2011-03-16

A Proteolytic Process to Simulate the Mechanics of Disc Dengeration in Bovine Cadaveric Tissue

Timothy A. Bishop
Brigham Young University - Provo

A Proteolytic Process to Simulate the Mechanics of Disc Degeneration in Bovine Cadaveric Tissue

Timothy Bishop

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Anton E. Bowden, Chair
Larry L. Howell
David T. Fullwood

Department of Mechanical Engineering

Brigham Young University

April 2011

ABSTRACT

A Proteolytic Process to Simulate the Mechanics of
Disc Degeneration in Bovine Cadaveric Tissue

Timothy Bishop
Department of Mechanical Engineering, BYU
Master of Science

Purpose. The present work hypothesized that proteolytic dissolution of intervertebral discs could induce biomechanical change comparable to the change observed in natural disc degeneration. A method to do such could be utilized for *in vitro* research where intersample differences in geometry and chemical makeup render it difficult to compare and aggregate results into generalized conclusions.

Methods. Forty-one bovine coccygeal intervertebral discs were isolated with individual functional spinal units. Samples were loaded in three modes: compression/tension, flexion/extension, and axial rotation. The anulus fibrosus of each disc was injected with 200µl trypsin or fetal bovine serum (control) and incubated for an allotted period: 30 minute, 60 minutes, or 180 minutes. Mechanical loading was repeated and the load-displacement responses before and after treatment were compared as were the differences between each time group.

Results. Significant change was observed in the discs' total range (stiffness), low range (laxity), and hysteresis. Changes in load-displacement response were observed to be correlated with both treatment and time.

Conclusions. Enzymatic degeneration of intervertebral discs shows promise as a means to further understanding of disc mechanics in varying levels of degeneration. In virtually all cases, the trypsinized discs exhibited the increased joint laxity and decreased stiffness that is associated with early stage, natural disc degeneration.

Keywords: Tim Bishop, biomechanics, intervertebral disc degeneration, trypsin

ACKNOWLEDGMENTS

For unquantifiable help, I thank my advising professor, Dr. Bowden. For valuable input, I thank my other committee members, Dr. Howell and Dr. Fullwood. For his ability to make any machine work as needed it and find the parts needed, I thank Kevin Cole. For friendship and good times, I thank all of the members of BABEL. Finally, for being everything to me, I thank my family. Without the help and support of any of these, this work would not be accomplished.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

LBP	Low Back Pain
IVD	Intervertebral Disc
NP	Nucleus Pulposus
AF	Anulus Fibrosus
FSU	Functional Spinal Unit
FBS	Fetal Bovine Serum

1. INTRODUCTION

1.1. Problem Statement

Throughout the world, virtually every person who lives to maturity will experience low back pain (LBP) at some point in their life. A substantial percentage of those suffer from chronic LBP. It is estimated that healthcare costs associated with LBP in the United States reach over $90 Billion annually [1]. Among the most common causes or risk factors of LBP are genetic inheritance [2, 3], age [4], heavy and repetitive loading [5], cigarette smoke [6], trauma and disease. Although LBP is believed to be highly related to disc degeneration, research has not found significant correlation [7, 8].

This thesis will present a method by which the mechanical changes associated with disc degeneration may be induced in cadaveric tissue. The process will allow researchers to study a single sample at varying levels of degeneration holding other important factors constant. The process described hereafter employs an enzymatic reaction with the chemical makeup of the intervertebral disc (IVD).

1.2. Chapter Layout and Summary

Chapter 2 presents a review of the background literature used as a foundation for this research. It describes the anatomy and physiology of the spine, and sets up the context for a discussion of IVD degeneration. It further presents the literature associated with the use of

animal models for human conditions and the biochemical basis of the treatment described here. Finally, the most similar, published, work is described and it is shown how this thesis expands the state of the art.

Chapter 3 duplicates a journal publication currently undergoing peer review and was presented in part in 2009 at an annual meeting of the Orthopaedic Research Society [9]. It describes a novel enzymatic technique for inducing mechanical disc degeneration in bovine cadaveric discs. Further background information is laid out including a detailed description of the methodology followed. The results indicate that applicability of the technique for extension to human cadaveric discs.

Chapter 4 adds thoughts and insights to the work presented in Chapter 3 which were left out due to paper length restraints. A deeper look into testing protocols and data analysis techniques which were investigated but ultimately discarded is presented. Further discussion of the results of the experimental work is presented. Greater explanation of metrics used is given. Other lessons learned throughout the research are also put forward to allow the reader to follow up this thesis with additional work.

Chapter 5 summarizes the contributions of this work. It also presents opportunities seen by the author as potential future work.

