non-linear constitutive model was fit to the creep data using GraphPad Prism 7 software (La Jolla, CA) to yield elastic ($\Psi$) and viscous ($\eta$) damping coefficients for the short-term ($\eta_1$ and $\Psi_1$) and long-term ($\eta_2$ and $\Psi_2$), as described previously.\textsuperscript{372} Tensile and compressive stiffness was determined using a linear fit of the loading force-displacement curve from 60-100% of the 35\textsuperscript{th} cycle. Torsional stiffness was calculated from a linear fit of the loading torque-rotation curve of the 35th cycle. Torque range and axial range of motion (RoM) was calculated as the peak-to-peak torque and displacement, respectively. The constant-rate slow-ramp compression stiffness was determined using a linear fit of the slow-ramp load-displacement response.

**Macroscopic Evaluations and Histology of IVDs**

Separate samples (n=3/group) were fixed for 7 days in 10% neutral-buffered formalin, followed by decalcification in 12% formic acid. Samples were cut into 3-mm sagittal slices and images were captured for macroscopic evaluation by 3 blinded observers (R.B., J.W., and J.M.) using the Thompson grading scale (n=3/group) \[Data not showed\].\textsuperscript{373} These mid-sagittal slices were paraffin-embedded and 7µm sections were obtained. Staining was performed in accordance with methods form Leung et al.\textsuperscript{374} Briefly, sections were rehydrated via ethanol gradations and stained with 3% Alcian blue (pH 1.0) for 8 min, 0.1% safranin-O for 6 min, 0.25% tartrazine for 10 sec, 0.001% fast green for 4 min. Micrographs were acquired using an Axio Vert.A1 microscope with AxioVision SE64 Rel. 4.9.1 software (Zeiss, San Diego, CA. Micrographs were then qualitatively evaluated
for endplate Integrity, AF Morphology, AF/NP Demarcation, NP Matrix Homogeneity, and NP Matrix Stain Intensity.

**Statistical Analysis**

Results are represented as mean ± standard error of the mean (SEM) and significance was defined as (p≤0.05). Statistical analysis was performed using Prism 7 software. Comparisons were performed using a one-way ANOVA with Holm-Sidak method for multiple comparisons (MR imaging Index, DHI, ELISA) or Dunnett’s post-hoc (FSU kinematics and Thompson grading) analysis. Pfirrmann scoring was evaluated via a Kruskal-Wallis test with Dunn’s multiple comparisons.

**RESULTS**

All animals tolerated the intradiscal C-ABC injection procedure at 6 weeks and underwent discectomy to remove the degenerated IVD material prior to implantation of the novel AF and NP implants. **Figure 68** illustrates the implantation procedure and the recovered sample following the final endpoint of the study.

All animals recovered from anesthesia, and began weight bearing and eating within one hour postoperatively. For the following 11 weeks, no animals showed no signs of complication including hind end weakness or pain response upon palpation of the lumbar region and recovered as expected. **Figure 69** illustrates the study design, while **Tables 20&21** depict the treatment and sample allocations for the treatment study.
Figure 69: Animal study design overview and surgical approach. Animal study progressed over 17 weeks beginning with MR imaging and IVD degeneration (Degen) induction at week 0. At week 6, degenerate IVDs (2 of 3) were repaired with AFRP
and ABNP implants (non-seeded or seeded with hADSCs). Implants remained in vivo for 11 weeks prior to study termination.

Table 20: Representative table depicting the total sample sizes of each animal and their respective treatments per IVD level. D: C-ABC injected degenerate IVDs through 17 weeks, R: repaired IVDs with acellular implants, R+: repaired IVDs with hADSC seeded implants, V: vehicle control (200uL of BSA injection only), and U: uninjured control.

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<td>V</td>
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<td>V</td>
<td>U</td>
</tr>
<tr>
<td>Total # IVD per Disc Level</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
</tr>
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</table>
Table 21: Representative table depicting the sample allocation and sample size per outcome measure separated by disc level. Each outcome measure illustrates the distribution of different animal samples based on treatment.

<table>
<thead>
<tr>
<th>Disc Level</th>
<th>L1/2</th>
<th>L2/3</th>
<th>L3/4</th>
<th>L4/5</th>
<th>L5/6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome Measure</td>
<td>Time-Point</td>
<td>Animal ID</td>
<td>Sample Size</td>
<td>Animal ID</td>
<td>Sample Size</td>
</tr>
<tr>
<td></td>
<td>17 weeks</td>
<td>F</td>
<td>n=2</td>
<td>B, D, I</td>
<td>n=2</td>
</tr>
<tr>
<td>Histology</td>
<td>6 weeks</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>10 weeks</td>
<td>E, G, H</td>
<td>n=3</td>
<td>E, G, H</td>
<td>n=3</td>
</tr>
<tr>
<td>Biochemical</td>
<td>6 weeks</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Intradiscal C-ABC Injection Results in Significantly Reduced MR Imaging Index, Increased Pfirrmann Grades, and Modic Scores of Degeneration in IVDs

T2-weighted MR images illustrated a progressive darkening of the NP-region at the week 6 time-point for the C-ABC induced degenerate IVDs (Fig.1). Following implantation and 11 weeks in vivo, Normalized MRI Index of IVDs with repair only implants demonstrated a further significant decrease in NP hydration (67.30±11.88%; p=0.033) compared to week 0 values. Conversely, IVDs with repair + hADSC implants demonstrated a trend of restoration in NP hydration at the same week 17 time-point (92.84±13.21%) with respect to their week 0 values. No significant differences were observed for MRI Index between the repair groups and their respective week 6 time-points.
Figure 70: Longitudinal image tracking of sheep lumbar IVDs. A) Representative graphs depicting quantitative MR image analysis for normalized MRI Index for IVDs with repaired implants. Solid black lines connecting groups indicate a significant difference compared to week 0 values (p≤0.05).

