INTERVERTEBRAL DISCS: METABOLIC RESPONSES TO LOAD, INJURY, AND PRO-INFLAMMATORY STIMULATION

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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INTERVERTEBRAL DISCS: METABOLIC RESPONSES TO LOAD, INJURY, AND PRO-INFLAMMATORY STIMULATION

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ABSTRACT

Introduction: Intervertebral disc (IVD) degeneration is a significant cause of back pain and disability. Factors such as trauma and magnitude and frequency of loading are associated with IVD degeneration. However, the contributions of mechanical loading, injury, pro-inflammatory stimulation, or any/all of these factors combined, are not fully known. Excessive/abnormal loading is thought to contribute to degeneration through disruption of the extracellular matrix and pro-inflammatory/degradative responses of the tissue. The objective of this study was to determine how magnitude of loading with or without injury and/or pro-inflammatory stimulation affects the metabolic response of a whole-organ rat tail IVD explant model.

Methods: Endplate-IVD-endplate whole-organ explants were harvested from the tails of rats. Injured groups were punctured with a 20G needle to the center of the nucleus and stimulated with or without 10ng/mL IL-1β. Explants were cultured in the Flexcell system at various loading magnitudes for 3 days. Media was collected for biomarker analysis and tissue for extracellular matrix composition analysis.

Results: Uninjured explants had increased pro-inflammatory, anti-degradative, and degradative biomarker release with the application of load. There was also an increased release of glycosaminoglycan (GAG) to the media and decreased tissue GAG with load. With injury, there was a general decrease in pro-inflammatory and anti-degradative biomarker release and increased GAG released to the media with the application of load. In injured and IL-1β stimulated explants, there was increased pro-inflammatory and anti-degradative biomarker release to the media, decreased degradative activity, and decreased GAG release to the media with the application of load.

Discussion: These results suggest that, with uninjured and non-inflamed discs, loading is associated with relevant pro-inflammatory and degradative responses in a magnitude-dependent manner. Additionally, loading may counteract the inflammatory and degradative responses in injured discs associated with pro-inflammatory cytokine stimulation. These differences have clinical importance as they can help in the development of methods to mitigate risk for IVD degeneration. Further study is required to elucidate the mechanisms underlying IVD responses to load and/or injury with or without cytokine stimulation and translate these findings to the clinical setting.

Chapter 1: Literature Review

Introduction:

Intervertebral disc (IVD) degeneration is a significant contributor to back pain, and 70-85% of all people will suffer from back pain at some point in their lives.[1,2] Back pain resulting from IVD degeneration is a significant cause of disability and decreased quality of life for patients.[3] Further, the costs for treatment and the financial losses associated with disability for those suffering from IVD degeneration have been calculated to be over $100 billion annually in the US.[4,5] Unfortunately, there is no cure for IVD degeneration, and current therapies only relieve symptoms and do not restore IVD tissue composition, architecture, or function.[3,6]

Intervertebral Disc Morphology and Function:

Intervertebral discs lie between two vertebrae along the length of the spinal column. The human spine contains 23 intervertebral discs: 6 cervical, 12 thoracic, and 5 lumbar.[7] IVDs are composed of three distinct tissue types: the annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplates. The annulus fibrosus forms a fibrous ring around the inner, gelatinous nucleus pulposus. Together, the AF and NP are superiorly and inferiorly bordered by a cartilage endplate, which regulates diffusion of nutrients into, and waste out of, the NP.[8] As the NP and inner AF of the disc are avascular, these portions of the IVD receive nutrients via diffusion from blood vessels in the adjacent vertebrae that terminate at the cartilage endplate. Additionally, very few nerve endings penetrate the IVD with the dorsal root ganglia innervating only the superficial fibers of the annulus fibrosus. In terms of biomechanical functions, intervertebral discs are designed to absorb compressive forces applied to the spine, allow spinal mobility, and provide stability to the spine.[8,9]

The annulus fibrosus is divided into outer and inner components.[7,10–12] This distinction is not visible to the naked eye, but it can be delineated microscopically and histologically.[12] The outer annulus fibrous is composed primarily of type I collagen, proteoglycans, elastic fibers, and water.[8–11] The type I collage fibrils are arranged at oblique angles in 15-25 lamellae with collagen fibers in one lamellae organized at approximately perpendicular angles to the collagen fibers in the adjacent lamellae.[8,10,13,14] Within the collagen type I fiber, collagen type V core proteins act to define the diameter of the collagen

type I fibrils.[15,16]

These lamellae are connected and stabilized by translamellar cross-bridges containing aggrecan, versican, collagen type VI, and elastic fibers.[11,18–20] The recoil properties of the elastic fibers aid in collagen crimp patterns during compression and relaxation and help in returning the IVD to its pre-loaded orientation.[18,19] Among these cross-bridges, aggrecan and versican are thought to act as lubricating agents.[18] Decorin and biglycan are also considered to play critical roles in the integration of elastic fibers to the extracellular matrix.[21] As these bridges span upwards of eight lamellae, the collagen type VI is thought to mesh into the existing collagen network of the lamellae through decorin and biglycan linkages.[18,22] The collagen fibrils themselves are cross-linked via decorin and biglycan to protect against cleavage of collagens, and fibromodulin expression has been seen to coincide with fibrillogenesis and extracellular matrix organization.[11] These small leucine rich proteins also act as signaling molecules that control proliferation, differentiation, ECM synthesis, and ECM degradation.[23] As the AF approaches the nucleus pulposus and transitions into the inner annulus fibrosus, the collagen content shifts towards higher collagen type II, type IX, and type XI, and the collagen fibrils become less organized.[10,17]

The nucleus pulposus is a gel-like structure in the center of the IVD.[8,9] Its roles include shock absorption, load distribution, and local tissue lubrication.[9] The NP is composed of predominately type-II collagen, proteoglycans, water, and chondrocyte-like cells.[8,13,14,24] However, there is no uniform organization of the collagen fibers of the NP. The cells of the NP have integrin receptors that interact with laminins and fibronectin, which bind to collagen fibers in the ECM.[23,25–28] These integrins facilitate interactions between the cells and the surrounding matrix: regulating cell signaling, modulating cell survival and proliferation, and protein production as well as responses to environmental stimuli.[17,28,29] The nucleus pulposus is composed of 70-90% water by wet weight and has higher water and proteoglycan contents compared to the AF and cartilage endplates.[8,17] This high water and proteoglycan content enables the NP to distribute approximately equal forces to the surrounding annulus fibrosus when the disc is compressed allowing the IVD to effectively absorb shock.[17]

The superior and inferior aspects of the disc are adjoined to cartilage endplates (CEPs).[30–32] The CEP in the lumbar region of the spine has a mean thickness of 0.77 ± 0.24mm and minimal thickness at the center measuring 0.54 ± 0.12mm and occupy 90% of the surface area of the interface between the IVD and

vertebral bodies.[14,31] The outer two-thirds of the annulus fibrosus is not covered with the CEP as it is anchored to the bone of the surrounding vertebral body via limited interwoven bundles of collagen.[32] The CEP is composed of uniform, homogeneous hyaline cartilage similar to that of articular cartilage containing type-II collagen, proteoglycans, and water. In contrast, the pericellular matrix in the CEP is more randomly organized than articular cartilage as there is no columnar organization of the cells as seen in the deep zone of articular cartilage.[30] Instead, the collagen fibers are arranged parallel to the vertebrae and NP.[30] The CEP's pericellular matrix consists of collagen type III and collagen type VI.[14] Vertebral capillaries terminate at the endplate, therefore nutrients must diffuse through the CEP in order to reach the NP and inner AF.[8] The primary roles of the CEP include exchange of nutrients into and waste out of the IVD and transmission of compressive loads to the disc through maintenance of NP pressure.[30]

Intervertebral Disc Degeneration:

Intervertebral disc degeneration (IVDD) may occur due to numerous conditions including trauma, genetics, lifestyle, aging, or atherosclerosis.[33,34] Excessive or repetitive loading, herniation, or spinal surgery may accelerate degeneration.[33,35] IVDD is thought to initially develop in the NP, starting with loss of proteoglycan and water content in conjunction with increased type-I collagen content.[2,13,33,36,37] These alterations result in a more fibrous NP and loss in distinction of the boundary between AF and NP.[2,33,36,38] As a result, the disc experiences a loss of structural integrity as the NP can no longer effectively resist axial compression.[33,37] This change in NP structure often results in a loss of disc height and increased tensile forces on the annulus fibrosus.[33,39–42] Overloading of the AF leads to tissue failure in the form of disc protrusion and/or herniation.[33,36] Another factor that is believed to contribute to the changes observed in the NP is calcification of the cartilage endplates.[39,43] Calcification leads to reduced nutrient and waste exchange in the disc and reduced cell viability in the NP and CEP.[14,39,43] The decrease in the viable cell population decreases the tissues' abilities to maintain normal tissue matrix composition, which further contributes to the progression of disc degeneration.[37]

Loss of notochord cells in the NP is another consistent characteristic of IVD degeneration. Two mechanisms of disease for this cell loss have been proposed.[44] One mechanism of notochord cell loss is thought to be related to normal development and maturation with contributing to initial matrix synthesis and then undergoing apoptosis or necrosis. The other proposed mechanisms is a process by which

notochord cells serve as NP progenitor cells and undergo terminal differentiation to give rise to cartilage-like NP cells. In IVDs, notochord cells have been reported to inhibit angiogenesis by suppressing VEGF expression in endothelial cells. Regarding degeneration, progressive loss of large vacuolated notochord cells is seen after birth, wherein the NP becomes populated by small cartilage-like NP cells, which is thought to be associated with IVDD. Additionally, compressive stress of 1.3MPa in rat tails led to notochord cell disappearance after 7 days when smaller, round, chondrocyte-like cells clustered, while AF puncture induced transformation of notochord cells into a chondrogenic and subsequently fibrocartilaginous phenotype.[45]

Clinically, IVD degeneration is most often diagnosed based on related symptoms of pain and dysfunction in conjunction with loss of disc height or loss of water content as determined by radiographs and/or MRI.[46] However, physical changes in IVDs as determined via imaging methodologies do not always manifest in clinical disease and the development of pain, as high-intensity zones in MRIs of lumbar spines were found in patients that were asymptomatic.[3,47] Therefore, there is a critical need to determine which morphologic components of IVD degeneration are directly related to pain and dysfunction such that they require treatment. Further, there is a need to better characterize the pathophysiology of IVD degeneration in order to develop novel diagnostic and prognostic methodologies to determine which patients will develop symptoms during development and progression of IVD degeneration.

