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An Investigation of the Relationship Between Intervertebral Disc Puncture, Inflammation, and Tissue Mechanics Using a Rat-Tail Model.

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**An Investigation of the Relationship Between Intervertebral Disc Puncture,
Inflammation, and Tissue Mechanics Using a Rat-Tail Model.**

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Under the supervision of

Dr. Diane Gregory

Submitted to the Department of Kinesiology and Physical Education, in fulfillment of the

requirements for the degree of

Masters of Kinesiology

Wilfrid Laurier University

Abstract

Introduction: The intervertebral disc (IVD) is composed of the annulus fibrosus (AF), which surrounds and contains the nucleus pulposus (NP). It is hypothesized that when the IVD becomes injured, in the case of IVD herniation, a localized innate immune response is initiated. Although the presence of pro-inflammatory cytokines in injured IVDs has been well documented, the extent to which inflammation affects the mechanical properties of the IVD remains poorly understood. The purpose of this study was to determine the effect of IVD damage (via puncture) and inflammation (via lipopolysaccharide (LPS) exposure) on the mechanical and structural properties of the IVD.

Methods: Four functional spinal units (FSUs) (bone-IVD-bone) were dissected and removed from the tails of 20 Sprague Dawley rats (80 FSUs in total). In half of the FSUs, a puncture model (using a 19G needle) was used to mimic IVD herniation. Each FSU (two punctured and two non-punctured) was subsequently cultured in media to support cell viability. Further, 10ug/mL of LPS was added to the media of one punctured and one non-punctured FSU. FSUs were then cultured for either 24 hours or 6 days after which they were removed and mechanically tested in order to determine changes in IVD mechanics as a result of exposure to LPS and/or IVD puncture. All FSUs were subjected to cyclic mechanical loading in compression/tension using a displacement-controlled protocol. From the force displacement data obtained, the neutral zone (NZ) size (mm) and stiffness (MPa/mm) were calculated and compared. Four additional FSUs cultured for 6 days were subsequently used for histological analyses in order to visually identify changes to the structural properties. Media was collected and analyzed in order to quantify concentrations of pro-inflammatory cytokines (TNF α , IL-6) and chemokines (MCP-1, MIP2, RANTES).

Results: When collapsed across damage and media condition, NZ size increased and stiffness decreased from 24 hours to 6 days. Further, there was an increase in NZ size as a result of puncture, which is indicative of an increase in joint laxity. Interestingly, there was a decrease in NZ stiffness in the punctured FSUs observed at 24 hours however by 6 days, punctured FSUs were more stiff than the non-punctured FSUs indicating the presence of a potential reparative mechanism in the IVDs. All cytokines and chemokines measured in the media, regardless of condition, peaked at 24 hours and dropped off by 6 days. There was an interaction of media (LPS versus no LPS) and day (24 hours versus 6 days) where the FSUs placed in LPS had a higher concentration of TNF α and RANTES at 24 hours but no significant difference at 6 days. Comparatively, IL-6 concentrations were higher in the control group at 6 days with no changes at 24 hours. Histological analysis showed proteoglycan staining in the puncture tract, AF inward bulging and disorganization in the control media/puncture group. Moreover, in the LPS/no puncture FSU there was a significant lack of proteoglycan staining in the NP.

Discussion and Conclusion: This study demonstrated that punctured FSUs might initiate a reparative mechanism within the IVD that is not present in non-punctured FSUs. Further, the findings of this study indicate that the inflammatory profile created by LPS is different than the inflammation that might ensue as a result of IVD herniation. The increase in TNF α and RANTES in the LPS group and the increase in IL-6 in the control group is different than what has been found in previous research and may be due to differences in the regulatory mechanisms of TNF α and IL-6 or due to differences in the dose dependent response to LPS exposure

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1. List of Abbreviations

- **AF: Annulus fibrosus**
- **ANOVA: Analysis of variance**
- **DAMP: Damage-associated molecular pattern**
- **DDD: Degenerative disc disease**
- **ECM: Extracellular matrix**
- **G: Gauge**
- **IAF: Inner annulus fibrosus**
- **IVD: Intervertebral disc**
- **LBP: Low back pain**
- **LPS: Lipopolysaccharide**
- **NP: Nucleus pulposus**
- **NZ: Neutral zone**
- **OAF: Outer annulus fibrosus**
- **PAMP: Pathogen-associated molecular pattern**
- **ROM: Range of motion**
- **TLR: Toll like receptor**
- **VEP: Vertebral endplates**

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4. Introduction

The intervertebral disc (IVD) is composed of the layered annulus fibrosus (AF), the gelatinous nucleus pulposus (NP) and the cartilaginous vertebral endplates (VEP). The VEPs separate the IVD from the adjacent vertebrae at both the cranial and caudal ends. Injury to the IVD has been acknowledged as a significant contributor to the development of low back pain (LBP), which remains a major health care issue worldwide (Andersson, 1999; Freemont, 2009; Hoy *et al.*, 2012; Luoma *et al.*, 2000). Only the outer one-third of the AF is vascularized leading to poor recovery of the IVD if damaged. IVD herniation, which is the migration of the NP through fissures of the damaged AF, is one of the most common methods of injuring the IVD. Herniation leads to a significant disturbance in the IVD's homeostatic environment leading to disruptions in its ability to maintain its biomechanical function (Kadow, Sowa, Vo, & Kang, 2014). It is hypothesized that when the NP and AF are displaced as a result of injury, a localized innate immune response is provoked. This response causes the resident cells to produce pro-inflammatory cytokines and chemokines at the site of injury leading to inflammation and additional tissue damage which further compromises the IVD's biomechanical function (Werling, Jungi, & Bern, 2003). The aim of this research paper was to gain a better understanding of how IVD damage and exposure to an inflammatory environment, affect the biomechanical and structural integrity of the IVD. LBP as a result of IVD herniation remains a complex and poorly understood area of research. Understanding how the mechanical properties of the IVD are affected as a result of exposure to inflammation will help further the body of knowledge with the hope of developing adequate treatment and rehabilitation options for those suffering from LBP.

5. Review of the Literature

5.1 Prevalence of LBP

LBP is the most common and costly reoccurring condition affecting 70-80% of the population at least once in their lifetime (Andersson, 1999). Worldwide, LBP poses as a huge health care burden as it is the leading cause of limitations in daily activities for individuals under the age of 45 years (Hoy *et al.*, 2012). This translates to severe consequences economically, where international annual indirect costs associated with LBP are between 1-28 billion dollars (Dagenais, Caro, & Haldeman, 2008; Gore, Sadosky, Stacey, Tai, & Leslie, 2012). Although most incidences of LBP resolve by themselves, a significant percentage of individuals develop chronic LBP where pain persists for longer than three months. In fact, a recent review by Hoy *et al.* (2012) reported that over 20% of people worldwide develop chronic LBP and almost 40% report a lifetime prevalence of reoccurring episodes. Consequentially, LBP constitutes as a major international health care issue with treatment options remaining marginally effective.

5.2 Causes of LBP

Degeneration of the lumbar IVDs has been associated as a significant contributor in the development of LBP. A study by Luoma *et al.*, (2000) found that the 4-year prevalence of LBP associated with IVD degeneration was 81.1%. Although there is little consensus on the direct causes of IVD degeneration, it is often referenced as a multifaceted issue involving a combination of genetic, biological, nutritional and mechanical factors (Adams & Roughley, 2006; Kadow *et al.*, 2014). It is still largely unknown why some cases of IVD degeneration are asymptomatic while others lead to LBP; unregulated inflammation within

the joint, however, is thought to be a contributing factor (Freemont, 2009; Molinos *et al.*, 2015; Wuertz & Haglund, 2013).

IVD herniation is the result of a sudden or progressive displacement of IVD material beyond the limits of the IVDs boundaries and can severely accelerate the degenerative process (Fardon *et al.*, 2014). Additionally, the healthy intact IVD is thought to be an immune privileged site, meaning it is isolated from the adaptive immune system (Clouet *et al.*, 2009; Erwin & Hood, 2014). However, upon injury through herniation or degeneration, the inner contents of the IVD can become exposed to adaptive immunity. The innate immune response is the result of resident cells producing pro-inflammatory cytokines and chemokines at the site of injury, which can compromise the IVD's homeostatic environment (Wuertz & Haglund, 2013). Recent research has developed interest in evaluating the effects of the immune response that is activated through injury and its implications in the development of LBP (Gabr *et al.*, 2011; Murai *et al.*, 2010; Purmessur *et al.*, 2013). Despite recent developments in this area of research, the extent to which the immune response affects the mechanical properties of the IVD remains poorly understood.

5.3 Anatomy of the IVD

The IVD is a critical structure of the spinal column; it connects adjacent vertebrae and accounts for approximately 30% of the total vertebral height (Urban & Roberts, 2003). Its main functions are to transfer load, dissipate forces, and allow minor movement along the spinal column (Urban & Roberts, 2003). A healthy lumbar IVD has an average cross-sectional area of $1727 \pm 549 \text{ mm}^2$ and a IVD height of $8.93 \pm 0.13 \text{ mm}$ (O'Connell, Vresilovic & Elliot 2006). The IVD is a heterogeneous fibrocartilaginous joint composed of

three regions: the AF, the NP and the cartilaginous VEP (Figure 1). The IVD is defined as an anisotropic structure as it exhibits different mechanical properties when loaded in different directions (Galante, 1967; Rodgers & Cavanagh, 1984). Each structure has a distinct cellular, compositional, and morphological make-up that gives the IVD its unique mechanical properties. In addition, the IVD is mostly an aneural and avascular structure since both nerves and blood vessels are present in only the outer one third of the AF (Erwin & Hood, 2014). As a result, the transfer of molecules is highly dependent upon diffusion through the layers of the AF and the VEP.

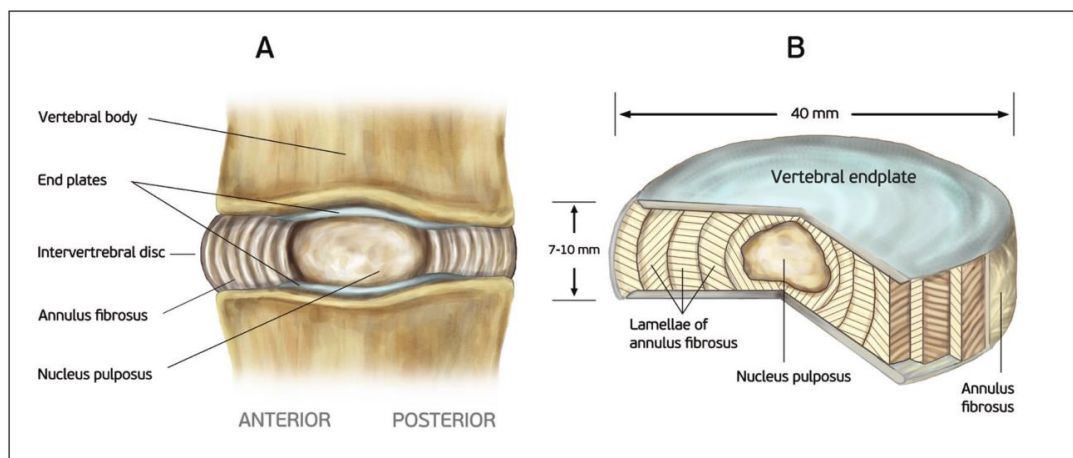


Figure 1: The anatomy of the IVD **A)** showing the VEPs, AF and NP **B)** showing the lamellae as retrieved from Tomaszewski, Saganik, Gładysz, & Walocha, (2015), reproduced with permission.

5.3.1 AF Function, Cellular Composition and ECM

The AF is a dense structure derived from the mesenchyme during embryonic development (Kerr, Veras, Kim, & Séguin, 2016). The AF acts to surround and contain the NP due to the 15-25 concentric lamellae that make up its structure (Figure 1). The AF can be further broken down into the outer annulus fibrosus (OAF) and the inner annulus fibrosus (IAF).

The OAF is comprised of elongated fibroblast-like cells which synthesize type I collagen fibres, proteoglycans, and elastin (Table 1). These components, combined with small concentrations of water, make up the OAF's extracellular matrix (ECM) (Freemont, Watkins, Le Maitre, Jeziorska, & Hoyland, 2002; Zhao, Wang, Jiang, & Dai, 2007). The type I collagen fibres are oriented parallel to one another, alternating in direction in each successive lamellae (Pezowicz, Robertson, & Broom, 2005) and act to resist tensile forces. Collagen fibres in the OAF are strongly attached to the cranial and caudal vertebrae providing strength within the IVD.

The IAF is commonly referred to as the transition zone between the OAF and the NP since its composition reflects characteristics seen in both structures (Erwin & Hood, 2014). The IAF is composed of water and spherical chondrocyte-like cells, which synthesize primarily type II collagen fibres as well as type I collagen fibres (to a lesser extent), proteoglycans and elastin (Table 1) (Freemont *et al.*, 2002; Guilak *et al.*, 1999; Zhao *et al.*, 2007). The presence of type I collagen fibres decreases in concentration within the IAF closer to the NP. These collagen fibres are more weakly attached to the vertebra compared to within the OAF (Adams, 2015). In healthy young adults, the differentiation between the NP and the AF is highly distinguishable. However, as a natural consequence of aging, the anatomical differentiation between both structures becomes less apparent (Erwin & Hood, 2014). The visible disruption in the anatomical boundary can be attributed to biological and mechanical changes that occur in the cellular make-up of the IVD as a result of aging and injury (Figure 2).

5.3.2 NP Function, Cellular Composition, and ECM

The NP is a gelatinous, highly disorganized structure derived from the notochord during embryonic development (Hunter, Matyas, & Duncan, 2004). In humans, the concentration of notochordal cells is highest during early childhood, but these cells are gradually replaced with chondrocyte-like cells as the individual ages (Adams & Roughley, 2006; Tomaszewski *et al.*, 2015; Weiler *et al.*, 2012). The spherical chondrocyte-like cells within the NP synthesize type II collagen fibres and a rich concentration of the proteoglycan aggrecan (Table 1). The type II collagen fibres range in sizes from 30-500µm, are highly unorganized, and are found interspersed throughout the gelatinous network (Tomaszewski *et al.*, 2015). Proteoglycans are proteins surrounded by glycosaminoglycan groups and are critical in water retention (Pattappa *et al.*, 2012; Zhao *et al.*, 2007). Water is abundant within the NP as a result of the high concentration of the hydrophilic aggrecan (a type of proteoglycan) molecules (Erwin & Hood, 2014). Aggrecan maintains hydration of the NP by attracting water molecules and enables the IVD to generate hydrostatic pressure and resist compressive forces when placed under load (Boxberger, Sen, Yerramalli, & Elliott, 2006; Pattappa *et al.*, 2012). The age-related loss of proteoglycan concentration is due in part to the progressive conversion of notochord cells to chondrocyte-like cells. This adaptation leads to a decrease in proteoglycan synthesis and thus a loss in the IVD's ability to effectively maintain its hydrostatic properties (Figure 2) (Urban & Roberts, 2003).