Prior to implantation (week 6) with repair only and repair + hADSCs implants, IVDs which received the C-ABC injection demonstrated a significant increase in Pfirrmann grade (2.00±0.76; p<0.001) and Modic Type (0.50±0.19; p=0.019) compared to week 0 values. Following implantation and 11 weeks in vivo, the Pfirrmann grade and Modic Type continued to worsen for repaired IVDs with repair only (3.50±0.29; p<0.001 and 1.25±0.25; p=0.003, respectively) and repair + hADSCs (3.00±0.41; p=0.003 and 0.75±0.25; p=0.024, respectively) compared to week 0 values.
Table 22: Representative tables depicting quantitative MR image analysis for A) Pfirrmann grading and B) Modic Type changes over the three time-points. *Bold indicates significant difference compared to week 0 values (p≤0.05).

### A  
**Table: Pfirrmann Grade**

<table>
<thead>
<tr>
<th>IVD Treatment</th>
<th>0 Week Post C-ABC Injury</th>
<th>6 Week Post C-ABC Injury</th>
<th>17 Week Post C-ABC Injury</th>
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</thead>
<tbody>
<tr>
<td>Degen C-ABC</td>
<td>1±0</td>
<td>1±0</td>
<td>1.25±0.25</td>
</tr>
<tr>
<td>Repair + hADSCs</td>
<td>1±0</td>
<td>2±0.41*</td>
<td>3±0.41*</td>
</tr>
<tr>
<td>Repair Only</td>
<td>1±0</td>
<td>2±0.41*</td>
<td>3.5±0.29*</td>
</tr>
<tr>
<td>Uninjured</td>
<td>1±0</td>
<td>1±0</td>
<td>1±0</td>
</tr>
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### B  
**Table: Modic Type**

<table>
<thead>
<tr>
<th>IVD Treatment</th>
<th>0 Week Post C-ABC Injury</th>
<th>6 Week Post C-ABC Injury</th>
<th>17 Week Post C-ABC Injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degen C-ABC</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>Repair + hADSCs</td>
<td>0±0</td>
<td>0.5±0.29*</td>
<td>0.75±0.25*</td>
</tr>
<tr>
<td>Repair Only</td>
<td>0±0</td>
<td>0.5±0.29*</td>
<td>1.25±0.25*</td>
</tr>
<tr>
<td>Uninjured</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

**Intradiscal C-ABC Injection Results in Significant Reductions in IVD Height**

Normalized DHI values of degenerate IVDs illustrated a significant decreased at the week 6 time-point following the C-ABC injection for both IVDs with repair only (65.88±6.32%; p=0.021) and with repair + hADSCs (64.67±2.29%; p=0.001) compared to the week 0 values. IVDs with repair only implants were not able to restore the IVDs DHI...
to week 0 values, but instead maintained the significant loss of DHI (67.07±5.76%; p=0.018) that was seen at week 6. Conversely, IVDs with repair + hADSCs demonstrated a significant increase in DHI (79.91±5.69%; p=0.047) compared to the week 6 implantation DHI. Additionally, this restoration in DHI was found to be non-statistically different from the week 0 values.

Figure 71: Longitudinal image tracking of sheep lumbar IVDs. A) Representative graphs depicting quantitative radiograph image analysis for normalized disc height index (DHI) for IVDs with repaired implants. Solid black lines connecting groups indicate a significant difference compared to week 0 values (p≤0.05).

Intradiscal C-ABC Injection Results in Significant Alterations in Functional Spinal Unit Kinematics

Kinematic creep loading (Fig) of FSUs containing repaired IVDs with repair only and repair + hADSCs demonstrated a significantly increased creep displacement (0.67±0.06 mm; p=0.001 and 0.59±0.10 mm; p=0.026) compared to FSUs with uninjured
IVDs (0.33±0.03 mm, respectively) (Fig). Additionally, long-term elastic ($\Psi_2: 574.52 \pm 59.01 \frac{N}{mm}$; $p = 0.016$ and $552.14 \pm 39.30 \frac{N}{mm}$; $p = 0.033$, respectively) and viscous ($\eta_2: 9.18 \pm 1.35 \times 10^5 \frac{Ns}{mm}$; $p = 0.001$ and $9.46 \pm 1.04 \times 10^5 \frac{Ns}{mm}$; $p = 0.001$, respectively) damping coefficients of FSUs with degenerate IVDs were significantly lower compared to FSUs with uninjured IVDs ($\Psi_2: 766.75 \pm 69.98 \frac{N}{mm}$ and $\eta_2: 2.07 \pm 0.19 \times 10^6 \frac{Ns}{mm}$, respectively) (Figs).
Figure 72: Kinematic testing of FSUs with treated IVDs. A) Loading scheme for FSU testing depicting creep, axial cyclic tension-compression, axial torsion, and slow constant-rate ramp testing. B) Representative graph depicting the creep response of a sheep IVD and associated creep parameter measures. Graphs depicting C) step and...
creep displacement, D) viscous damping and E) elastic damping coefficients, F) compressive stiffness, G) tensile stiffness, H) axial range of motion, I) slow ramp compressive stiffness, J) torque range, and K) torsional stiffness values of uninjured, vehicle and degenerate (degen) sheep IVDs, respectively. Solid lines connecting groups indicates significant difference (p≤0.05) compared to uninjured controls.