There are currently no treatments available for patients with IVD degeneration that can fully restore tissue composition, structure, and function.[3,6,48] Initial treatments are often focused on symptomatic pain relief, including NSAIDs, physical therapy, chiropractic care, and opioids.[3,49] The goals of these treatments are to reduce pain and strengthen the 'core musculature' to increase support and stability of the spinal column.[3] If patients do not experience relief with these treatments, then more invasive treatments including epidural injection consisting of anesthetics, steroids, or both are a commonly employed as a next attempt to provide the patient symptomatic relief. If these treatments do not result in relief of symptoms, then surgical interventions including microdiscectomy, laminectomy, or full discectomy with spinal fusion are considered based on symptoms, anatomic site(s), and severity of degeneration and disruption. These surgical methods involve removal of herniated tissue, impingement relief, and spinal stabilization. While all these treatments can provide symptom relief in the short term, none are able to restore the structure and

function of the IVD. Further, there is high patient to patient variability in long-term outcomes, with many patients requiring further surgical interventions to the operated or adjacent IVDs segments after initial surgical intervention.[50] Therefore, there is a critical need to study the pathophysiology of IVD degeneration to allow for the development of novel treatment strategies to restore the structure and function of the degenerative IVD.

Degradative enzymes

In healthy IVDs , there is a homeostatic balance among production of degradative enzymes and inhibitors to maintain tissue physiology, composition and structure. However, during IVD degeneration, it is theorized that there is a shift in this balance towards higher degradative enzyme activity, resulting in breakdown of NP and AF and compromising structure and function in progression towards IVD degeneration.[51,52] Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are known to play major roles in extracellular matrix degradation.[52,53] MMP-1, MMP-8, and MMP-13 are collagenases and cleave intact collagen fibrils.[54–56] MMP-1 cleaves intact collagen type-I, II, III and XI; MMP-8 cleaves collagen type-I, II, and III with a preferential affinity for collagen type-I; and MMP-13 preferentially cleaves collagen type-II. MMP-2 is a gelatinase and degrades denatured collagen fibrils such as collagen types-I, II, III, V, and XI. MMP-3 is a stromelysin which digests non-collagenous matrix proteins like aggrecan, fibronectin, decorin, and other proteoglycans;[55] and MMP-14 is a membrane-type MMP known to activate other MMPs such as MMP-2.[56–58]

Aggrecanases (ADAMTS) comprise a class of degradative enzymes with affinity for proteoglycans, and ADAMTS-4 and ADAMTS-5 have been extensively studied in relation to cartilage degradation during osteoarthritis.[59] Previous studies have indicated that ADAMTS 4 can degrade aggrecan, biglycan, decorin, cartilage oligomeric protein (COMP), and fibromodulin, while ADAMTS-5 can degrade aggrecan and biglycan.[60–62] Further, ADAMTS-5 is constitutively produced by cartilage tissue, indicating a role for the enzyme during normal tissue turnover, while ADAMTS-4 production is induced during the development and progression of OA.[60] In discs, ADAMTS-4 gene expression has been reported to be upregulated in degenerative IVDs, and ADAMTS-4 expression levels correlated with degeneration severity grade of the IVD.[53,63,64] However, the data for ADAMTS-5 expression during IVD degeneration is less

clear, with some studies indicating a significant increase of ADAMTS-5 expression in degenerative discs, and others reporting no significant differences between degenerative and healthy IVDs.[53,63,65] In the IVD, ADAMTS cleave aggrecan and the resulting fragment is able to diffuse within the tissue but is restricted from diffusing out due to the CEP and AF.[66] Thus, aggrecan fragments accumulate in the NP, and the fragments have been implicated as a contributing factor for progression of IVDD.

Tissue inhibitors of metalloproteinases (TIMPs) bind to MMPs and ADAMTSs at a 1:1 ratio to inhibit the activity of the degradative enzymes.[52] There are four known TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) and all but TIMP-4 have been shown to be produced by the NP and AF of the IVD. The production of TIMP-1 is upregulated during IVDD, and it is known to inhibit various collagenases and gelatinases with a strong affinity for MMP-3.[58] The production of TIMP-2 is also increased during IVDD, and has been shown to have a strong affinity for MMP-2 and ADAMTS. The production of TIMP-3 is downregulated during IVDD and has been shown to have a strong affinity for ADAMTS.[52,53,58,60,64,67]

Mechanical loading

The human spine experiences load at all times based on gravity and muscle forces with different extremes of load based on posture, activity, and body composition.[37] Compared to sitting, standing reduces pressure in the disc by around 30% and reclining is believed to reduce pressure and load on discs by another 36%.[68] These values depend on IVD surface area and volume, body weight, and body position.[68] It has also been determined that load on the L3-4 segments in the sitting and standing position with 20 degrees of flexion was 250% of total body weight.[36] It has been reported that healthy adult human spines sustain 0.1-0.3 MPa of compression during daily activity and up to 2 MPa of compression when lifting a 20kg weight with a rounded back.[69] Due to the avascular nature of the NP and inner AF tissues, the application of these loads to the IVD are required for the transport of nutrients and removal of waste products from the tissues.[6] However, excessive or abnormal compressive forces have been associated with accelerated degeneration and altered composition of IVDs.[35,52]

The extracellular matrix composition and structure of the annulus fibrosus, nucleus pulposus, and cartilage endplate are all important for mechanical performance of the IVD. The cartilage endplates serve to diffuse loads across the surface of the disc and transmit the mechanical forces to the NP due to its stiff nature and its capability to support high pressures and generate osmotic pressures from the CEP's fixed

charge density.[30] The high proteoglycan and water contents of the NP allow the tissue to be compressible and are responsible for the IVD's ability to withstand and recover from compressive loads.[8,9,13] The AF withstands tensile stresses.[8,21] The ringed structure and high collagen content of the AF, in conjunction with the translamellar cross-bridges, allow the AF to resist the complex tensile and compressive loading scenarios that occur during movement.[11,22,70–72]

As patients age, the composition and structure of IVDs change significantly, resulting in a reduction in the tissues' ability to withstand loads.[33] During degeneration, proteoglycan and collagen contents decrease in the CEP, causing it to thin, lose water content, and calcify.[73] In human thoracolumbar CEPs, calcium expression was significantly increased in Pfirrmann grade 3 and 4 discs compared to grade 2 discs.[73] This calcification is associated with occlusion of vascular canals, which then diminishes nutrient delivery.[74] Moderate to severe degeneration is associated with increased collagen fibril tangles and disarrangement, which increase ECM stiffness.[75] The nucleus pulposus becomes more cartilaginous, resulting in a substantial loss in the swelling pressure in the NP, and the ability of the NP to resist compression.[76] As the ability of the NP to resist pressure decreases, more forces are transmitted to the AF which can contribute to the degradation of the AF and weakening of the IVD unit.[33,36] Over time, the ability of the tissue to absorb energy enabling it to withstand repeated axial compression diminishes.[8]

While physiology loading is required to maintain IVD health, excessive and/or repetitive loads can contribute to development and progression of IVDD.[35] Previous studies have reported that as load increases the proteoglycan content of the tissue decreases, and the expression of type-I and type-II collagen increases.[77] Exposure to vibration and injury have also been shown to affect mechanical function of the disc and can lead to low back pain and degeneration.[35,78] It has been observed that cumulative exposure to jackhammers, chainsaws, rotary cultivators, as well as driving automobiles, motorcycles, buses, tractors, and heavy construction equipment are associated with increased risk for experiencing low back pain.[78] An automobile has a typical load frequency of 5 Hz, which is high enough to increase risk for mechanical damage to resonating structures like the IVD. After disc herniation, the progression of degeneration may be accelerated as patients often experience altered range of motion, which then alters the mechanics of the tissue.[33]

Mechanical stress also influences anabolic and catabolic factors in the tissue.[76] Static compression has been shown to accelerate catabolic responses and induce IVD degenerative changes *in vitro* similar to those observed clinically.[52] These changes include altered cell synthetic activities and apoptosis and ECM water content proteoglycan content.[76] Increased magnitude, frequency, and duration of tensile loading are associated with increased MMP-1, MMP-3, and TIMP-1 production.[52] The expressions of MMP-2 and TIMP-1 are increased as the duration of compression is increased. Further, MMP-3, MMP-13, ADAMTS-4, and TIMP-1 expression is elevated as the magnitude, frequency, and duration of compressive loading increase. Further, compressive loads in the range of 2-4 MPa, compared to 0.7-1 MPa, resulted in a decrease in MMP-3 gene expression and decreased TIMP-1 gene expression. Therefore, the effects of load magnitude, frequency, and duration on metabolic responses of the IVD are dynamic and load-dependent. However, the contributions of load to disease mechanisms in IVDD are still incompletely characterized and require further study.

Inflammatory stimulation

While an inflammatory phase is necessary for effective wound healing, persistent inflammation can damage tissues and contribute to pain.[3] The inflammatory cytokines IL-1β and TNF-α are believed to play prominent roles in IVDD.[79] A previous study reported increased production of IL-1β by tissues from herniated and degenerative IVDs compared to normal tissues, and production increased as severity of degeneration increased.[79] Further, the expression of IL-1β has been reported to increase with increasing age and severity of IVD disease.[52,79] The production of TNF-α is localized to the site of herniation after disc herniation, indicating a role for TNF-α in the inflammatory cascade of IVD degeneration after disc injury.[79] Further, it has been reported that there is an increase in TNF-α expression associated with increased patient age and IVDD severity, indicating a role for TNF-α in chronic IVDD and progression of disease.

Based on the increased production of IL-1β and TNF-α by degenerative IVD tissues, they have been used individually or together to stimulate IVD tissues as a pro-inflammatory insult for *in vitro* culture studies.[52,79,80] IVD tissues stimulated with IL-1β decreased matrix synthesis, increased production of degradative enzymes, increased production of PGE2, and increased production of pro-inflammatory cytokines and chemokines.[6,52,79–82] Stimulation of IVD tissues with TNF-α also increased expression of degradative enzymes and pro-inflammatory cytokines and chemokines, increased cell death, and decreased

production of ECM molecules.[6,79,83–85] Further, treatment of human and rabbit nucleus pulposus cells with IL-1β and TNF-α together shifted metabolic responses of the cells toward degradation by reducing the TIMP-1:MMP-3 ratio.[52]

While IL-1β and TNF-α are considered primary drivers of a pro-inflammatory environment for the IVD, other inflammatory mediators have been reported to be present and contribute to IVDD.[86–90] The production of IL-17 was increased in herniated human disc tissue, where it has been reported to promote inflammation, chemotaxis, and angiogenesis.[91] Further, IL-17 can activate and mobilize neutrophils, and upregulate the expression of MMP-1 and MMP-13 in disc chondrocytes.[86] The production of IL-18 is upregulated during IVDD, and has been linked to a shift in IVD ECM composition by increasing collagen type I and II gene expression and MMP-13 gene expression.[87] Increased IFN-γ production has been observed in IVDD tissues, and reported to stimulate macrophages, and upregulate the expression and production of degradative enzymes including cathepsin B, cathepsin H, cathepsin L, and cathepsin D, and reduce IVD cell proliferation.[88] Finally, the production of PGE2 is upregulated during IVDD, and has been reported to stimulate pain; promote vasodilation, swelling and edema; attract and activate neutrophils and macrophages; increase IL-10 and inhibit IFN-γ; and decrease the gene expression of aggrecan.[89,90] Taken together, these data highlight the importance of inflammation to the development and progression of IVDD, and the complexity of the signaling pathways activated during its pathogenesis.