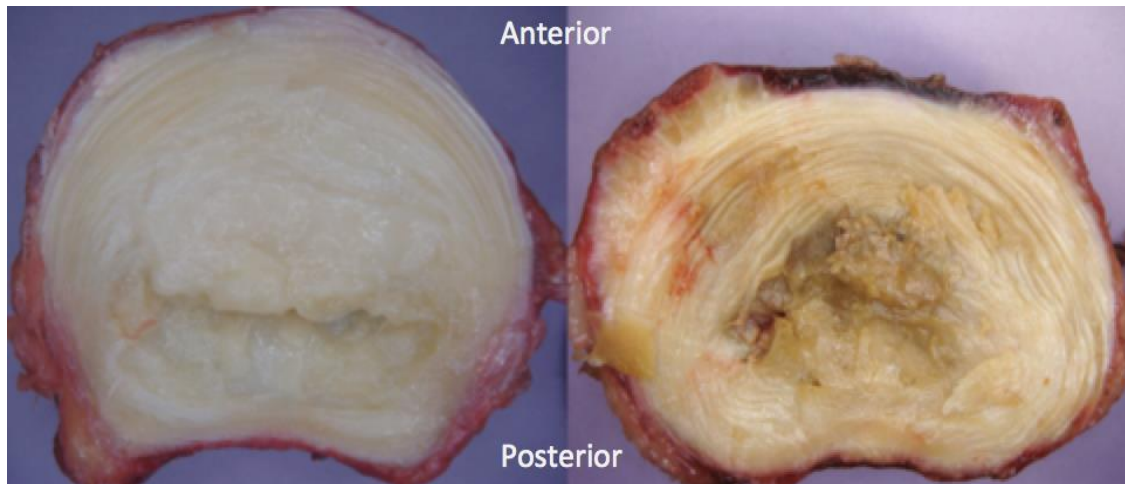


Figure 2: A representation of a healthy human IVD (left) where the highly organized AF surrounding the gelatinous NP are distinguishable and a degenerated IVD (right) where the AF/NP border is severely disrupted, the NP is severely dried out and the AF layers are deteriorated. As retrieved and reproduced with permission from Dr. Gregory.

5.3.3 VEP Function, Cellular Composition, and ECM

The VEP separates the AF and the NP from the vertebral bodies at both the cranial and caudal ends (Figure 1). The VEP is composed of horizontally organized chondrocyte cells that synthesize type I and type II collagen fibres and proteoglycans. (Table 1) (Lotz, Fields, & Liebenberg, 2013). In healthy adults, the VEP is avascular and aneural and is a critical structure of the IVD (Raj, 2008). The relative size of the VEP varies in thickness (between 0.1 and 2.00mm) depending on its position and level within the vertebral column. The VEP is thinnest above/below the NP and thickest in the outer portions of the IVD above/below the AF (Erwin & Hood, 2014; Lotz *et al.*, 2013). Due to its thinness above the NP, the VEP mediates the diffusion of small molecules from the NP to the vascularized vertebral bodies (Lotz *et al.*, 2013). The absorption of nutrients such as glucose and oxygen are able to diffuse in and waste products are able to diffuse out of the avascular IVD (Lotz *et al.*, 2013). Additionally, the VEP acts to dissipate forces evenly along the vertebral bodies by helping to contain the pressurized NP.

Table 1: Composition of the ECM of human lumbar IVDs as retrieved from Pattappa *et al.*, 2012; Tomaszewski, Saganiak, Gładysz, & Walocha, 2015.

	OAF	IAF	NP	VEP
Human lumbar IVD	Type I collagen (50-70% dry weight), Proteoglycan (10-20%), Elastin (2%), Water (60-80%)	Type I/II collagen (50-70% dry weight), proteoglycan (10-20%), elastin (2%) and water (60-90%)	Type II collagen (20% dry weight), proteoglycan aggrecan (50%) and water (70-90%)	Type II collagen, proteoglycan and water (50-60%)

5.4 Mechanical Properties of the IVD in Compression and Tension

5.4.1 The Force-Displacement Relationship and Mechanical Neutral Zone

The complex interaction of each structure (AF, NP, VEP) within the IVD is what allows it to respond dynamically when loaded in 6 degrees of freedom (Palepu, Kodigudla, & Goel, 2012). It is important to understand the mechanical properties of the spine as many accounts of degeneration and low back pain have been attributed to altered mechanics within the IVD (Raj, 2008; Vergroesen *et al.*, 2015). Tension is characterized as the loading of tissue where forces acting in the same plane, but in opposite directions, pull the structure apart (Rodgers & Cavanagh, 1984). As previously described, the AF experiences a considerable amount of tensile force as it deforms to withhold and contain the pressurized NP. Compression is defined as the loading of a tissue where forces acting in the same plane but in the opposite direction drive the structure together (Rodgers & Cavanagh, 1984). The NP and the IAF conversely are loaded in compression due to the hydrostatic pressure within the NP. The relationship between magnitude of loading and resulting deformation is referred to as the force-displacement relationship. In biological tissues, such as the IVD, the force-displacement relationship is used to examine recoverable (termed the elastic region)

and non-recoverable (termed the plastic region and indicates damage/failure) deformation due to load (Panjabi, 1992; Rodgers & Cavanagh, 1984) as well as tissue properties such as stiffness and range of motion. Tissues have a force-displacement relationship in all degrees of freedom. Generally, the NP exhibits an isotropic force-displacement relationship, as its primary function is to resist compressive forces throughout its neutral zone region (Guilak *et al.*, 1999). Contrastingly, the AF exhibits anisotropic properties due to its more heterogeneous structure as it responds to loads (Guilak *et al.*, 1999).

The neutral zone (NZ) is the mechanical term used to define the IVD's range of motion (ROM) that causes the least amount of tissue resistance (Figure 3) (Panjabi, 1992; Smit, van Tunen, van der Veen, Kingma, & van Dieën, 2011) and its range is often used as an indicator of joint flexibility or laxity. Since IVDs have a force-displacement relationship for all degrees of freedom, IVDs also have a NZ for all degrees of freedom, including tension-compression (Barbir, Michalek, Abbott, & Iatridis, 2010; Boxberger, Sen Sounok, *et al.*, 2006; Panjabi, 1992). The effects of spinal instability in degenerated IVDs as a result of changes to the NZ have been studied in previous work (Mimura *et al.*, 1994; Beazell, Mullins & Grindstaff, 2010) and changes to the NZ have been recognized as a better indication of joint instability compared to changes in full ROM (Panjabi, 1992; Oxland & Panjabi, 1992). Since the NZ is defined as a region of low resistance, increases in size and a decrease in stiffness within this region can result in hypermobility of the IVD joint. A hypermobile joint can result in the activation of compensatory mechanisms (increased muscle activation, altered mechanical loading) in an attempt to stabilize the joint further compromising the IVD and its mechanics.

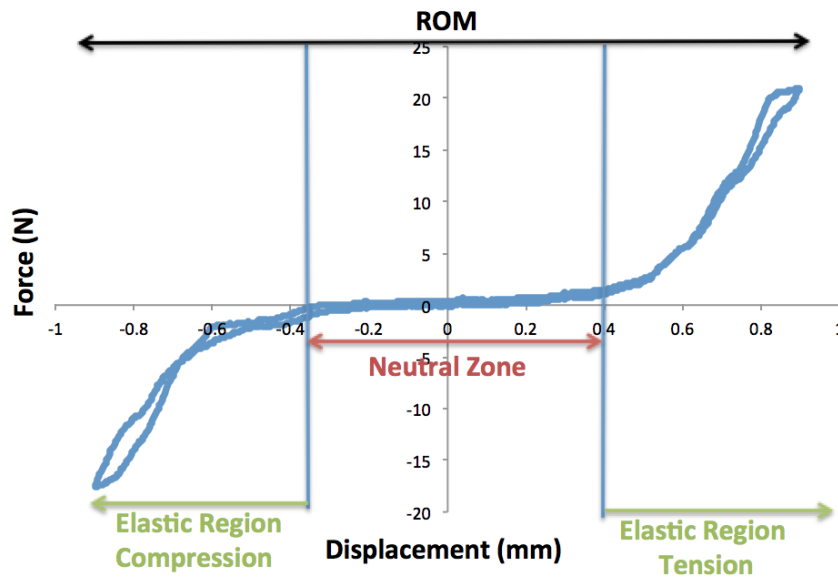


Figure 3: A force-displacement graph illustrating the ROM of one full compression/tension cycle as well as the elastic regions (region of high stiffness without failure) and NZ size (region of low stiffness).

5.5 The Rat Tail Model

5.5.1 Rat-tail model as a suitable model for IVD research

Animal models are commonly used in IVD research as they offer an accessible and affordable alternative to cadavers. Small animals have been prevalent in studies evaluating the effects of injury-induced degeneration (Masuda *et al.*, 2005; Sobajima *et al.*, 2005; Zhao *et al.*, 2007), altered mechanics (J J MacLean, Lee, Alini, & Iatridis, 2005; Sarver & Elliott, 2005; Yurube *et al.*, 2012), and genetic factors (Chan, Song, Sham, & Cheung, 2006) on the IVD. In particular, the rat-tail model has been popular in IVD studies as the tail is cost effective, requires minimal invasive surgery, and is an easily accessible and reproducible way to study IVDs (Ching, Chow, Yao, & Holmes, 2003; Iatridis, Mente, Stokes, Aronsson, & Alini, 1999; MacLean *et al.*, 2003; Zhang *et al.*, 2011). The IVDs between the caudal vertebrae Co2-Co12 are commonly used in research, as they are the most anatomically

similar to each other in shape and size with minimal spinal processes (Hebel & Stromberg, 1976).

It is evident that the size and composition of rat-tail IVDs vary considerably from humans IVDs. Nevertheless, comparisons of IVD geometry of the rat caudal IVD against those of the human lumbar have been reported and are summarized in Table 2 (Boxberger, Sen, *et al.*, 2006; Elliott *et al.*, 2008; Showalter *et al.*, 2012).

Table 2: Comparison of IVD geometry between the human lumbar and rat-tail. Standard deviations depicted in parentheses. Retrieved and modified from Boxberger, Sen, Yerramalli, & Elliott, 2006; Elliott *et al.*, 2008; Showalter *et al.*, 2012.

	IVD Area (mm ²)	IVD Height (mm)	NP Size (mm ²)	Water Content AF/NP
Human IVD	1727 (549)	8.93 (0.13)	479 (110)	76.5%/80%
Rat tail IVD	8.86 (3.54)	0.94 (0.09)	3.30(1.55)	70.4%/83.5%
Relative percentage	0.5%	10.5%	0.7%	N/A

In addition, the cellular composition, particularly the preservation of notochordal cells within the NP, is known to be vastly different between rat IVDs and human IVDs. In general, rat IVDs maintain their concentration of notochordal cells much longer throughout their lifespan than do humans. This has significant implications on the rat IVD's ability to maintain its mechanical integrity and decreases its susceptibility to injury. However, rats are considered to have reached full skeletal maturity between 2-3 months of age and is therefore a common time frame to sacrifice the rats for research (Ching *et al.*, 2003; Han *et al.*, 2008; Iatridis *et al.*, 1999).

5.5.2 Biomechanics of the Rat Tail IVD

Elliott & Sarver (2004) exposed rat caudal IVDs to 20 cycles of cyclic loading in compression and tension with peak loads reaching 3.5N applied at a rate of 1Hz. Once normalized for IVD size and geometry, they concluded that the compressive stiffness (2-4MPa) in the rat were similar to the compressive values (3-9MPa) of humans reported in previous studies (Gardner-Morse & Stokes, 2004; McGlashen, Miller, Schultz, & Andersson, 1987). In addition, the study by Lotz & Chin, (2000) have projected that in normal in vivo conditions, rat caudal IVDs are exposed to 0.4MPa of force when lifting and moving their tail. When differences in surface area are account for, this amount of force is comparable to the forces experienced by humans in standing upright posture (Schultz, Belytschko & Andriacchi, 1973). This study establishes that although obvious differences between the human lumbar and rat caudal IVDs exist, rats still demonstrate similar mechanical properties when size and geometry are accounted for.

In 2010, Barbir, Michalek, Abbott, & Iatridis tested the NZ size and stiffness of rat caudal IVDs. Three caudal FSUs (Co5/Co6, Co7/Co8, Co9/Co10) from 34 rats were excised and were subject to cyclic compression/tension for 20 cycles at 1Hz with a force of 6.25N. Their findings are as follows: neutral zone stiffness: 0.42 ± 0.09 N/mm, neutral zone size 0.82 ± 0.05 mm and cumulative height loss: 0.02 ± 0.0 mm. Moreover, they found that IVDs treated with enzymes that break down the ECM of the NP resulted in an increase in NZ size and a decrease in stiffness with no changes to the compressive and tensile stiffness (Barbir *et al.*, 2010). These findings provide baseline measures for the rat caudal IVD and indicate that changes to the NP result in significant variations in NZ size and stiffness.

5.6 Injury to the IVD

Due to the high and constant demand placed on the IVD, it is not surprising that it is highly susceptible to injury. IVD degeneration is a complex and poorly understood deterioration of the IVD marked by a disruption in the balance of the tissues' anabolic and catabolic activity. Degeneration can lead to altered dissipations of force throughout the IVD, which has severe effects on spine kinetics (Iatridis, Michalek, Purmessur, & Korecki, 2009). Research continues to evaluate the differences that constitute the natural changes associated with aging (Adams, 2015; Zhao *et al.*, 2007), and the accelerated degenerative process that can occur as a result of herniation (Han *et al.*, 2008; Issy *et al.*, 2013). There are a multitude of factors that are believed to predispose or hasten IVD herniation/ degeneration such as altered mechanical loading patterns, genetics, nutrition and inflammation (An *et al.*, 2004; Chan *et al.*, 2006; Kadow *et al.*, 2014; Urban & Roberts, 2003).