2. BACKGROUND

This chapter sets the context for the work presented in this thesis. Spinal anatomy is complex and, when it does not function properly, a person may be impaired by pain or loss of physical ability. Each component in the spine plays a specific and important role. With age and a number of other correlaries, spinal components degenerate and experience biochemical, biomechanical, geometric, and other changes. Much research has been done to better understand those changes as well as the origins thereof. An emerging focus of study includes the consequences of degenerative changes. The method described in this thesis is able to help the study of the consequences of degeneration by artificially inducing biomechanical changes.

2.1. Spinal Anatomy and Intervertebral Discs

Spinal anatomy includes vertebrae, IVDs, musculature with accompanying tendons, and ligaments. Vertebrae may be divided into two major parts: the vertebral body and the posterior elements. The vertebral body is a large cylinder that gives strength in bearing a person's body weight. It is highly vascularized and the marrow is among the most active blood-forming tissues in an adult [10]. The posterior elements include many bony processes used as moment arms to assist ligaments and muscles to control spinal movement. In the posterior is also a direct interaction between adjacent vertebrae at the zygopophyseal or facet joints. Between the anterior

vertebral body and the posterior elements is a protected channel, vertebral canal, through which runs the spinal cord.

Muscles attach to the posterior processes and act to maintain erect posture as well as extend or rotate the spine. To help protect the spine from excessive motion and stabilize the joints, there are six major groups of ligaments commonly referred to in literature. Ligaments attach along the length of the spinal columna as well as between spinous processes. The intertransverse ligament found in literature is not universally present and some argue is not a true ligament [11]. The anterior longitudinal ligament and posterior longitudinal ligament run along the length of the spinal column on the anterior and posterior of the vertebral bodies, respectively. The ligamentum flavom runs along the posterior of the vertebral canal further protecting the spinal cord. The intraspinous and supraspinous ligaments attache between and along the posterior edges of the spinous processes, preventing excessive flexion. Each constituent plays an important role in keeping a back functioning properly and pain-free.

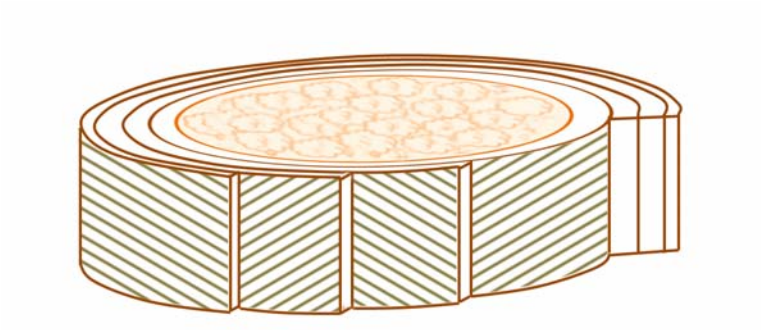

Figure 2-1. The AF consists of concentric lamellae of highly organized collagen fibers.

The function of an IVD includes allowing controlled motion, cushioning impact and bearing loads. The IVD is a symphysis type joint between two vertebrae, greatly restricting any translational motion between two, adjacent vertebrae while still allowing limited rotation and

bending. It can be divided into three major structural elements: endplates of hyaline cartilage which articulate directly with the vertebral bodies, the anulus fibrosus (AF) and the nucleus pulposus (NP).

The endplate is the connection point between IVD and vertebral body. It is an important part in allowing and controlling nutrient diffusion from the highly vascularized vertebra into the avascular IVD. The AF is made up of strong collagen fibers (mostly type I collagen) in annular lamellae oriented in alternating orientations of approximately 30° from the horizontal (Figure 2-1). Encapsulated within the center of the AF is the gelatinous NP which consists of a hydrated proteoglycan gel in a loose matrix of randomly oriented collagen type II and elastin fibers [12].

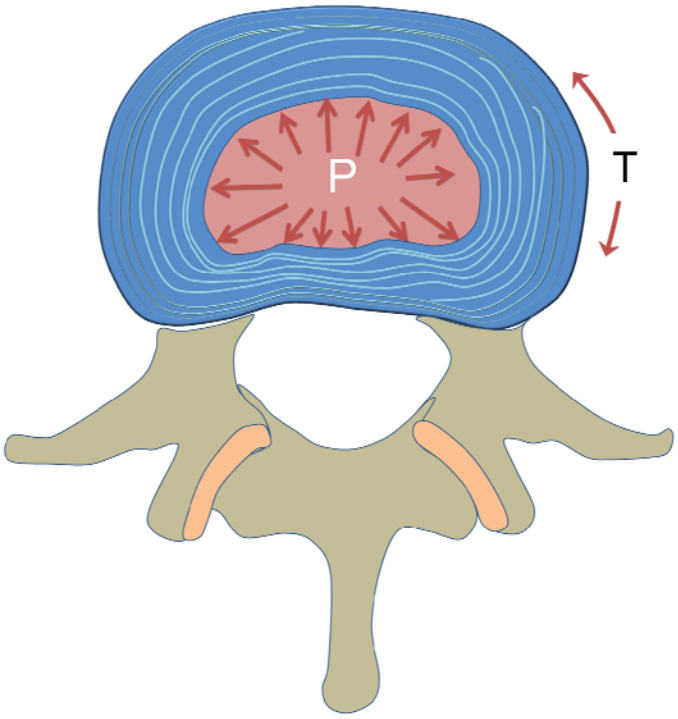

Figure 2-2. Pressure in the NP forces the AF into tension to resist compressive loads.