Models of IVD Degeneration

In vivo Animal models

It is common to use animal models for the study of IVDD, and mice, rats, rabbits, sheep, pigs, cows, and dogs can be effective models for hypothesis-driven research in this area.[2,92] Mouse and rat models have advantages over large animal models, including high genetic characterization, the ability to make genetic knockouts to study specific aspects of disease, lower costs, less spacious housing requirements, and high reproducibility.[2,42] Rabbit models are similar to rodent models in terms of costs and housing requirements, and the ability to create genetic knockouts, but the genetic characterization is not as thorough for rabbit models as it is for the rodent models.[2,42,92] However, rabbit IVDs have a more homology to human IVDs than rodent IVDs based on the facet joints and paravertebral muscles and ligaments.[42] Size, anatomy, and biomechanics of ovine and canine IVDs are more similar to the human IVDs than are the

small animal IVDs.[42,93,94] Further, similar to humans, sheep and chondrodysplastic dogs do not retain notochordal cells throughout life, and experience disc calcification with age.[42] Additionally, sheep and dogs allow for the use and evaluation of similar surgical protocols as used for the treatment of humans patients. Importantly, canine patients experience spontaneously occurring IVDD that closely mimics what is seen in humans with respect to causes, symptoms, diagnostics, treatment options, management, and outcomes.[95]

A general limitation for most animal models is associated with the maintenance of a notochordal cell population in the NP through skeletally maturity.[42,92] These notochordal cells influence proteoglycan metabolism, hyaluronan production, and serve as potential progenitor cells.[42] In human IVDs, the notochordal cell population is maintained through development, but then replaced by chondrocytic cells by adulthood.[42,92]

Structurally, there are differences between human and animal IVDs as well.[2,41,42,92] The size of the IVD is notably smaller for rabbit, mouse, and rats.[41,42,92] Further, the smaller disc height in animals aids diffusion of nutrients into the NP.[42,92] Whereas the diameter of human discs increases from the cervical to lumbar region, quadrupedal animals have approximately equal diameter discs throughout the spinal column.[92] Additionally, human discs are convex on both the upper and lower surfaces, whereas calves and pigs only have convex surfaces on the cranial side of the discs.

Another major limitation for all of the aforementioned animal models is differences in mechanical loading profiles of these quadrupeds compared to bipedal humans.[42] While loading profiles differ, muscle contraction and ligament tension are significant contributors to loads experienced by IVDs in both species, and loading of the IVD may even be greater in quadrupedal animals due to increased complexity involved in stabilizing the horizontally-aligned spine of large animals.[42,92] Conversely, small quadrupedal animals, such as, mice, and rats, no not require extensive forces to stabilize their spine. Intradiscal pressure of animal IVDs might be similar to humans due to the smaller diameter, but are subjected to markedly smaller loads than human IVDs.[92] While animal and human species have more limited axial rotation in the lumbar spine compared to thoracic and cervical spine, the absolute range of motion in human lumbar discs is greater than calf, sheep, and pig lumbar discs.[92]

Considering relative advantages and disadvantages, rodent and rabbit models are best suited for mechanistic and screening studies, while large animal models are good models for studying structure,

geometry, biochemistry, and biomechanics of discs, and, especially those using dogs, are ideal for preclinical translational studies aimed at diagnostic, preventative, and/or therapeutic strategies for IVDD.[93] [2,42,92] However, limitations must be considered when translating findings from an animal model to clinical application.[92]

In vitro culture models

While animal models allow for the study of IVDD development and progression, the complexity of potential confounding factors can make it difficult to control variables in order to study specific aspects of disease. Further, in order to address the 3 R's (reduce, refine, replace) of ethical use of research animals, alternative models for screening novel strategies prior to study in animal models are needed. Therefore, numerous *in vitro* models have been developed for the study of IVDD pathobiology.[41,93,94,96–113] These *in vitro* models include monolayer cell culture, 3D cell culture, tissue explant culture, and whole organ explant culture.

Monolayer and 3D cell cultures of AF and NP cells are commonly used to analyze metabolic responses to specific stimuli. Advantages of monolayer cell culture include availability of cells for culture, limiting animal use for studies, and ease of performing gene expression and intracellular signaling studies.[92,114] However, as cells are passaged during culture, there is a loss of cellular phenotype, significantly affecting the cellular responses to stimuli.[115,116] Further, the effects of extracellular matrix interactions with cells are lost or diminished, even in 3D cultures.[92,114] Monolayer cell culture models have been used to study responses of normal and degenerative IVD cells to various stimuli, including the pro-inflammatory cytokines IL-1β and TNF-α.[40,81,116–123] Results from these studies indicate that these cytokines can decrease synthesis of ECM and increase production of degradative enzymes and other inflammatory cytokines and chemokines. Additionally, monolayer and 3D cultures are often used to assess cellular responses to various biomechanical loads and for tissue engineering.[124–128]

Compared to cell cultures, whole-organ explant cultures provide a more physiologic model because cells are not removed from their specialized ECM.[41,92] Whole-organ models have been utilized to study the influences of load, various injection types (saline, trypsin, mesenchymal stem cells, IVD cells, or BMP-2, for example), cytokine stimulation, or injury on the metabolic responses, structure and composition, and mechanical properties of the IVD.[41,43,69,77,81,93,94,97,99,100,109,112,129] These studies typically

use IVDs harvested from tails of animals, with bovine and rat being most commonly used. While rat tail IVD explant studies typically utilize the entire IVD unit with the boney end plates attached, the majority of bovine tail IVD explant studies have been performed with boney endplates removed.

To simulate injury in explant models, stab or needle puncture models are most common.[33,41,43,48,109,129,130,135] Models utilizing needle puncture have been successful in causing reproducible degenerative-like changes over time including reduced disc height, water content, and GAG content. Decreased NP volume, annular layer disorganization, inward inner annulus fibrosus bulging, and annular tears are also observed.[41,136] These changes are noted to have progressive mechanical, biological, and biochemical consequences that mimic human IVDD.[48] After various studies on needle size and the effects on degeneration, it has been determined that a needle size of greater than 40% of the disc height is necessary for significant disc changes including reduced axial stiffness.[41,43,137]

In rat tail models, 18, 21, and 25-gauge (G) needles were used to determine which best produced desired degenerative changes.[2,48,135,137] In these studies, puncture injury resulted in the depressurization of the NP, causing mechanical disruption of the disc. Over time after injury, the 18G and 21G resulted in a significant reduction in disc height, significant changes in tissue histopathology grade, decreased NP area, and loss of distinction between NP and AF.[2,48,135] The injury caused by the 25G needle did not result in these same changes during the study period. Based on the data from these studies, the 21G needle is consistently used for the rat tail IVD stab/injury model studies. A modification of the stab model includes the aspiration of a portion of the NP after puncture.[43] With this technique, there was an observed decrease in water and GAG contents in the NP. These models provide insight into the role of acute injury on the development and progression of IVDD and allow for initial testing of novel treatments.

As outlined, load is an important factor for maintaining IVD health and function. Therefore, numerous models have been developed using human, bovine, ovine, caprine, rabbit, and rat explants to analyze the effects of load on the IVD.[69,77,94,109,129,131–134,138,139] In addition to species differences, IVD explant models vary based on inclusion of boney and cartilage endplates, inclusion of cartilage endplates only, or with boney and cartilage endplates removed. These explant variations affect diffusion and biomechanical properties. As such, culture duration and loading parameters may be affected based on maintenance of cell viability and tissue integrity, respectively.

Duration, magnitude, and frequency of the load applied to IVD during explant culture are important factors to consider when developing a loading protocol of IVDs as these variables significantly influence cell health, functions, and responses.[140] To model physiologic loading , many studies will apply a cyclic compressive load that ranges from 0.1 to 0.5MPa at a low frequency of ~0.2Hz for 8-16 hours/day.[141,142] This level of load has been shown to maintain cell viability and metabolism during explant culture.[69,132,133,140,143] However, when load is increased to levels in the 1-2 MPa range and\or frequency to 1Hz, there is a significant decrease in cell viability and ECM integrity in association with an upregulation of gene expression and/or synthesis of MMPs and aggrecanases.[52,94,129,131,133,144]

Taken together, well-designed whole organ explant model studies can provide insight into the development and progression of IVDD related to inflammatory, degradative, traumatic, and loading stimuli. As such, these models can act as an effective bridge between cell-based studies and preclinical animal models for programmatic research aimed at diagnostic, preventative and therapeutic strategies for IVDD.

Conclusion

IVD degeneration is a multifaceted disease with trauma, herniation, genetics, lifestyle, and smoking being contributing factors to its development and progression.[34] Unfortunately, the pathogenesis and pathophysiology of the disease are still incompletely characterized, and the precise mechanisms that lead to debilitating back pain have not been fully elucidated. *In vitro* models provide a powerful tool to control variables in order to target mechanisms and pathways. Whole organ IVD explant models maintain the complex tissue architecture, composition, fluid flow dynamics, and cell distribution within the tissue, making them attractive models for bridging cell culture studies to animal models in a comprehensive translational approach to addressing the major unmet needs in optimizing care of patients suffering from intervertebral disc disease.[41]

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Chapter 2: Effects of Cyclic Compression on Intervertebral Disc Metabolism in a Whole-Organ Rat Tail Model

Introduction:

Intervertebral disc (IVD) degeneration is a significant cause of back pain and disability, costing an estimated $100 billion annually in direct and indirect costs in the US.[1–3] There are numerous factors associated with intervertebral disc degeneration (IVDD) and increased risk of back pain, including aging, trauma, genetics, lifestyle, and magnitude and frequency of applied loads.[4–7] However, mechanisms of disease for IVDD are still incompletely characterized.

