ABSTRACT

Title of Document: UTILIZATION OF PNEUMATIC ARTIFICIAL MUSCLES TO STUDY EFFECTS OF LOAD HISTORY ON THE INTERVERTEBRAL DISC

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Degenerative disc disease is commonly linked with low back pain, a major musculoskeletal disorder contributing to an annual socioeconomic impact of over $100 billion. The intervertebral disc (IVD) plays a critical role in spinal load bearing and many of the mechanisms of its degeneration are still unknown. This study focused on eliciting gene expression changes of the Nucleus Pulposus (NP), the inner region of the IVD critical to load support using an *in vivo* rat model. First, pneumatic artificial muscles (PAMs) were calibrated and integrated into a small loading device as an actuation mechanism. Next, various load histories were then applied on IVDs and gene expression was determined by qRT-PCR. Results show that discs with increased intradiscal pressure led increased expression of genes common to the NP. This study contributes to the better understanding of how load history alters IVD health and validates a device for future long term studies.

UTILIZATION OF PNEUMATIC ARTIFICIAL MUSCLES TO STUDY EFFECTS OF LOAD HISTORY ON THE INTERVERTEBRAL DISC

By

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Without you, my work would not have been possible. Thank you to…

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My committee members, for taking the time to read my paper and challenge my findings. Your suggestions for revision and insightful questions helped me realize what is important about my work and what I should focus on discussing for parts of this paper.

My family and friends. Thank you for your continued help, support, and guidance as I move on to future adventures...

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Chapter 1: Introduction

Low back pain is the leading musculoskeletal disease that contributes to overall years lived with disability worldwide, affecting more than 632 million people[1]. Low back pain and intervertebral disc degeneration negatively affects quality of life for nearly 10 percent of the world. The disease has a major socioeconomic impact of over $100 billion per year and affects people of all ages [2]. Though the direct cause of low back pain is still unknown, there is a high correlation with the incidence of degenerative disc disease, in which biological and mechanical changes to the disc occur. These changes are multifaceted; some common indicators are herniation, bulging, and loss of disc height [3]. There are links between degeneration and factors such as genetics, nutrition, age, and prior load history. However, many of the mechanisms of degeneration are still not fully understood, and much more research is necessary to understand what is happening both mechanically and biologically within the disc.

1.1 Intervertebral Disc Anatomy and Function

Intervertebral Discs (IVDs) are located between spinal vertebrae, and are major cartilaginous joints that are responsible for load bearing and flexibility in the spine. The disc is comprised of several distinct regions: the nucleus pulposus (NP), the annulus fibrosus (AF), and endplates. Unlike articular cartilage, the IVD has a very complex design, where a gelatinous core is supported within a fibril structure. The NP constitutes the gelatinous inner core, a viscous gel which is comprised mainly of type II collagen and proteoglycans, mainly aggrecan. This inner core is surrounded by fibrocartilaginous layers called the annulus fibrosus. This AF is created mainly by type I collagen, and is comprised of concentric lamellar rings that circumferentially surround the nucleus pulposus. The proximal and distal end plates form cartilaginous caps on the disc and are a main path of nutrient transport. As the disc is avascular, simple transport phenomena are relied upon for nutrient supply to the disc.

The NP consists mainly of chondrocytes, though there are also cells of notochordal nature that begin dying during adolescence. They produce an extracellular matrix (ECM) which is responsible for bearing a high percentage of the load. Proteoglycans such as aggrecan contain large numbers of glycosaminoglycan (GAG) attachments for enhanced hydration account for roughly half of its dry weight. These GAGs are brush-like structures that are highly negatively charge and work to attract positively charged cations [3]. The high concentration of ions due to these proteoglycans in the matrix induce water influx to the disc to equilibrate osmotic pressure (Figure 1).This induced osmotic pressure is utilized to resist compressive loads until age, load history, degradative enzymes, or injury alter the structure of the NP's ECM, and therefore the fluidic environment inside it. The other major component, roughly one quarter of dry weight, of the NP's ECM is type II collagen, which is presumed to provide tensile strength to the nucleus [3]. Other collagens and proteins further organize the inner core of the disc and account for the remaining dry weight of the tissue [4]. The AF contains cells that begin like chondrocytes towards the inner parts of the disc, and gradually become more fibroblastic as they near the outer edges of the lamellae. These lamellae are designed primarily of type I collagen, and are designed to resist tension caused by the deformation of the nucleus under loading. As the lamellae get closer to the nucleus, an increasing number of proteoglycans can be found [4]. These structures are important to retain all tissues within the IVD, and divert outward forces from the nucleus circumferentially. The endplates serve as attachment to the cortical bone of vertebral bodies, and is comprised primarily of hyaline cartilage. It is designed primarily for nutrient transport to the avascular disc and to enable fluidic exchange during loading [5]

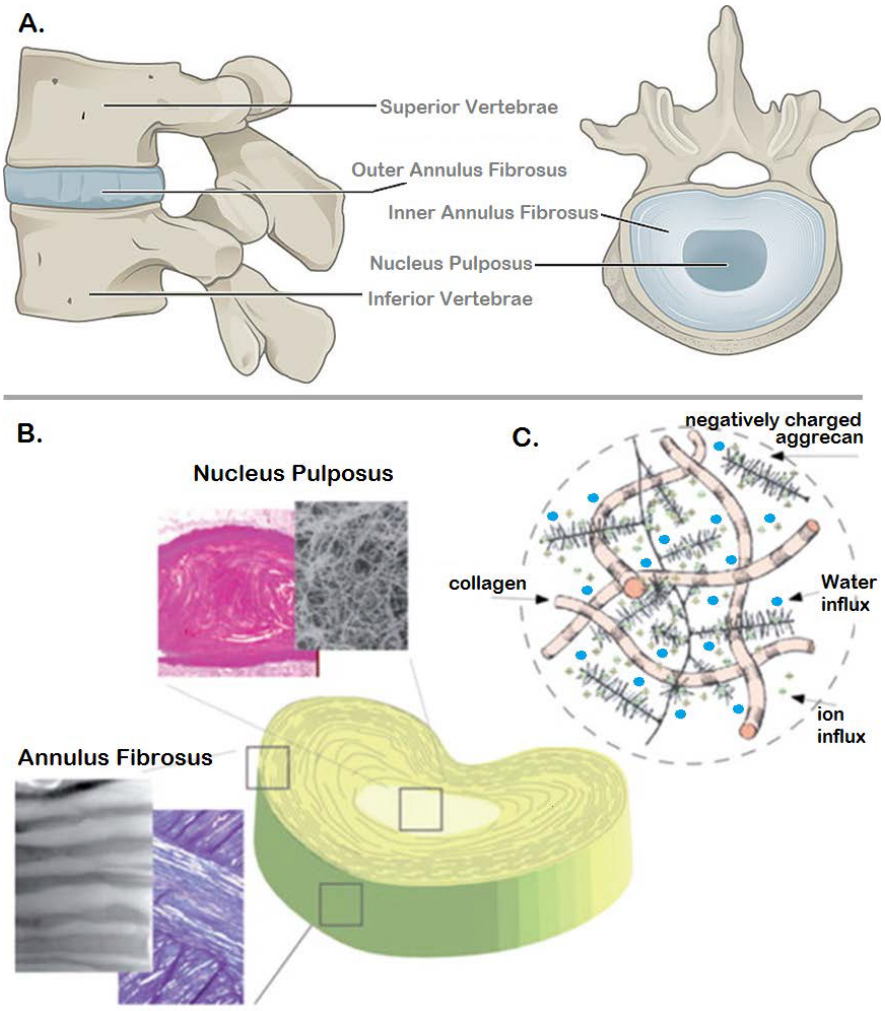

Figure 1.1 Depicts the anatomical location of the IVD with respect to vertebrae (A), bright field and histological images of the nucleus and annulus (B), and a molecule schematic of the osmotic pressure generation within the disc (C). Proteoglycans work to attract cations, leading to locally high ion concentrations which promote influx of water. Adapted from Whatley et al, 2012

The IVD functions as ligaments, holding the spinal vertebrae together, and act like joints to provide flexibility to the spine. The subregions within the disc appear to be organized to maintain load bearing responsibility during spinal motion. The AF resists biaxial shear and tensile stress within and between lamellae, while the NP undergoes hydrostatic pressure

changes. However, it appears the ability of the subregions to stay within physiologic ranges of stress and strain change based on health and load history. With increased resistance to load, the disc progressively stiffens, allowing segment's behavior to accommodate free mobility within a certain range of motion, limited by facet joints of the spine. When the hydrostatic pressure from deformation overcomes the osmotic pressure within the disc, water is expelled from the nucleus. Dependent on load and rate, when load is removed hydration levels return.

The disc's hydration is vital to IVD biomechanics, and can change due to age, injury, loading, or genetics. It is known that starting in the early 20s, there is a decline in the concentration of aggrecan within the disc, limiting the disc's resistance to load by reducing the maximum osmotic pressure of the disc [6]. Genetic factors that control GAG structure, proteoglycan degrading enzymes, and ECM production also contribute to variations in disc health [7]. The NP then begins to undergo a phase change, shifting from the viscous, gel-like material, to a firmer, more fibrous core. They type II collagen dominance of the nucleus begins to shift towards type I collagen. Proteoglycan degradation severely damages the swelling capacity, forcing the well distributed hydrostatic loading to become uneven, creating pressure peaks throughout the disc [8]. MRIs have been used as a detection tool, using the loss of hydration as an early sign without performing invasive measures [9,10,11,12]. Overall, the function of the disc drastically changes. There have even been suggestions that stiffer NP may transfer axial load to the AF, instead of acting as a circumferential tensile rings [8]. The process of degeneration is still not fully understood due to its very complex nature. To further understand the breakdown of the disc during degeneration, research must be done to investigate the healthy disc as well. Over time, the NP's ability to swell diminishes, leading the elastic properties of the region to be dominant. It is still unknown whether adult NP cells dismantle their surrounding ECM or if they are unable to maintain tissue composition by producing adequate amounts of matrix proteins.

1.2 The Role of Load History in IVD Degeneration

Prior work from out lab has established a link between different loading scenarios and the amount of hydrostatic pressure that may be generated in the disc during loading events. It was discovered for discs placed under pre-stress and then an exertion load, that discs with lower levels of pre-stress were able to generate higher intradiscal pressure (Figure 1.2) [27]. When discs are placed under constant stress, such as a fixed load being applied between the vertebrae, they experience phenomena known as creep. During creep, as water is expelled from the NP, the balance between hydrostatic and osmotic pressure changes, slowing the transport of water out of the region, causing continuous compression of the disc until water transport has reached equilibrium. These loading scenarios could be thought of as people undergoing different levels of activity, such as lounging around the house all day (lower pre-stress) versus working a day in construction lifting materials and remaining upright (higher pre-stress), followed by a trip to the gym to lift the same amount of weight.