In an ordinary, healthy disc, neither the collagen fibers of the AF nor the NP are able to withstand much compressive load alone. When combined the disc acts as a pressure vessel: the NP effects hydrostatic pressure when compressed thus inducing tension in the fibers of the AF (Figure 2-2). Normal compressive loads on a disc are related to the position in the spine. As the lumbar spine is below more of a persons mass, it bears greater load than is required of the thoracic and cervical spine.

2.2. Disc Degeneration

Most people do not worry about disc degeneration until the moment that it causes them pain. According to the American Academy of Orthopaedic Surgeons (AAOS), over $90 billion in healthcare costs were caused by LBP in 2007. It is the second leading cause of doctor's visits behind only the common cold, and LBP is the most common reason for workers' compensation accounting for one quarter of all claims [1]. Disc degeneration is defined to be "an aberrant, cell-mediated response to progressive structural failure" [13]. Contrasted with a general weakening of the structure of the disc, pain is associated with the structural failure.

Common forms of structural failure in the AF include delamination, radial fissures, and peripheral rim lesions. Delamination, or circumfrential tearing, is the separation of collagen fiber layers (lamellae) from one another. Radial fissures are tears in the AF that allow the NP to move radially and herniate in severe cases. Radial fissures are often developed through high pressure bending (lifting a heavy box off of the floor) and are most often located in the posterior or posterolateral portions of the disc [14]. Peripheral rim tears are most likely caused by trauma. They are most often located in the anterior part of the disc and may be associated with osteoarthritic bone growth [15].

Disc degeneration can be classified morphologically using the Thompson scale, a ranking of 1-5 where 1 is a healthy disc and 5 is a completely degenerated disc [16]. Disc degeneration has also been correlated with water content in the IVD which can be measured with magnetic resonance imaging (MRI) [17, 18]. The Pfirrman scale gives a MRI (quantitative) based metric of degradation [19].

2.3. Cadaver Testing

Human cadaveric spines are difficult to acquire and are unique in many qualities: age, disc health, geometry, etc. Thus it is less meaningful to compare intersample results than it would be if other conditions could be held constant. Larger sample sizes are required to reach statistical significance than is always possible or desired. Those studies that have been done using human cadaveric specimens are commonly performed with weights applied on a moment arm and the distance traveled being recorded, but no follower load is applied to better mimic physiologic conditions. Functional spinal units (FSUs) are categorized by degeneration level and by vertebral level in the spine.

The effects of degeneration on spinal motion have been recorded in several studies. It is difficult to find an authoritative study because of all of the varying assumptions in the experimental setup and methodology. Krismer et al and Tanaka et al showed that range of motion in axial rotation increases with Thompson grade of degeneration by 18-42% per grade (8.5 Nm applied torque) or 29-71% per grade (5.6 Nm applied torque) respectively [20, 21]. Brown et al also reported a decrease in general joint stiffness by about 22% in initial degenerative states with a restiffening of joints in later stages of degeneration [22]. It is possible that a lack of significant change in the mechanical response in flexion/extension and lateral bending is due to unphysiologic loading conditions without a follower load.

2.4. Animal Models

Because of the expense, regulations, and ethics associated with human subjects and cadaveric specimens, animal substitutes are often used. Many species have been used, but among the most common are cows, sheep, pigs, dogs, rabbits, rats, and mice. There are problems associated with using animal models: chemical make-up of the discs differ from human discs, geometries are incongruent, and loading conditions are very different due to posture [23, 24]. Notochordal cells which are developmental in embryos and quickly disappear in humans after birth remain prevalent in many animals throughout their lives, which is believed to signifcantly reduce the occurrence of disc degeneration. The percentage of aggrecans, proteoglycans, and collagen types also vary by species. Where humans have smaller vertebrae and discs in the cervical and thoracic spines and the size varies significantly within the lumbar region, quadripeds have a consistent sized spinal column. Although the spine of a quadriped does not need to support body weight directly, it requires more muscle force to maintain horizontal posture and it is hypothesized that compressive loading on a large quadriped such as calf or lamb, may be greater than that on a human spine.