Based on the exquisite design and function of the IVD, its biology and biomechanics are inextricably linked in both health and disease. Due to the avascularity of the inner annulus fibrosus (AF) and nucleus pulposus (NP), dynamic loading of the IVD is essential to maintain the health of the organ by promoting the diffusion of nutrients through the cartilage endplate into the disc.[8–10] Previous studies have reported wide ranges of compressive forces on human IVDs measured *in vivo* for various activities and positions including laying supine (0.1-0.2MPa), sitting with an unsupported back (0.46MPa), relaxed standing (0.5MPa), standing in a flexed forward position (0.8-1.5MPa), climbing stairs (0.5-0.7MPa), jogging (0.35-0.95MPa), or lifting and twisting (>2MPa).[11,12] Further, the weight of the patient and the strength of the supporting muscles of the spine are believed to play prominent roles in the level of forces experienced.[11] While these forces are required to maintain the health of the IVD, excessive and abnormal compressive forces and exposure to high frequency vibrations have been associated with increased rates of IVDD in patients.[4–7,11,12]

The ability of healthy IVDs to absorb energy from repeated axial compression, also known as hysteresis, decreases as the duration of the applied load increases.[13] Therefore, as the duration and/or frequency of the applied load increases, the ability of the IVD to resist and recover from the load decreases, which may contribute to the development and progression of IVDD clinically. Additionally, with increased age and during the development of IVDD, there is a decrease in NP hydration and an increase in NP collagen type I content[15,16] This shift towards a more fibrocartilaginous NP decreases the shock-absorbing

properties of the IVD. Furthermore, excessive mechanical strain has been shown to induce extracellular matrix (ECM) breakdown of the IVDD, and a downregulation in the gene expression of ECM components aggrecan and collagen type II.[5,6,17] Together, these changes may result in a significant alterations in metabolic responses of the IVD to loading associated with normal movement, leading to disc failure.[7,15] Despite having these data, the effects of loading on metabolic responses of the IVD that may contribute to development and progression of IVDD are still incompletely characterized.

Therefore, this study was designed to determine how magnitude of cyclical compressive loading affects the inflammatory and degradative metabolism of the IVD using a whole organ rat tail IVD explant model. It was hypothesized that increased magnitude of loading will result in corresponding significant increases in production of pro-inflammatory and degradative biomarkers. We further hypothesized that, as magnitude of compressive load increases, there will be a significant decrease in tissue proteoglycan (GAG) and collagen (HP) content compared to unloaded controls.

Methods:

Tissue Harvest and Culture

With Animal Care and Use Committee approval (ACUC #9435), tails were collected from six skeletally mature Sprague Dawley rats euthanized for reasons unrelated to this study. Soft tissues were aseptically dissected from the caudal vertebrae and whole organ explants (n=36) consisting of cranial body half, cartilage endplate, IVD, cartilage endplate, and caudal body half were created using a scalpel, rongeurs, and a bone cutter. The boney endplates were trimmed with a diamond bone saw to obtain flat, parallel surfaces for loading. Whole organ explants were then randomly assigned to one of the following culture groups (n=6/group): [1] No load (0MPa, 0Hz) [2] Low load (0.5MPa, 0.5Hz), or [3] High load (1.0MPa, 0.5Hz). Explants were cultured in 5ml of supplemented Dulbecco's Modified Essential Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA). The medium was supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1x MEM N-E Amino Acid solution, 1x insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1x penicillin streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified). Whole organ explants were cultured in BioPress compression plates at 37°C and 5% CO_2, and

load was applied using the Flex-Cell compression plus unit (FX-5000C Compression System, Flexcell International Corporation, Burlington, North Carolina). Compressive load was applied using a half sine wave profile for 3 hours on and 1 hour off for 4 cycles (total of 16 hours), followed by an 8-hour rest period. In order to mimic the consistent load experienced by the IVD during normal movement during the day, a minimum load of 0.25MPa was applied to the tissue during load, such that the magnitude of load applied to the tissue during the loading period ranged from 0.25MPa to either 0.5MPa or 1.0MPa depending on load group (**Figure 2-1**). After 3 days, media were collected and stored at -20°C for biomarker analyses and tissues were processed for analysis of proteoglycan (GAG) and collagen (HP) content as outlined below.

Media Biomarker Assays

Media were analyzed for IFN-γ, IL-1β, IL-4, IL-6, TIMP-1, TNF-α, and VEGF using the Rat Magnetic Luminex Assay kit (R&D Systems, Minneapolis, MN, USA), and RANTES, IGF-1, MMP-8, and FGF-basic using the Mouse Magnetic Luminex Assay kit (R&D Systems, Minneapolis, MN, USA). MIP-1α, IL-10, MCP-1, and GRO-α concentrations were analyzed using the Rat Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA). Testing for nitric oxide (NO) production was done using the fluorescent reagent diaminonaphthalene (DAN)[18] assay and prostaglandin E2 (PGE2) content was determined using the PGE2 Express EIA kit (Cayman Chemical, Ann Arbor, MI, USA). The level of MMP activity was detected using the fluorescent general MMP activity assay (AnaSpec, Fremont, CA). Glycosaminoglycan (GAG) content release to the media was assessed using the DMMB.[19] All assays were performed following the manufacturer's instruction or as previously described.

Tissue Analysis

IVD tissues were trimmed from the vertebrae using a scalpel. Tissue was weighed then digested overnight in a 0.6mg/ml papain digest solution at 65°C. The GAG content of the tissue digest was determined using the DMMB assay[19] and collagen content was determined using the hydroxyproline assay, as previously described.[20] To determine the level of GAG and collagen in the tissue, the GAG and HP values were normalized to the wet weight of the tissue. To determine if load effected the balance between

proteoglycan and collagen in the tissue, the GAG:HP ratio was determined. Finally, to determine the effect of load on the release of proteoglycans from the tissue, the percent GAG in the tissue was determined by dividing total tissue GAG by the sum of total tissue and media GAG.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp). Data was tested for normality using the Shapiro-Wilk test, and significant differences between groups were determined using either a one-way ANOVA and a Tukey's post-hoc analysis, or a Kruskal-Wallace test and Mann-Whitney U-test post-hoc analysis, based on the normality of the data, with significance set at $p<0.05$. In order to account for multiple comparisons, a Bonferroni Correction for multiple comparisons was performed.

Results:

Media Inflammatory and Degradative Biomarker Concentrations (**Figure 2-2**)

IFN-γ ($p<0.035$), IL-6 ($p<0.032$), IL-1β ($p<0.003$), and MMP-8 ($p<0.034$) levels were all significantly higher in the 0.5MPa loaded group compared to the no-load control group after 3 days of culture. The levels of TIMP-1 ($p<0.015$) and PGE2 ($p<0.001$) were significantly higher in the 1.0MPa group compared to the no-load control group after 3 days of culture. Further, the production of PGE2 was significantly ($p<0.001$) higher in the 1.0MPa group compared to the 0.5MPa group after 3 days of culture. There were no significant differences between groups for production of IL-4, TNF-α, VEGF, IGF-1, FGF-basic, MIP-1α, GRO, NO, and MMP activity after 3 days of culture.

Tissue Extracellular Matrix (**Figure 2-3**)

The 1.0MPa loaded group released significantly more GAG to the media ($p<0.012$) compared to the no-load control group. Additionally, percent-GAG in the tissue was significantly lower in both the 0.5MPa ($p<0.001$) and 1.0MPa ($p<0.013$) loaded groups compared to the no-load control. There were no significant differences observed between groups for tissue GAG content, HP content, or GAG:HP ratio.

Discussion:

To the author's knowledge, this is the first study to measure the release of specific pro-inflammatory, anti-inflammatory, degradative, and anti-degradative biomarkers during *ex vivo* culture of whole organ IVD explants in response to compressive loading. Dynamic loading is considered a critical component of IVD health as it is the primary mechanism for delivery of nutrients into the IVD and removal of waste out of the IVD.[10] However, the data from this study indicate that there are also magnitude-dependent pro-inflammatory, extracellular matrix, and degradative responses by the IVD to cyclical compressive load. As such, the metabolic responses by the rat tail IVDs to load in this *ex vivo* model may characterize mechanisms of disease for repetitive compressive load contributing to the development and progression of IVD degeneration.

Previous studies have reported increases in IL-6, IL-1β, and IFN-γ gene expression in degenerative and herniated human discs.[21,22] The data from the present study indicate that these pro-inflammatory responses can be stimulated in normal IVDs through application of cyclical compressive load. The increase in production of these biomarkers in response to load is consistent with previous studies analyzing changes in IL-6 and IL-1β gene expression in response to static and dynamic loads using *ex vivo* explant models.[23–25] However, the data from the present study indicated that lower magnitude load elicited more robust pro-inflammatory responses by the IVD compared to higher magnitude load. The IVD explants cultured at 0.5MPa load, but not at 1.0MPa load, significantly increased the release of IL-1β, IL-6, and IFN-γ from the tissue compared to the no-load control. Nevertheless, there was no statistically significant differences between loaded groups. It is possible that the responses to load are time-dependent such that additional assessment timepoints are needed to more fully characterize the effects of load-level in the dynamics of pro-inflammatory cytokine release by the IVD during culture.

The significant increase in the release of PGE2 in the 1.0MPa group supports the concept that there is a significant pro-inflammatory response by IVDs subjected to higher magnitude of compressive load. This load-dependent PGE2 response is likely mediated, at least in part, by IL-1β as it has been reported to increase PGE2 production by the IVD.[26] Previous work reported a significant increase in release of PGE2 by articular cartilage after impact injury with level of PGE2 release related to magnitude

of the impact applied and related loss of cell viability.[27] Therefore, it is possible that the load-dependent PGE2 response in the 1.0 MPa group may also be directly related to traumatic loading and associated cell death. Further study is required to fully characterize the mechanisms driving PGE2 production by IVDs in response to loading in order to determine its relevance for clinical application.

The data from this study also indicate a potential divergence in production and regulation of degradative enzyme activity by the IVD in response to load magnitude. In agreement with gene expression data from a previous study, the production of TIMP-1 was increased by the IVD in the 1.0MPa group.[28] Further, the data from this study indicate that there may be a threshold in load magnitude that must be reached before TIMP-1 production by the IVD is affected, since production of TIMP-1 was only significantly different in the 1.0MPa group and not the 0.5MPa group. Therefore, these data indicate that as the magnitude of load applied to the IVD increases, there are pathways activated in the disc to potentially counteract an increase in degradative enzyme activity and tissue degradation. Additionally, this finding may explain why there is conflicting data in the literature regarding TIMP-1 gene expression in human degenerative IVDs, with one study reporting a significant increase and another study reporting no significant change in TIMP-1 gene expression in IVDD.[22,28,29] It is likely that some of the differences in TIMP-1 expression observed in clinical samples may be due to patient variability. However, further study is required to determine how repetitive compressive load affects production of TIMP-1 by IVDs and how these changes in metabolic responses relate to the development and progression of IVDD.

Release of the degradative enzyme, MMP-8, was significantly increased in the 0.5MPa group compared to the no-load control. This increase in MMP-8 production was not enough to result in a significant increase in total MMP activity compared to the other groups. However, it does indicate load-dependent responses in degradative enzyme prodcution including shifts in specific MMPs such as MMP-8. A previous study reported that MMP-8 gene expression increased significantly in human degenerative IVDs, and that MMP-8 gene expression had a positive correlation to IVD histomorphological degeneration.[29] The data from the present study support this finding and indicate that repetitive compressive load may be a mechanism for increased MMP-8 production during the development and progression of IVDD.