Axial cyclic kinematic loading of FSUs containing repaired IVDs with both repairs only and repair + hADSCs demonstrated a trend of restoration in compressive stiffness (1876.10±272.46; p=0.858 and 2056.10±296.23; p=0.722, respectively) compared to FSUs with degenerate IVDs (1372.60±221.50 N/mm; p=0.65) with respect to FSUs with uninjured IVDs (1933.70±155.52 N/mm). FSUs with both repair treatments also demonstrated a restoration in tensile stiffness (396.80±63.01 N/mm; p=0.098 and 363.49±81.07 N/mm; p=0.093) compared to FSUs with degenerated IVDs (339.60±58.46 N/mm; p=0.034) with respect to FSUs with uninjured IVDs (584.71±81.70 N/mm). Additionally, FSUs with both repair treatments demonstrated a restoration in torsional stiffness (2.64±0.17 Nm/°; p=0.428 and 2.60±0.11 Nm/°; p=0.141, respectively) compared to FSUs with degenerated IVDs (2.45±0.12 Nm/°, p=0.021) with respect to FSUs with uninjured IVDs (2.78±0.03 Nm/°). Conversely, FSUs with both repair treatments demonstrated a significant increase in the axial range of motion (0.94±0.11 mm; p=0.003 and 1.05±0.23 mm; p=0.032) and torque range (16.84±0.72 Nm; p=0.008 and 16.25±1.08 Nm; p=0.016) compared to FSUs with uninjured IVDs (0.45±0.06 mm and 19.57±0.41 Nm, respectively).
Repair of C-ABC Injection Induced Degenerate IVDs Demonstrated Significant Changes in IVD Morphology and Microarchitecture

Qualitative histological analysis illustrates significant differences from uninjured and repaired IVDs. Qualitatively, repaired IVDs revealed significant alterations and disruption of tissue architecture compared to uninjured and degenerate IVDs in the AF (Figure 73), NP (Figure 74), and CEP (Figure 75) regions of the IVD. Prominent intravertebral herniations (Schmorl’s nodes) were observed in all evaluated repaired IVDs (Figure 76) similar to those found previously in the degenerate model IVDs. These consistently exhibited several abnormalities compared to control IVDs: 1) failure and displacement of the CEP into the subchondral bone, 2) extrusion of marked amounts of NP into the vertebrae, 3) apparent remodeling around the herniated tissue, and 4) thickening of the vertebral bone. The AF of repaired IVDs also showed a notable change in morphology, in contrast to the control IVDs that showed normal convex AF orientation and degenerate IVDs that showed abnormal concave AF orientation. Staining of AF tissue resembled tissue typically found in scar tissue with little to no organization. Higher magnifications revealed changes in distinct regions of the IVD. The ability of the CEP to retain stain was severely diminished by C-ABC injection, like those found in degenerate IVDs. Additionally, thinning and permeabilization of the CEP was seen through microscopic NP expulsion and reduction in CEP thickness. Of note, the intensity and frequency of Schmorl’s node formations were less frequent than those found in degenerate IVDs.
Figure 73: Qualitative histological comparison of treatment groups for the AF region of the IVD. Representative histological images of AF lamellae from A-C) uninjured control, D-F) degenerate control, G-I) repair only, and J-K) repair + hADSCs IVDs depicting differences in lamellar thickness and organization (Red – AF Lamellae). Scale bar = 500 µm. *FAST Staining:* Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.
Figure 74: Qualitative histological comparison of treatment groups for the NP region of the IVD. Representative histological images of NP tissue from A-C) uninjured control, D-F) degenerate control, G-I) repair only, and J-K) repair + hADSCs IVDs depicting differences in illustrating the difference in glycosaminoglycan staining intensity (blue/green = glycosaminoglycan). Scale bar = 500 µm. FAST Staining: Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.
Figure 75: Qualitative histological comparison of treatment groups for the CEP region of the IVD. Representative histological images of the cartilaginous end-plates from A-C) uninjured control, D-F) degenerate control, G-I) repair only, and J-K) repair + hADSCs IVDs depicting differences in illustrating the difference in illustrating differences in integrity and thickness. Scale bar = 500 µm. *FAST Staining:* Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.
Figure 76: Qualitative histological comparison of Schmorl’s node formation in A) degenerate, B) repair only, and C) repair + hADSCs IVDs. Representative histological images of the surrounding cartilaginous end-plates, NP tissue, and bone depicting protrusion of NP tissue into the adjacent vertebral body bone. Scale bar = 500 µm. 

*FAST Staining: Greenish-blue = glycosaminoglycan / NP, Red = CEP/AF, Yellow = vertebral bone, and Light Green = outer AF.*
DISCUSSION

Treatment of degenerate IVDs with AFRP and ABNP implants, both seeded and non-seeded) demonstrated potential in the restoration of many outcome measures. Cyclic axial and torsional kinematics demonstrated that the replacement of the degenerate IVD tissue was not dependent on cell-response as seen with restorations in compressive, tensile, and torsional stiffness for both treatment groups. Conversely, only implants seeded with hADSCs demonstrated a restoration in imaging modalities for both DHI and MRI Index compared to degenerate controls with respect to uninjured IVDs. Histological analysis of repaired IVDs (scaffolds only and seeded) illustrated similar morphological features to that of degenerate IVDs. The AF region of the repair demonstrated unorganized collagen repair similar to scar tissue. The CEP’s of repaired IVDs demonstrated similar thinning and loss of stain compared to degenerate IVDs; however, the formation and density of Schmorl’s node formations were less frequent than those found in degenerate IVDs. Cytokine concentrations of repaired IVDs demonstrated a decrease in AF tissue regions and an increase in NP tissue regions for pro-inflammatory cytokines. The increase in cytokine production within the NP region could be contributed to the formation of Schmorl’s nodes, which in turn breaks the CEP barrier. The destruction of this cartilaginous barrier could allow for blood vessels within the vertebral bone to provide a direct systemic response to the surrounding tissue.