2.5. In Vitro Research

Empirical data has shown that degeneration decreases the stiffness of the AF and increases that of the NP [13, 25-29]. As an IVD ages, the biochemical changes reduce its ability to retain water in the NP. As the hydration is reduced, the loose proteoglycan matrix is compacted and loses its ability to maintain hydrostatic pressure. The AF then is too often loaded in compression rather than tension and a general reduction in stiffness is observed.

The IVDs' ability to adapt to loading conditions is also decreased in a degenerated state [30]. In a normal, healthy IVD, cell health is maintained by nutrient transfer as a disc is

naturally compressed and decompressed. As an IVD's water content is reduced either through the aging process or through structural failure, the nutrient transport method fails. It has been observed that blood vessels, which normally are not present, are prevalent in painful, degenerated discs [31] and often are accompanied by pain signaling nerves (nocioceptors) [32, 33]. These means, by which a body attempts to repair itself, may be the origin of the pain which impairs a person with disc degeneration.

Studies have shown that disc degeneration can be recreated through mechanical methods. Loading a cadaveric sample repetitively or beyond physiologic boundaries has been used to recreate delamination, radial fissures and peripheral rim lesions [13, 34]. However, loading only in compression will cause fracture of the endplate and vertebral body before radial tears will allow herniation [35]. These changes in mechanical behavior are not easily controlled and the location of these mechanically induced symptoms is locally unpredictable. Additionally, it is virtually impossible to functionally isolate a particular functional spinal disc of a multisegment specimen using these techniques, thus practical applications are limited.

2.6. Proteases/Chondroitinase

Proteases have proven to be effective at breaking down the localized protein structures in IVDs. Papain and chymopapain are derived from the latex of papaya fruit. Papain has been used as a meat tenderizer in South America. Trypsin and chymotrypsin are produced in the pancreas of many vertebrate animals to assist in the digestion process. Chondroitinase ABC is produced by a bacterium, *Proteus Vulgaris*, and is relatively newer in research applications [36]. In the 1960's, a method was proposed and implemented to inject protease into prolapsed or herniated discs to reduce pressure on the spinal cord [37]. The practice was later ended due to the risk of neural damage resulting in paralysis and severe allergic reactions to chymopapain [38-40].

2.7. Previous Protease Degeneration Work

Mwale et al injected trypsin into the NP of bovine tail specimens and tested the mechanical changes in compression. The treatment resulted in reduced average disc stiffness under compressive loads [41]. Trypsin was also used to effectively reduce the pressure within an IVD to allow for an injection of another treatment designed to allow for tissue regeneration by Roberts et al, and fetal bovine serum (FBS) was used to inhibit trypsin activity after the incubation time [42]. No work has been done to prepare a method by which enzymatic degeneration is employed in the AF to emulate mechanical degeneration in IVDs with comparison to loading in bending and rotation as well as compression.

3. PROTEOLYTIC DISC DEGENERATION[1]

This chapter presents experimental work wherein was hypothesized that proteolytic dissolution of IVDs could provide a suitable biomechanical analog to natural disc degeneration. Such a model could be utilized for *in vitro* research where intersample differences in geometry and chemical makeup render it difficult to compare and aggregate results into generalized conclusions.

Forty-one bovine coccygeal IVDs were isolated with individual FSUs. Samples were loaded in three modes: compression/tension, flexion/extension, and axial rotation. The AF of each disc was injected with 200µl trypsin or FBS and incubated for an allotted time period. Mechanical responses before and after treatment were compared as were the differences between each time group.

Significant change was observed in the discs' total range (stiffness), low range (laxity), and hysteresis. Changes in load-displacement response were observed to be correlated with both treatment and time. The observed results indicate that enzymatic degeneration of IVDs shows promise as a means to further understanding of disc mechanics in varying levels of degeneration. In virtually all cases, the trypsin treated discs exhibited the increased joint laxity and decreased stiffness that is associated with early, natural disc degeneration.

[1] The contents of this chapter have been submitted for publication

3.1. Introduction

Despite the lack of clinical correlation between the severity of lumbar pain symptoms and the severity of disc degeneration [7, 8], over 90% of surgical spinal procedures are performed consequential to the degenerative process [43]. For clinicians, treating disc degeneration is complicated because of the multifactorial traits of this pathology, including changes in morphology, biochemical composition and mechanical environment of the disc and surrounding tissue.

Early degenerative changes in the NP include breakdown of polar aggregating proteoglycans with subsequent loss of hydration and disc height [26, 44, 45]. Changes in the NP are closely correlated with compositional and structural changes in the AF including loss of hydration and proteoglycan content, cracks, delamination, a reduced number of layers, collagen fiber reorientation [30], and increased layer thickness [28]. With advanced disc degeneration, the levels of the majority of matrix molecules are decreased, with the exception of biglycan and fibronectin [44]. Degenerated discs have more abundant nerve supply than normal discs, and the nerves in discs appear to be capable of conducting pain signals [32, 33, 46].