Application of compressive load was associated with significant effects on tissue GAG

metabolism in the present study. The 1.0MPa group released significantly more GAG to the media, and both the 0.5MPa and 1.0MPa group had significantly lower percent GAG in the tissue compared to the no load control. This indicates that there is a shift towards higher GAG production and release by the IVD, increased GAG degradation and release by the IVD, or both in response to repetitive compressive loading. While the tissue GAG content and the GAG:HP ratio of the loaded IVDs were lower than the unloaded control, which could potentially indicate that increased tissue ECM degradation is occurring in the loaded groups, this difference did not reach statistical significance during the timeframe of this study. It is possible that during a longer culture period the difference in tissue GAG content of the loaded and unloaded explants would reach significance. However, previous studies reported that dynamic compressive load applied to rat tail IVDs *in vivo* and *in vitro* resulted in significant increases in tissue GAG content, which would indicate that the increase in GAG released to the media may be a result of increased synthesis by the IVD.[6,28] Further study is required to understand the roles for load in the dynamics of tissue GAG production and degradation in IVD health and disease.

As with any in vitro study, there are inherent limitations to consider when interpreting the data. The tissues have been removed from the *in vivo* environment, and the changes in the production of biomarkers by the IVD may be more indicative of tissue responses to the *in vitro* culture conditions and not reflective of the *in vivo* response to load. These IVDs were harvested from rat tails, and therefore the responses and response to load of a rat tail IVD may not be similar to a human lumbar or cervical IVD. The smaller size of rat tail IVDs allows for easier fluid flow to the NP during culture compared to larger IVDs from other species, therefore responses due to nutrient exchange within the IVD may not be reflected in this model. While the compressive loads applied to the IVDs are similar to loads applied *in vivo*, the magnitude of the load *in vitro* may not be truly reflective of what the IVD experiences *in vivo*. In this study, loading magnitudes of 0.5MPa and 1.0MPa applied at a frequency of 0.5Hz were chosen due to data in the literature. There have been studies on human intradiscal pressure during various activities that state physiologic loading to be between 0.1.0MPa and 2.0MPa, and frequency of physiologic loading to be between 0.2Hz and 1.0Hz, as well as other rodent studies using 0.5MPa, 1.0MPa, and 0.5Hz.[9,11,12,30] The 0.5MPa of load is representative of relaxed standing or relaxed sitting, both with a straight back, and the 1.0MPa of load is representative of standing while flexed forward, standing up from a chair, and/or lifting a

20kg object close to the body, which are common movements humans experience.[12] However, the dynamics of loading were uniaxial, and is not reflective of the complex loads applied to the IVD *in vivo*. Additionally, this *ex vivo* model involves the removal of the surrounding tissue layers, which influence the load experienced by the IVD *in vivo*. Therefore, the loads experience by the IVD are not directly translatable to the load experiences *in vivo*. Finally, the explants were cultured for only 3 days, therefore the data from this study may only reflect the acute response of IVDs to load and are not reflective of the IVDs response to the chronic application of load.

Conclusion:

With these limitations in mind, the data from this study indicate that repetitive compressive loading of whole organ IVDs is associated with relevant pro-inflammatory (IL-1β, IL-6, INF-γ, and PGE2) and degradative (MMP-8 and TIMP-1) responses in a magnitude-dependent manner. Characterizing the mechanistic pathways that drive these changes in tissue metabolism may provide insight into the development and progression of IVDD. Characterizing these pathways may allow for the development of novel methodologies to mitigate occupational, environmental, and activity-related risk factors associated with IVDD. Further, these studies may allow for the development of novel preventive and therapeutic strategies for IVDD clinically. Further study is required to determine the precise mechanisms for the magnitude-dependent variance in the production of pro-inflammatory and degradative biomarkers by IVDs associated with repetitive compressive loading.

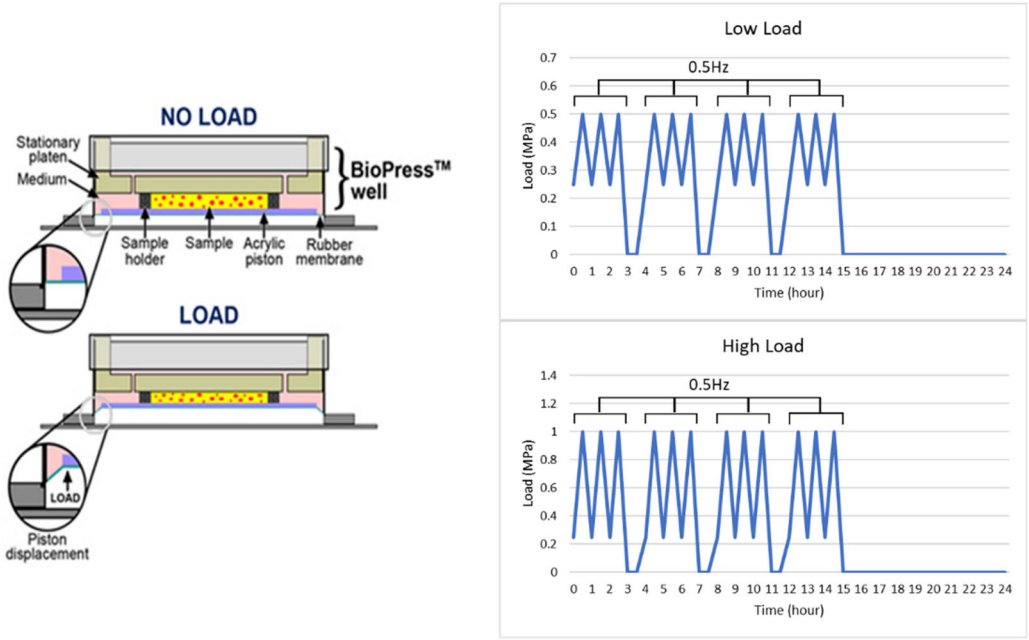

Figure 2-1: Loading system and schematic of the loading protocols utilized in this study.

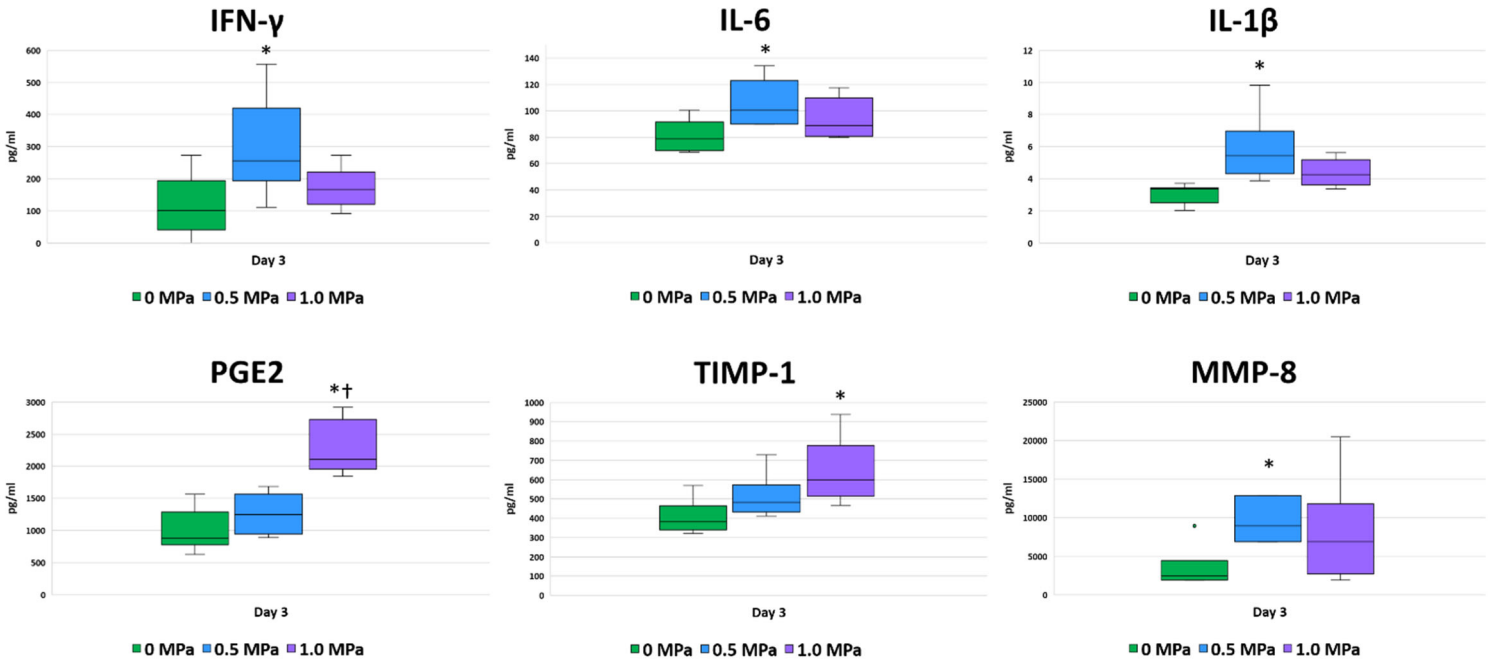

Figure 2-2: Media biomarker concentrations in the 0 MPa, 0.5 MPa, 1.0 MPa groups for IFN-γ (pg/ml), IL-6 (pg/ml), IL-1β (pg/ml), PGE2 (pg/ml), TIMP-1 (pg/ml), MMP-8 (pg/ml). (*) significantly different than corresponding control. (†) significant difference than 0.5 MPa.