The response of these repair implants serves as a “worst-case” scenario as the environment that they were implanted into was a severe case of IVD degeneration. Tissue engineering approaches for pre-formed NP and AF repair implants are designed to be
implanted during early-stages (mild to moderate) of disc degeneration. Therefore, further analysis of these implants is warranted due to their potential to provide mitigation/regeneration of these severe degenerate IVDs.
CHAPTER XII

CONCLUSIONS & RECOMMENDATIONS FOR FUTURE STUDIES

Coupled with our previously reported findings that illustrate the simplicity and scalability of our patch formation method concurrent with its ability to support cell cytocompatibility, the studies performed within this dissertation suggest our AFRP may serve as an ideal surrogate to replace and regenerate the native human AF tissue. Overall testing results demonstrated similar mechanical properties to native human AF tissue illustrating its benefit of being a mechanically competent biomaterial without the need of in vitro cellular extracellular matrix enhancement. Additionally, the cytocompatibility of the AFRP demonstrated its potential to allow for tissue regeneration and remodeling. Lastly, the AFRP demonstrated its potential to survive the severe degenerate environment and contribute to the potential restoration of these degenerated IVDs *in vivo*. Therefore, AFRPs may improve patient outcomes and potentially lower health care costs for those with IVDH by allowing for the utilization of less aggressive discectomy, minimizing the reoccurrence of IVDH. Additionally, the AFRP may permit the use of early-stage interventions of IVDD including nucleus arthroplasty biomaterials.

Future studies should include the development of a full-thickness annular plug to replace the full-void of missing annular tissue caused either by tissue failure or surgical intervention. Additionally, further characterization of the ovine model should be conducted including: the mechanical, biochemical, and histological of healthy ovine IVDs.
Furthermore, a concentration study of this ovine model is warranted to determine a less aggressive approach which would be more optimal for NP arthroplasty procedures.
APPENDICES
Appendix A

AFRP Formation via Sewing Machine
Appendix B

Changes in Thermal Denaturation Temperatures Following Crosslinking

Representative graph depicting the changes in thermal denaturation temperatures (°C) between native AF, fresh and decellularized porcine pericardium, and crosslinked pericardium tissues.
Appendix C

Percent Mass Loss Following Accelerated Collagenase Degradation of AFRPs

Accelerated Collagenase Degradation

% Mass Loss

NC AFRP, Native AF, 6mM EDC, 30mM EDC, 0.2% GLUT, 0.6% GLUT
Appendix D

Impact of Inflammation on Human and Ovine Adipose-Derived MSCs for Osteogenic, Chondrogenic, and Adipogenic Differentiation

A

B

C

D

E

F

346
Appendix E

Effects of Inflammation on Human AD- and AM-MSCs Differentiation into Adipocytes

A

B
AFRP scaffolds were cultured TNF-α and IL-1β cytokines in concentrations found in late-stage degeneration IVDs. Differentiation potential of AD-MSCs seeded on AFRPs (n=3/time-point) were evaluated using RT-PCR for putative AF profiles Collagen I, V, XII; P4HA1; and Decorin. RNA isolation was performed as described above. Expression of AF markers was compared to their relative gene transcript levels were compared to day 3 transcript levels cultured under non-INF culture conditions (Table 23). Results herein are described as relative fold change ± SD, and statistical changes were evaluated on the ΔCt values. The production of Col I was significantly down-regulated at day 3 (0.554±0.134; \( p=0.020 \)) and statistically up-regulated at days 8 (2.969±0.349; \( p=0.006 \)) and 15 (2.266±0.501; \( p=0.030 \)) compared to non-INF day 3 controls (Figure 77A). Col V production was also statistically down-regulated at days 3 (0.609±0.232; \( p=0.012 \)) and 8 (0.597±0.302; \( p=0.020 \)) compared to non-INF day 3 controls (Figure 77B). Lastly, the gene transcript levels of early collagen production marker P4HA1 was significantly up-regulated at days 3 (1.828±0.486; \( p=0.013 \)), 8 (3.190±0.572; \( p=0.003 \)), and 15 (2.573±0.692=; \( p=0.018 \)) compared to non-INF day 3 controls (Figure 77D). No statistical changes were observed in gene markers Col XII or Decorin at either time-points.
Figure 77: Representative images of the relative fold change ± CI ($2^{-\Delta\Delta Ct \pm 2SD}$) of AFRPs cultured in INF and Non-INF culture conditions. At days 3, 8, and 15 RNA isolated from AFRP scaffolds were analyzed for AF putative gene profiles: A) Collagen I, B) Collagen 12, C) Collagen 5, D) P4HA1, and E) Decorin.
Table 23: Effects of TNF-α and IL-1β on gene expression values (fold change) for select AF putative gene markers of Day 3, 8, and 15 AFRPs. Fold change was calculated as $2^{-\Delta Ct}$. 95% confidence interval is calculated as $2^{-\Delta Ct \pm 2SD}$. Statistical difference was calculated using a paired student’s t-test performed at the ΔCt level (Ct target – Ct internal control [18s]) to non-INF day 3 controls. Statistical significance (p≤0.05) is denoted with a bold * and red text.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Day</th>
<th>Fold Change</th>
<th>95% CI ($2^{-\Delta Ct \pm 2SD}$)</th>
<th>Average ΔCt ± SD</th>
<th>p-value (vs. Day 3 Non-INF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col 1</td>
<td>3</td>
<td>0.554</td>
<td>(0.497, 0.616)</td>
<td>4.377 ± 0.095</td>
<td>0.020*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.969</td>
<td>(2.245, 3.93)</td>
<td>1.953 ± 0.336</td>
<td>0.006*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.266</td>
<td>(1.517, 3.384)</td>
<td>2.343 ± 0.492</td>
<td>0.030*</td>
</tr>
<tr>
<td>Col 5</td>
<td>3</td>
<td>0.609</td>
<td>(0.506, 0.732)</td>
<td>10.310 ± 0.164</td>
<td>0.012*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.597</td>
<td>(0.469, 0.761)</td>
<td>10.337 ± 0.254</td>
<td>0.020*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.173</td>
<td>(0.038, 0.792)</td>
<td>12.127 ± 1.897</td>
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<tr>
<td>Col 12</td>
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<td>0.847</td>
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<tr>
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<td>8</td>
<td>1.519</td>
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<td>9.353 ± 0.463</td>
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<tr>
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<td>15</td>
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<td>(0.913, 3.097)</td>
<td>9.207 ± 0.731</td>
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</tr>
<tr>
<td>P4HA1</td>
<td>3</td>
<td>1.828</td>
<td>(1.238, 2.698)</td>
<td>8.870 ± 0.344</td>
<td>0.013*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.190</td>
<td>(2.017, 5.403)</td>
<td>8.067 ± 0.458</td>
<td>0.003*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.573</td>
<td>(1.479, 4.475)</td>
<td>8.377 ± 0.600</td>
<td>0.018*</td>
</tr>
<tr>
<td>Decorin</td>
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<td>0.633</td>
<td>(0.310, 1.291)</td>
<td>6.410 ± 0.630</td>
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</tr>
<tr>
<td></td>
<td>8</td>
<td>1.209</td>
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<tr>
<td></td>
<td>15</td>
<td>0.835</td>
<td>(0.435, 1.603)</td>
<td>6.010 ± 0.517</td>
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Appendix G