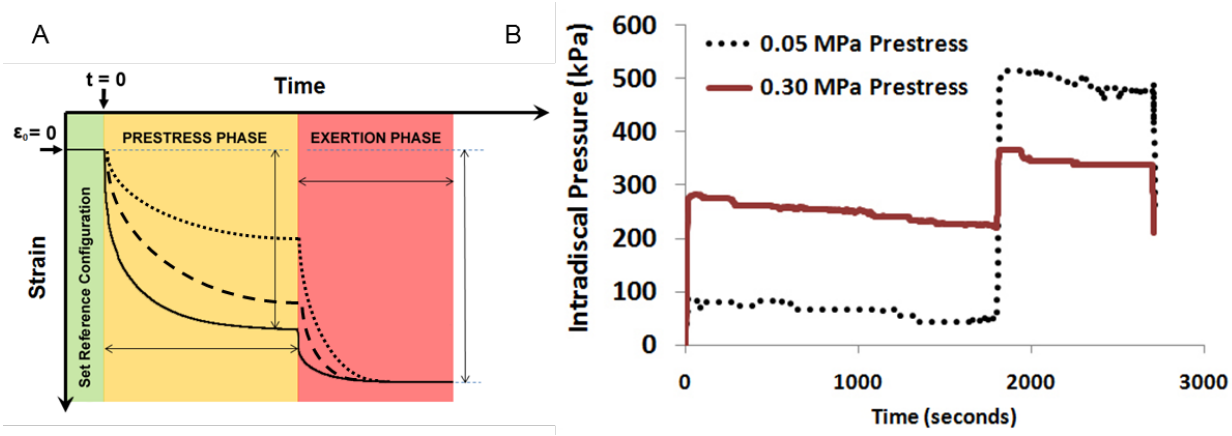

Figure 1.2 shows a schematic of static loading scenarios creating different levels of pre-stress affecting strain on the intervertebral disc (A), and a plot of pressure experienced within an intervertebral disc experiencing 0.05 MPa and 0.30 MPa pre-stress followed by an exertion load of 0.50 MPa. Adapted from Hwang et al, 2007.

The contributions of load history and its effects after loading scenarios are one of the lesser understood factors on disc mechanics. Most past loading experiments have used long

term cyclic loading, but the concept of load changes considers how prior loads affect future loading and the constantly changing hydration within the disc. Most exploration of this concept was performed examining only extreme cases of disc hydration for comparison [13]. A number of other dynamic loading experiments have presented unexpected gene expression results, suggesting that load history and prior load conditions are an important factor to disc health [14]. The nucleus is clearly an important feature of the disc, while the general function and mechanical abilities of the disc are well known the biological causes are not fully understood. Since degenerative disc disease is characterized by a loss of hydration, it is clear that retention of water after loading is a major cause of changing mechanical responses given similar loading scenarios. After water is expelled from the NP due to pressurization by the disc, several factors contribute to the amount of rehydration that can occur. The frequency between loading events dictates the amount of time available for water transport to occur, and the proteoglycan content dictates the ionic concentration differential that creates osmotic pressure to induce transportation back into the NP. Understanding how different scenarios affect the biology of the disc are important in understanding how hydration and pressure generation in the disc elicit different cellular responses and remodeling of the disc due to loading.

1.3 Rat Tail Loading Models of Disc Degeneration

In order to study disc mechanics and IVD degeneration, a wide variety of research models have been examined for their utility in the past. Common models have involved human cadaveric spines, multiple large animals such as bovine and ovine, and small animal models using rat, rabbit, and mice [13,15,16,17,18,19]. Cadaveric tissue has the obvious disadvantage of the inability to easily harvest tissue and examine biologic response to applied loads. Small animal models are generally easier to work with, and have been well explored in terms of in vitro cellular behavior. Previous in vivo studies have relied on small animal models for well controlled and easily manipulated environments. Rat and mouse models in particular have been used to

study effects of in vivo static loading on discs, as caudal vertebrae in the tail are easily accessible for loading and instrumentation. Prior studies have shown that cellular response of the AF to in vivo dynamic compression and immobilization has led to an overall downregulation of anabolic genes such as type I and II collagen, and upregulation of catabolic genes such as aggrecanase, collagenase, and stromelysin [18]. Long term application of 1 MPa cyclic compression shows stimulation frequency is important in the maintenance of proteoglycans and NP gene expression. As frequencies stay around 1 Hz, typical phenotype is maintained; much higher or lower frequencies, as well as longer loading durations show upregulation of genes associated with ECM remodeling [18, 21]. Others have shown that even with increasing proteoglycan content, IVDs undergo greater decreases in disc height and stability, possibly explained by increasing proteoglycan density correlation with induced cellular apoptosis [15, 16]. Studies stimulating degenerative changes using static compression suggest compaction of NP cells related to apoptosis and other catabolic processes [36,37,38,39]. Based on studies of load history's effects on NP stress, the ability to pressurize appears to be linked to the discs hydration and stress state [27]. The fluidic nature of immature NP allows effective hydrostatic pressure generation, though this can have undesired effects. With hydrostatic pressure as low as 1 MPa, increased collagen degradation has been seen with loading applied at high frequencies [40, 41]. This high sensitivity to distortion and pressure suggests better understanding mechanical stress exposures on the disc and stress distribution within the disc will help understand changes during growth and aging.

Current rat tail loading devices used for IVD studies have a number of limitations. A major limitation of many devices is the requirement for the animal to be placed under anesthesia and loaded using a benchtop materials testing system. Limits of anesthesia prevent such devices to be utilized for long term loading regimen not only due to potential toxic effects, but also due to immobilization of the animal during loading. Other devices are only capable of applying static loading scenarios which are unlike the physiologic dynamic loading experienced

during day to day activities, and many of the devices require permanent instrumentation and/or invasive surgeries [15, 17, 31]. The most notable loading device utilizes piston actuation and a removable Ilizarov-type frame that has the ability to apply long term dynamic loading scenarios to rat caudal discs [20]. However, the rigid actuation of the pistons restricts movement of the tail on loaded segments, and pistons must overcome friction to switch between loads, prohibiting smooth actuation, and potentially creating acute loading events.

1.4 Objectives

We aim to focus this study on the effects of load history on gene expression and to create a small loading device for future long term load history studies. In order to create a device appropriately weighted and sized, miniature pneumatic artificial muscles (PAMs) were used as the actuation device for out loading device. Experiments were performed to optimize and characterize the miniature PAMs to fully understand the relationship between PAM length, pressurization, and applied force. Once the actuation devices were characterized, a control system was created to determine how much pressure to apply to the PAMs to achieve a desired force as the disc in the rat tail undergoes creep. Once a control system is in place, viability of the actuators to apply both static and dynamic loading was determined. Components for the in vivo loading device are then 3D modeled using CAD software to determine device weight, followed by their creation. The device will be further used in the future to apply long term loading regimes on caudal rat discs. These experiments will aim to further explore the function and behavior of the disc, both biologically and mechanically. Further, this device was applied to caudal rat discs to explore the effect of short load history on gene expression responses. Expression of type I collagen, type II collagen, aggrecan, and sox9 was examined for three different load histories and compared with prior gene expression studies. Due to the limits of anesthesia, these loading regimes were limited to short durations due to the in vivo nature of the experiments.

The presented research will contribute knowledge towards full understanding of the IVD. The effects of load history are a critical aspect of the disc that have just begun to be explored. Cellular response within the nucleus, specifically how cells regulate their ECM, needs to be fully understood for the advancement of future IVD therapies. IVD research is currently in a stalled state, lagging behind the overall study of cartilage and joints. The current understanding of the disc and its main components is incomplete. Basic scientific research about the function, response, and behavior of the disc need to be further explored and understood before IVD research can move forward. Creating a device capable of applying long term loading scenarios to discs will further enable study of the effects of load history. The device can also be used to study potential therapies to overcome degenerative changes to the disc. The knowledge of the biological gene expression response of the NP in this research affects the general intradiscal response and interactions in other parts of the disc. This provides just one further step in the basic understanding of the disc, and provides better methods for exploring future IVD therapies.

Chapter 2: Creation of a Novel Dynamic Loading System

2.1 Introduction

Intervertebral disc mechanics have been widely researched in the past in an attempt to better understand the causes of low back pain, a musculoskeletal disorder experienced by more than 70% of people during their lifetime. The IVD is a complex type of fibrocartilage tissue, unlike better known articular cartilage. Articular cartilage varies in depth and has several zones in which collagen orients in different manners, whereas the IVD is comprised of the gelatinous NP which is surrounded by a fibrous structure called the AF. This complex structure increases the difficulty of understanding and predicting the disc's behavior. Many groups have attempted to understand the biological response of the disc to loading of disc cells *in vivo*, in scaffold, and in organ culture. The most common models of research are cadaveric or large animal based (bovine, ovine), with other studies focusing on small animals (rabbit, rat). For cadaveric tissue, it is nearly impossible to harvest live tissue to study the resultant biologic responses after loading, ruling out many of the models possibilities outside of studying disc mechanics post mortem. Our lab focuses on development of caudal rat disc models, as the discs are easily accessible via the tail. This study aims to take advantage of the rat tail model to develop a loading device for *in vivo* studies of biologic response to load with the ability to use for long term studies

Few rat models have been used to study the effects of long-term loading in the past. One of the most unique ideas applied in studying spinal stress in rats is forced hind limb ambulation of rats by forelimb amputation [22]. While creative, there is little control in the loading scenarios and the effects of degeneration are only due to the upright posturing of the animal. Wuertz et al. have created a piston-based ring device to attach to rat tails [21]. They have completed studies of loading up to 8 weeks. This was the model device of our own design with the goals of reducing weight, size, and cost, while maintaining the robust control abilities applied by Wuertz. This goal is to be achieved in a similar manner, cross drilling pins through vertebrae for loading with a ring based device.

Instead of using costly, rigid pistons, this device is based on Pneumatic Artificial Muscles (PAMs) which have many advantages for biologic applications. PAMs are a type of nonlinear McKibben actuator, commonly used in robotics and aerospace applications. These actuators which were developed by J. L. McKibben in the 1950s and are unequaled in actuation force per actuator weight [23]. These actuators consist of an inner elastic bladder which is surrounded by a woven fiber as illustrated in Figure 2.1 [24]. As pressure is applied, the elastic tube expands while geometrical constraints of the woven fiber simultaneously force the actuator to contract[25]. Unlike pistons that force rigidity in the area of applications, the flexibility of the PAMs allows biologic tissues to maintain their natural range of motion. In addition, PAM devices are able to transition smoothly between loads due to their elastic bladder. When pistons switch between loads, they need to overcome friction in the device before they begin to apply loads. As the force of friction is overcome, there is an acute loading event which may be unnatural and cause trauma in many biologic applications instead of the desired load, skewing results of the testing. Acute loading as friction is overcome may also cause cellular damage. Furthermore, PAMs are able to smoothly unload tissues, whereas an unloaded tissue may have to compete against friction in order to relax with piston based systems. The flexibility of PAMs also allows for a greater range of loading scenarios, such as overloading one side of the disc for potential future bending studies.