Current surgical treatments for lumbar related damage, namely spinal fusion and total disc arthroplasty, are problematic in the context of disc degeneration because they alter the mechanical stress fields experienced by adjacent discs [47-49]. Altered stress fields have been linked to accelerated disc degeneration in adjacent levels which further complicates the long-term well-being of the patient. Surgical treatments designed to function in a healthy environment are often operating in a degenerate environment.

Cadaver testing of spinal devices is an important part of the design process, providing key insights into device functionality and interaction with the surrounding tissue. However,

availability and cost issues dictate that most cadaver testing is done on spines from a broad cross-section of degenerative states. Thus, it has heretofore been impractical to experimentally quantify the reduction (or improvement) in efficacy of spinal treatments due to progressive degeneration of the surrounding tissue.

The goal of this research was to evaluate the changes induced in bovine cadaver segmental biomechanics by protease activity in the bovine tail disc. It was hypothesized that by controlling the timing of the protease action in the disc that duplication of the mechanical behavior of the degenerate disc could be achieved. This work provides an important precursor to the development of a human cadaver model of mechanical disc degeneration.

3.2. Methods

3.2.1. Preparation

Eighteen bovine coccygeal spines (ages between 20 and 25 months) were acquired from a local abattoir and kept frozen at -20ºC until testing. Muscle and adipose tissue were dissected, taking care to preserve each IVD and vertebrae. Discs were screened for testing based on their diameter (between 10 and 25 mm), and obviously irregular or damaged segments were rejected. Forty-one FSUs were isolated from the spines by cutting through adjacent discs. Hydration was maintained with phosphate buffered saline solution during dissection and a generous coating of petroleum jelly during testing.

Each FSU was potted in custom test fixtures that allowed application of prescribed angular rotations in the flexion-extension and axial rotation axes, as well as application of compressive loads. The vertebrae of the FSU were embedded in a two-part polyester resin (Bondo, 3M Corp, St. Paul, MN), and care was taken to align the centerline of the disc

horizontally with the fixture. A servohydraulic testing machine (Instron model 1321, Instron, Norwood MA) was used equipped with a 1000 lb linear load cell (Omega Engineering, Stamford, CT) during compression testing and a 20 Nm torque transducer (Omega Engineering, Stamford, CT) during angular rotation.

3.2.2. Testing

Each FSU was tested in three modalities: compression/tension, flexion/extension, and axial rotation. Testing order was randomly selected between specimens, but was consistent between subsequent tests on the same specimen. All testing was performed at room temperature. A summary of loading protocol is found in Table 3-1. Data from the final cycle in each testing modality was analyzed.

Table 3-1. Summary of test specifications for each mode of loading.

Mode	Number of Cycles	Speed	Range	Recorded Data
Compression	30	0.5 Hz	±400N	Displacement
Flexion/Extension	15	1.0 Hz	±15º	Torque
Axial Rotation	15	1.0 Hz	±3º	Torque

After testing in all three modalities, each FSU specimen was heated to 37° C for 15 minutes to facilitate the protease action of the trypsin injections. Each FSU was randomly assigned to one of four testing groups: 30 minute trypsin, 60 minute trypsin, 180 minute trypsin, or 180 minute control. The AF of each FSU was injected with 25µL of either 10x 2.5% trypsin or, in the case of the control group, FBS in eight equally spaced locations around the disc (a total of 200µL) with a 27ga needle (Figure 3-1). Needle size choice was based on reported work indicating that needles smaller than 27ga do not disrupt the mechanical properties of the disc

[50]. FBS has been used in controls in previous research for its non-reactivity with the disc and because it suppresses trypsin activity [42]. Ten FSUs were used as a control with an injection of FBS and incubated for three hours. The other 31 FSUs were injected with trypsin and allowed to incubate for 0.5, 1.0, or 3.0 hours at 37ºC. After incubation, the FSU was allowed 15 minutes to normalize to room temperature before being subjected to the same testing protocol described previously.