5.6.1 IVD Herniation

IVD herniation occurs as a sudden or progressive migration of the NP through radial fissures of the damaged AF (Fardon *et al.*, 2014; Gregory, 2011). IVD herniation can be the result of sudden trauma or a result of an accumulation of repetitive stress. The most common type of herniation is reported in slightly degenerated IVDs that are exposed to repetitive forces (Kadow *et al.*, 2014). Severely degenerated IVDs are less likely to herniate, as the NP is already too dehydrated and the hydrostatic pressure is not great enough to push the NP through the fissures of the AF (Figure 2) (Schmidt, Kettler, Rohlmann, Claes, & Wilke, 2007). The consequences of herniation are substantial due to the avascular nature of the IVD. Once injured, the IVD does not have the necessary means

to properly remove and replace the damaged tissue. This process leads to an accumulation of waste products within the inner portions of the IVDs, further exacerbating the degenerative process (Iatridis *et al.*, 2009). Research is still needed in order to determine why some herniated IVDs lead to painful symptoms while others do not. However, the effects of inflammation as a result of herniation are believed to have important implications in the onset of pain (Grange *et al.*, 2001; Risbud, Makarand and Shapiro, 2008; Wuertz & Haglund, 2013) and will be discussed later in this paper.

5.6.2 IVD Degeneration

Since degeneration is a complex interplay of many factors, coming to a true definition has proven difficult. A paper by Adams & Roughley (2006), proposed the following definition:

“disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerated disc is one with structural failure combined with accelerated or advanced signs of aging” (Adams & Roughley, 2006).

IVD degeneration is associated with many structural changes due to degradation of the ECM in the AF (layer disorganization), NP (dehydration) and VEP (calcification). One of the biggest and most definitive indicators for early IVD degeneration is a significant drop in proteoglycan concentrations within the NP. As previously discussed, the loss in proteoglycan concentrations is often due to the age-related conversion of notochordal cells to chondrocyte-like cells (Adams & Roughley, 2006). However, degeneration of the IVD can occur as a result of acute herniation from repetitive mechanical loading, which leads to a loss of pressurization of the IVD. Loss of NP pressurization has an immediate and direct affect on IVD mechanics (Michalek & Iatridis, 2011). The NP’s inability to maintain

pressurization leads to a drop in hydrostatic pressure and the capacity for the IVD to resist compressive forces becomes severely disrupted (Zonia & Munnik, 2007). Consequentially, compressive forces exerted along the spine are translated to the IAF and the OAF (Adams & Roughley, 2006). Long-term exposure to compressive forces through repetitive mechanical loading leads to biological changes in the AF's ECM towards catabolism further compromising the IVDs ability to withstand forces during movement (Lotz & Chin, 2000). In degenerated IVDs, when the AF is loaded in compression, there is an increase in type I collagen disorganization (Figure 2). Progressive deterioration of the AF causes clefs and fissures, predisposing the IVD to herniation (Gregory *et al.*, 2011).

5.6.3 Needle Puncture Model

The first puncture model was proposed by Smith & Walmsley, (1951) as a means to induce IVD degeneration in animal models since it mimics herniation. Since then, it has been a widely practiced and accepted method in *in vivo* and *in vitro* research. The puncture model can be classified into two general categories: the full AF puncture model and the partial AF puncture model (Masuda *et al.*, 2005). The full AF puncture model is the most commonly practiced method as it is easily reproducible and induces IVD degeneration faster than the partial AF model (Han *et al.*, 2008; Sobajima *et al.*, 2005). Many studies have evaluated the effects of needle puncture in IVD degeneration in animal models (Han *et al.*, 2008; Issy *et al.*, 2013; Keorochana *et al.*, 2010; Zhang *et al.*, 2011), and have demonstrated that AF injury causes immediate changes to IVD mechanics (Korecki, Costi, & Iatridis, 2008; Michalek, Buckley, Bonassar, Cohen, & Iatridis, 2010; Michalek & Iatridis, 2011). A review conducted by Elliott *et al.*, (2008), found that significant changes

in IVD mechanics, biochemistry and decreases in IVD height were found only in studies that used needle sizes that were greater than 40% of the overall IVD height.

Many *in vivo* and *in vitro* studies have evaluated the effect of varying needle sizes ranging from as large as 18G to as small as 30G (Han et al., 2008; Hsieh, Hwang, Ryan, Freeman, & Kim, 2009; Issy et al., 2013; Michalek, Buckley, et al., 2010). In general, in *in vivo* models, the larger needle diameters (18G and 20G) resulted in greater changes in IVD properties such as biomechanics, AF damage, and water loss approximately one week post-puncture, and that structural property changes remained 30 days post-puncture (Issy et al., 2013).

Inflammatory markers have also been examined in response to IVD puncture *in vivo* in rats. The study by Miyagi *et al.*, (2012) examined the effect of IVD puncture (24G) on the expression of pro-inflammatory cytokines. In the puncture injury group, cytokines were significantly elevated at 4 days (IL- β and IL-6) and 1 week (TNF α) post-puncture compared to controls.

It is evident that the puncture model causes significant and immediate changes to IVD mechanics in the rat-tail model. Based on these findings, a 19G needle was chosen to simulate IVD herniation as an 18G needle and 20G needle have been shown to cause histological (Han *et al.*, 2008; Issy *et al.*, 2013; Hsieh *et al.*, 2009) and biomechanical (Hsieh *et al.*, 2009) changes to the IVD. In addition, there is evidence that needle puncture causes significant increases in pro-inflammatory cytokines (Miyagi *et al.*, 2012).

5.7 The Immune Response and Inflammation

Damage to the IVD through herniation triggers an immune response leading to inflammation and further tissue damage (Ulrich, Liebenberg, Thuillier, & Lotz, 2007). It is

still unknown whether IVD degeneration is a cause or consequence of inflammation and to what degree it advances the degenerative process. The innate immune response is the acute onset of inflammation activated primarily by local cells as a result of tissue damage (Suffredini, Fantuzzi, Badolato, Oppenheim, & O'Grady, 1999). The innate immune response is particularly important in the IVD given that it is largely avascular and aneural. Recent studies have determined that damage to the NP and AF elicits a localized innate immune response that is independent of blood supply (Murai *et al.*, 2010). The damage associated with these structures activates signaling molecules known as alarmins or danger associated molecular patterns (DAMPs) (Manson, Thiemermann, & Brohi, 2012), which activate toll like receptors (TLRs) within the IVD (Werling *et al.*, 2003). Activation of TLRs play a pivotal roll in innate immunity by promoting the induction of cytokines, chemokines, and catabolic enzymes and have been highly reviewed in research of inflammatory joint diseases such as arthritis. Specifically, TLR2 and TLR4 receptors have been found in human IVDs on the cells of the AF, NP and VEP and activation of these receptors have been correlated with degree of degeneration (Klawitter *et al.*, 2014). Activation of the TLR pathway leads to a signaling cascade and the release of pro-inflammatory cytokines/chemokines within the IVD (Hirsiger, Simmen, Werner, Wanner, & Rittirsch, 2012; Sun *et al.*, 2013; Zhao *et al.*, 2007). The concentration and type of cytokine/chemokines secreted varies depending on the specific structure (AF, NP, VEP) degree of injury (intact, herniated and degenerated) and the receptors activated (Shamji, Setton, Jarvis, So, Chen, Jing, Bullock, Isaacs, Brown and Richardson, 2011; Risbud, Makarand & Shapiro, 2008).

5.7.1 LPS as an Inflammatory Stimulant in IVD Research

Recent work has confirmed that TLR-2 and TLR-4 within the IVD also respond to pathogen associated molecular patterns (PAMPS) from bacterial and viruses in addition to alarmins (DAMPs). In fact, studies have found that painful degenerated (Stirling, Worthington, Rafiq, Lambert, & Elliott, 2001) and herniated (Albert *et al.*, 2013) IVDs had higher concentrations of bacterial cultures compared to controls indicating that bacterial infection may contribute to the pathogenesis of degenerative disc disease (DDD). LPS is an endotoxin (a PAMP) found on the outer membrane of gram-negative bacteria, is immune stimulant and is pro-inflammatory. Its use in IVD research has become increasingly popular due to its recognition and activation of the TLR-2 and TLR-4 receptors (Bobacz, Sunk, Hofstaetter, Amoyo, Toma, Akira, Weichart, Saemann & Smolen, 2007; Kim *et al.*, 2013).

The study by Rajan, Bloom, Maidhof, Stetson, Sherry, Levine & Chahine (2013) found that TLR-4 expression was found to be in all three-cell types of the bovine IVD with the highest being in the VEP compared to AF and NP. Upon 24 hours of LPS exposure (0.01µg/ml, 0.1µg/ml, 1.0µg/ml and 10µg/ml) they found that cells within the IVD responded in a dose dependent manner with the most significant increase in TLR-4 activation occurring in the NP. Interestingly, cell viability dropped 20% upon 10µg/ml of LPS exposure in the NP and was unaffected in the VEP and AF. Increases of TNFα, IL-1β and IL-6 were observed in a dose dependent manner with peaks occurring at 1.0µg/ml followed by significantly lower levels of cytokines observed with 10µg/ml exposure. However levels of cytokines seen at 10µg/ml were still significantly greater compared to controls. LPS also caused a significant dose dependent down-regulation of aggrecan (NP and AF) and type II collagen (NP, AF, VEP).

Rajan *et al.* (2013) further examined the effect of injecting LPS directly into rat caudal IVDs using a 33G needle. They found through histological analysis that LPS resulted in significant changes in IVD morphology such as border disruptions, AF tears, and changes in NP cellularity compared to a PBS injection control. Increases of TNF α and IL-1 β were also quantified at 24 hours and 7-day post injection. In the present study, levels of TNF α and IL-1 β were higher at 24 hours versus 7 days post injection (in both LPS and PBS) but that LPS stimulation resulted in significantly higher increases in those cytokines after 24 hours. Interestingly, 7 days post injection there was still an increase in IL- β in the LPS group compared to controls but there was no difference in TNF α levels between groups. This suggests that TNF α might play a pivotal role in early inflammation whereas IL- β is more important in later stages of inflammation. Their findings indicate that TLR-4 receptors exist on all three major structures of the IVD and that upon LPS exposure both the expression of TLR-4 and concentration of cytokines within the IVD are increased in a dose dependent manner leading to a breakdown of the ECM.

5.7.2 Cytokine and Chemokine Released Through TLR4 Activation

Cytokines (TNF α and IL-6) and chemokines (MCP-1, MIP2 and RANTES) have been chosen as primary inflammatory markers in this project due to their up regulation through TLR-2 and TLR-4 activation and their importance in innate immunity. As previously mentioned, stimulation of the TLR-4 receptor with LPS resulted in increases of some of these cytokines/chemokines. In addition, some of these cytokines and chemokines have been found to be secreted by IVD cells themselves (Gruber, Hoelscher, Ingram, Bethea, Norton & Hanley, 2014; Risbud & Shapiro, 2008; Guerreiro, Laird, Gregory & DeWitte-Orr, 2017)

Cytokines are cell signaling molecules that mediate cell-to-cell communication and regulate immune responses by stimulating the movement of other inflammatory markers towards the site of injury (Borish & Steinke, 2003). TNF α is a pro-inflammatory cytokine responsible for innate immune cell activation and it is arguably the most commonly researched cytokine in relation to IVD degeneration (Evans *et al.*, 2005; Walter *et al.*, 2015; Purmessur *et al.*, 2013; Fujita *et al.*, 2012; Zhang *et al.*, 2016). Not only has TNF α been found to be elevated in herniated (Le Maitre *et al.*, 2007) and degenerated (Lee *et al.*, 2009; Holm *et al.*, 2009) IVDs but its ability to activate and increase other cytokines through feed forward mechanisms have also been established (Gruber *et al.*, 2014; Purmessur *et al.*, 2013). In addition, cells treated with TNF α have also resulted in decreases in aggrecan and type II collagen in IVDs (Seguin, Pilliar, Roughley & Kandel, 2005). Despite the evident implications of TNF α in IVD pathogenesis, treatment options targeting this cytokine have resulted in poor outcomes indicating that the activation and presence of TNF α in DDD is still not fully understood. IL-6 can act as either a pro or anti-inflammatory cytokine that is primarily known for activating acute-phase response proteins and it is thought to play an important roll in pain (Scheller, Chalaris, Shmidt-Arras & Rose-John, 2011) and has been found to be spontaneously produced in herniated IVDs (Kang, Georgescu, McIntyre-Larkin, Stefanovic-Racic, Donaldson & Evans, 1996). In innate immunity, chemokines are important secondary messengers, which are up regulated by resident cells within the IVD (Borish & Steinke, 2003). RANTES is a chemokine that is known for actively recruiting leukocytes to the site of injury and its implications in IVD degeneration has just recently gained more attention. Based on previous work done with human IVDs, it appears as though RANTES is most commonly elevated in painful IVDs (Kepler *et al.*, 2013; Gruber *et al.*,

2014a). Moreover, IVDs treated with IL-1 β /TNF α and Fetal bovine serum (used to create a degenerative phenotype) have been shown to cause significant increase in RANTES levels (Kepler et al., 2013). The chemokine MIP2 is a significantly less studied inflammatory mediator in IVD research yet it is very important in innate immunity due to its ability to recruit neutrophils (type of white blood cell). However, MIP2 has been found to be secreted by NP and AF cells upon decorin (a DAMP) and LPS exposure (Guerreiro, Laird, Gregory & DeWitte-Orr, 2017) indicating that this chemokine is up regulated through the TLR-4 receptor and could play a critical role in IVD degeneration and herniation. Finally, MCP-1 is a key chemokine involved in the recruitment of macrophages (Deshmane, Kremlev, Amini, & Sawaya, 2009) and infiltrated macrophages within the IVD are thought to be responsible for reabsorption of herniated tissue (Kikuchi, Nakamura, Ikeda, Ogata & Takagi, 1998). The study by Yoshida, Nakamura, Kikuchi, Takagi & Matsukawa (2005) used a rabbit model to evaluate IVD herniation and found that at 1-day post injury there was an elevation of TNF α and IL-1 β followed by an increase in MCP-1 and macrophages three days after injury (Yoshida *et al.*, 2005). Additionally, MCP-1 has been found to increase in both the AF and NP after TNF α exposure (Fujita *et al.*, 2012; Ohgi, Hunter, Pillus, & Rosenfeld, 2015) with its expression progressively decreasing with age (Fujita *et al.*, 2012). The presence of these cytokines within the IVD both with and without LPS exposure would help elucidate the IVD's ability to mount a localized inflammatory response and recruit other inflammatory cells important for the later stages of adaptive immunity.