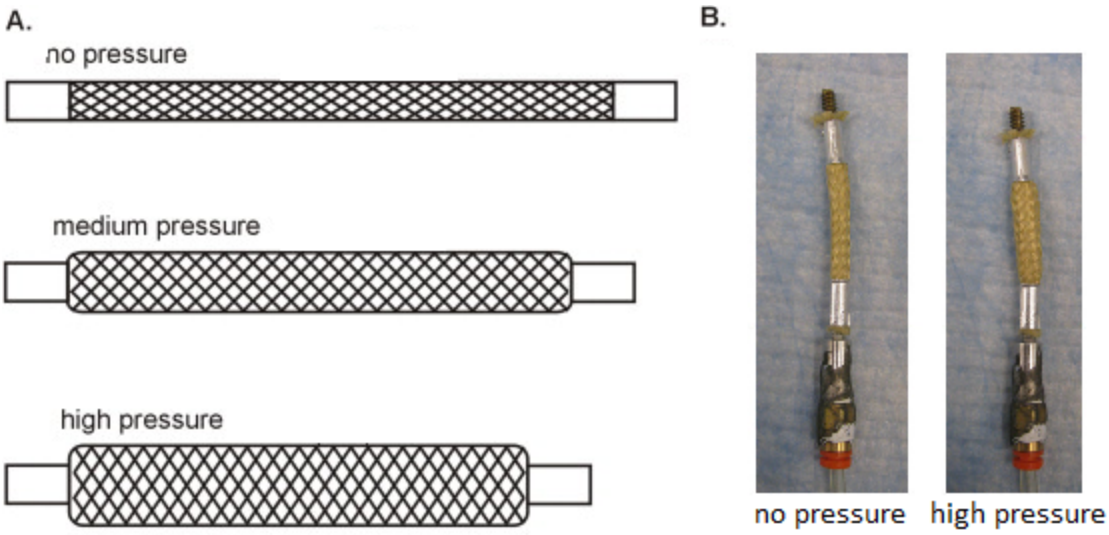

Figure 2.1 (A) shows the operation principle behind the actuation of PAM devices. (B) Shows the operation of one of the actual PAM devices used in this study, made with a latex bladder and Kevlar sleeving. Image adapted from De Volder et al. 2011; Hwang et al. 2011

Previous work in our lab was completed to create and characterize a variety of miniature PAMs, which to our knowledge, is the first attempt to develop miniature PAMs on a scale of less than inches. The PAM devices were placed between to plates, and a tail was affixed in line with a load cell to monitor the response of the system to applied loading (Figure 2.2). After characterization and calibration of the PAMs, they were placed under control by a pressure regulator and custom Labview (National Instruments, Austin, TX) program, and interface was developed to monitor the system as an 18N compressive force was applied to the disc. As the IVD is a viscoelastic tissue, it experiences creep, which is the tendency to deform over time, during loading when placed under a constant stress. This change in displacement creates the necessity of controlling the pressure into the system. However, the initial attempts to control load and compensate for viscoelastic creep in the tissue were unsuccessful (Figure 2.3). The device was unable to achieve the targeted load and overexerted force to accommodate for the

creep in the tissue, causing the force to steadily increase during the testing of the tissue. Much work was done to add to the system. Loading platen and load cell fixtures were redesigned to more closely mimic the layout of the system for *in vivo* purposes. A second pressure regulator was added to the control system to enable the PAMs to be treated as individual units to accommodate their different pressure responses. Finally, characterization of the PAMs was necessary to result in accurate force targeting and control for the duration of testing.

Unlike pistons, which have a linear relation between input pressure and applied force, PAMs need to consider the length of the device due to their nonlinearity. This non-linearity adds a level of complexity to the system as the relationships between force, pressure, and length need to be fully understood in order to maintain control of the system. This also creates a need for displacement feedback in the system, as load cells are too bulky to accommodate on the tail. A small DVRT, or displacement sensor, is therefore necessary to determine the change in length of the PAM devices to allow the system to compensate for changes in length during the duration of loading. This study is to verify the calibration of the PAM system and its control to determine its utility for *in vivo* testing. The ability to apply controlled loading is vital to study the effects of prior load history on degenerative changes associated with differing stress magnitudes and frequencies, making this system vital to further understanding of the complex effects of disc loading on cellular response and tissue mechanics.

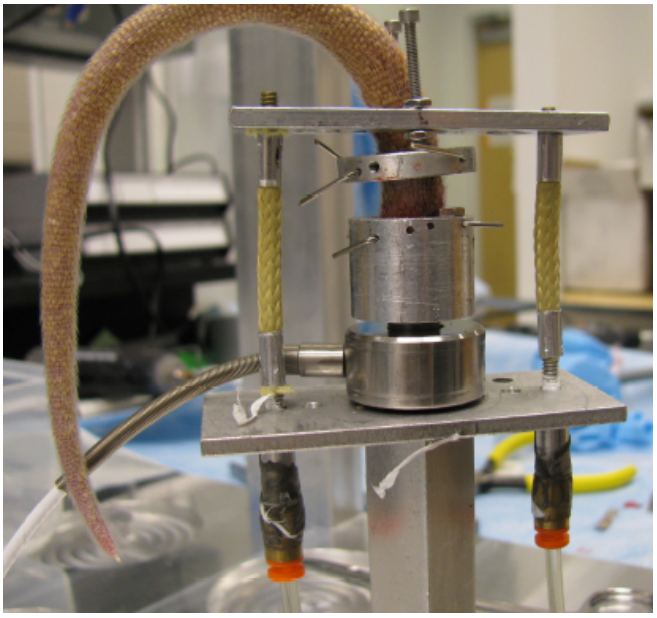

Figure 2.2 shows the original *in vitro* test setup of the PAM device.

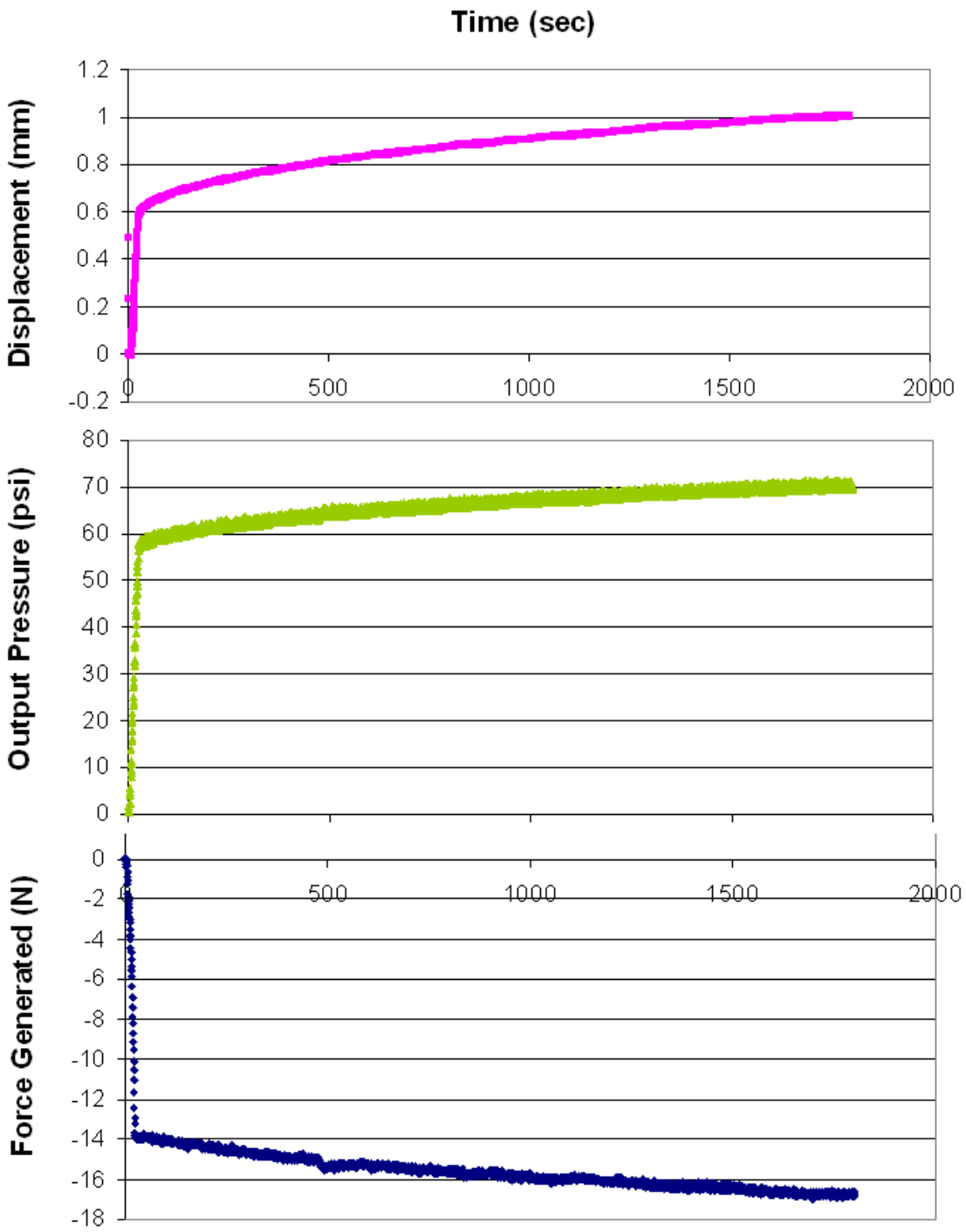

Figure 2.3 shows previous results of *in vitro* PAM device testing, showing inability to reach the targeted load and inability to properly respond to creep of the tissue, resulting in overexertion of pressure and steadily increasing resultant force

2.2 PAM Fixed Force Calibration

To determine the relationship between the three parameters of input pressure, PAM length, and actuation force, miniature PAM devices were mounted onto a Bose Electroforce materials testing system (LM-1, Bose Corp., Eden Prairie, MN) for calibration. Under displacement control, the PAMs were inflated to either 3N, 6N, or 9N and then compressed in length up to 1.0 mm in increments of 0.1 mm. At each increment of displacement, the pressure into the PAMs was increased until the original load was reached (Figure 2.4). At each displacement, the pressure required for each PAM to exert the targeted force was recorded. This test accounted for the three changing variables in the system to determine what type of pressure increase was necessary to reach targeted loads and then maintain them with changing displacement. Pressure versus displacement for each PAM at each force was fit with a linear regression, resulting in an equation that can dictate pressure necessary for a targeted force at given displacement. These equations are necessary to determine the pressure input necessary for reaching and maintaining forces during later verification of and experimentation on the system.