Biaxial Heat Map Tracking of Stress and Strain Distribution

Acellular AFRP

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</tr>
<tr>
<td>4%</td>
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<td></td>
</tr>
<tr>
<td>10%</td>
<td>Fast</td>
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Bovine AF

1% Slow

4% Medium

10% Fast
Day 28 bAFC

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<td><img src="image11" alt="Image" /></td>
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Day 56 hADSC

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<td>[Image]</td>
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Appendix H

Animal Study Model and Treatment ELISA Data for Inflammatory Cytokines

Seventeen weeks post-injection (pilot), AF and NP tissue from treated, degenerate, vehicle, and uninjured IVDs (n=6/group) were assessed for the pro-inflammatory cytokines IL-1β, TNF-α, MMP-1, MMP-13, ADAMTS-4, and IL-6. Prior to tissue isolation, the ovine IVDs were potted in functional spinal units and kinematic testing was conducted. During this mechanical testing, the IVDs underwent two freeze-thaw cycles prior to harvest. Tissues were isolated, cryo-pulverized, and homogenized on ice in lysis buffer (750uL/10mg of tissue; 100mM TRIS buffer (pH=7.4), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.5% Deoxycholic Acid, and 1x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). Homogenates were then agitated for 60 min at 4°C, and the supernatant was isolated via centrifugation (5 minutes at 10,000xg). Supernatants underwent Enzyme-linked immunosorbent assay (ELISA) for ovine IL-1β (AB Clonal Science, Inc., Woburn, MA) according to the manufacturer’s instructions, the results of which were normalized to the total protein quantified via Bicinchoninic Acid (BCA) assay (ThermoFisher Scientific, Waltham, MA).

Intradiscal C-ABC Injection Results in Significantly Increased Concentrations of Pro-Inflammatory Cytokines in AF Tissue of IVD Compared to NP Tissue

ELISA analysis of treatment groups demonstrated no significant differences in cytokine production within the AF or NP region for TNF-α, IL-1β, IL-6, MMP-1, MMP-13, or ADAMTS-4 (Figures 78 & 79, respectively). However, cytokine arrays
demonstrated a significant increase in all measured cytokines between week 6 degenerate explants and week 17 IVDs. Of note, quantification of the AF tissue region of the IVD demonstrated significantly higher production of similar cytokines measured in the NP tissue region of the IVD.

Figure 78: Representative graphs depicting the cytokine concentrations of A) TNF-α, B) IL-1β, C) IL-6, and D) MMP-1 within the AF tissue region of sheep IVDs. Graphical results are normalized concentrations from to total protein quantification
from BCA analysis. Dotted line separates degenerate explant tissue from week 6 and week 17 IVD samples.
Figure 79: Representative graphs depicting the cytokine concentrations of A) TNF-α, B) IL-1β, C) IL-6, D) MMP-13, and E) ADAMTS-4 within the NP tissue region of sheep IVDs. Graphical results are normalized concentrations from total protein quantification from BCA analysis. Dotted line separates degenerate explant tissue from week 6 and week 17 IVD samples.

Intradiscal C-ABC Injection Results in Significantly Increased Cytokine Concentrations in the NP Tissue Region of the IVD

ELISA analysis of repaired groups was compared to uninjured and degenerate controls for cytokine production within the NP or AF region for TNF-α, IL-1β, IL-6, MMP-1, MMP-13, or ADAMTS-4 (Figures 80 & 81, respectively). Within the NP region, significant increases were observed for both repaired only and repaired + hADSCs for TNF-α (p=0.043 and p=0.008, respectively), IL-1β (p=0.047 and p=0.049, respectively), MMP-13 (p=0.029 and p=0.061, respectively) compared to uninjured controls. Additionally, the repair only IVDs demonstrated significant increases in cytokine production within the NP region for IL-6 (p=0.036) and ADAMTS-4 (p=0.094) compared to uninjured controls. Within the AF region, repair + hADSCs demonstrated a trend of a decrease in cytokine production for both repaired only and repaired + hADSCs for TNF-α, IL-1β, IL-6, and MMP-1 compared to uninjured controls.
Figure 80: Representative graphs depicting the cytokine concentrations of A) TNF-α, B) IL-1β, C) IL-6, and D) MMP-1 within the AF tissue region of sheep IVDs. Graphical results are normalized concentrations from total protein quantification from BCA analysis. Dotted line separates degenerate explant tissue from week 6 and week 17 IVD samples.
Figure 81: Representative graphs depicting the cytokine concentrations of A) TNF-α, B) IL-1β, C) IL-6, D) MMP-13, and E) ADAMTS-4 within the NP tissue region of sheep IVDs. Graphical results are normalized concentrations from total protein quantification from BCA analysis. Dotted line separates degenerate explant tissue
from week 6 and week 17 IVD samples. Bold * indicates significant difference (p<0.05) compared to uninjured controls.