5.7.3 Effects of Inflammation on IVD Mechanics

Recent studies have determined that altered mechanical loading results in an increase in pro-inflammatory cytokines/chemokines (Gawri *et al.*, 2014) however few

studies have reviewed how the initial presence of inflammation changes mechanics. At the time of this literature review, the only known study to report the effects of inflammation on IVD biomechanics was the study conducted by Walter *et al.*, (2015). The aim of the study was to investigate whether TNF α could penetrate an intact IVD, how long the inflammatory markers persisted within the IVD, how TNF α affected the structural and compositional makeup of the IVD, and if the presence of TNF α had an influence on the mechanical properties of the IVD. They found that TNF α was able to penetrate intact bovine caudal IVDs when subject to 8 hours of dynamic loading (0-0.8MPa at 0.1Hz) but not during 24 hours of static loading (0.2MPa), and that concentrations were highest in the NP. In addition, the presence of TNF α within the IVD caused an increase in IL-1 β and IL-6 compared to time matched controls. The presence of these pro-inflammatory cytokines further altered the biomechanical integrity of the IVD, causing an increase in joint stiffness. Through histological analysis, the NP demonstrated signs of aggrecan degradation and a more fibrous structure (indicative of increased collagen) in the IVDs treated with TNF α . This study suggests that the presence of inflammatory cytokines, specifically in the NP, causes a disruption in its ECM. This finding is further supported by the work of Purmessur *et al.*, (2013) that demonstrated that IVDs exposed to TNF α for 7 days elicited many of the catabolic changes associated with IVD degeneration and injury such as a loss of proteoglycans and break down of collagen I and II. The noted breakdown of aggrecan, which is critical in maintaining hydrostatic pressure and thus the mechanical integrity of the IVD, was found to cause an irreversible increase in joint dynamic stiffness (Walter *et al.*, 2015). Interestingly, there was no change in height loss between all groups indicating that the increase in joint stiffness was likely not associated to fluid loss. They hypothesized that

the altered mechanical loading was the result of a shifting of forces to the AF as a result of the loss of NP pressurization.

6. Objectives

The purpose of this study was to determine the effect of IVD damage (via puncture) and inflammation (via LPS exposure) on the mechanical and structural changes of the IVD at two separate time points. Four specific objectives were set:

Objective 1: determine if changes in the biomechanical properties of the cultured IVDs change over time

Objective 2: determine the effect of a 19G puncture on the compression-tension biomechanical properties of the IVD

Objective 3: determine if the presence of inflammation through LPS exposure alters the compression-tension biomechanical properties of the IVD

Objective 4: determine if either IVD puncture or LPS-induced inflammation results in structural changes of the IVD using histological analyses.

7. Hypotheses

1) It was hypothesized that IVDs placed in culture would destabilize, marked by an increase in NZ size and decrease in NZ stiffness that would worsen over time.

2) It was hypothesized that punctured IVDs would destabilize, marked by an increase in NZ size and a decrease in NZ stiffness.

3) It was hypothesized that destabilization of the joint would be observed in the IVDs exposed to LPS which would be greater than the destabilization observed in the IVDs exposed to puncture due to a higher concentration of pro-inflammatory markers.

4) It was hypothesized that puncture exposure in the IVDs cultured for 6 days would result in AF disorganization and a loss of proteoglycan (aggrecan) concentration in the NP indicative of ECM tissue degradation caused by the puncture wound. Further, it was hypothesized that exposure to LPS would cause similar but more disruptive changes in the AF and NP compared to the changes seen by the puncture wound, indicating a catabolic breakdown of its ECM.

8. Methods and Materials

8.1 Experimental Specimens

Twenty mature Sprague Dawley rats (aged 2-4 months) were obtained from the Animal Care Facility (under existing animal use protocols) at Wilfrid Laurier University in Waterloo Ont. All rats were control subjects from other studies being conducted at Wilfrid Laurier University and were euthanized in a slow fill CO₂ chamber. Immediately following euthanasia, the tail (vertebral level Co1 to Co10) was removed and skinned using an #11 blade scalpel. Four functional spine units (FSU; vertebrae-IVD-vertebra) were excised from each tail yielding 80 FSUs in total. Due to the variability in size in some of the rat-tails, the four FSUs most similar in size within each rat-tail were chosen; typically these FSUs were from Co2-Co8. FSUs were excised by cutting through adjacent IVDs while keeping the middle IVD intact (Figure 4). The entire dissection and removal of the FSUs was completed prior to culturing in an enclosed and sterile biosafety cabinet (Class II-Type A2, Thermo Scientific, Waltham, Massachusetts, United States) to eliminate the chance of contamination. The biosafety cabinet was sterilized with 10% bleach solution and ethanol.

8.2 Experimental Design

The experimental designed consisted of four conditions cultured for two different time points. The four conditions were: 1) non-punctured IVD, control media; 2) non-punctured IVD, LPS media (described in section 8.4); 3) punctured IVD (described in section 8.3), control media; 4) punctured IVD, LPS media. The two time points were 24 hours and 6 days (Table 3). A quasi-randomization protocol was used to decrease the effects that IVD level could have on the outcome measures. The quasi-randomization was chosen over a full randomization as it allowed for more accurate comparisons in cytokine and chemokine concentrations (Table 4).

Table 3: Summary of the experimental design including both media conditions (Control/LPS) and puncture conditions (Punctured/Non-Punctured) and the two time point conditions (24 hours and 6 days), (N=80).

	24 Hours		6 Days	
	Punctured	Non Punctured	Punctured	Non Punctured
LPS Media	n=8	n=8	n=12	n=12
Control Media	n=8	n=8	n=12	n=12

Table 4: Summary of the quasi-randomization protocol used in order to decrease the effects that IVD level may have on the outcome measures.

	FSU Level			
	Co2	Co4	Co6	Co8
Rat #1	Control Media/ Non-Punctured	LPS Media/ Non-Punctured	Control Media/ Punctured	LPS Media/ Punctured
Rat #2	Control Media/ Punctured	LPS Media/ Punctured	Control Media/ Non-Punctured	LPS Media/ Non-Punctured

8.3 IVD Puncture Protocol

A full AF puncture model was used to simulate IVD herniation in vitro in two of the four IVDs in each rat tail (Michalek, Funabashi, & Iatridis, 2010). Specifically, the IVD was located through palpation and a sterile single use 19G needle was inserted dorsally into the IVD, twisted 360 degrees, and then removed (Figure 4) (Han *et al.*, 2008; Issy *et al.*, 2013). A 19G needle was chosen as its needle diameter (1.1mm) is greater than 40% of the average rat-tail IVD height ($0.94\pm 0.09\text{mm}$) (Elliott *et al.*, 2008) and has been shown to cause significant and immediate damage to the IVD (Han *et al.*, 2008; Issy *et al.*, 2013).

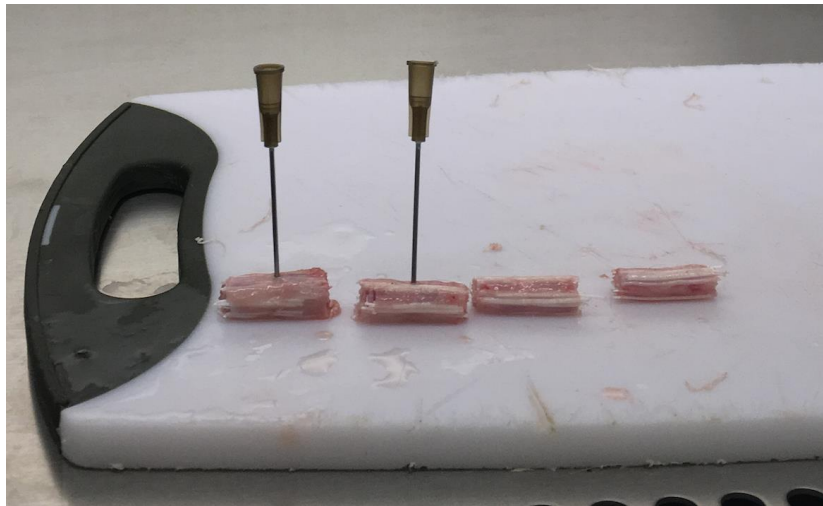


Figure 4: Example of a 19G needle being inserted dorsally and twisted 360 degrees into the IVDs Co3 and Co5.

8.4 Media Compositions

Following the puncture protocol, two FSUs (one punctured and one non-punctured) from each tail were placed in a control media containing DMEM (contains amino acids, salt, glucose and vitamins), 1% penicillin-streptomycin (reduces bacterial infection), 10% fetal bovine serum (provides growth factors needed to grow and stay alive) and 25mM HEPES free acid solution (helps buffer the solution) (Sowa, Coelho, Vo, Pacek, Westrick, &

Kang, 2015). The remaining punctured and non-punctured FSUs were placed in a media solution containing the same concentrations as the control media with the addition of 10µg/mL of LPS in order to create an inflammatory environment (Comalada *et al.*, 2003; Rajan *et al.*, 2013). All FSUs were placed in an incubator set at a constant temperature of 37°C for either 24 hours (n=32) or 6 days (n=48).

8.5 Cytokine and Chemokine Concentrations

At the 24-hour and 6-day mark, 250µl of media was removed using a sterile pipette from all the FSUs cultured for 6 days in order to determine the cytokine and chemokine profiles of the media. No media was removed/analyzed for the FSUs cultured for only 24 hours. Removal of the media at both times points was conducted in a ventilated and sterile biosafety cabinet sterilized with 10% bleach and ethanol. Cytokine (TNFα and IL-6) and chemokine (MCP-1, RANTES, MIP-2) concentrations were quantified using a 5-plex inflammatory cytokine and multiplex array reader and was conducted separately in Dr. DeWitte-Orr's lab (Wilfrid Laurier University).

8.6 Biomechanical Testing

Following the culture period and prior to mechanical testing, the medial/lateral and anterior/posterior size (mm) of the IVDs were measured using a digital calliper. The ligaments and tendons surrounding the IVD were removed as fully as possible without damaging the IVD. Additionally, incisions were made circumferentially around the vertebrae to detach the surrounding tissue that could have influenced IVD mechanics during clamping. To ensure consistency, all FSUs were mounted into the mechanical testing system (UStretch, Cellscale, Waterloo, Ontario) with the dorsal part of the specimen facing downward (Figure 5A&B). All 80 FSUs were subjected to uniaxial preconditioning in

compression and tension in order to determine the individual displacement limits and to decrease the effects of super hydration on the viscoelastic properties of the IVD. Following preconditioning, the FSUs were subjected to 20 phases of cyclic mechanical loading in compression and tension (rate of 0.1mm/sec) using a displacement controlled protocol as it has been previously shown that 15-20 cycles are required to produce repeatable force-displacement curves (Elliott *et al.*, 2008; Elliott & Sarver, 2004).

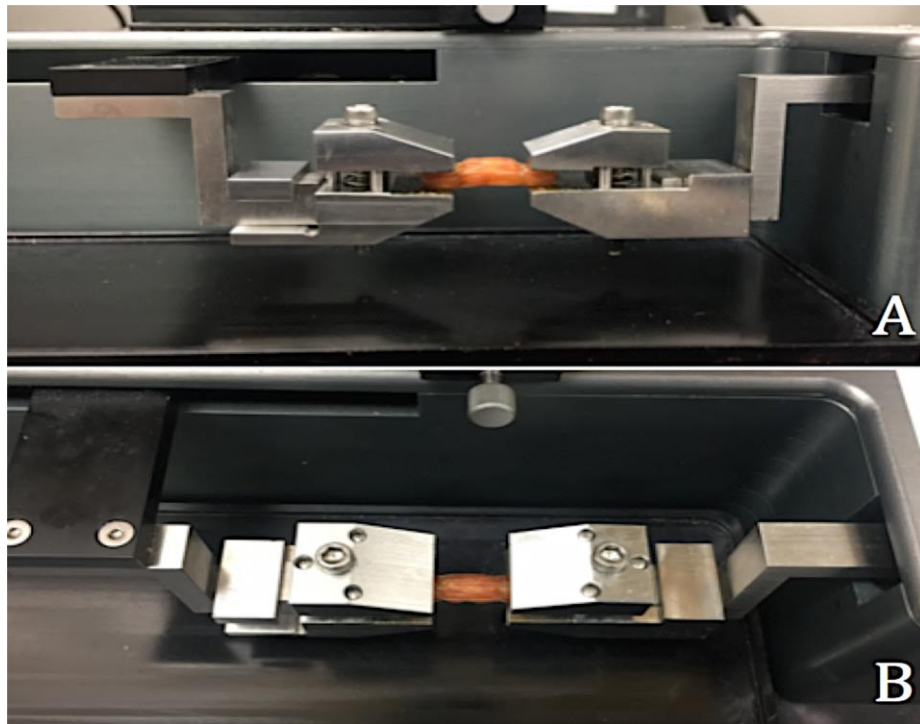


Figure 5: **A)** A sagittal view of the specimen and a **B)** frontal view of the specimen mounted in the UStretch (Cellscale, Waterloo, Ontario). The UStretch was used to place all 80 FSUs through a preconditioning cycle followed by 20 cycles of cyclic mechanical loading in compression and tension using a displacement-controlled protocol.

The force data obtained from the UStretch software were normalized to stress by dividing the force (N) by the cross sectional area of the IVD (stress reported in MPa). The cross sectional area was calculated using the medial/lateral and anterior/posterior measurements obtained prior to mechanical testing using a digital calliper. Measuring IVD

height using a digital calliper provides an inaccurate measurement of IVD height due to the shape of the IVD (Figure 1). In order to obtain accurate measurements of IVD height an X-ray imaging system is required. Since access to an X-ray imaging system was not possible, it was not feasible to accurately measure IVD height and therefore displacement data could not be normalized to strain. Once normalized, the neutral zone (NZ) size (mm) and stiffness (slope of the linear portion of the stress-displacement graph; MPa/mm) of the last three cycles in both compression and tension were calculated, averaged and compared across all conditions. NZ size was determined by fitting a linear line to the data with a cut off value of $R^2=0.77$ (Figure 6A). A cut off value of $R^2=0.77$ was used to determine the inflection points of the linear region within the full ROM of the IVD. Of the 160 trials, (compression=80; tension=80) 20 trials (compression=10;tension=10) fell below the 0.77 cut off value. Values below $R=0.77$ were evaluated on a case by case basis to determine if the low R value was due to the data going into a non-linear region or due to variability of that particular data set. Stiffness was determined as the slope of the linear line fitted to the data between the inflection points (Figure 6B).