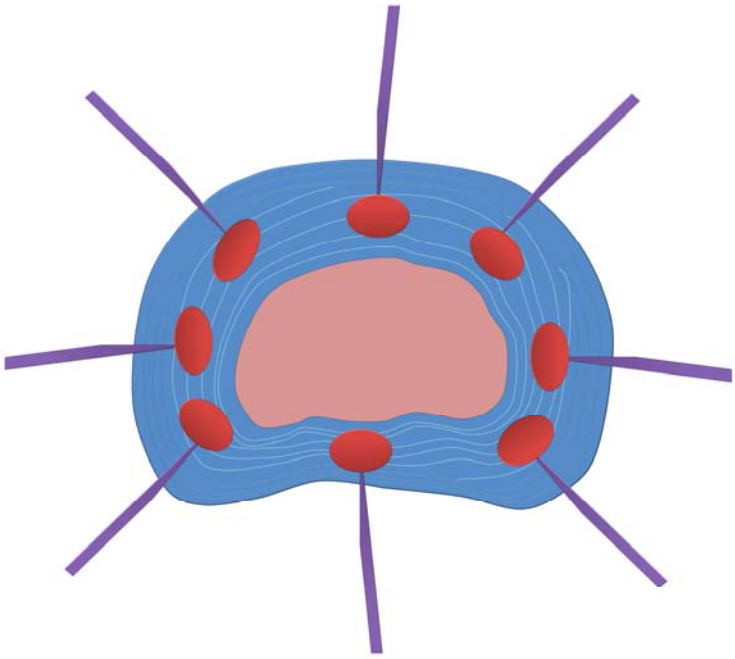

Figure 3-1. Injections of trypsin or FBS (control) were made into the AF at eight equi-spaced locations around the disc.

3.2.3. Analysis

Three characteristics of the load-deflection curves were identified for comparison: total range, low range, and hysteresis (Figure 3-2). The flexibility data was transformed into a uniform coordinate space. Resultant changes in each of the three characteristics were analyzed using a paired t-test for means. Differences between test groups (30 minute, 60 minute, 180

minute, control) were analyzed using single factor ANOVA. Data points falling more than two standard deviations from the group mean were excluded from the statistical analysis.

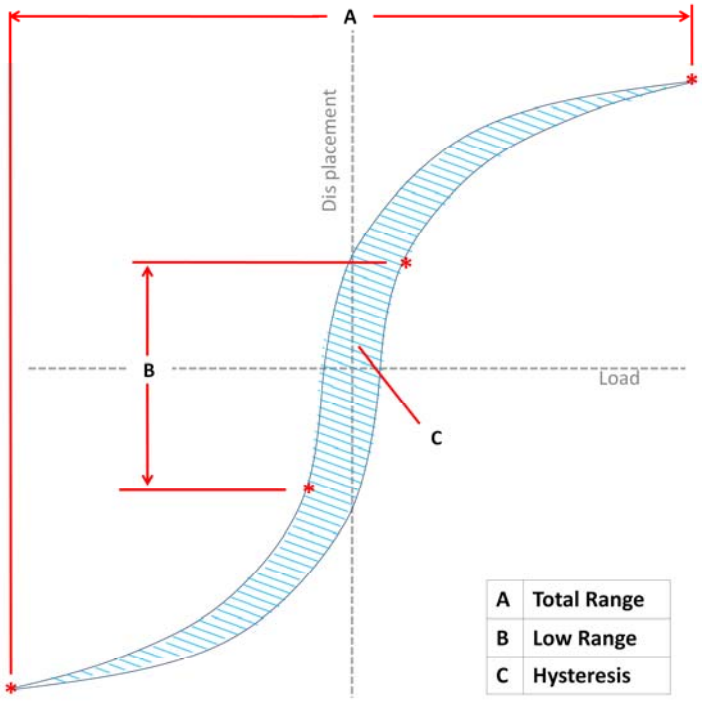

Figure 3-2. Quantitative metrics evaluated to determine changes in segmental biomechanics before and after protease treatment. Starred points indicate the maximum loading conditions and inflection points where it was determined that the response transitioned between low and high ranges. The measure of hysteresis, C, was taken as the numerically derived area between loading and unloading curves.

3.3. Results

3.3.1. Observed Changes Within Groups

Changes in the load-deflection responses of each test group following treatment are shown in Figure 3-3, Figure 3-4, and Figure 3-5 and p-values demonstrating the significance of the changes are shown in Table 3-2 with the average percent change. In compression loading, the total range for all trypsin groups showed significant decreases, while that for the control group did not. An increase in the low range and hysteresis for all groups was observed.

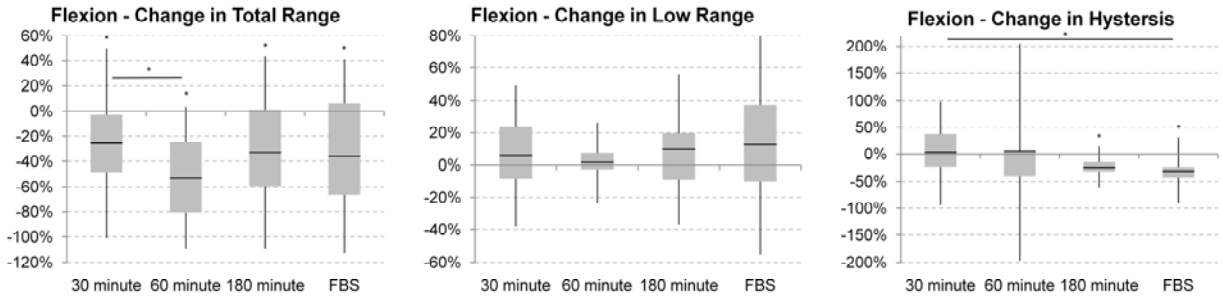

Figure 3-3. Typical changes in segmental biomechanics following protease treatment loaded in flexion-extension as a percent change. A black line marks the mean change. The gray box indicates the range of the data in the 2nd and 3rd quartiles. The whiskers extending from the boxes indicate a positive or negative 2 standard deviations from the mean rather than any particular data points. An asterisk above a whisker indicates that the change in the response of that group and loading reached statistical significance ($p \leq .05$). An asterisk above a line between two groups indicates significant difference between those groups.