Cytokine production of IVD pro-inflammatory cytokines showed elevated concentration levels in all study groups: uninjured, vehicle, and degenerate IVDs. This could be contributed to a systemic effect due to an inflammatory response that was caused by the C-ABC injection.
Appendix I

Surgical Tracking Sheets of Ovine Study

Clemson University

Preliminary evaluation of a novel nucleus pulposus replacement and annulus fibrous patch in an ovine model of intervertebral disc degeneration: Pilot Study

PSRL Study ID: SRL 16-15

Disk Degeneration Necropsy Record

Date: 02/27/2017

Sheep Ear Tag #: 6993

Weight: 71 kg

1. Perform euthanasia immediately prior to MRI.

2. Perform MRI with the following sequences:
   - Sagittal T1
   - Sagittal T2
   - Sagittal STIR

3. Perform necropsy and remove spine from T11 to L7 en bloc.

4. Remove extraneous tissue from the spine.

5. Following en bloc removal of spine, perform annulotomy and nucleotomy of specific degenerated IVDDs.

   Annulotomy, nucleotomy and repair of L1/L2 IVD: YES ☑ NO ☐

   Annulotomy, nucleotomy and repair of L2/L3 IVD: YES ☑ NO ☐

   Annulotomy, nucleotomy and repair of L3/L4 IVD: YES ☑ NO ☐

5. Following procedures, place nucleus pulposus and annulus fibrosis material from degenerated IVDDs into individual containers with storage solution.

6. Ship samples fresh overnight on ice to the following address:

   Attn: Jeremy Mercuri
   Laboratory of Orthopaedic Tissue Regeneration & Orthobiologics
   313 Rhodes Engineering Research Center
   Clemson University
   Clemson, SC 29634
   (864) 656-9978

Comments:

Procedure performed by [Signature] and [Signature] 02/27/2017

Rev. 02/27/2017
Clemson University
Preliminary evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pilot Study
PSRL Study ID: SRL 16-15

Disk Degeneration Necropsy Record
Date: 6/7/2017

Sheep Ear Tag: 9617- Weight: 65 kg

1. Perform euthanasia immediately prior to MRI: ☑
2. Perform MRI with the following sequences:
   1. Sagittal T1
   2. Sagittal T2
   3. Sagittal STIR
3. Perform necropsy and remove spine from T11 to L7 en bloc: ☑
4. Remove extraneous tissue from the spines: ☑
5. Following en bloc removal of spine, perform annulotomy and nucleotomy of specific degenerated IVDs: ☑
   - Annulotomy, nucleotomy and repair of L1/L2 IVD: YES ☑ NO ☑
   - Annulotomy, nucleotomy and repair of L2/L3 IVD: YES ☑ NO ☑
     Placed 3 nucleus replacements
     Placed 5x8 mm annuloplasty patches
   - Annulotomy, nucleotomy and repair of L4/L5 IVD: YES ☑ NO ☑

5. Following procedures, place nucleus pulposus and annulus fibrosus material from degenerated IVDs into individual containers with storage solution: ☑

6. Ship samples fresh overnight on ice to the following address:
   Attn: Jeremy Mercutz
   Laboratory of Orthopaedic Tissue Regeneration & Orthobiologics
   313 Rhodes Engineering Research Center
   Clemson University
   Clemson, SC 29634
   (864) 656-0978

Comments:

Procedure performed by: [Signatures] 6/7/2017

Rev. 02/27/2017
Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/22/2017

Sheep Ear Tag # CH43

Surgeons: Dr. H. Seim III, Dr. J. Easley, Scrub Asst. F. Brown, E. Bisa, A. McFarland
Surgery Techs: Kimberly Lebock, Other: Cassie Bennett, A. McFarland
Anesthetists: L. March, O. Other

SURGICAL TECHNIQUE

1. Position of Sheep: Lateral recumbency.
2. Wood clipped from left lateral lumbar region.
3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography.
4. Retropertitoneal approach to L1-L4 lumbar vertebrae ventral to transverse processes.
5. L1-L4 vertebral body exposure.
6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc.
7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc.
   ID of NPR: Non-Swede.
   # of NPRs implanted into disc: 3
8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire.
   # of knots: 8

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc.
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:
    ID of NPR: Swede.
    # of NPRs implanted into disc: 5
11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire.
    # of knots: 8

Closure:
1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern.
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern.
3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern.

Comments or Intraoperative Complications (describe):
No dermaband used, placed only suture to secure.

Procedure performed by: [Signature]
Rev. 03/21/2017
Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/2/2017

Sheep Ear Tag #: 367, Age (approx): 3+, Sex: 

Surgeons: Dr. H. Stein III [ ], Dr. J. Easley [X], Scrub Asst:

Surgery Techs: Kimberly Labsock [X], Other:

Anesthetists: L. Mangin [X], Other:

Initiation of Disk Degeneration

1. Position of Sheep: Lateral recumbency:

2. Wool clipped from left lateral lumbar region:

3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography:

4. Retrophleboguinal approach to L1-L4 lumbar vertebrae ventral to transverse processes:

5. L1-L4 vertebral body exposure:

6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc:

7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc:

   ID of NPR: Scrubbed, # of NPRs implanted into disc:

8. Close window defect of the L2-L3 annulus fibrosus with APRP with 4-0 Fiberwire:

   # of knots: 3

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc:

10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:

    ID of NPR: Scrubbed, # of NPRs implanted into disc:

11. Close window defect of the L3-L4 annulus fibrosus with APRP with 4-0 Fiberwire:

    # of knots: 3

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern:

2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern:

3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern:

Comments or Intraoperative Complications (describe):

Procedure performed by: [Signature]

Rev. 05/21/2017

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Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/1/2017

Sheep Ear Tag #: 6416

Age (approx): 3+

Surgeons: Dr. H. Seim III [ ] Dr. J. Easley [x] Scrub Asst: [ ]

Surgery Tech: Kimberly Lehnock [x] Other: [ ]

Anesthetists: L. Mangia [x] Other: [ ]

Surgical Technique

1. Position of Sheep: Lateral recumbency: [x]

2. Wool clipped from left lateral thoracic region: [x]

3. Locate T11/L1 disk space and L1 vertebral body utilizing radiography: [x]

4. Retroperitoneal approach to L1-L4 vertebrae ventral to transverse processes: [x]

5. L1-L4 vertebral body exposure: [x]

6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc: [x]

7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc: [x]

   ID of NPR: 955G6X72P0

   # of NPRs implanted into disc: 1

8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire: [x]

   # of knots: 6

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc: [x]

10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc: [x]

   ID of NPR: 955G6X72P0

   # of NPRs implanted into disc: 1

11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire: [x]

   # of knots: 6

   [Note: More than one Fiberwire was placed beyond the defect to ensure stability.]

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern: [x]

2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern: [x]

3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern: [x]

Comments or Intraoperative Complications (describe):

Video from camera combined with cast video

Procedure performed by: [Signature] and [Signature] 05/22/2017

Rev. 05/21/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/25/2017

Sheep Ear Tag # 598

Age (months): 12

Surgery: Dr. H. Sieim III, Dr. J. Beans

Scrub Assistant: Pate, Nurse: Meldahl

Surgery Tech: Kimberly Lohbeck

Other: Alex P. Fender, T. Waple, Mason

Anesthetists: L. Maagin, Other

SURGICAL TECHNIQUE

Initial: Position of Sheep: Lateral recumbency.

1. Wool clipped from left lateral lumbar region.

2. Locate T13/L1 disk space and L1 vertebral body utilizing radiography.

3. Retropereitoneal approach to L1-L4 lumbar vertebral ventral to transverse processes.

4. L1-L4 vertebral body exposure.

5. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc.

6. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotimized disc.

7. ID of NPR: S1000

8. # of NPRs implanted into disc: 1

8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire.

9. # of knots: 8

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc.

10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotimized disc.

ID of NPR: S1000

# of NPRs implanted into disc: 1/2

11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire.

# of knots: 8

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern.

2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern.

3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern.

Comments or Intraoperative Complications (describe):

Procedure performed by: 

Rev. 05/21/2017

5/25/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-01

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/2/2017

Sheep Ear Tag #: 44-3
Age (approx.): 3+
Surgeons: Dr. H. Seim III [ ], Dr. J. Easley [ ] [Scrub Asst.]
Surgery Techs: Kimberly Lebhec [Other:]
Anesthetists: L. Maing [Other:]

SURGICAL TECHNIQUE

1. Position of Sheep: Lateral recumbency:
2. Wool clipped from left lateral lumbar region:
3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography:
4. Retroperitoneal approach to L1-L4 lumbar vertebral ventral to transverse processes:
5. L1-L4 vertebral body exposure:
6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc:
7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc:
   ID of NPR: 1
   # of NPRs implanted into disc: 1
8. Close window defect of the L2-L3 annulus fibrosus with AFPR with 4-0 Fiberwire:
   # of knots: 3
9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc:
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:
    ID of NPR: 2
    # of NPRs implanted into disc: 1
11. Close window defect of the L3-L4 annulus fibrosus with AFPR with 4-0 Fiberwire:
    # of knots: 3

Closure:
1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern:
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern:
3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern:

Comments or Intraoperative Complications (describe):

Procedure performed by:

Rev. 05/21/2017

65 / 2 /2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Sheep Ear Tag #: C98

Surgical: Dr. H. Sein III □ Dr. J. Easley □
Scrub Asst: K. S. Sein III
Surgery Techs: Kimberly Lebock □ Other: Alex M. Good
Anesthesiats: L. Mangan □ Other □

Date: 5/23/2017

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Initiation of Disk Degeneration

1. Position of Sheep: Lateral recumbency: □
2. Wool clipped from left lateral lumbar region: □
3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography: □
4. Retropertioneal approach to L1-L4 lumbar vertebrae ventral to transverse processes: □
5. L1-L4 vertebral body exposure: □
6. Locate L1-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc: □
7. Following subtotal nucleotomy of L1-L4 disc, place NPR implantation into nucleotomized disc:
   ID of NPR: S2202 □ of NPRs implanted into disc: 1
8. Close window defect of the L1-L4 annulus fibrosus with AFRP with 4-0 Fiberwire:
   # of knots: □
9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc: □
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:
    ID of NPR: A2202 □ of NPRs implanted into disc: 1
11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire:
    # of knots: □

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern: □
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern: □
3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern: □

Comments or Intraoperative Complications (describe):
- Pelvic cavity entered around L1/L2.
- Postoperative care of sheep following NPR implantation:
- Bile leak (animal toxic no noted); no parenteral fluids
- Adjacent vertebrae noted and treated for life

Procedure performed by: [Signature]
Rev. 05/21/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/23/2017

Sheep Ear Tag #: 95385; Age (approx.): 3+;
Surgeon: Dr. H. Selin [ ]; Dr. J. Easley [x] Scrub Aide: [ ]
Surgery Team: Kimberly Lebock [ ] Other: [ ]
Anesthetists: L. Mangis [x] Other: [ ]