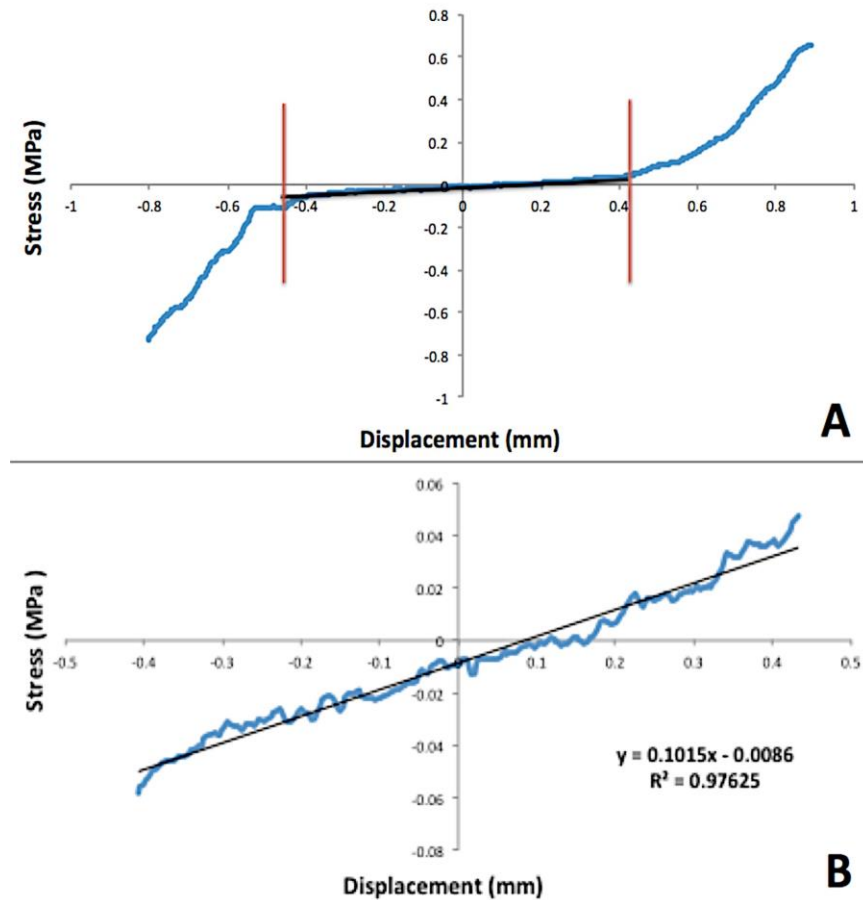


Figure 6: Representative FSU's **A**) full ROM in compression/tension where the NZ size (red) was calculated by fitting a linear line to the data (black line) and **B**) isolated NZ showing size (0.84mm) and stiffness (0.1015MPa/mm)

8.7 Histological Analysis

Upon completion of the 6-day culture period four FSUs (one from each condition) were prepared for histological analysis. Each FSU was decalcified in Cal-EX (Fisher Scientific) for 48 hours, then imbedded in formalin for 24 hours and sent to the Ontario Veterinary College Lab Services (Guelph, Ontario, Canada) for processing and staining using a combination stain (Gruber, Ingram, & Hanley, 2001). This combination staining method used Weigert's hematoxylin, picrosirius red, and alcian blue to stain for type I collagen (stained red), type II collagen (stained purple), and proteoglycans (stained blue).

8.8 Statistical Analysis

A three-way analysis of variance (ANOVA) was used to determine the main effect of damage (punctured/non-punctured), media condition (LPS/control media) and time point (24 hours/6 days) on NZ size and stiffness. An $\alpha=0.05$ was used to determine significance.

A second three way repeated measures ANOVA was used to determine the main effect of damage, media condition, and time point (repeated measure) on cytokine and chemokine concentrations. An $\alpha=0.05$ was used to determine significance.

Student's T-tests were used in order to determine statistical significance between all significant interactions. An $\alpha=0.05$ was used to determine significance.

9. Results

9.1 Biomechanical Testing

9.1.1 Effect of Time Point

When collapsed across damage and media condition, there was a main effect of time point on the NZ size such that the FSUs placed in media for 6 days ($\bar{x}=0.79\text{mm}$; $SD=0.14\text{mm}$) had a bigger NZ size, indicating increased joint laxity, compared to 24 hours ($\bar{x}=0.67\text{mm}$; $SD=0.14\text{mm}$), $p<0.0001$ (Figure 7A & Table 5). Additionally, there was also a main effect of day on NZ stiffness where the FSUs placed in media for 24 hours ($\bar{x}=0.19\text{MPa/mm}$; $SD=0.10\text{MPa/mm}$) were stiffer than the FSUs placed in media for 6 days ($\bar{x}=0.11\text{MPa/mm}$; $SD=0.06\text{MPa/mm}$); $p<.0001$ (Figure 7B & Table 5).

Table 5: Summary of the main effects on the biomechanical and cytokine/chemokine profiles as a result of exposure to the three experimental conditions: time conditions (24 hours and 6 days); puncture conditions (punctured/non-punctured) and media conditions (control media/LPS media).

Condition	NZ Stiffness	NZ Size	Cytokine/Chemokine Profiles
Day (24 hours/ 6 days)	↓6 days vs. 24hrs p<0.0001	↑6 days vs. 24hrs p<0.0001	↑TNF α , IL-6, MCP-1, MIP2 and RANTES were higher at 24 hours vs. 6 days. p<0.0001
Damage (Puncture/ Non- punctured)	No significant change	↑Punctured vs. non-punctured p=0.002	No significant change
Media Condition (Control media /LPS Media)	No significant change	No significant change	↑TNF α and RANTES in the LPS group. p<.0001 ↓IL-6 in the LPS group. p=0.0002.

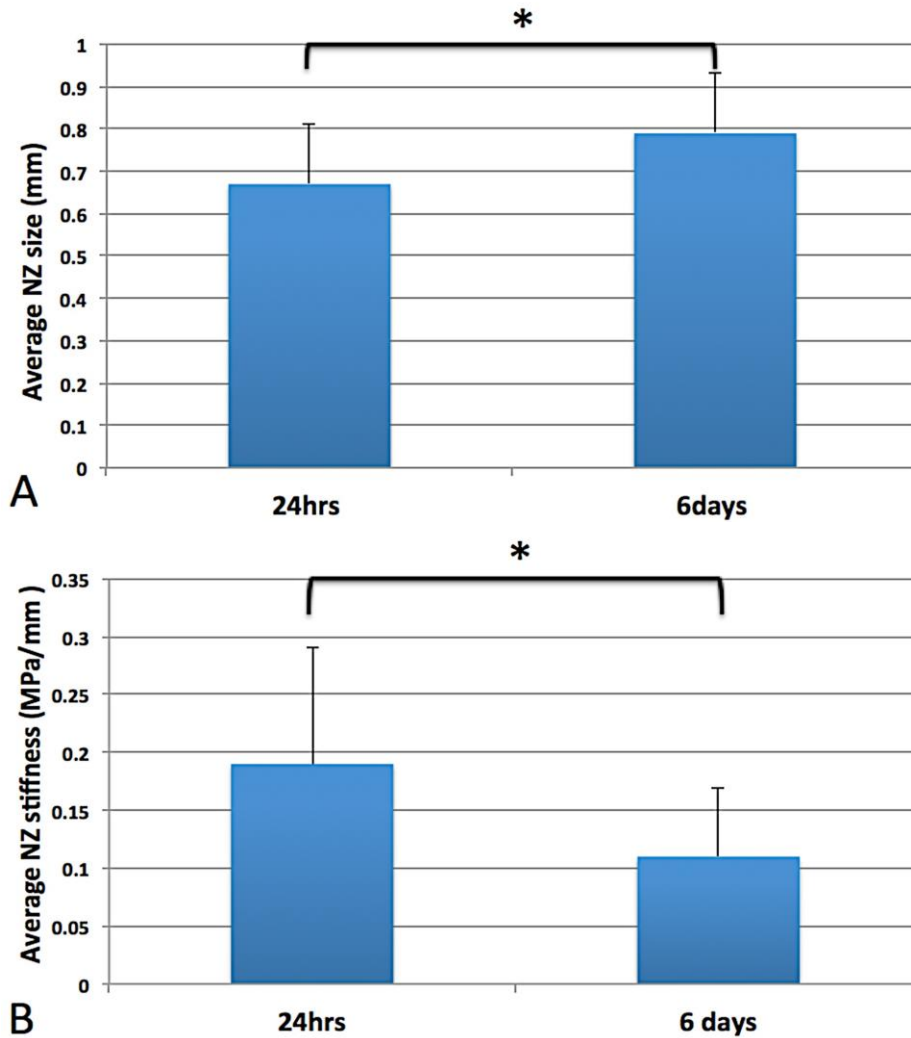


Figure 7: **A)** Significant main effect of time point on NZ size such that the FSUs placed in media for 6 days resulted in a bigger NZ size (mm); $p < 0.0001$. **B)** Significant main effect of day on NZ stiffness where the FSUs placed in media for 6 days were less stiff (MPa/mm) than those cultured for 24 hours; $p < 0.0001$. Errors bars denote standard deviations.

9.1.2 Effect of Damage

When collapsed across media and time point, there was a significant main effect of damage on NZ size such that the punctured FSUs ($\bar{x}=0.77\text{mm}$; $\text{SD}=0.14\text{mm}$) had a bigger NZ size compared to the non-punctured FSUs ($\bar{x}=0.71\text{mm}$; $\text{SD}=0.15\text{mm}$), $p=0.0022$ (Figure 8 & Table 5).

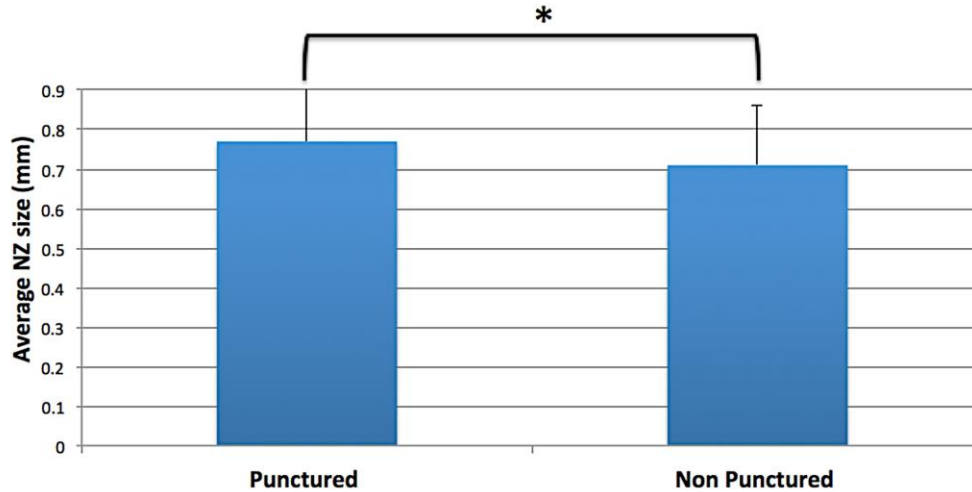


Figure 8: Significant main effect of damage on NZ size such that the punctured FSUs had a bigger NZ size (mm) compared to the non-punctured FSUs; $p=0.0022$. Error bars denote standard deviations.

9.1.3 Interactions

There was a significant interaction between time point and damage such that at 24 hours the punctured FSUs ($\bar{x}=0.17\text{MPa/mm}$; $\text{SD}=0.08\text{MPa/mm}$) were less stiff than the non-punctured FSUs ($\bar{x}=0.21\text{MPa/mm}$; $\text{SD}=0.11\text{MPa/mm}$) while conversely, after 6 days in culture, the punctured FSUs ($\bar{x}=0.13\text{MPa/mm}$; $\text{SD}=0.07\text{MPa/mm}$) became more stiff than the non-punctured FSUs ($\bar{x}=0.10\text{MPa/mm}$; $\text{SD}=0.05\text{MPa/mm}$), $p=0.0032$ (Figure 9).

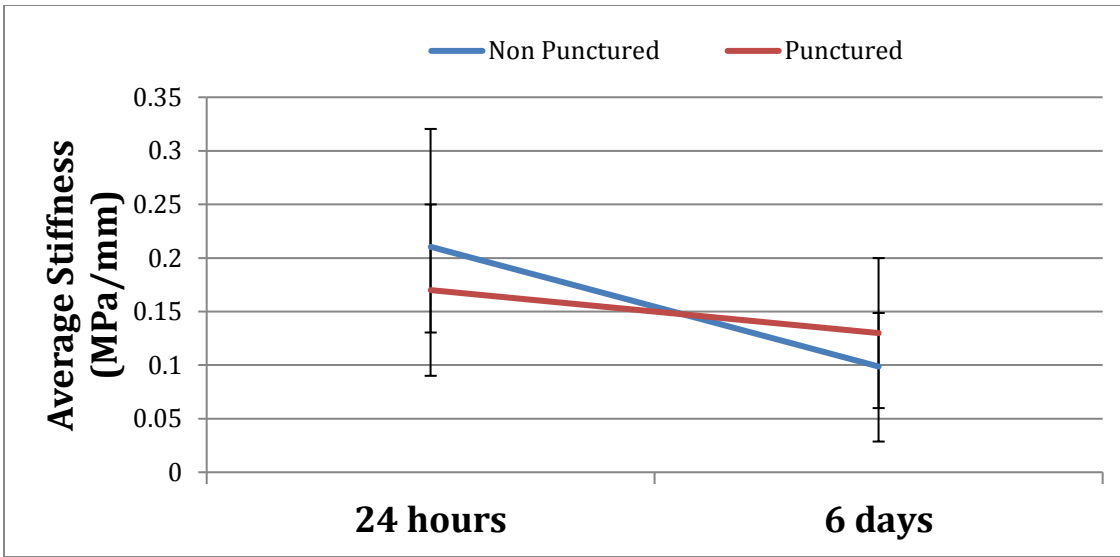


Figure 9: The interaction between damage and time point for average stiffness (MPa/mm); $p=0.0032$. Errors bars denote standard deviations.