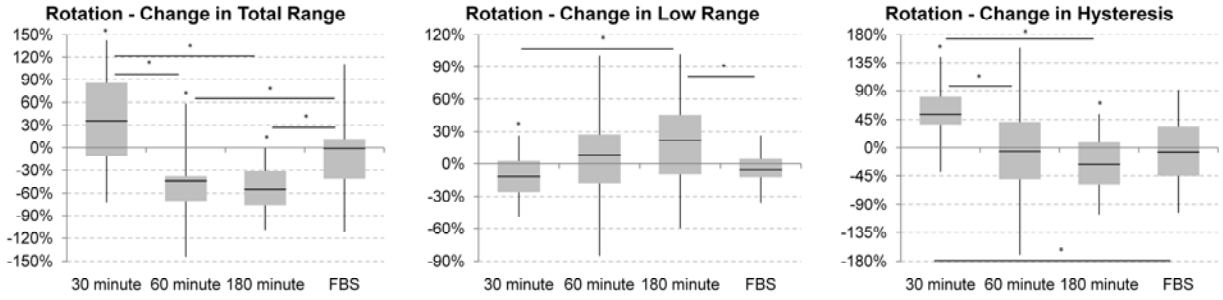

Figure 3-4. Typical changes in segmental biomechanics following protease treatment loaded in axial rotation as a percent change.

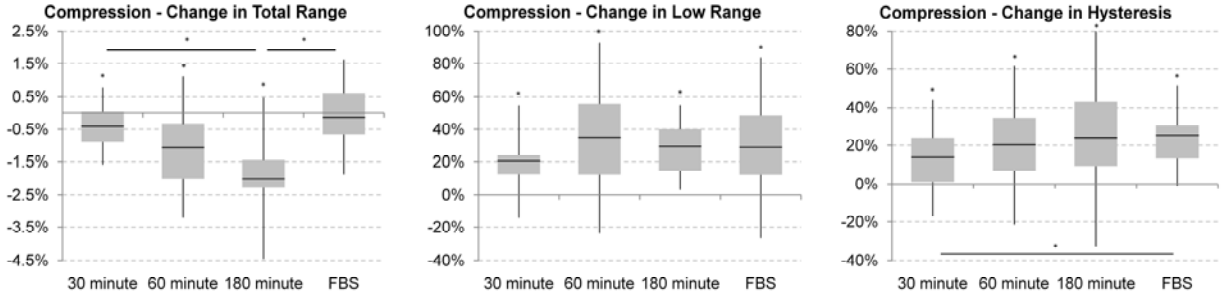

Figure 3-5. Typical changes in segmental biomechanics following protease treatment loaded in compression as a percent change.

Table 3-2. P-values of the significant changes observed between before and after treatment tests. The average change is presented above the associated, statistical p-value (i.e. The 30 minute trypsin group reduced the total range of loading by an average of 25% with significance demonstrated by p=0.0307).

Flexion/Extension

	Total Range	NZ Range	Hysteresis
30 Trypsin	-25% 0.0307	5.6% 0.2179	2.2% 0.4441
60 Trypsin	-53% 0.0002	1.4% 0.3787	4.1% 0.4497
180 Trypsin	-33% 0.0162	9.5% 0.1138	-24% 0.0079
180 FBS	-36% 0.0116	12% 0.1405	-30% 0.0092

Axial Rotation

	Total Range	NZ Range	Hysteresis
30 Trypsin	35% 0.0348	-11% 0.0519	52% 0.0045
60 Trypsin	-43% 0.0170	7.6% 0.3180	-5.2% 0.4226
180 Trypsin	-54% 0.0002	21% 0.0655	-26% 0.0342
180 FBS	-0.4% 0.4922	-5.0% 0.1834	-6.2% 0.3555

Compression

	Total Range	NZ Range	Hysteresis
30 Trypsin	-0.4% 0.0359	20% 0.0023	13% 0.0107
60 Trypsin	-1.0% 0.0069	35% 0.0022	20% 0.0098
180 Trypsin	-2.0% 0.0007	29% 0.0001	24% 0.0127
180 FBS	-0.1% 0.4175	29% 0.0044	25% 0.0024

In flexion-extension, the total range decreased for all groups with a spike of effects in the 60 minute trypsin group. Hysteresis significantly decreased in both the control and the 180 minute trypsin group.