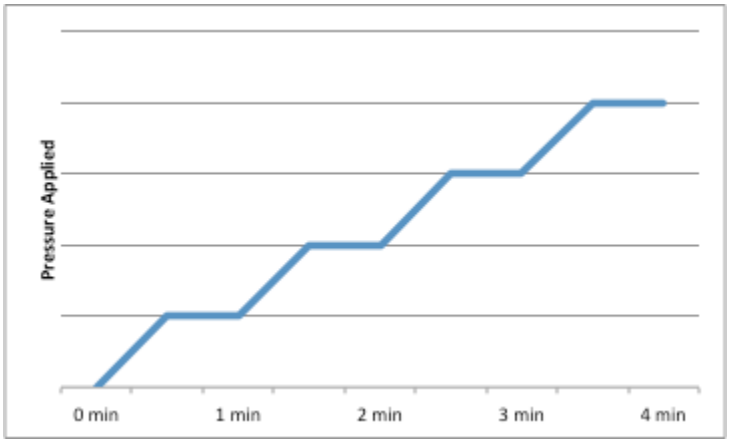

Figure 2.4 is a sample of applied pressure versus time during PAM calibration. Displacement is increased for every step up in pressure.

2.3 *Ex Vivo* Viscoelastic Feedback Testing

Prior to *in vivo* studies, the PAM system needed to be tested to ensure stable forces can be maintained under displacement control. For purpose of verifying the system's utility, a load cell was attached in line with isolated spinal motion segments, with two half inch, Kevlar braided PAMs used for actuation. Motion segments had Kirschner wires cross drilled through them to fix the segments between a loading ring and a custom load cell attachment (Figure 2.5). Half hour tests of an 18N load were run to determine the ability of the system to hold a load while the IVD experienced creep. The magnitude of 18N corresponds to roughly 1.5 MPa of stress on the IVD and was chosen for the short tests as it is the highest load magnitude wished for targeting with *in vivo* testing. It has been estimated that 1MPa (about 300% of body weight) and higher constitute harsh exertion loads. This value has been used in the past as the basis of many biomechanics experiments [14, 26]. With the larger magnitude of stress, the tissue is expected to undergo the most creep and is therefore best able to determine the ability of the system to maintain a load under displacement control.

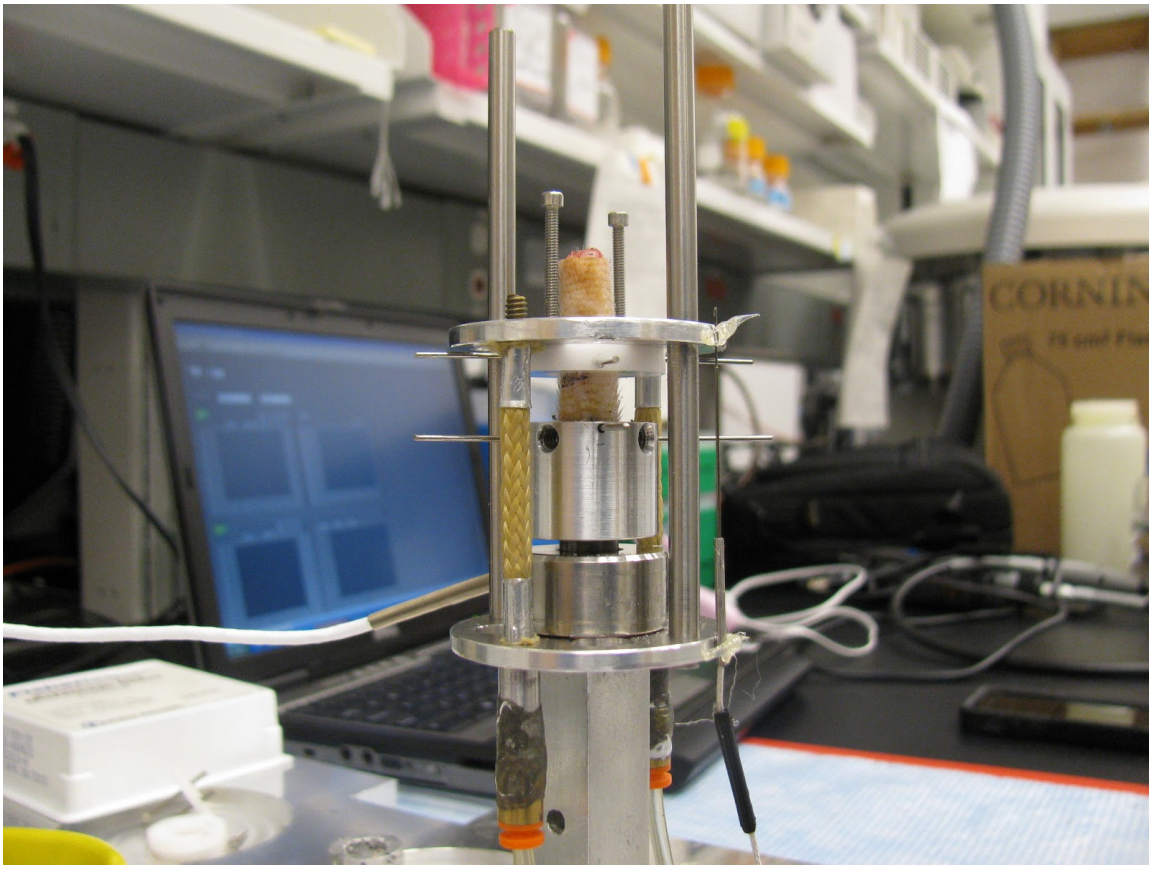

Figure 2.5 is a picture of the *ex vivo* test setup

A custom Labview program was created to enable displacement control of the PAM system. A small DVRT that is mounted in parallel with the PAMs on the loading frame is used to determine the change in displacement that the IVD has experienced, in turn, determining how much compression the PAMs have undergone. Based on the change of length the PAMs have undergone, the pressure required to exert the desired force can be applied. Prior lab work had unsuccessfully averaged the PAM characteristics and applied pressure from a single regulator based on creep to target and maintain 18N. Since the two PAMs in our system have different pressure intercepts for each force, it was determined that adding a second regulator would be beneficial to the control of the system. New work to modify the control program incorporates a second pressure regulator to allow each PAM to be individually controlled based on the

displacement of the whole system. The program uses feedback from the DVRT and applies it to the characterized equations fit during calibration to determine the pressure output necessary to hold a desired force (Figure 2.6). A voltage applied to the pressure regulators input is then modified in real time while monitoring the pressure output of the regulator until the actual pressure into each PAM is equal to the desired pressure to generate the necessary force. For verification purposes, a load cell with a custom fixture to allow for cross pins to be affixed to caudal vertebrae was placed between the plates holding the PAM devices and DVRT sensor. The input voltage and pressures of each regulator, the DVRT's position, and the force output of the load cell were recorded for the duration of testing.

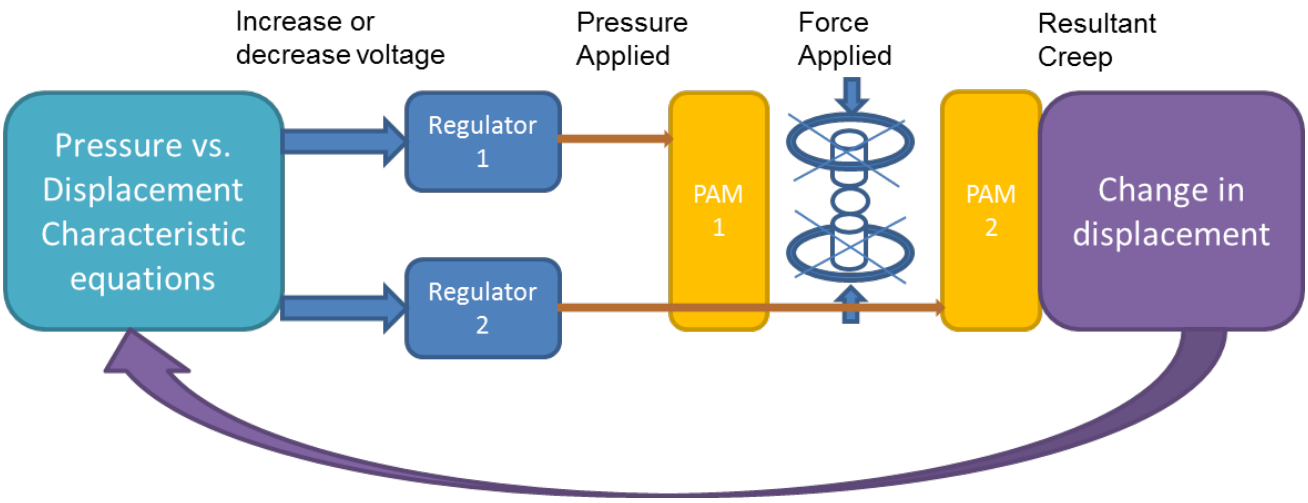

Figure 2.6 A flowchart describing the control of the PAM actuation system

After completion of several static loading protocols, a more biologically relevant cyclic loading regimen was tested to determine the flexibility of PAMs for dynamic load application. As short loading experiments demonstrated that tissue creep had ended before 30 minutes of static load application, applying a cyclic load after a short testing scenario seemed probable under the fixed displacement at the end of the test. To apply the cyclic loading, the Labview program was customized to modulate pressure after the static load was held for 30 minutes. In order to control the amplitude of cyclic loading, the desired pressure drop at a fixed displacement to

cycle between 18N and 9N was calculated from the characterization curves. Once the pressure drop was known, the program was designed to increase and decrease the pressure of a known drop at 1Hz for several minutes to validate the control system.

2.4 *Ex Vivo* Loading Results

The PAM actuation system is dependent on the feedback of the displacement parameter to adjust the regulated pressure to achieve a desired force. Once the relationship between the parameters was determined, it was input into a custom Labview program to allow for automatic control during various loading scenarios. The goal of this experiment is to ensure the system is able to hold a constant force while compensating for the constant change of viscoelastic creep during testing with only displacement feedback as a control.

Prior results of *ex vivo* testing were inconsistent with what was hoped to be accomplished. Originally, the system was unable to reach 18N, exerting only 13N during initial compression. The system, thought to able to actively track and respond to creep during testing, overcompensated for the amount of creep occurring, resulting in the load applied to the tail steadily increasing for the duration of testing. With this modified loading system, as the displacement of the system continued to increase in magnitude, the program was able to accurately compensate with a rise in pressure, holding a steady force for the duration of testing (Figure 2.7). The system was able to quickly reach the targeted 18N and readily hold a stable load for 30 or more minutes. From previous and current work on the PAMs, prior to *in vivo* testing, these systems should be tested *ex vivo* to ensure that they have been calibrated correctly, otherwise the targeted load may not be achieved, or the system may either overestimate or underestimate the creep of the system, resulting in an inability to maintain control during the test.