9.2 Cytokine and Chemokine Profiles

9.2.1 Effect of Time Point

When collapsed across damage and media condition, there was a significant main effect of time point on cytokine and chemokine concentrations. Absolute values of all five cytokines/chemokines (TNF α , IL-6, MCP-1, MIP 2 and RANTES) were lower at 6 days compared to 24 hours (Figure 10). The percent decrease was 36.81% for TNF α , 15.49% for IL-6, 32.67% for MCP-1, 6.23% for MIP2 and 91.54% for RANTES (Table 5).

Table 6: Summary of the percent drop in cytokine and chemokines concentrations between 24 hours and 6 days. All changes were significant to $p<.0001$

	24 hours	6days	% Drop
TNF α	38.71pg/ml	24.46pg/ml	36.81%
IL-6	17847.90pg/ml	15082.95pg/ml	15.49%
MCP-1	3772.89pg/ml	2540.38pg/ml	32.67%
MIP2	1424.29pg/ml	1335.56pg/ml	6.23%
RANTES	209.91pg/ml	17.36pg/ml	91.54%

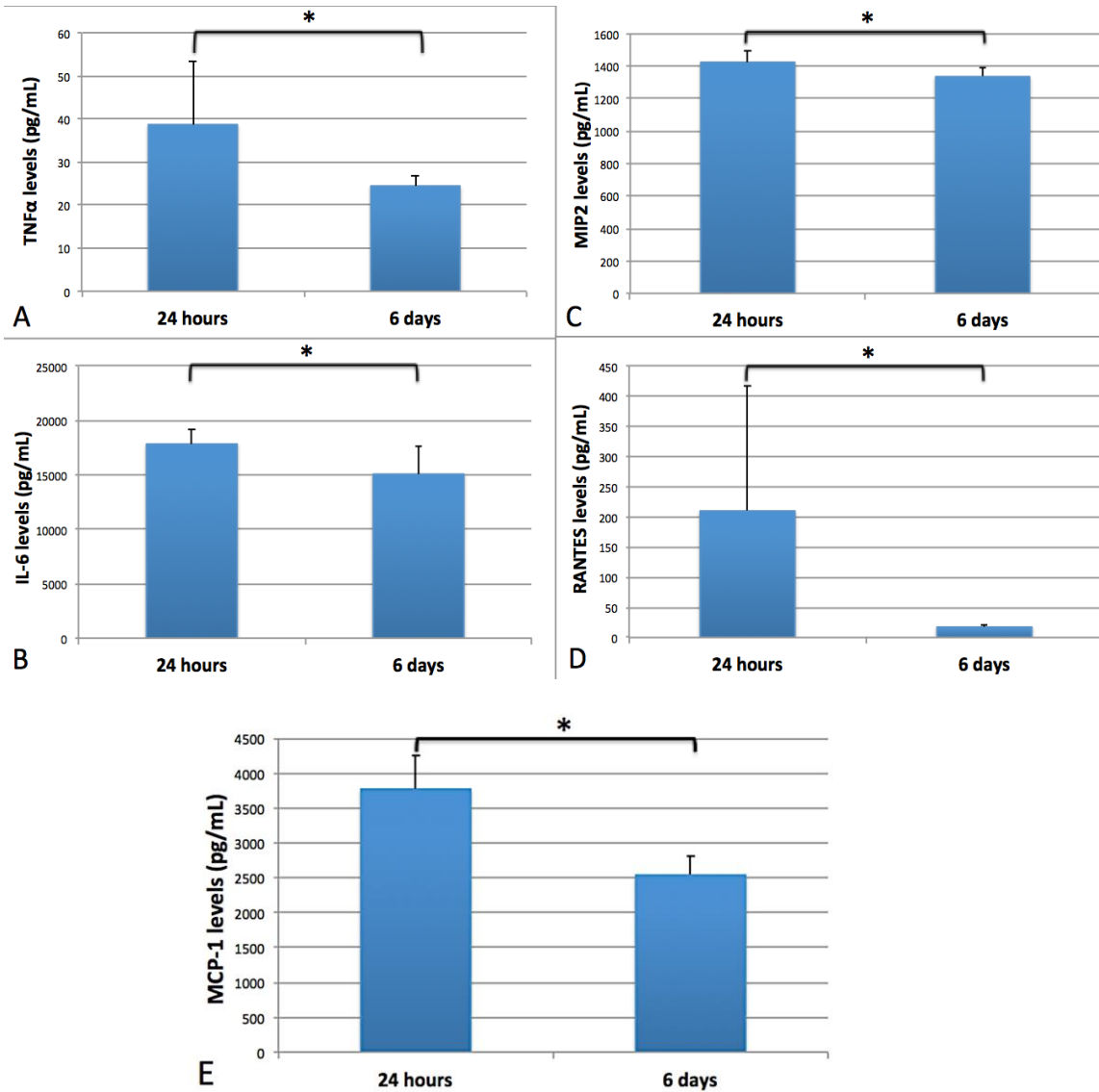


Figure 10: Significant main effect of time point on levels of **A)** TNF α ; $p < 0.0001$ **B)** IL-6; $p < 0.0001$ **C)** MIP2; $p < 0.0001$ **D)** RANTES; $p < 0.0001$ and **E)** MCP-1; $p < 0.0001$. Errors bars denote standard deviations.

9.2.2 Effect of Media Condition

When collapsed across damage and time point, there was a significant main effect of media condition where TNF α was elevated in the LPS media group (absolute value=37.11pg/mL; SD=7.86) compared to control media (absolute value=26.06pg/mL; SD=4.97), $p < 0.0001$. Similarly, RANTES was elevated in the LPS media group (absolute value=183.79pg/mL; SD=104.25) compared to control media (absolute value=43.49pg/mL;

SD=41.10), $p < .0001$ (Figure 11A&B). Conversely, IL-6 was lower in the LPS media group (absolute value=15536.90pg/mL; SD=1949.39) compared to the control media (absolute value=17394.03pg/mL; SD=1286.77), $p=0.0002$ (Figure 11C).

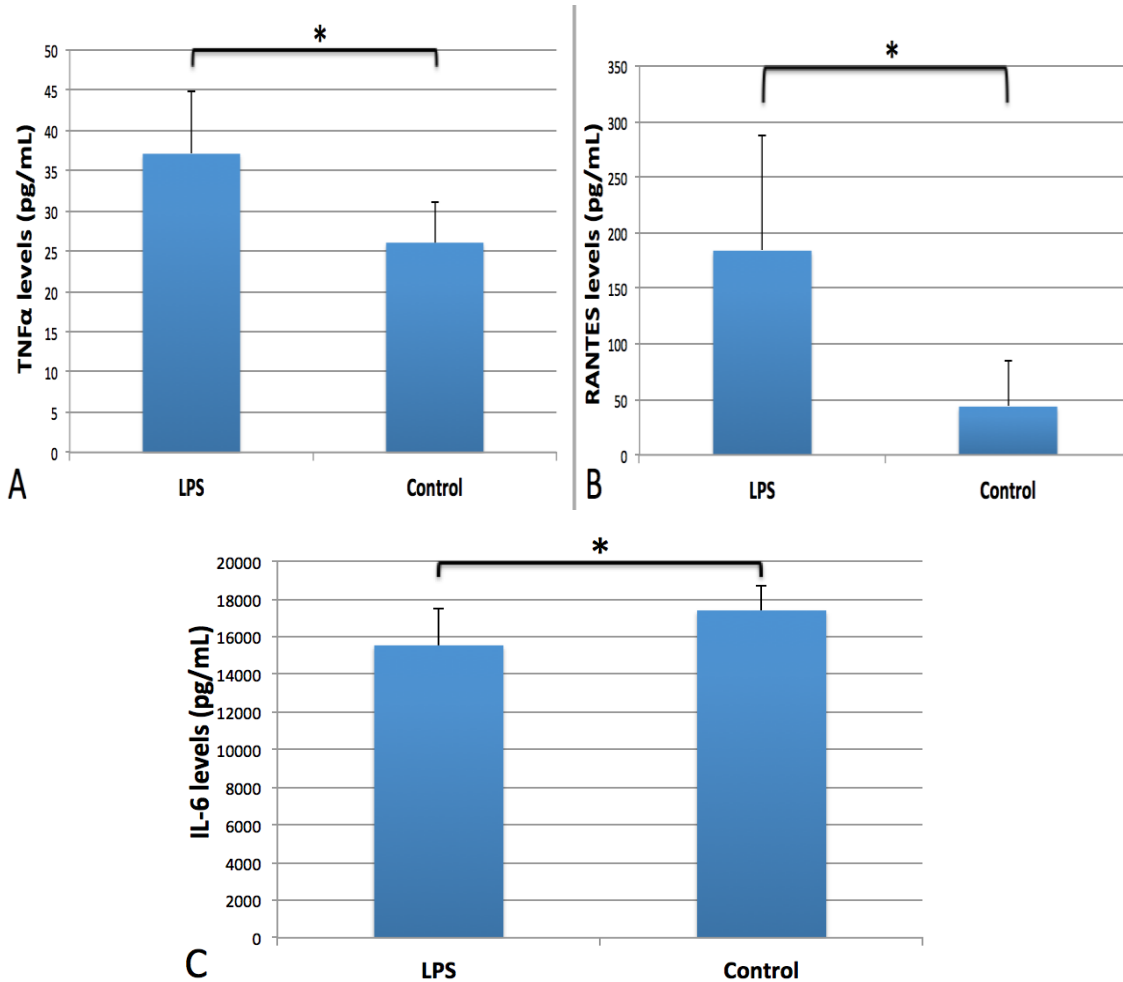


Figure 11A: Significant effect of media on the cytokine TNF α and **B:** the chemokine RANTES where absolute increases were higher at 24 hours compared to 6 days, $p < .0001$. **C:** Significant effect of media on the cytokine IL-6 where in the control group the absolute increase was higher at 17394.0 compared to 15536.9 in the LPS group, $p=0.0002$. Errors bars denote standard deviations.

9.2.3 Interactions

There was a significant interaction between time point and media condition such that both RANTES ($p < 0.0001$) and TNF α ($p < 0.0001$) concentrations were higher for FSUs cultured with LPS compared to the control media at 24 hours, but with no difference in

levels between the media types at 6 days (Figure 12A&B). Additionally, a significant interaction between time point and media condition was also observed for IL-6 ($p < 0.0001$). However, in the case of IL-6, concentrations between LPS and control media were not different at 24 hours, but at 6 days, FSUs cultured with LPS showed lower levels of IL-6 compared to the control media (Figure 12C).

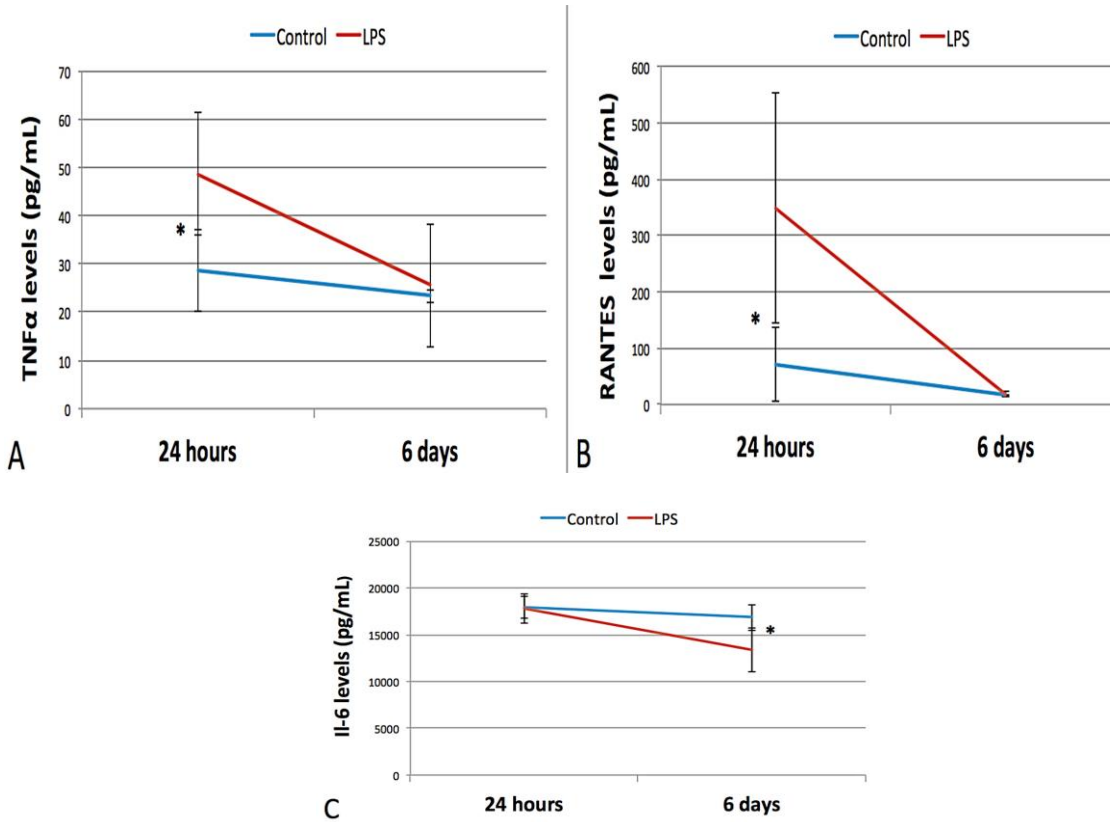


Figure 12: Graph illustrating the interaction of day (24hours/6days) and media (LPS/Control) on **A)** TNF α absolute increases; $p < 0.0001$ **B)** RANTES absolute increases; $p < 0.0001$ and **C)** IL-6 absolute increases; $p < 0.0001$. Asterisk indicates significance below $p = 0.05$. Errors bars denote standard deviations.

9.3 Histological Findings

Half of the IVD were not cut far enough into the imbedded FSUs and were therefore unusable for histological analysis. Two of the four conditions (no puncture/control media and puncture/LPS media) resulted in images that were primarily endplate and did not

allow for any analysis of the integrity of the NP/AF (images not shown). The IVD exposed to puncture/ control media resulted in an obvious tract wound with evidence of NP material extrusion (Figure 13A&B). Additionally, the AF and NP border appeared to be disrupted with slight appearances of delamination or AF cell disruption and inward bulging of the IAF (Figure 13A&C). Interestingly, despite the small size of the NP, there seemed to be a substantial concentration of proteoglycans and an attempted encapsulation of the opposing needle injury site (Figure 13A&C). The size of the NP was irregularly shaped and took up about half of the area of the IVD. The IVD exposed to no-puncture/ LPS media had an NP that took up over half of the surface area of the IVD with significantly lower levels of proteoglycan staining within the NP indicating a loss of material (Figure 14A&B). The border between the NP and AF seemed to be minimally disrupted with very little proteoglycan staining into the IAF and AF (Figure 14A&C). The AF seemed to be minimally disrupted with some instances of AF delamination (Figure 14A&D).