1. 2.07

SURGICAL TECHNIQUE

1. Position of Sheep: Lateral recumbency: [x]
2. Wool clipped from left lateral lumbar region: [ ]
3. Locate T13-L1 disk space and L1 vertebral body utilizing radiography: [x]
4. Retropereitoneal approach to L1-L4 lumbar vertebral ventral to transverse processes: [x]
5. L1-L4 vertebral body exposure: [x]
6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc: [x]
7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc: [x]
   ID of NPR: [ ] # of NPRs implanted into disc: [1/2]
8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 FiberWire: [x]
   # of knots: [6]

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc: [x]
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc: [x]
    ID of NPR: [ ] # of NPRs implanted into disc: [1]
11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 FiberWire: [x]
    # of knots: [6]

Closure:
1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern: [x]
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern: [x]
3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern: [x]

Comments or Intraoperative Complications (describe):

Procedure performed by: [Signature] and [Signature]
Rev. 05/21/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/18/2017

Sheep Ear Tag #2510:

Age (approx.): 2

Surgeons: Dr. H. Sein III Dr. J. Easley Scrub Asst: Kyle Scrub Tech: Kimberly Lebsock Other: Alvi McFarland 4 Way Verity Dissector

Anesthesiata: L. Mangin Other:

SURGICAL TECHNIQUE

Initiation of Disk Degeneration

1. Position of Sheep: Lateral recumbency:
2. Wool clipped from left lateral lumbar region:
3. Locate T13-L1 disk space and L1 vertebral body utilizing radiography:
4. Retroprenumeral approach to L1-L4 lumbar vertebrae ventral to transverse processes:
5. L1-L4 vertebral body exposure:
6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc:
7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc:

ID of NPR: X/A

# of NPRs implanted into disc: X/A

8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire:

# of knots: X/A

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc:
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:

ID of NPR: X/A

# of NPRs implanted into disc: X/A

11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire:

# of knots: X/A

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern:
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern:
3. Close skin with 2-0 Nylon suture in a Ford-interlocking pattern:

Comments or Intraoperative Complications (describe):

Animal was euthanized prior to surgery. Histopath and cultures sample taken from L1-2 ZPD. Arteries and nerves sampled for analysis. Easton L12, L23, L34 obtained for gomori.

Procedure performed by: 

Rev. 05/21/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5 / 24 / 2017

Sheep Ear Tag #OAH; Age (months): 3+

Surgeons: Dr. H. Seism III ☐ Dr. J. Eagle ☑ Scrub Anea: A+ × ☑ Dr. __________

Surgery Techs: Kimberly Lebock ☑ Other: ______________

Anesthetists: L. Margot ☐ Other: ______________

SURGICAL TECHNIQUE

Initiation of Disk Degeneration

1. Position of Sheep: Lateral recumbency: ☑
2. Wool clipped from left lateral lumbar region: ☑
3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography: ☑
4. Retropreperitoneal approach to L1-L4 lumbar vertebral column to transverse processes: ☑
5. L1-L4 vertebral body exposure: ☑
6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc: ☑
7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc: ☑

ID of NPR: ______________ # of NPRs implanted into disc: __________

8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire: ☑

# of knots: __________

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc: ☑
10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc: ☑

ID of NPR: ______________ # of NPRs implanted into disc: __________

11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire: ☑

# of knots: __________

Closure:
1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern: ☑
2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern: ☑
3. Close skin with 2-0 Nylon suture in a Ford interlocking pattern: ☐

Comments or Intraoperative Complications (describe):

Procedure performed by: ___________________________ ___________________________ 5 / 24 / 2017

Rev. 05/21/2017
Clemson University

Evaluation of a novel nucleus pulposus replacement and annulus fibrosus patch in an ovine model of intervertebral disc degeneration: Pivotal study

PSRL Study ID: SRL 17-03

Nucleus Pulposus Replacement and Annulus Fibrosus Repair Operative Record

Date: 5/24/2017

Sheep Ear Tag # CML X

Ass (approx): 3+

Surgeons: Dr. H. Sein III [ ] Dr. J. Edley [ ] Scrub Asst: ALEX [ ] Other:

Surgery Techs: Kimberly Leabock [ ] Other:

Anesthetist: L. Mangin [ ] Other:

SURGICAL TECHNIQUE

1. Position of Sheep: Lateral recumbency.

2. Wool clipped from left lateral lumbar region.

3. Locate T13/L1 disk space and L1 vertebral body utilizing radiography.

4. Retroperitoneal approach to L1-L4 vertebral body ventral to transverse processes.

5. L1-L4 vertebral body exposure.

6. Locate L2-L3 disk space and perform a subtotal nucleotomy of the intervertebral disc.

7. Following subtotal nucleotomy of L2-L3 disc, place NPR implantation into nucleotomized disc:
   ID of NPR: [ ] # of NPRs implanted into disc: [ ]

8. Close window defect of the L2-L3 annulus fibrosus with AFRP with 4-0 Fiberwire:
   # of knots: [ ]

9. Locate L3-L4 disk space and perform a subtotal nucleotomy of the intervertebral disc.

10. Following subtotal nucleotomy of L3-L4 disc, place NPR implantation into nucleotomized disc:
    ID of NPR: [ ] # of NPRs implanted into disc: [ ]

11. Close window defect of the L3-L4 annulus fibrosus with AFRP with 4-0 Fiberwire:
    # of knots: [ ]

Closure:

1. Close dorsal abdominal musculature with 0 absorbable suture in a continuous pattern.

2. Close subcutaneous tissue with 2-0 absorbable suture in a continuous pattern.

3. Close skin with 2-0 Nylon suture in a Ford interlocking pattern.

Comments or Intraoperative Complications (describe):

Procedure performed by: [Signature] and [Signature]

Rev. 05/21/2017
REFERENCES


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