During the dynamic testing trials, the system was able to accurately cycle between the targeted 18N and 9N forces (Figure 2.8). Instead, the pressure drop calculated resulted in a

slight variation between the desired and targeted force. It is also noticeable that the system experiences inconsistency between the peaks and valleys present in the magnitude of the force. Since the system relied on the observation of a constant displacement by the end of testing to calculate the pressure drop, slight variances in disc displacement due to relaxation and re-exertion of the load created small deviations in the desired load. Control of the cyclic loading scenario should be modified to account for the actual displacement of the disc which will require more sophisticated cyclic control methodology than is currently employed.

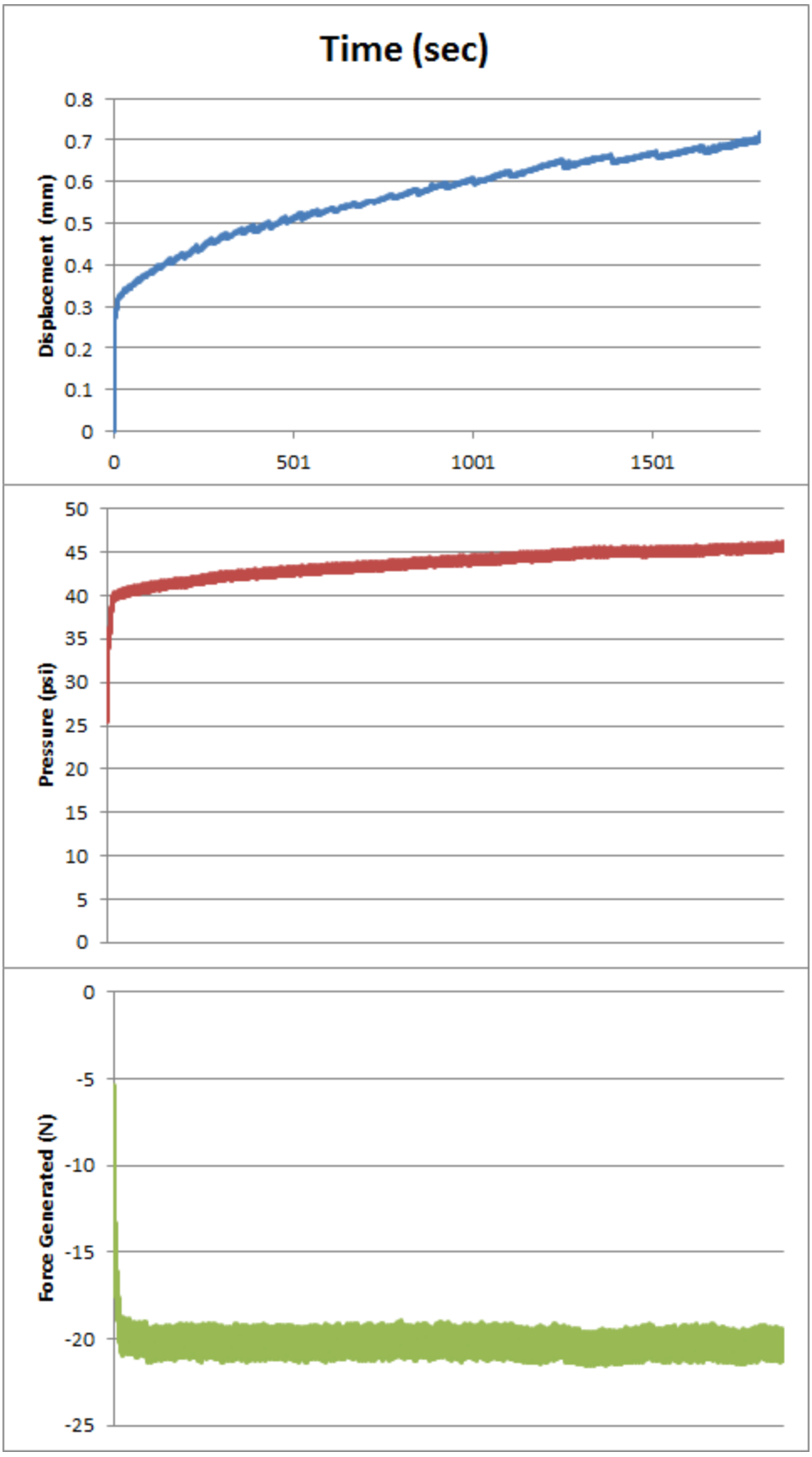
Time (sec)
Displacement (mm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
501
1001
1501
Pressure (psi)
50
45
40
35
30
25
20
15
10
5
0
Force Generated (N)
0
-5
-10
-15
-20
-25

Figure 2.7 shows *ex vivo* testing demonstrated an accurate portrayal of the targeted force. The system was also able to increase pressure to the system to compensate for the creep of the tissue, resulting in a steady load for the duration of the testing. PAMs should be testing ex vivo to ensure accurate calibration before proceeding to live testing to ensure the system control is accurate.

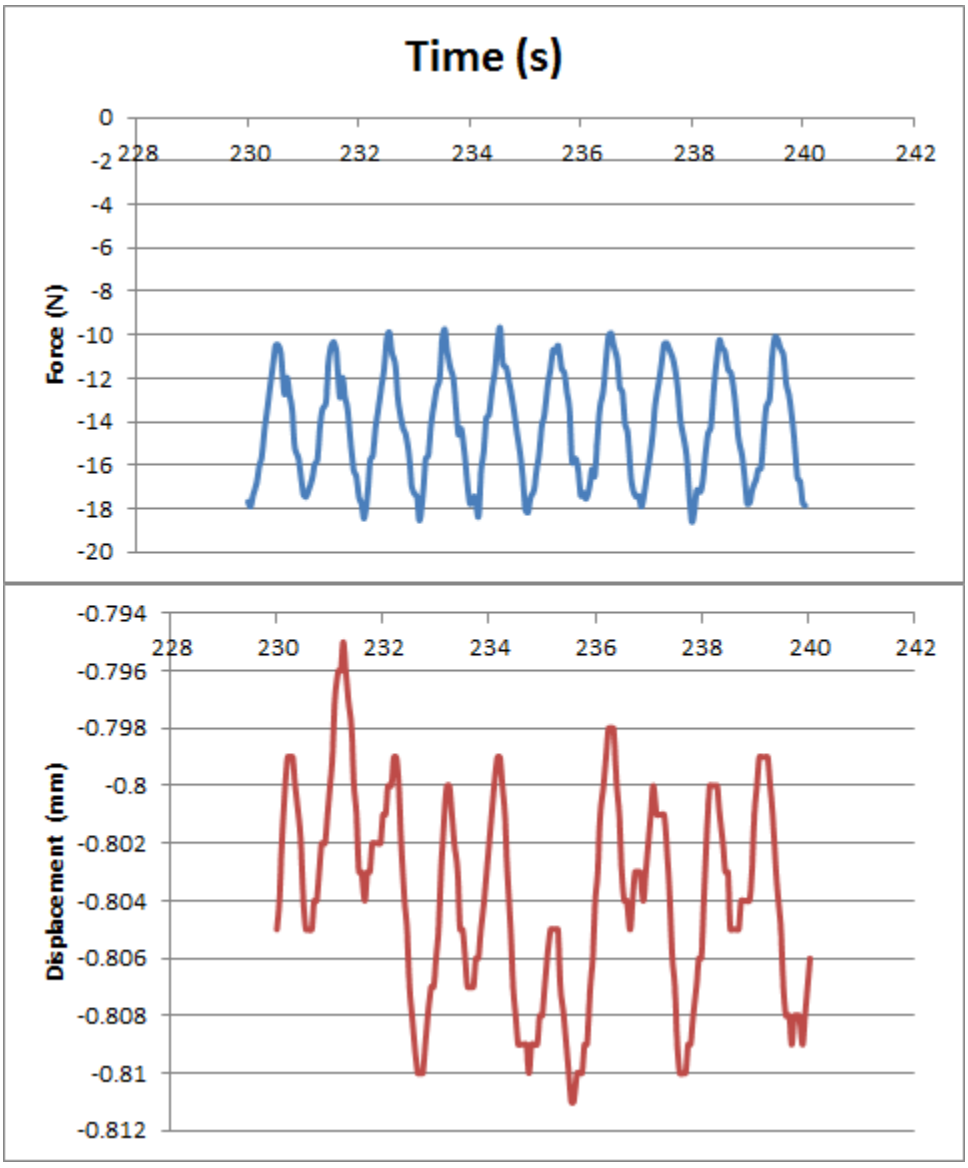

Figure 2.8 shows cyclic loading of the PAMs at 1 Hz between 18N and 9N. Slight inconsistencies in the peaks and valleys of loading exist; Control methods for the cyclic loading should be reevaluated and applied for smoother loading.

2.5 Discussion

This study demonstrates successful implementation of a flexible, frictionless, and lightweight actuator to apply both static and dynamic compressive loads for use in an animal model of intervertebral disc loading. Due to the nonlinear nature of the PAM devices, extensive characterization of the devices is necessary to apply load profiles quickly and precisely [25]. To complete this, the relationships between applied force, inflation pressure, and PAM length need to be thoroughly understood. Previous work in the our lab characterized the PAMs both by measuring force during length changes at constant pressure and during pressure changes at fixed lengths. Current work was completed to characterize the pressure changes necessary to maintain a force given fixed displacement. These characterization experiments allowed for a full understanding of the interaction of PAMs and enabled accurate control of the system's actuation to target the desired loads.

We were able to successfully apply a constant force on an excised motion segment, while the intervertebral disc experienced creep. The displacement feedback control of the system was both active and successful in tracking the creep of the tissue, allowing for compensation in the system to maintain loading. The intended force of 18N was attained and then maintained for the duration of testing via attenuation of the pressure inlet to the PAMs as they changed in length. This ability to hit the desired load and hold it stable demonstrate proper calibration of both the slope and intercept for each of the two PAMs used in load application. Prior to *in vivo* testing, *ex vivo* calibration and verification of the PAM systems as a set should be performed to maintain accuracy of the systems.

Previous work in the lab utilized only a single pressure regulator to control two different PAMs, leading to instability in the maintained load and inability to reach the targeted load. Addition of a second regulator and its control were necessary for functionality of the device as the PAMs used require very different input pressures to output the same amount of force. Future work may seek to build more miniaturized PAMs with comparable characteristics, which would allow matching pairs of actuators to be fed from a single pneumatic regulator. Ability to use a single regulator for each loading device would allow for easier expansion of the system for loading multiple discs simultaneously, as well as allowing for simpler control of the system.