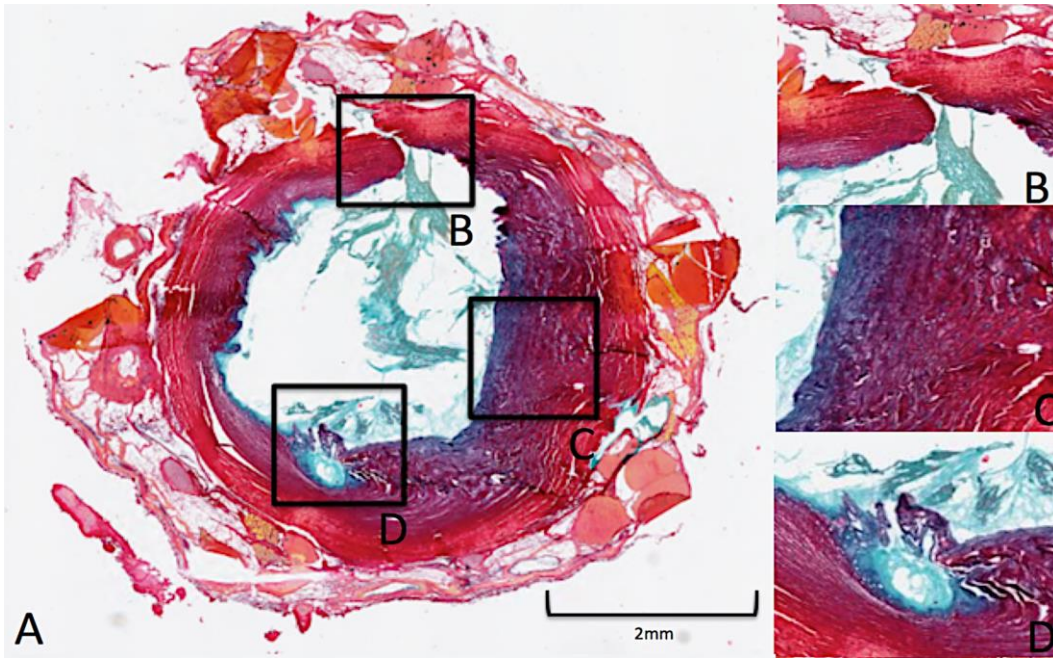


Figure 13: A) Full image of an IVD exposed to puncture/control media using the combination stain (Gruber et al., 2002) where the B) presence of the puncture tract C) AF/NP border and D) annular repair are visible. Type I collagen is stained red, type II collagen is stained purple and proteoglycans are stained blue. 2mm scale bar shown.

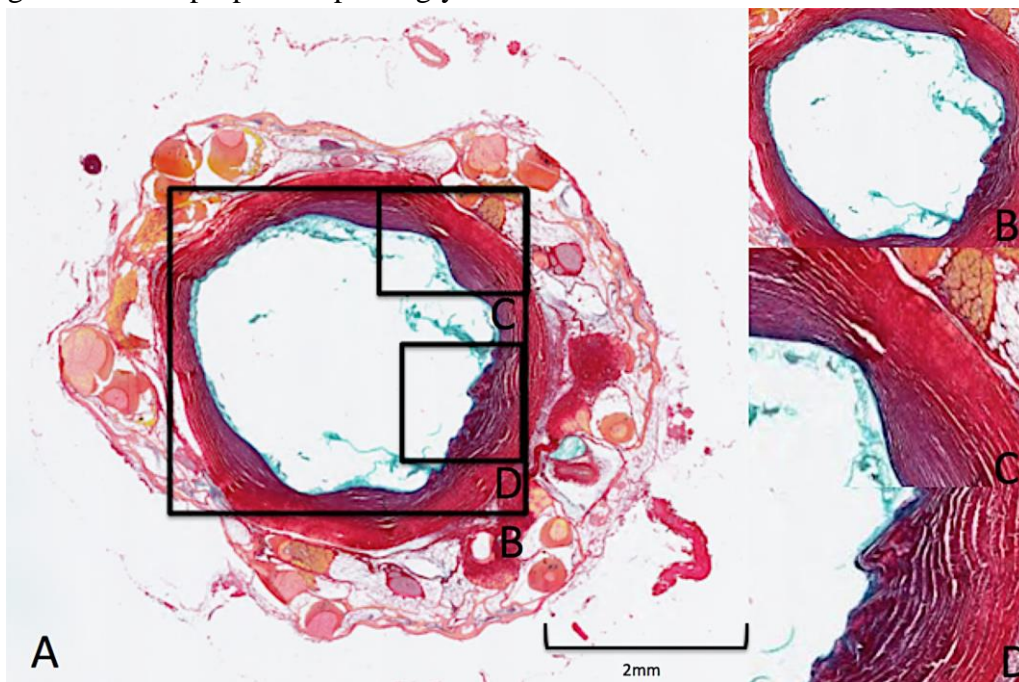


Figure 14: A) Full image of an IVD exposed to no-puncture/LPS media using the combination stain (Gruber et al., 2002) where the B) size and loss of NP material C) AF/NP border and D) annular integrity of the IVD are visible. Type I collagen is stained red, type II collagen is stained purple and proteoglycans are stained blue. 2mm scale bar shown.

10. Discussion

10.1 Revisiting the Purpose and Hypotheses:

This study aimed to establish how exposure to an inflammatory environment and damage through IVD puncture affect the mechanical and structural properties of the IVD using a rat-tail model. First, it was hypothesized that FSUs placed in culture would experience a destabilization of the joint that would increase from 24 hours to 6 days post-culture. There was an increase in NZ size and a decrease in NZ stiffness from 24 hours to 6 days regardless of condition, indicating an increase in joint laxity. Second, it was hypothesized that punctured IVDs would experience an increase in NZ size and a decrease in NZ stiffness. Punctured IVDs, regardless of condition, had an increase in NZ size compared to non-punctured IVDs. Interestingly, while a destabilization of the joint was observed at 24 hours marked by decreased NZ stiffness, by 6 days, punctured IVDs were actually stiffer than non-punctured IVDs indicating the presence of some sort of repair or re-stabilization. Third, it was hypothesized that exposure to LPS would result in a destabilization of the joint that would be greater in magnitude compared to puncture due to a higher concentration of pro-inflammatory cytokine/chemokines. While the current work did observe higher concentrations of most cytokine/chemokines, this did not translate into a destabilization of the joint. However, a trend towards destabilization was observed ($p=0.09$) that might have been found to be significant if exposure to LPS was longer. Last, it was hypothesized that IVD puncture would cause FSUs cultured for 6 days to experience an increase in AF disorganization and a loss of proteoglycan/aggrecan concentration in the NP and that exposure to LPS would cause similar but more disruptive changes in the AF and NP. Unfortunately, due to issues with histological analyses, this hypothesis could neither be

confirmed nor rejected. However, for the IVDs that were stained successfully, it appeared that the puncture resulted in a higher concentration of proteoglycans in the NP and an attempted encapsulation of the opposing needle injury site. Interestingly, the IVD exposed to LPS only appeared to be minimally disrupted.

10.2 Increased Mechanical Laxity Over Time

After six days in culture, all IVDs, regardless of exposure to LPS or puncture, had an increase in the compression-tension NZ size and a decrease in NZ stiffness compared to after 24 hours, which is indicative of an increase in joint compliance. These changes in mechanics can most likely be attributed to the inevitable tissue breakdown and cell remodelling that ensues as a result of being excised and left in culture. Specifically, loss of proteoglycan content leading to dehydrated NP cells and annular disorganization has been reported in cultured IVDs which has been further positively correlated with a decline in the mechanical integrity (Mimura, Panjabi, Oxland, Crisco, Yamamoto & Vasavada, 1994).

10.3 Mechanical Changes due to IVD Puncture

IVD puncture was also found to significantly alter IVD mechanics. Specifically, IVDs exposed to the 19G needle puncture, regardless of time and media condition, experienced an increase in NZ size compared to non-punctured IVDs. This increase in NZ size is likely due to the loss of pressurization within the NP that occurs upon puncture injury (Elliott *et al.*, 2008; Han *et al.*, 2008; Issy *et al.*, 2013; Korecki *et al.*, 2008). These mechanical changes have been supported in a study by Johannessen *et al.*, 2006, where progressive removal of the NP, while keeping the AF intact, resulted in an increase in NZ size and ROM in compression and tension, similar to the current study (Johannessen, Cloyd, Connell, Vresilovic, & Elliott, 2006). They concluded based on their findings, that during

compressive and tensile loading, changes observed within the NZ region are a result of disruptions in the NP. In addition, a study by Cannella *et al* (2008) found that a decrease in IVD height and an increase in NZ size were correlated with increased denucleation of the NP. Thus, they concluded that the increase in NZ size was the result of a transfer of forces to the AF when the NP can no longer maintain the hydrostatic pressure that it is meant to withstand in normal conditions (Cannella *et al.*, 2008). Since the AF is designed to withstand primarily tensile forces, the shift towards bearing compressive forces throughout its NZ region can lead to catabolic changes in the AF's ECM further exacerbating the damage caused by the puncture wound (Hsieh *et al.*, 2009). Evidence of architectural and cellular disruptions within the AF and NP in rat caudal IVDs after needle puncture (Han *et al.*, 2008; Issy *et al.*, 2013) and stab incision (Rousseau *et al.*, 2007) have been reported previously and are likely due to this transfer of loading from the NP to AF. In addition, through histological analysis, there were notable morphological changes observed in the FSU exposed to punctured/control media, such as AF layer disorganization and the potential inward bulging of the IAF into the NP space. The effects of spinal instability as a result of changes to the NZ have been studied in previous work (Mimura *et al.*, 1994; Beazell, Mullins & Grindstaff, 2010) and changes to the NZ have been recognized as a better indication of joint instability compared to changes in ROM (Panjabi, 1992). Since the NZ is defined as a region of low resistance, increases in size and a decrease in stiffness within this region can result in hypermobility of the IVD joint.

Interestingly, NZ stiffness in the punctured versus non-punctured IVDs did not follow the same pattern as NZ size. In particular, a significant interaction between damage and time point showed that similar to NZ size, stiffness was lower in the punctured IVDs at

24 hours, but by the 6-day mark, punctured IVDs were actually stiffer than non-punctured IVDs. What is of particular interest about these findings is that despite the catabolic effects that ensue as a result of damage early on (increased joint laxity at 24 hours), as well as the time dependent decrease in mechanical integrity described above, there seems to be an anabolic response triggered in an attempt to heal itself. This could indicate that there is a reparative mechanism that is induced within the IVD as a response to the depressurization that occurs from the puncture wound. Instances of self-repairing mechanisms within the AF (fibrous capping) and NP (increases in cell number) have been seen in previous work (Korecki *et al.*, 2008; Rousseau *et al.*, 2007; Ulrich *et al.*, 2007). Research by Ulrich *et al.*, (2007) found through histological analysis that a fibrous cap was visible around the annular injury in IVDs exposed to a single stab incision, which the authors hypothesized was in an attempt to potentially stabilize the injury (Ulrich *et al.*, 2007). Although not present at the insertion site, this potential mechanism could have occurred in this study, as evidence of the attempted encapsulation of the opposing injury wound (Figure 13A&D). In one study, granulation of the fibrous cap occurred indicating that the tissue developed microscopic blood vessels and became innervated, which possibly plays an important role in the development of discogenic pain (Freemont *et al.*, 2002). Interestingly, increased proteoglycan production both in the NP and AF has been observed in rat tail (Rousseau *et al.*, 2007; Ulrich *et al.*, 2007) and rabbit (Lipson & Muir, 1981) IVDs upon exposure to a puncture or stab. The increased production of proteoglycans may be triggered immediately after puncture injury, which could temporarily minimize the natural degeneration that ensues over time. Evidence in support of this hypothesis was observed through histology where the IVD exposed to the puncture/control media condition resulted in an increase in

proteoglycan staining (indicative of an increase in NP material) compared to the IVD exposed to non-punctured/LPS condition. The increase in proteoglycan concentrations is likely an internal attempt to re-pressurize the NP, which as previously stated is crucial for its ability to bear load in compression. Increases in proteoglycan concentration particularly in the IAF and the NP are believed to inhibit further infiltration of the granular tissue allowing it to stay isolated to the periphery (Melrose, Roberts, Smith, Menage, & Ghosh, 2002).

While previous work has detected increased inflammatory cytokines and chemokines in response to puncture, the current study did not. This is likely because in previous work, cytokine levels were measured along the puncture track rather than in the media surrounding the FSU. For example, a study by O'Neill *et al.*, (2004) found that porcine IVDs exposed to a stab incision resulted in an inflammatory profile that was anabolic and favourable for tissue repair (increase in IL-8 and a decrease in IL-1 β) resulting in a significant reduction in a degenerative phenotype (O'Neill *et al.*, 2004). Perhaps a similar profile would have been observed in the puncture track in the current study thereby corroborating the finding of increased stiffness (compared to non-punctured IVDs) due to repair at the 6-day mark. However, in the study by Ulrich *et al.*, (2007) where they observed the fibrous cap following AF stab, cytokine levels determined through enzyme linked immunosorbent assays were either not present (IL-6 and TNF α) or found in very low concentrations (IL-1 β) at 4, 7 and 14 days post injury, similar to the findings of the current study. Future work should examine cytokine/chemokine concentrations within the punctured IVD as well as examine structural changes that occur at the site of puncture.