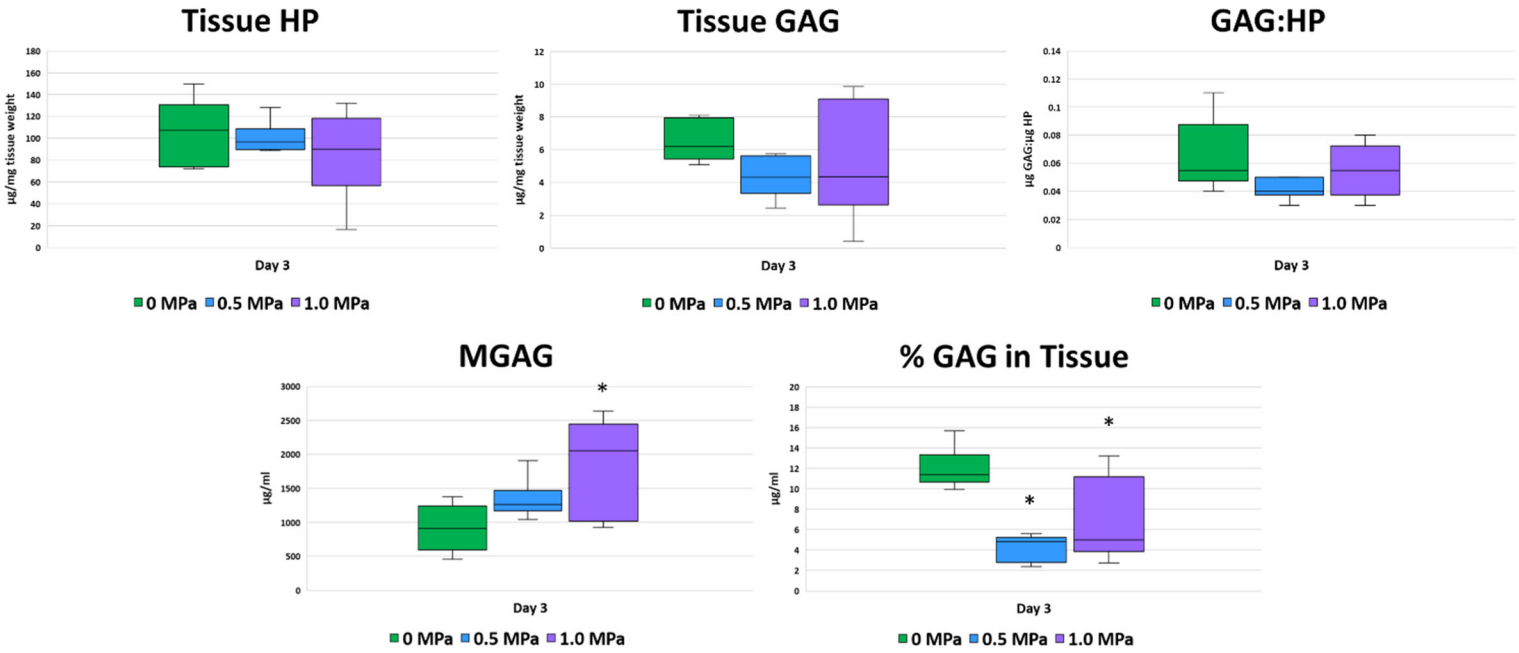

Figure 2-3: Tissue extracellular matrix concentration in the 0 MPa, 0.5 MPa, 1.0 MPa groups for Tissue HP (µg/mg tissue weight), Tissue GAG (µg/mg tissue weight), GAG:HP (µg GAG: µg HP), MGAG (µg/ml), and % GAG in Tissue (µg/ml). (*) significant difference compared to 0 MPa.

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Chapter 3: Effects of Cyclic Compression and Pro-inflammatory Stimulation on Injured, Whole-Organ Rat Tail Intervertebral Discs

Introduction:

Intervertebral disc (IVD) degeneration is a significant cause of back pain and disability, costing an estimated $100 billion annually in direct and indirect costs in the US.[1–5] There are numerous factors associated with intervertebral disc degeneration (IVDD) and increased risk of back pain, including aging, trauma, genetics, lifestyle, and magnitude and frequency of applied loads.[2,6] However, mechanisms of disease for IVDD are still incompletely characterized.

Based on the exquisite design and function of the IVD, its biology and biomechanics are inextricably linked in both health and disease. Due to the avascularity of the inner annulus fibrosus (AF) and the nucleus pulposus (NP), dynamic loading of the IVD is essential to maintain its health by promoting diffusion of nutrients through the cartilage endplate into the disc.[2,3] However, the ability of healthy IVDs to absorb energy from repeated axial compression, also known as hysteresis, decreases as the duration of the applied load increases.[7] Therefore, as the duration and repetition of a load applied to the IVD increases, the magnitude of load on the IVD may also increase, resulting in altered metabolic responses by the tissue that could lead to local inflammation and degradation. Further, tissue changes to the structure and composition of AF and NP due to aging or injury can significantly affect the ability of the IVD to resist loads applied during movement, further exacerbating the inflammatory and degradative responses of the IVD to load.[8–10] Importantly, acute injury to the IVD that results in the compromise of the AF and decompression of the NP rapidly and significantly affects the ability of the IVD to resist loads applied during normal activities of daily living.[6,9,11] However, the mechanisms driving injury-related inflammatory and degradative metabolic responses of the IVD to load have not been well characterized.

The *ex vivo* IVD puncture model has been used in numerous studies to analyze the responses of the IVD to injury.[7,9,10,12–19] These models cause reproducible degenerative-like changes over time similar to

those reported clinically, including progressive alterations in disc height, histological changes in IVD organization and architecture, biochemical composition, and biomechanical properties. Previous studies using a bovine caudal IVD injury model, reported that high frequency loading of injured IVDs resulted in increased MMP-7 and MMP-13 gene expression, and increased release of CCL5 (RANTES) to the media compared to uninjured low frequency loaded samples.[17,18] While these studies provide insight into some of the inflammatory and degradative pathways activated by the application of load to the injured disc, the metabolic responses of injured IVDs to repetitive compressive loads with variation in load magnitude and frequency are still incompletely characterized.

In addition to physical damage that occurs to the IVD during degeneration, there is a significant inflammatory response by the IVD during degeneration.[20] To mimic the inflammatory environment, numerous *in vitro* models have stimulated cells, tissue explants, and IVD explants with the pro-inflammatory cytokines IL-1β and TNF-α.[21–30] These studies indicate that stimulation of IVD tissues and cells with IL-1β or TNF-α results in significant increases in gene expression and/or production of degradative enzymes (MMP-3 and MMP-13) as well as inflammatory cytokines and chemokines, nerve growth factors, and substance P. Further, cytokine stimulation significantly decreased the expression, production and tissue composition of extracellular matrix components collagen type I, collagen type II, and aggrecan. While these data elucidate significant effects of cytokine stimulation on the metabolism of the IVD, the effects of repetitive compressive load and injury on responses of the IVD to cytokine stimulation are still incompletely characterized.

Therefore, this study was designed to determine how magnitude of cyclical compressive load and cytokine stimulation affect the inflammatory and degradative metabolic responses of injured IVDs using a whole organ rat tail IVD explant model. It was hypothesized that as magnitude of load increased, there would be a significant increase in cytokine-stimulated production of pro-inflammatory and degradative biomarkers by IVD explants. We further hypothesized that as magnitude of compressive load increases, there will be a significant decrease in tissue proteoglycan (GAG) content due to cytokine stimulation compared to unloaded controls and controls without cytokine stimulation.

Methods:

Tissue harvest and culture

With Animal Care and Use Committee approval (ACUC #9435), tails were collected from 8 skeletally mature Sprague Dawley rats after euthanasia for unrelated reasons. Soft tissues were dissected aseptically from the caudal vertebrae with a scalpel and rongeurs, and whole organ explants consisting of the cranial body half, cartilage endplate, IVD, cartilage endplate, and caudal body half were isolated using bone cutters (n=36). The boney endplates were trimmed using a diamond bone saw to obtain flat, parallel surfaces for loading. Each disc was injured by penetrating the posterolateral AF with a 20G needle, entering the approximate center of the NP, and aspirating a portion of the NP by moving the plunger of a 1 mL syringe to 0.1 mL. Injured whole organ explants were then randomly assigned to one of the following groups (n=6/group): [1] 0.5MPa, 0ng/mL IL-1β (0.5-0), [2] 0.5MPa, 10ng/mL IL-1β (0.5-10), [3] 1.0MPa, 0ng/mL IL-1β (1-0), [4] 1.0MPa, 10ng/mL IL-1β (1-10), [5] 0.0MPa, 0ng/mL IL-1β (0-0), or [6] 0.0MPa, 10ng/mL IL-1β (0-10). Explants were cultured in 5 mL of Dulbecco's Modified Essential Medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 1mM sodium pyruvate, 2mM L-glutamine, 0.5mg/mL ascorbic acid, 1 x MEM N-E Amino Acid solution, 1% insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1x penicillin streptomycin-amphotericin B (all components from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified). Explants were cultured in BioFlex plates at 37°C and 5% CO_2, and load was applied using the Flex-Cell compression plus unit (FX-5000C Compression System, Flexcell International Corporation, Burlington, North Carolina). Load was applied using a half sine wave profile for 3 hours at 0.5Hz and unloaded for 1 hour for a total of 16 hours, followed by an 8-hour rest period. In order to mimic the consistent load experienced by the IVD during normal movement during the day, a minimum load of 0.25MPa was applied to the tissue during load, such that the magnitude of load applied to the tissue during the loading period ranged from 0.25MPa to either 0.5MPa or 1.0MPa depending on load group (**Figure 3-1**). After 3 days, media were collected for biomarker analyses and tissues were processed for analysis of proteoglycan (GAG) content.

Media Biomarker Assays

Media were analyzed for IFN-γ, IL-4, TIMP-1, TNF-α, and VEGF using the Rat Magnetic Luminex Assay kit, and RANTES, IGF-1, and FGF-basic using the Mouse Magnetic Luminex Assay kit (R&D Systems, Minneapolis, MN, USA). IL-6, MIP-1α, IL-10, MCP-1, and GRO concentrations were also determined using the Rat Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA). Prostaglandin E_2 content was determined using the PGE2 Express EIA Kit (Cayman Chemical, Ann Arbor, MI, USA), and nitric oxide content was detected using the fluorescent reagent diaminonaphthalene (DAN) assay.[31] General MMP activity levels were determined using the fluorescent general MMP activity assay (AnaSpec, Fremont, CA). Glycosaminoglycan (GAG) released to the media was analyzed using the DMMB assay as previously described.[32]

Tissue Analysis

Using a scalpel blade, IVD tissues were trimmed from the boney vertebrae. Tissues were then weighed and digested overnight. Digestion was performed using 1 mL of 0.6 mg/mL papain digest solution at 65°C. The GAG content of the tissue digest was determined using the DMMB assay.[32] Values were normalized to the wet weight of the tissue during analysis.

Statistical Analysis

A Shapiro-Wilk test was performed to test normality of the data. The distribution of the data was not normally distributed; therefore, the data was analyzed using non-parametric methodologies. To determine significant differences between cytokine treated and non-cytokine treated samples at each load level, a Mann-Whitney Rank Sum test was performed. To determine significant differences between load groups based on cytokine stimulation and between all groups, a Kruskal-Wallace test and Mann-Whitney U-test post-hoc analysis was performed. Significance was set at $p<0.05$ after a Bonferroni correction for multiple comparisons was performed.