PAMs were also able to successfully apply cyclic loading to an intervertebral disc with some success. Though the desired upper and lower limits of 18N and 9N for loading were not accurately targeted, the actual loads of 18N and 10N only differ on the lower bound. Control of the cyclic loading was based on observations that the tissue's displacement had reached near equilibrium by the end of a short duration static load. It is likely that the inability to accurately decrease the load fully to 9N is due to slight relaxation of the disc when the initial stress is lessened. For future dynamic loading scenarios, the LabView control program should be modified to account for any variances in displacement to allow for more accurate loading.

Chapter 3 *In Vivo* Gene expression testing

3.1 Introduction

Biological and mechanical dysregulation of the IVD is commonly related to aging as well as degenerative disc disease. This disease is characterized by a loss of mechanical properties due to the breakdown of extracellular matrix, limiting the hydration and health of the tissue. The IVD then becomes more compromised; inability to retain water within the tissue leads to a continuous breakdown, further compromising the disc. Many of these extracellular changes are still not fully explained, but many of the previous experiments to determining the effects of load history on the IVD have linked them to a variety of unexplained biological processes.

Within healthy IVDs, the inner gelatinous core of the disc, the nucleus pulposus (NP), maintains a vital role to the load bearing capabilities of the disc. The NP of healthy tissue is able to maintain its hydration, supporting this role, but disc disease causes a breakdown of extracellular matrix, hindering the disc's ability to retain water. The NP's hydration allows the tissue to pressurize under load; this ability to disperse applied force has led to studies heavily emphasizing the fluid content and pressure of the disc. Both instantaneous stiffness and viscoelastic properties are both influenced by the discs hydration and the hydration level in the disc changes in response to load. As a viscoelastic material, these physical parameters are all influenced by the recovery time given to the tissue.

Understanding the mechanics of how the disc functions is necessary to provide insight into chemical reactions and changes within the disc. Many factors including the hydration of the disc have been shown to influence the health of the extracellular matrix. Prior work in our lab has shown that load history influences current and future disc behaviors, specifically in terms of hydration. The lab has directly measured and shown dependence between load history and intradiscal pressure [27]. These changing pressures within the disc influence the overall hydration

of the disc, as well as other mechanical factors, changing the disc's response to future loading scenarios. As these conditions within the disc change, the cellular environment will change, impacting the disc's cellular activity. The metabolic behavior of cells is known to be influenced by both chemical and physical factors in their environments.

IVD response to changing environments and load histories has been extensively studied *in vitro* or in organ culture. Some groups have performed in vitro biomechanical testing by excising discs and determining the physical and chemical changes of the disc after testing such as the loss of disc height and water content [28]. Other groups have created bioreactors to culture whole bovine intervertebral discs and induce biomechanical loads for up to several weeks to determine the gene expression response to loading [29]. Another interesting approach to whole tissue culture has been to encase rabbit discs within alginate and maintained in culture for up to a month at a time which found the method to be viable for future studies [28]. However, a large number of problems with studying biologic responses to loading in organ culture or in vitro exist. Studies of whole organ culture typically find poor cell viability within the nucleus pulposus, not only making gene expression analyses difficult, but affecting the normal tissue conditions which may affect biomechanical properties. However, a few groups have monitored gene expression responses or tissue behavior to load history *in vivo.* Many rat and mouse models have induced static compression *in vivo*, some using springs and loading fixtures implanted onto tails or directly to the spine [31]. Most notably, loading frames have been created for attachment to rat's tails to induce chronic static or dynamic loading scenarios for up to 8 weeks to examine physical and chemical changes to the disc [20, 21, 28].

This study both creates an Ilizarov-type fixation device for loading via PAMs as previously discussed, and examines the gene expression changes associated with differently applied short term load histories. Examining these cellular responses is important to understand how stress levels affect matrix distribution and important in determining what levels of stress are detrimental and how they lead to changes on level of the whole tissue.

3.2 Creation of an Ilizarov-type frame for loading

Ilizarov type frames are commonly applied for orthopaedic applications, specifically fracture healing, and are primarily comprised of a circular frame that is affixed to bone via surgical pins [32, 33]. This type of frame is used for its beneficial fracture healing versus conventional fixation methods, and was a great fit for a load frame to apply to caudal discs within the rat tail. As the loading frame is external, there is easy access to add and remove attachments as necessary. The major goals of this frame were to remain as lightweight and small as possible so as to not aggravate a rat during long term studies. Custom washers to be fixed onto the caudal vertebrae were created with Delrin, a lightweight, sterilizable plastic. These washers had two sets of holes drilled radially to allow for kirschner wires to pass through and affix the washers onto a tail, and two threaded holes onto which loading platens could be attached (Figure 3). The loading frames were created out of carbon fiber, a high strength, lightweight material. These frames had several attachment points created to allow fixation of the PAM actuators, connection to the washers affixed to vertebrae, and guide rods to ensure uniaxial loading. The carbon fiber frames also had several large holes of material removed from within the washer's surface, reducing the weight of the device by more than 50%. These parts were designed, modeled, and analyzed in Creo Parametric 3.0. The pieces were individually fabricated in the Wind Tunnel Machine shop (UMD, CP), and weighed within 5% of their expected values. An assembly drawing of all the pieces was then created to determine the overall weight and size of the loading system to ensure its weight and size were both small enough so as to not disturb a rat during the time the device would be affixed. The device was calculated to be well under 10% of our typical rat's body weight. Several trial surgeries were performed where the device was instrumented onto rats. The rats maintained body weight and showed no other signs of distress while the device was affixed for as long as 1 month.

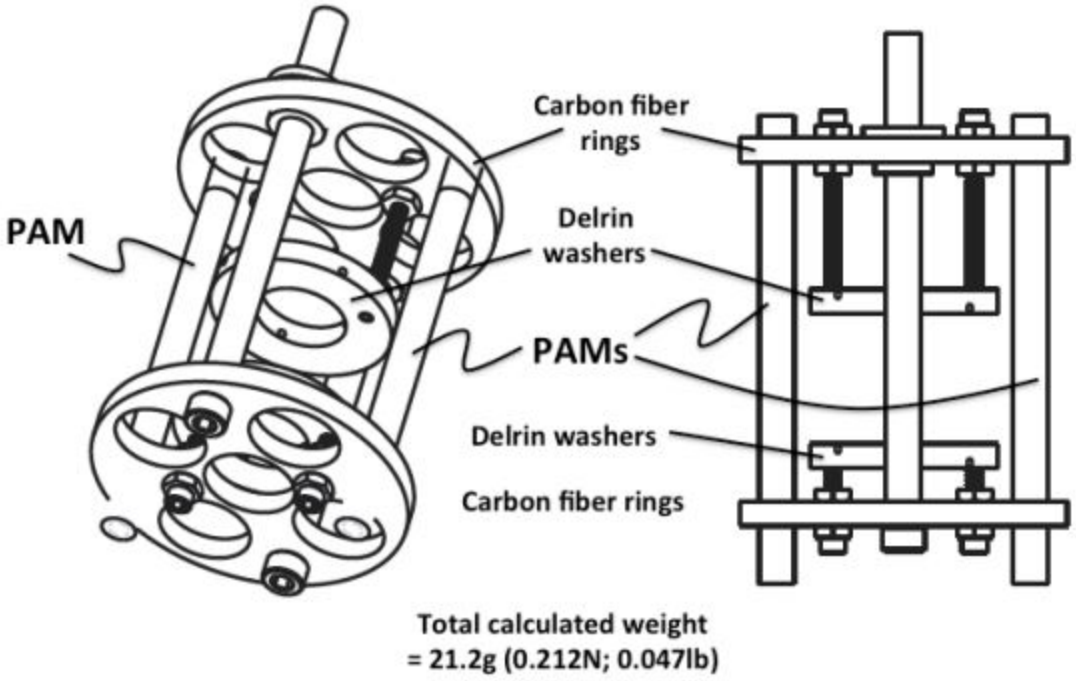

Figure 3.1 Perspective engineering drawing (left) and side view (right) of the entire assembled device containing Delrin washers to hold pins, carbon fiber rings to apply loading, and pneumatic artificial muscles (PAMs) to generate force. The total calculated weight (based on each component's masses) is 21.2g.

3.3 Short Term Loading

Skeletally mature Sprague-Dawley male retired breeders (9-18 month old) were anesthetized using isoflurane gas via inhalation and prepared for surgery. The rats were provided buprenorphine analgesic was provided for pain relief before surgery. Caudal c4-5 vertebrae were targeted using radiographic confirmation (Fluoroscan III, Hologic Inc., Bedford, MA), and Delrin washers were attached via drilling two sets of holes through the vertebrae in which custom Kirschner wires were inserted, affixing the washers to the tail (Figure 3.2). The custom carbon fiber rings were then affixed to the washers, and PAMs were attached to the device (Figure 3.3). For the short history loading groups, 3 groups of interest were chosen, shown in figure 3.4. The first group (n=4) consisted of an hour of axial load at 0.5 MPa, followed

by an additional hour of loading at 1.5 MPa. The second group was loaded for the first hour at 1.0 MPa, followed by the same additional hour loading at 1.5 MPa. The third group was to act as a time average stress control of the first group, maintain 1.0 MPa for 2 hours of loading. These experimentally applied static loads would be similar to stacking weight onto one's head and standing still as stress is applied to the discs. While this is an unlikely scenario compared to typical dynamic load application during spinal movement and ambulation, understanding how different stress levels and the ability of the disc to pressurize is important to understand the effects of dynamic loading. Due to the nature of dynamic loading and the time dependent properties of the disc, it is hard to understand the cellular response to a dynamic environment, where the hydration and stress levels of the disc are continually changing. Static loading allows for greater control of hydration and stress within the disc to better understand changes on a cellular level.

As previously mentioned, loads of 1.0 MPa (300% of body weight) and higher constitute harsh load exertions. We also know that greater preloads following exertion lead to a loss in disc hydration. These load profiles were chosen to examine the effects of different known pressures within the disc on the biologic response. The 0.5 MPa was chosen as the lower preload partially due to constraints on the PAM system, which according to our calibrations cannot consistently produce under 2N of compressive force each, so a load higher than 4N was chosen to enable preload of the system. This force is to be compared with a group experiencing 1.0MPa of preload, or a load that is known to produce hard conditions, and one that will decrease the ability to pressurize during challenge loading of 1.5MPa. These loading scenarios apply generally known environments on the cells that have been previously explored in our lab to study their effect on cellular behavior [35]. The variation in preload will allow for consideration related to the hydration of the disc based off of previous load, allowing us to examine how past loading history will affect future response of the disc. Also, these protocols consider a group that withstands an equal amount of stress over the duration of testing as the 0.5 MPa preload group

by holding 1.0 MPa for the full two hours. This group allows us to determine the response of load history when compared to the response of stress within the disc, as we expect hydration and pressure in the disc to affect the biologic response to load.