10.4 Inflammatory Marker Profiles in Response to LPS

The increase observed in all five cytokines and chemokines at 24 hours compared to 6 days indicates that the cells of the FSU produce and release significant magnitudes of inflammatory markers independent of a blood supply relatively quickly after excision and that this release becomes more controlled by the 6 day mark. These findings are supported by previous work that showed that independently cultured AF, NP and VEP released inflammatory markers (Rajan *et al.*, 2013). Further, cultured AF cells treated with 10 μ g/ml of LPS showed increased levels of RANTES, MIP-2, MCP-1 and IL-6 peaking at 18 hours post exposure while NP cells treated with the same concentration of LPS had increased levels of MCP-1 and MIP-2 peaking between 22 and 24 hours (Guerreiro *et al.*, 2017) followed by a decrease (suggesting increased control of production/release). These findings indicated that the cells within the IVD are able to mount a localized innate immune response relatively quickly when exposed to a PAMP. Contrastingly, in this experiment, FSUs exposed to LPS resulted in significant increases of TNF α , which was not produced by cultured AF and NP cells. One possible explanation for this is that the increase of TNF α at 24 hours could have been produced by the cells within the VEP, in the surrounding ligaments/tendons/muscles, or by resident macrophages still viable in the IVD and not solely by the AF and NP cells themselves. Another interesting finding in the inflammatory marker profile was the decrease in IL-6 with LPS exposure compared to control. This decrease is contrary to the majority of the literature examining the inflammatory response to LPS exposure in IVD cells (Guerreiro *et al.*, 2017; Burke *et al.*, 2003; Rand, Reichert, Floman & Rotshenker, 1997) that found IL-6 to increase following LPS exposure. There are a few possible explanations for this contrary finding in FSUs. First, FSUs, rather than

isolated IVD cells, were exposed to the LPS. As mentioned above, exposed cells in the FSU would have not only included AF and NP cells, but also VEP, tendon, and muscle cells as well. It is possible that non-IVD cells respond differently to LPS exposure resulting in a down regulation of IL-6. Second, IL-6 can act as either a pro-inflammatory cytokine or an anti-inflammatory cytokine (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). Thus it is plausible that IL-6, produced by these other resident cells, is acting in an anti-inflammatory fashion and is therefore decreased with LPS exposure. However, generally, IL-6 in the IVD has a pro-inflammatory or catabolic effect given its presence in herniated (Kang, Georgescu & McIntyre-Larkin, Racic, Donaldson & Evans, 1996) and degenerated (Burke *et al.*, 2003; Wuertz & Haglund, 2013) IVDs. Additionally, a study by Studer *et al.*, (2011), found that human NP cells exposed to 100ng/mL of IL-6 resulted in a down regulation in proteoglycan production and matrix protein gene expression (aggrecan and collagen II) indicating a catabolic break down of the IVDs ECM. Third, IL-6 production may be heavily dependent on the dose of LPS (Studer, Vo, Sowa, Ondeck, & Kang, 2011). In a study by Rajan *et al.*, (2013), a dose dependent increase in the cytokines TNF α , IL-6 and IL-1 β was observed with exposure of up to 1.0 μ g/ml of LPS, but with levels dropping off after 10 μ g/ml of LPS exposure. Therefore, it may also be possible that the decrease in IL-6 with LPS exposure was due to the high concentration used in the current study. In addition, there has been evidence to show that there may be a negative feedback or inhibitory loop that exists between TNF α and IL-6. This speculation is based on the work of Kiu *et al.*, (2007), that found that LPS-induced TNF α resulted in inhibition of IL-6 signaling in mouse macrophages. Further, the work by Aderka, Le & Vilcek, (1989), found that IL-6 inhibited LPS-induced TNF α in cultured human monocytes and in mice.

Monocytes are white blood cells and are found within the VEP of the IVD and could have resided in the surrounding tissue around the IVD. This hypothesis could potentially account for why there was no difference in IL-6 levels at 24 hours, when TNF α was elevated, followed by a higher concentration of IL-6 in the control group compared to the LPS group at 6 days. Evidently these findings cannot be directly inferred to the results observed in this study however it indicates that there may be a very important and complex relationship between the regulatory mechanisms of these two cytokines that warrants more careful consideration. Further work is necessary in order to determine the differences in cytokines released by the cells of resident tissues and by the cells of the IVD in order to confirm the above hypotheses.

10.5 The Catabolic and Anabolic Responses to Puncture and Inflammation

As described above, in many studies exposure to LPS increases pro-inflammatory cytokines/chemokines, which typically results in a catabolic response. Although not found to be significant, there was a trend towards increased laxity (marked by decreased joint stiffness) in the FSUs exposed to LPS ($p=0.09$). It is possible that no significant changes to stiffness were observed due to the concentration of LPS exposure being too large, not long enough, or due to an insufficient sample size. However there is the possibility that LPS exposure and consequently inflammation in the IVD is catabolic in nature and has a destabilizing effect on the joint. IVD puncture, on the other hand, did not significantly alter cytokine/chemokine concentrations, but did result in an immediate destabilization at 24 hours followed by a slight abruption of destabilization after 6 days, suggesting that IVD puncture does initiate some type of anabolic response. The difference between the catabolic and anabolic response observed could be due to the following two main reasons. First, the

cytokine/chemokine profiles were affected (increase in pro-inflammatory markers) by LPS exposure but not with IVD puncture. Previous research has revealed that exposure to TNF α , such as the case with LPS exposure, is both pro-inflammatory and catabolic in nature. A study by Kang *et al.*, (2015); found that injection of TNF α into a porcine IVD induced early stage IVD degeneration in both the NP (cellular disorganization) and AF (annular fissure formation and granulation). Further, a study by Millward-Sadler, Costello, Freemont, & Hoyland, (2009) found that human IVD tissue after 48 hours of exposure to TNF α resulted in a down regulation of gene expression for both collagen type I (in normal and degenerated samples) and type II (normal samples) (Millward-Sadler *et al.*, 2009). Therefore, it is possible that this pro-inflammatory, catabolic response of the cytokines produced in the FSUs exposure to LPS resulted in the observed trend towards increased joint laxity. This hypothesis is weakly supported by the increase in NP size and decrease in staining in the no-puncture/LPS group. Histology points towards evidence of NP degradation however exposure to LPS may have needed to be longer in order to cause a resulting change in mechanics. However, the study by Purmessur *et al.*, (2013) found that intact bovine FSUs exposed to TNF α for 7 days resulted in a significant loss of proteoglycan and type I and II collagen breakdown. Although TNF α levels were low, there was some evidence of reduced NP (and thus low proteoglycan content) in the no puncture/ LPS IVD (Figure 14A&B) Unfortunately it cannot be determined with certainty if the loss of NP material was due directly from TNF α as Purmessur *et al.*, (2013) suggested or due to exposure from LPS. Second, it is plausible that IVD cells mount a localized innate immune response to damage (isolated mostly to the puncture site) that is very closely regulated by the cells within the IVD and is typically anabolic and reparative in nature. A puncture wound results in a

controlled release of localized (within the puncture track and therefore potentially not detected in the surrounding media) inflammatory markers that promote an anabolic or healing response to the damage. It is important to note that, while punctured IVDs were stiffer than non-punctured IVDs at the 6-day mark, the punctured IVDs were still less stiff at 6 days than they were at 24 hours. This indicates that the increase in anabolic activity that occurred was enough to slow down the destabilization of the FSU, but not halt it or reverse it altogether. Unstable joints are not only at an increased risk of further injury but may be subject to compensatory mechanisms via surrounding structures and muscle activation that could further alter the mechanics of the spine (Panjabi, 1992). The changes in mechanics that occur both within the IVD and within its surrounding structures have serious implications to the development of degenerative disc disease and LBP (Panjabi, 1992; Yamamoto, Panjabi, Crisco & Oxland, 1989). It is still unclear if NZ size or stiffness is a more accurate indicator of joint instability and how these mechanical parameters change in different ranges of movement. Therefore, more work is needed in this area in order to determine a causal link between the anabolic and catabolic mechanisms that occur within the IVD as a result of injury (through puncture and inflammation) and their effect on IVD mechanics.

In general, the findings of this study indicate that the damage and inflammation created by LPS is different than the damage and inflammation that might ensue as a result of IVD herniation. It is hypothesized that herniation results in inflammation through detection of a DAMP (i.e. the cells of the IVD are detecting a resident molecule that is damaged) rather than a PAMP, such as LPS. It is postulated, based on the findings of this work, that the damage and inflammation mounted as a result of needle puncture is mostly

compensatory in nature and the IVD is somewhat equipped at being able to restore some function. Such possible differences merits attention in future work.

10.6 Limitations

This study is not without its limitations. First, due to the cost associated with running multiplex and the availability of rats, the 24 hours and 6 day cytokine/chemokine data were determined from IVDs cultured for 6 days. As a result, the specific inflammatory profiles of the 32 FSUs cultured for 24 hours is not known and differences may have existed between those rats and those cultured for 6 days. In addition, it cannot be fully confirmed that the levels of cytokine and chemokines present within the media are a direct result of NP and AF cell production. Since the FSUs were cultured with remnants of connective tissue, ligaments and vessels, it is possible that these biologically active tissues contributed to the up regulation of some of the inflammatory markers, most specifically TNF α . More work is needed in order to determine the differences in the inflammatory profile created by the cells of the IVD and by the cells that surround the IVD.

Second, histological analyses of the cultured FSUs provided very poor information in regards to the cellular and morphological changes occurring within the IVD. This issue can be attributed to the small sample size, the potential dehydration of the NP that occurred during processing and the insufficiency of slicing deep enough into the FSU. Further, it is still unclear how the up regulation of these specific inflammatory markers affects the cellular and enzymatic activity within the IVD. Therefore, a histological and immunochemical look at how the presence of these cytokine and chemokines influences changes in the catabolic and anabolic mechanisms of the IVD are a very interesting and critical area for future study.

Finally, these findings are far from being able to be inferred to that of human IVDs. There are evident limitations to using both the rat-tail model and the puncture model. In rats, the preservation of notochord cells within the NP is longer than in humans leading to differences in mechanical loading patterns (Alini *et al.*, 2008). Further, the loading patterns of the rat-tail are different than the loading patterns experienced by the human lumbar spine (Alini *et al.*, 2008) although when size and geometry are accounted for the forces are comparable in magnitude (Elliott & Sarver, 2004). Finally, there are likely differences in how the cells of the IVD in rats and the cells of the IVD in humans respond to an inflammatory stimulant such as LPS. A more complete overview of the cytokines and chemokines found within both the rat caudal and human lumbar IVDs is still needed. Despite these drawbacks, the rat caudal IVD is a common inexpensive and easily accessible way of studying tissue mechanics. Particularly for this research project, the rat caudal IVD was a suitable method due to the high cost associated with the media when culturing the FSUs.

Although the needle puncture model is a widely accepted method of producing IVD herniation and degeneration in both *in vivo* and *in vitro*, it is a highly simplified method that does not accurately encompass all aspects of the disease. In addition, the puncture model elicits IVD herniation by depressurizing the IVD through an outward to inward mechanism. However, most instances of IVD herniation are slow and progressive and occur through an inward to outward mechanism. Further, the puncture model is often critiqued for its ability to rapidly create a degenerative phenotype that would otherwise not be seen in normal physiological conditions. Therefore, the mechanical changes observed as a result of exposure to puncture damage and the inflammatory environment are simplified and are

specific to the model chosen in this study. In addition, the massive damage associated with cutting through the adjacent IVDs during excision in comparison to the damage inflicted by the 19G needle is an evident limitation of this study and must be revised in future studies. More work is needed in order to explore how exposure to these experimental conditions would affect the mechanics of other animal and human models. Despite its limitations, this study provided a basic understanding of tissue mechanics, which is critical in helping lay the foundation and provide context for future work in this area. Understanding the complex interaction between IVD puncture, inflammation, and mechanics is a critical step in the overall knowledge base of IVD damage and degeneration.

11. Conclusion

This study demonstrated that FSUs placed in culture experienced an increase in joint compliance marked by an increase in NZ size and a decrease in NZ stiffness, which was exacerbated at 6 days compared to 24 hours. Further, there was an effect of damage such that the punctured IVDs, regardless of condition, had a bigger NZ size compared to the non-punctured IVDs. Conversely, LPS did not significantly affect IVD mechanics, although a trend towards destabilization was observed. It is plausible that upon exposure to a longer duration or varying dose of LPS there would have been a destabilizing affect of the joint. Based on these findings, it appears as though the mechanical responses that ensue as a result of damage through puncture and inflammation are different in nature, which contradicts our original hypotheses.

In regards to the effect of IVD puncture, an increase in joint compliance was observed at 24 hours, which is in keeping with previous research; however at the 6-day time point, there appeared to be a decelerated destabilization as joint stiffness was higher in the

punctured IVDs compared to non-punctured. This may be an indication of a reparative mechanism within the IVD in response to the puncture. Similarly, the attempted conservation of NZ stiffness as a result of puncture injury could be the result of a controlled anabolic response that occurs within the IVD. The mechanisms to which this may occur could not be fully established within this study but could be due to an increase in proteoglycan production in the NP and IAF and a fibrous capping of the annular injury.

These findings also support the growing body of research that states that IVDs and their surrounding tissue are able to sense changes in their homeostatic environment as a result of LPS exposure and release pro-inflammatory cytokines and chemokines, which peak at 24 hours and then decrease by 6 days. Finally, after exposure to LPS, there was a significant increase in TNF α and RANTES but a decrease in IL-6. There were higher levels of TNF α and RANTES in the LPS group at 24 hours with no significant difference at 6 days. Conversely, there were no significant changes of IL-6 levels at 24 hours, yet by 6 days, there was a higher concentration in the control group compared to the LPS group. These findings are different from what has been shown in previous research and may be due to the differences in the regulatory mechanisms of these cytokines or due to differences in their dose dependent response to LPS exposure. Further studies are warranted in order to elucidate this relationship. Nevertheless, increasing evidence is emerging that shows the important implications of inflammation in IVD pathologies. Future work should aim to isolate the distinct differences that exist between the cytokines released by the IVD cells themselves and the surrounding tissue. Moreover, a more comprehensive overview of how changes in inflammation and mechanics occur over time is still necessary as there may be variations that may exist that were not acknowledged in this project. Finally, there is a need

to develop a controlled *in vitro* method of studying IVD puncture and the resultant inflammation in cultured IVDs cells.

IVD herniation and associated LBP remains a poorly understood pathology that comprises a complex interplay of biological, physiological, and mechanical factors. Due to the high personal and economic burden associated with LBP, the need for effective treatments options is essential. The association between pain, inflammation and mechanical loading patterns has been well studied in other diseases such as rheumatoid and osteoarthritis however this relationship has not yet been studied in IVD herniation. Current treatment options targeting inflammatory markers such as TNF α remain highly ineffective. Therefore, continued research is needed in order to comprehend the complex interaction that exists between inflammation, damage and biomechanics in order to develop adequate treatment options for those suffering from IVD herniation and LBP.

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