Results:

Inflammation-related biomarker concentrations (**Figure 3-2**)

Release of IL-6 to the media was significantly higher in the 0-0 and 1-10 groups compared to the 1-0 group ($p \leq 0.009$) and in the 0-0 group compared to the 0-10 group ($p=0.002$). IL-6 release to the media was also significantly lower in the 0-10 group compared to the 0.5-10 ($p=0.003$) and 1-10 ($p=0.003$) groups. The release of MIP-1α to the media was significantly lower in the 0-10 group compared to the 0-0 ($p=0.002$), 0.5-0 ($p=0.011$), 0.5-10, ($p=0.015$), and 1-10 ($p=0.012$) groups. The release of MCP-1 to the media in the 0-0 and 1-10 groups was significantly higher than the 0-10 ($p \leq 0.025$), and 1-0 ($p \leq 0.026$) groups. The release of GRO to the media in the 0-10 group was significantly lower than the 0-0 ($p=0.002$), 0.5-10 ($p=0.001$) and 1-10 ($p=0.024$) groups, and significantly higher in the 0.5-10 group compared to the 0.5-0 ($p=0.009$) group. IGF-1 release to the media was significantly lower in 0-10 group compared to the 0-0, ($p=0.002$), 0.5-0 ($p=0.008$), 0.5-10 ($p=0.001$), and 1-10 ($p=0.033$) groups, and significantly higher in the 0.5-0, 0.5-10, and 1-10 groups compared to the 1-0 group ($p \leq 0.026$). The release of FGF-b release to the media in the 0-10 and 1-10 groups was significantly higher than the 0-0 group ($p \leq 0.032$). The release of VEGF to the media was significantly lower in the 0-10 group compared to the 0-0 ($p=0.002$), 0.5-10 ($p=0.007$), 1-0 ($p=0.007$), and 1-10 ($p=0.015$) groups, and the 0-10 group approached significance compared to the 0-0 ($p=0.06$) and 0.5-0 ($p=0.055$) groups.

Degradation-related biomarker concentrations (**Figure 3-3**)

The release of TIMP-1 to the media was significantly lower in 0-10 group ($p \leq 0.018$) compared to the 0-0, 0.5-0, and 1-10 groups. Further, TIMP-1 was significantly lower in the 0.5-10 group compared to the 0-0 ($p=0.035$) and 0.5-0 ($p=0.026$) groups. Levels of general MMP activity in the media were significantly higher in the 0-10 group compared to the 0-0 ($p=0.002$), 0.5-0 ($p=0.015$), 0.5-10 ($p=0.021$), and 1-10 ($p=0.005$) groups. Further, general MMP activity was significantly higher in the 1-0 group compared to the 1-10 ($p=0.002$) and 0.5-0 ($p=0.021$) groups.

Proteoglycan content (**Figure 3-4**)

The proteoglycan (GAG) content in media and tissue, and percent GAG in the tissue (tissue GAG/tissue GAG plus media GAG) were determined for each sample. The tissue GAG content in the 0-10 group was significantly lower than the 0-0 ($p=0.002$), 0.5-10 ($p=0.012$), and 1-10 ($p=0.009$) groups. Additionally, media GAG was significantly higher in the 0-10 group compared to 0-0 ($p=0.002$), 0.5-10

(p=0.011), and 1-10 (p=0.011) groups. Media GAG content was also significantly higher in the 1-0 group compared to the 0.5-0 group (p=0.007). Finally, percent GAG in the tissue was significantly higher in the 0-0 (p=0.002), 0.5-10 ($p \leq 0.017$) and 1-10 (p=0.006) groups relative to the 0-10 group.

Discussion:

To the author's knowledge, this is the first study to measure the release of key pro-inflammatory, anti-inflammatory, degradative, and anti-degradative biomarkers during *ex vivo* culture of injured whole organ IVD explants in response to pro-inflammatory stimulation and compressive loading. The data from this study indicate that there is a significant pro-degradative response by injured IVDs to pro-inflammatory stimulation. Further, that repetitive compressive loads, at the magnitude and frequency used in this study, appeared to counteract this initial increase in the IVDs degradative response to inflammation. However, the data also suggest a more complex and counterintuitive inflammatory response by the IVD explants to cytokine stimulation and load.

The release of pro-inflammatory (IL-6, MIP-1α, MCP-1) biomarkers to the media was lower in the unloaded and cytokine stimulated group (0-10) compared to the unloaded and unstimulated control (0-0) after 3 days of culture. Further, while not statistically significant, the release of GRO was higher in the 0-0 group compared to the 0-10 group. These data indicate that injured IVDs may have a muted pro-inflammatory response to cytokine stimulation. This is in contrast to previous studies that found increased gene expression and protein production of these pro-inflammatory biomarkers by NP and AF cells after treatment with various inflammatory stimuli.[20,21,33–35] A previous puncture model study using murine IVD explants found that injured IVDs significantly increased production of IL-6 compared to uninjured controls at day 3 and 21 of culture.[19] Therefore, it is possible that puncture injury to the IVD in the present study stimulated a significant inflammatory response by IVD explants, and IL-1β resulted in a negative feedback mechanism within the tissue. However, this study was not designed to analyze these mechanisms, and additional studies are required to determine the consistency and potential mechanisms for this observation.

The data from the present study also revealed interesting differences between cytokine stimulated and unstimulated samples in response to compressive load magnitude. With the application of increased

magnitude of load in unstimulated samples, the production of IL-6 and MCP-1 decreased significantly. However, in the cytokine stimulated groups, the production of IL-6, MCP-1, and Gro-α significantly increased as level of load applied to the tissue increased. These data indicate that in the absence of pro-inflammatory stimulation, application of load to the injured IVD may decrease acute pro-inflammatory responses to injury. However, the application of load may exacerbate the acute pro-inflammatory responses by the inflamed IVD. Further study is required to determine how application of compressive load may affect the dynamics of pro-inflammatory biomarker production by IVDs after injury.

Production levels of the growth factor, IGF-1, and the chemokine, GRO, were highest in the 0.5-10 group. Further, the concentrations of these two biomarkers were similar within each group. These data indicate a potential link in the production of IGF-1 and GRO by the IVD in response to injury, pro-inflammatory stimulation, and load. Additionally, the production of these biomarkers appears to have a threshold mechanism, where production increases up to a specific level of load, but then decreases as load further increases. IGF-1 has been reported to stimulate the proliferation of NP cells, decrease apoptosis, and increase proteoglycan synthesis.[36–38] Therefore, these data indicate that after injury and with pro-inflammatory stimulation, the application of load at specific levels may initiate an attempted healing response by the IVD, and this response may involve a novel mechanism through the interaction of IGF-1 and GRO. The data from this study further indicate the importance of load in the pro-inflammatory stimulation of IGF-1 production. In the no-load samples, IGF-1 production was significantly decreased in IL-1β treated compared to untreated IVDs. However, in the 0.5 and 1.0 MPa load samples, the production of IGF-1 was significantly higher in the IL-1β stimulated compared to the unstimulated IVDs. Further study is required to determine how the production of these proteins are regulated by the IVD, and how the stimulation of the IVD with these proteins affects its metabolism and composition with and without injury.

The dynamics of growth factor release by IVD explants in the present study indicate that IGF-1, VEGF, and FGF-b each have unique production responses to injury, load, and pro-inflammatory stimulation. The production levels of IGF-1 and VEGF were significantly lower in the unloaded cytokine stimulated group (0-10) compared to the unloaded unstimulated control (0-0), while the production of FGF-b was significantly higher in the 0-10 group compared to the 0-0 group. Additionally, while the application

of load did not significantly affect the production of VEGF, IGF-1 production was highest in the 0.5 MPa loaded groups, and FGF-b was highest in the 1.0 MPa loaded groups. Previous studies have indicated that VEGF increases vascular infiltration into the disc, and IGF-1 and FGF-b increases cellular proliferation and proteoglycan production by IVD cells.[36,38–41] However, the dynamics of production regulation by, and mechanistic effect on, the IVD by these growth factors are still incompletely characterized. These data indicate that, acutely after injury, the IVD significantly decreases the production of VEGF and IGF-1 in response to pro-inflammatory stimulation, but application of load to the IVD is associated with recovery in production of these growth factors by the IVD. Furthermore, the stimulation of growth factor production by the IVD acutely after injury is potentially determined by the magnitude of the load applied to the tissue, with IGF-1 stimulation occurring at lower magnitudes and FGF-b stimulation occurring at higher magnitudes of load. Further study is required to determine how growth factors are regulated in the IVD based on these parameters, and how the dynamics of this regulation relates to the development and progression of IVDD.

The data from the present study indicate that pro-inflammatory stimulation of the injured IVD significantly increased tissue degradation acutely after injury. The production of TIMP-1 was significantly reduced in the 0-10 and 0.5-10 groups compared to the 0-0 control, indicating a loss of MMP activity regulation in these groups. Further, the level of general MMP activity was significantly higher in the 0-10 group compared to all other groups, which corresponded with a significantly higher release of GAG to the media and lower tissue GAG content and percentage of GAG in the tissue. However, the application of load to the IVD decreased general MMP activity in the media compared to the unloaded 0-10 group, and caused no significant differences in tissue GAG content compared to the unloaded, unstimulated control, indicating that load may aid in maintaining GAG content in the tissue of an injured disc. This is in agreement with previous studies that indicated increased MMP production, GAG release, and decreased tissue GAG content by IVDs after injury and\or IL-1β stimulation.[10,12,42–44] These data indicate the importance of loading to the health of the IVD, and indicate that moderate loading of the disc after injury may counteract the shift towards degradation that occurs in the IVD during the development and progression of IVD that occurs after injury. Clinically, this implies that after an acute injury to the IVD with or without surrounding inflammation, controlled loading, such as through therapy, may help maintain

tissue extracellular matrix components and limit degradative effects compared to keeping the injured disc immobile.

There are limitations to consider when interpreting these data. The tissues have been removed from the *in vivo* environment, and the changes in the production of biomarkers by the IVD may reflect responses to the *in vitro* culture conditions. In addition, species differences between rat tail and human IVDs may exist, including the smaller size of rat tail IVDs facilitating fluid flow to the NP during culture and the relative magnitude of the load applied being representative of those experienced *in vivo*. Further, the dynamics of loading were uniaxial and soft tissue components of functional spinal units were not included in the model, which oversimplifies the complexity of loading experienced by the spine. Finally, the explants were cultured for only 3 days, therefore the data from this study may only reflect the acute responses of injured IVDs to load and cytokine stimulation, and are not reflective of the IVDs responses to chronic application of these stimuli.

Conclusion:

With these limitations in mind, the data from this study indicate that after acute IVD injury, the application of physiologic dynamic compressive loading may counteract inflammatory and degradative metabolic responses associated with pro-inflammatory cytokine stimulation. Further, there are magnitude-dependent responses to load by injured and inflamed IVDs with respect to production of specific growth factors. Characterizing the pathways that drive these acute changes in injured IVDs may provide insight into the early stages of IVD degeneration. Understanding how load affects the IVD after injury may provide insight into the development of novel methodologies to mitigate risk for IVD degeneration. Further study is required to fully elucidate the mechanisms underlying IVD responses to load and cytokine stimulation observed in this study, and to translate these findings to the clinical setting.