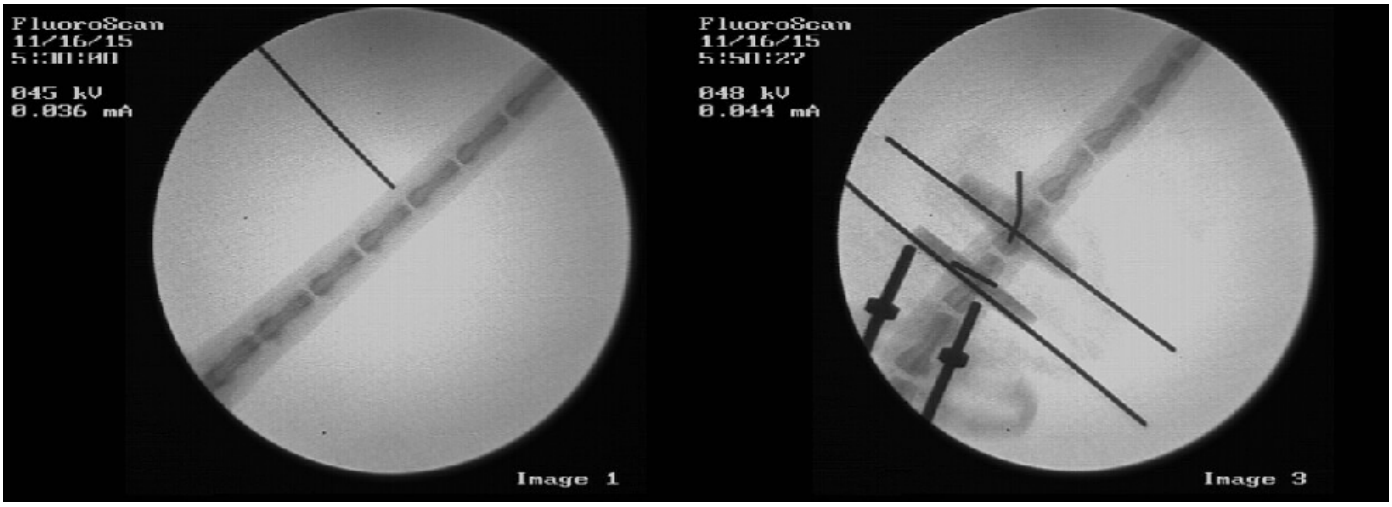

Figure 3.2 Radiographic images of: confirmation of caudal vertebrae c4-5 pre-surgery (left) and Delrin washers surgically attached to vertebrae via kirschner wires as confirmation of correct device placement

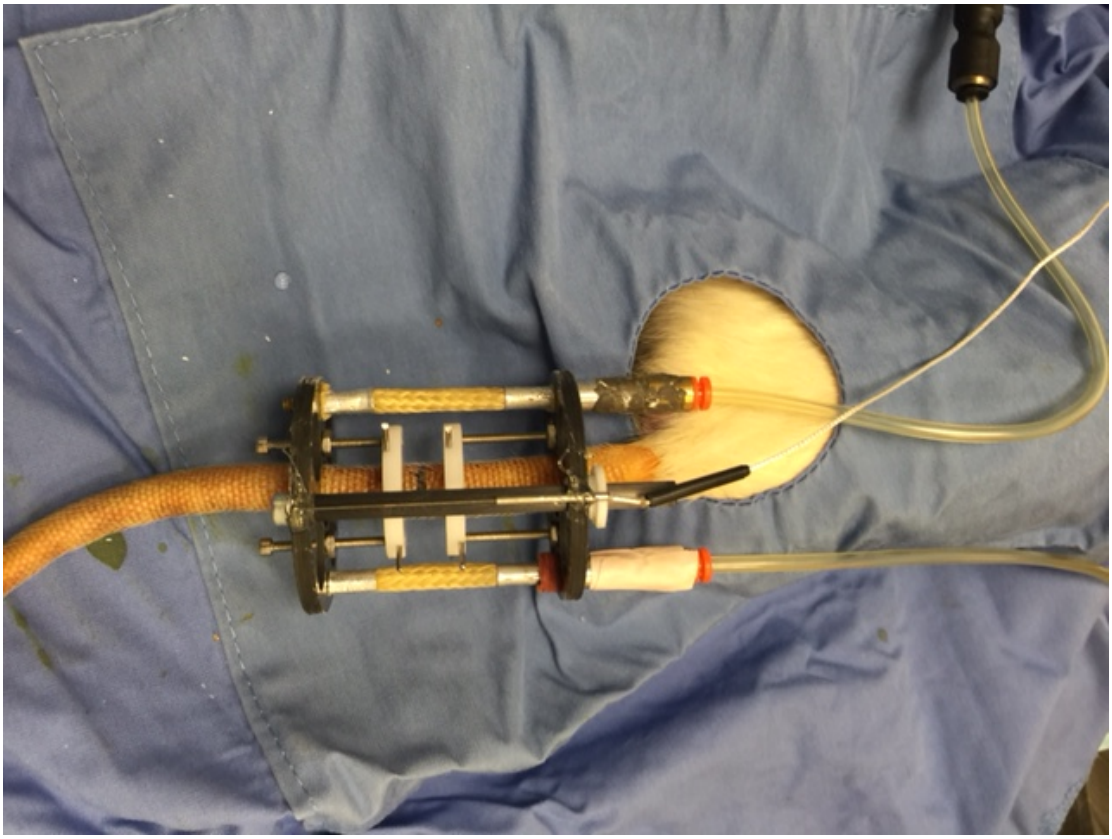

Figure 3.3 A fully assembled loading device attached to rat caudal c4-5

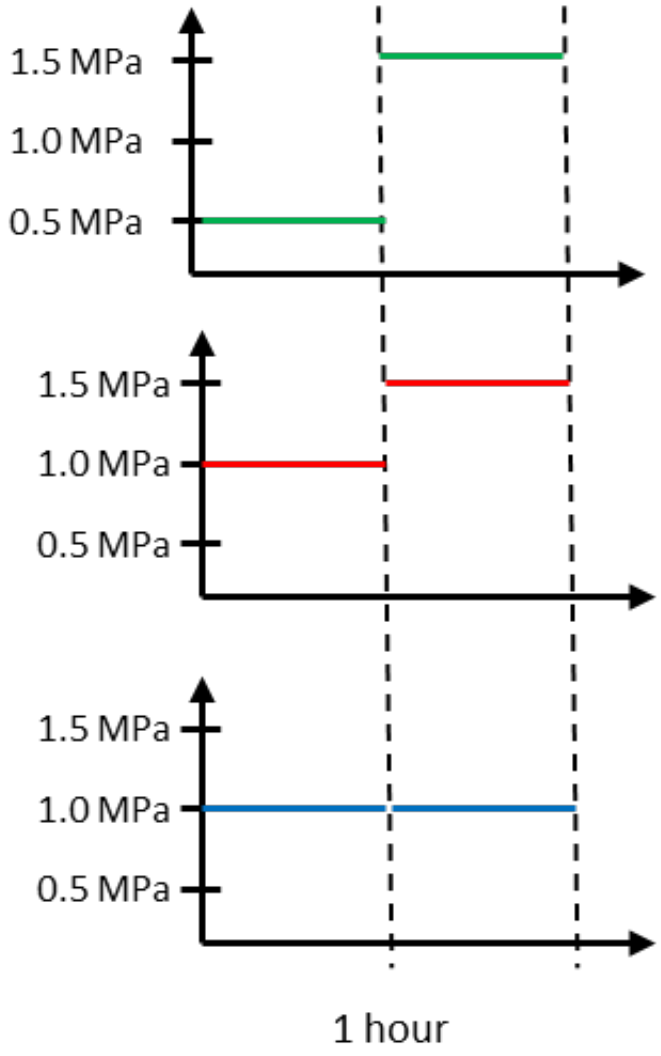

Figure 3.4 Load protocols for the short term *in vivo* tests

The applied force to achieve desired stress was estimated using measurements from radiographs and an approximate circular cross section of disc. The custom Labview program previously described was used to apply load based on displacement control using PAMs after the system was experimentally verified. After each group completed its duration of loading, the rat was euthanized followed by harvest of the NP for gene expression analysis via qRT-PCR. Experimental disc c4-5 was harvested along with the adjacent c3-4 to act as an internal control. Rats remained under anesthesia for the duration of surgery and loading protocols. All protocols

and procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park.

NP tissue was harvested from targeted IVDs after experiments were completed and placed in 350 µl of lysis buffer and flash frozen in liquid nitrogen. An RNA isolation kit (RNEasy Micro, Qiagen, Valencia, CA) was used to isolate RNA from the sample. Samples were then transcribed in an RT reaction and qRT-PCR was performed with SsoFast EvaGreen Supermix in triplicate using iCycler (Bio-Rad Laboratories, Hercules, CA) to quantify gene expression. The following genes were targeted for quantification along with 18s as a housekeeping gene: collagen I, collagen II, aggrecan, and sox9.

Table 3.1 Primer sequences used in the qRT-PCR reactions

Gene Name	**Primer Forward Sequence** **Primer Reverse Sequence**	**GenBank** **Ascension #**
Rat 18s	5’ CGC GGT TCT ATT TTG TTG GT 3’ 5’ AGT CGG CAT CGT TTA TGG TC 3’	X01117
Rat Type II Collagen A+B	5’ GTG AGC CAT GAT CCG C 3’ 5’ GAC CAG GAT TTC CAG G 3’	NM_012929
Rat Aggrecan	5’ GGA CTG GGA AGA GCC TCG A 3’ 5’ CGT CCG CTT CTG TAG CCT GT 3’	NM_022190
Rat Sox-9	5’ AAT CTC CTG GAC CCC TTC AT 3’ 5’ TTC CTC GCT CTC CTT CTT CA 3’	XM_343981

Rat Type I Collagen	5’ GCC CAG AAG AAT ATG TAT CAC CAG A 3’ 5’ GGC CAA CAG GTC CCC TTG 3’	NM_053304

Real-time qRT-PCR data was relatively quantified using the ΔΔCt method [34]. The ΔΔCt method is one which compares relative gene expression levels first to an internal control gene and then to a reference sample. Briefly, the Ct values for each gene triplicate are averaged, and the value for internal control gene 18s is subtracted from each gene of interest to give a ΔCt value. Then, subtraction of the ΔCt value for the internal control disc (unloaded disc c3-4) from the experimental discs (loaded c4-5) leads to a ΔΔCt value. These ΔΔCt values for each gene are then transformed to express relative mRNA level changes through the exponential relation $2^{\Delta\Delta Ct}$. Data can then be reported as the average value of the range of calculated fold difference, which accounts for standard deviation of the value as ΔΔCt+SD and ΔΔCt-SD. Statistical analyses were performed using an independent T-test to compare experimental gene expression with each control group.