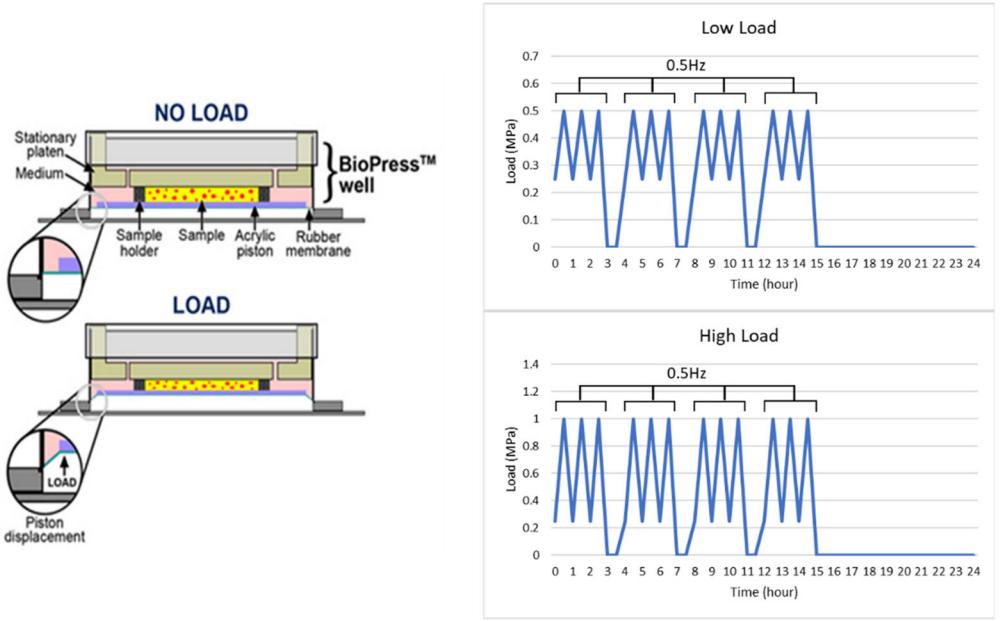

Figure 3-1: Loading system and loading protocols.

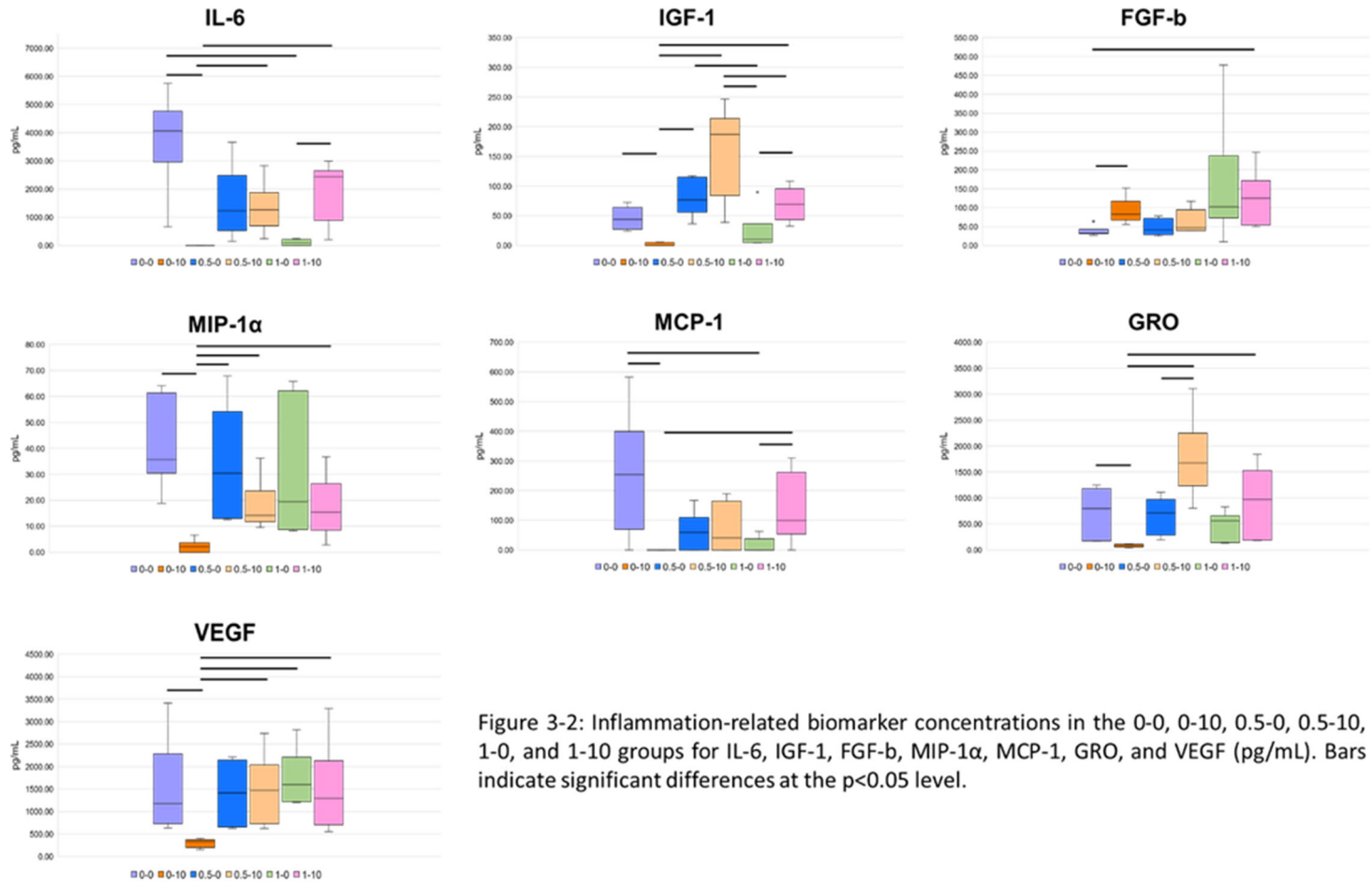

Figure 3-2: Inflammation-related biomarker concentrations in the 0-0, 0-10, 0.5-0, 0.5-10, 1-0, and 1-10 groups for IL-6, IGF-1, FGF-b, MIP-1α, MCP-1, GRO, and VEGF (pg/mL). Bars indicate significant differences at the $p<0.05$ level.

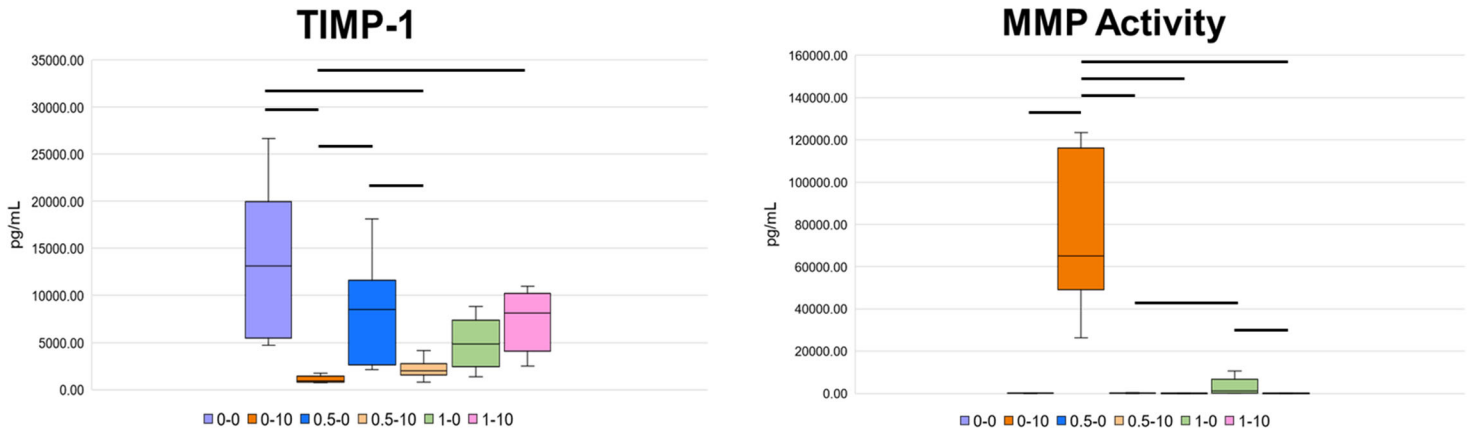

Figure 3-3: Degradation-related biomarker concentrations in the 0-0, 0-10, 0.5-0, 0.5-10, 1-0, and 1-10 groups for TIMP-1 and MMP activity (pg/mL). Bars indicate significant differences at the $p<0.05$ level.

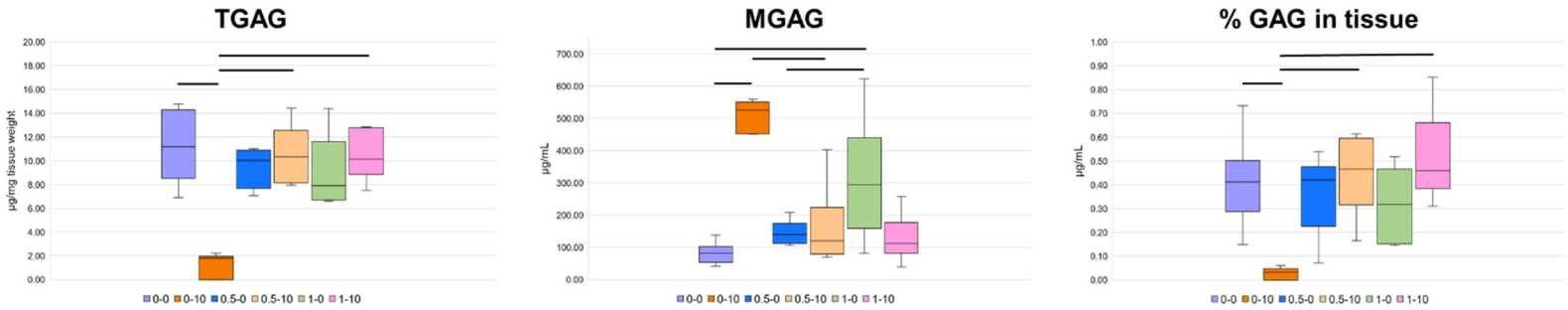

Figure 3-4: Proteoglycan content in the 0-0, 0-10, 0.5-0, 0.5-10, 1-0, and 1-10 groups for tissue GAG (µg/mg tissue weight), media GAG (µg/mL), percent GAG in the tissue (µg/mL). Bars indicate significant differences at the $p<0.05$ level.

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