3.4 Short Term Loading Gene Expression

The relative gene expression is used here to determine the biological response to the different applied load histories tested *in vivo*. Figure 3.2 shows the quantitative results of qRT-PCR shown as units of gene expression fold difference compared to control on a logarithmic scale. The group preloaded with 0.5 MPa of stress showed consistently higher expression of all the genes examined in the study when compared to control or other loading groups. The 2 hour 1.0 MPa load group experienced the same total stress as the 0.5 MPa preload, however relative gene expression was much lower than the preloaded group. The 1.0 MPa preloaded group also showed decreased gene expression than the 0.5 MPa preload group, though noticeably sox9 and collagen 1 expression were also decreased compared to the 1. MPa hold. The low sample

sizes (n=4) yielded poor statistical significance between gene expression, but many trends hold throughout the group giving the ability to explain or suggest behavior of the disc. These groups are meant to show the immediate biologic response to given loads and known cellular environments short term, to help suggest what long term changes may present themselves during extended studies.

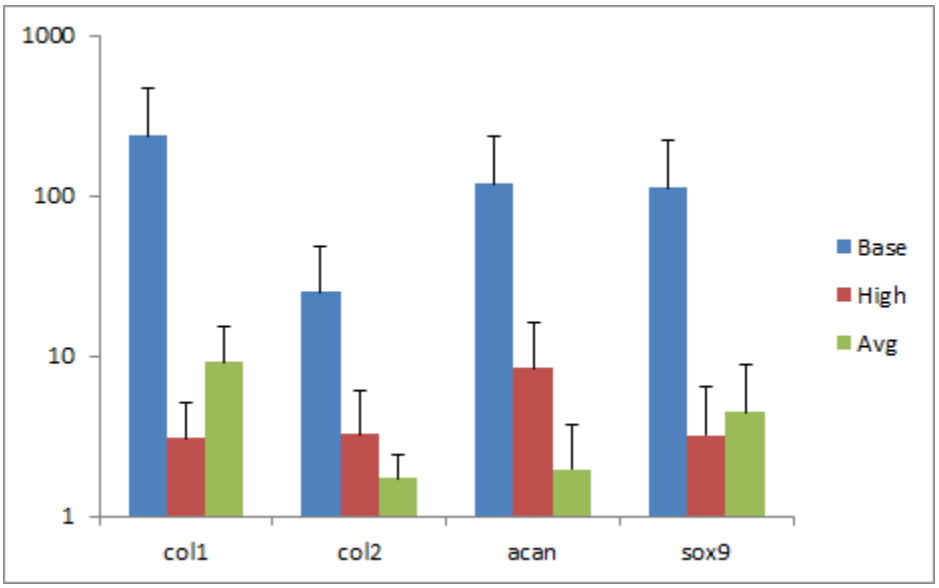

Figure 3.5 Real time qRT-PCR was performed for each of the short term loading scenarios. Experimental groups were compared to each control group using an independent T-test.

3.5 Discussion

The NP cells' biological responses to different loading scenarios are examined in this section using the relative gene expression changes in each loading scenario. The quantitative results of real time PCR are shown in figure 3.2 in units of fold difference on a logarithmic scale for all genes examined in the loading groups. The group consisting of an hour of preload at 0.5 MPa followed by an hour at 1.5 MPa showed consistently increased gene expression when compared to both the group with a higher preload and the group with a constant stress held

over two hours. The increased expression between the 0.5 MPa group and the 2 hour hold group is an important difference, as they experience the same average stress for the same duration of loading. This change shows that the gene expression is related to the load history of the groups as opposed to being related to the overall stress placed on the disc. It has previously been shown that load history influences the hydration and mechanical behavior of the IVD, specifically that low prestress conditions followed by an exertion load result in an increased intradiscal pressure when compared to higher prestress loads [27]. There is also a consensus that some loading on is necessary for regulation and maintenance within the NP.

The 0.5 MPa group has the highest expression of type II collagen, aggrecan, and sox9, all genes normally expected present in the healthy NP. As previously mentioned, the NP is comprised mainly of aggrecan and type II collagen in healthy tissue, this work shows loading of the disc generates a cellular response to maintain the ECM. The high magnitude of hydrostatic pressure experienced by this group works to increase the expression of ECM components responsible for maintaining osmotic pressure within the disc. In comparison, the other groups experiencing lower hydrostatic pressures showed about 10-fold less expression. The low pressure change experienced in the NP shows an inability to stimulate expression of important ECM components to maintain the osmotic pressure within the disc.

Prior loading experiments in our lab have targeted either 0 or 0.5 MPa of one-hour preload followed by one-hour of 1.0 MPa exertion loading. These results yielded higher expression with the 0.5 MPa preload, though there was only about 10-fold difference in gene expression compared with the nearly 100-fold seen in these experiments with 0.5 MPa preload followed by 1.5 MPa exertion. The large difference in expression is likely due to the increase of pressurization that would be expected with the increased magnitude of challenge loading. Interestingly, the prior group with no preload followed by exertion was unable to stimulate as large an increase of expression as the 0.5 MPa preload group, the opposite effect of what is seen with the new loading profiles. The new work shows the group with higher preload inducing

less gene expression. This suggests that the cells within the NP need to sense some pressure to produce genes, as the non-preloaded group from the old work showed little gene upregulation.

It is believed that hydrostatic pressure should stimulate buildup of ECM proteins, as seen here, suggesting that there may some remodeling or restructuring of the ECM. This suggests pressure buildup had some effect to signal cells to regenerate and reorganize the surrounding matrix. Both the preload groups showed increased type I collagen, the more fibrous elements of the disc which could be expected under any loading scenario, however the drastic increase presented here may be due to the high magnitude of loading experienced by the disc.

From these short term results, there are a few hypotheses that could be made of long term loading with these profiles. The inability of the cells to produce matrix as aggressively with higher preload suggests that the NP of these tissue will undergo more rapid degenerative changes. With less proteoglycan and type II collagen production in these groups, the ability of the disc to hydrate, causing height reduction of the disc and inability of the NP to pressurize. The results from the 1.0 MPa hold group suggest that changing pressure within the disc is necessary to produce a cellular response, as the static pressure from the 2-hour hold group induces the least production of aggrecan and type II collagen.

Many things were learned about the NP's biological response to load history from these experiments. There are noticeable differences between the low and high preload groups, showing that preload and exertion loads change the behavior of the disc. The 2 hour hold group showed that the unique response to preload and exertion is not resultant from the total stress placed on the disc, but the changing hydrostatic environment. This study proved NP cells are sensitive to subtle environmental changes imposed by different load histories. Discs were better stimulated to produce vital ECM components while experiencing a lower preload followed by the same exertion, suggesting changes in cellular response are due to pressure differentials rather than the peak stress experienced by the disc. This increased production of ECM suggests a

greater ability to cope with harsh exertion loads experienced by the disc, and better ability to hydrate during relaxation. The hydration and osmolarity of the disc during loading should be further studied to determine the total response of the cell's to their environment. This study begins to translate some physical effects previously seen to biological changes experienced by cells' gene expression. There are many contributing factors to the gene expression of cells with many differing triggers and signaling pathways. More studies should be performed to understand that signaling that generates response, but it is clear that load history does in fact change the mechanical and biological behavior of the disc.

Chapter 4: Conclusions and Future Work

The main goals of this project were to further explore and understand the biological and mechanical responses of the IVD. There is still much work to be completed to fully understand the disc and the cause and effects related to degeneration. The disc's complex environment still contains many unknown parts that contribute to the disc's behavior. The work presented in this thesis works to create a device for future *in vivo* studies and shows some initial data to understand the acute cellular response to loading, furthering the relatively unknown biologic response to load history.

Most *in vivo* studies are limited to anesthetic constraints during live animal experiments, limiting studies to short durations of loading. To combat this effect, a long term loading device was designed using miniature PAMs for actuation. The actuation coupled with a previously studied pin and ring configuration was used to compress caudal rat discs *ex vivo* to calibrate and validate the function of the control system. The ability of the LabView program to maintain a stable force during viscoelastic creep was assessed and the validity of the displacement control was confirmed.

Once the control of the device was validated, pilot studies with the PAM actuation system were completed to elicit the biologic response of the NP to load. We found that a lower preload with high exertion load generated a larger cellular response to production of anabolic genes such as type II collagen and aggrecan when compared to groups with a higher preload or groups experiencing the same stress during loading. This shows the disc's ability to respond to different loading regimes causing subtle changes in the cellular environment. This study also confirms that ability of the PAM actuation system to deliver load to a particular target and the ability to elicit a response, leading the way to future studies utilizing the PAMs to study disc

degeneration. It appears that higher levels of preload corresponding to heavier activity are unable to produce adequate pressurization to elicit cellular response to harsh exertion loads. This inability to elicit a response is then further detrimental.

However, there is still much work to be done. We have verified the system works, but much still needs to be completed for long term studies on rats without anesthesia. Pilot testing has confirmed the ability of the pin and ring system to remain undisturbed on rats for over a month with no effects on the rat's health, suggesting the ability to use this system for long term loading. However the necessary tubing and wiring presents a problem for entanglement and constriction of movement during long term studies. Several steps can be taken to overcome this problem, and others may exist as well.

To alleviate the requirement of two sets of tubes running to two pressure regulators, it may be possible to create more miniature PAMs with characteristics equal (or equal enough) to each other to allow for the same calibration equations. This would allow for a single regulator to pass one tube to the system through the rat's cage which could then be split to each PAM connected to the tail in a manner similar to how Wuertz et al. attached their piston based system. Another option could be to create a rotating joint that would attach to a central point on the cage, which would have fixed ends going to the pressure regulators, while allowing the tubes within the cage freedom to move as the rat desires. Another option we have considered is creating a new type of cage for the testing which would act to keep the rat in one place and have the cage move around a turntable, allowing for exercise while keeping the rat in one location, preventing the entanglement of tubing and subsequent constriction of motion.

More work can also be done in the future to create control methods within the LabView program to allow for a wider range of loading scenarios and magnitudes. Determining how different loads affect cellular and morphological changes in the disc will play a vital role in understanding the mechanisms of degeneration. These loading methodologies can work with the long term load history studies to fill the gaps of knowledge and gain a better understanding

of the basic science affecting the IVD. The system could also be expanded with multiple PAM devices to allow for experiments to be run in parallel, increasing the amount of data that could be collected and decreasing the amount of time required.

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