In axial rotation, significant increases in the total range and hysteresis occurred in the 30 minute trypsin group whereas in the 60 minute and 180 minute trypsin groups the opposite effect was observed. The low range decreased in the 30 minute trypsin group but then increased in both the 60 and 180 minute trypsin groups. The control group showed no significant change from the treatment in any of the three characteristics loaded in axial rotation.

3.3.2. Observed Changes Between Groups

Across group comparisons of the same three characteristics of the load-deflection curves demonstrated statistical significance as shown in Table 3-3. In compression, the decrease of the total range in the 60 minute trypsin group was greater than that in the 30 minute group but was greater in the 180 minute trypsin group than the 60 minute group. The change in hysteresis was significantly greater in the control group than the 30 minute trypsin group.

Table 3-3. P-values of differences between treatment groups. The difference, displayed above the p-value, is defined as the average change in the row group subtracted from the average change in the column group. For example, in the Total Range table for Flexion/Extension, the average normalized change in the 60 minute trypsin group was 27 percentage points less than that of the 30 minute trypsin group with statistical significance ($p < .05$).

Flexion/Extension

Total Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	-27% 0.0469	-7.4% 0.3380	-10% 0.2802
60 Trypsin		20% 0.1124	17% 0.1505
180 Trypsin			-3.0% 0.4355

Axial Rotation

Total Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	-78% 0.0024	-89.3% 0.0002	-35% 0.0881
60 Trypsin		-11% 0.2814	43% 0.0539
180 Trypsin			53.9% 0.0093

Compression

Total Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	-1% 0.0678	-1.6% 0.0016	0% 0.2221
60 Trypsin		-1% 0.0468	1% 0.0350
180 Trypsin			1.9% 0.0015

Low Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	-4.2% 0.3169	3.9% 0.3524	6.6% 0.3049
60 Trypsin		8.1% 0.1943	11% 0.2019
180 Trypsin			2.7% 0.4180

Low Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	19.1% 0.1343	32.6% 0.0201	6.5% 0.2176
60 Trypsin		13.5% 0.2524	-13% 0.2257
180 Trypsin			-26.1% 0.0429

Low Range	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	14.4% 0.0977	8.8% 0.1135	8.3% 0.2130
60 Trypsin		-5.6% 0.3025	-6% 0.3200
180 Trypsin			-0.5% 0.4817

Hysteresis	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	1.9% 0.4784	-26% 0.0995	-32% 0.0525
60 Trypsin		-28% 0.2426	-34% 0.1727
180 Trypsin			-6.1% 0.3235

Hysteresis	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	-57.6% 0.0407	-78% 0.0005	-59% 0.0090
60 Trypsin		-21% 0.2411	-1% 0.4881
180 Trypsin			19.8% 0.1706

Hysteresis	60 Trypsin	180 Trypsin	180 FBS
30 Trypsin	6.6% 0.2177	10% 0.1598	12% 0.0543
60 Trypsin		4% 0.3741	5% 0.2847
180 Trypsin			1.2% 0.4552

In flexion-extension, the change in total range was greater in the 60 minute trypsin group than in the 30 minute group. Measured change in hysteresis was significantly greater in the control group than in the 30 minute trypsin group.

In axial rotation, decreases were observed in total range of both the 60 minute and 180 minute trypsin groups to be greater than changes in the 30 minute and control group. The increase in the low range of the 180 minute trypsin group was significantly greater than both the 30 minute and control groups. The increase in hysteresis in the 30 minute group was significantly different than in the other three groups as each of their changes was in the opposite direction.

In cases where significant change was observed in the three trypsin treatment times, one of three patterns was observed: 1) an initial jump to some level for the 30 minute group, the 60

minute group then exceeded that change, but the 180 minute trypsin group showed a retreat in the magnitude of change tending back towards the levels of the 30 minute tests, 2) an initial movement in the 30 minute group and then a nearly linear trend in the opposite direction through the 60 and 180 minute groups, and 3) a nearly linear pattern where the 30 minute group showed the least magnitude change, the 180 minute group showed the greatest change and the 60 minute group was in between the other two in magnitude of change.

3.4. Discussion

3.4.1. General Effects

In virtually all cases, there was a distinguishable change in mechanical properties after 30 minutes of trypsin incubation. In several cases this first change was exceeded with the 60 minute treatment; in other cases the change at 60 minutes was in the opposite direction as that observed at 30 minutes. These results are consistent with an initial effect resulting from the injection of fluid and partially explain the changes noted in the control group injected with FBS. When treated with trypsin for 180 minutes, the changes tended to be in the same direction as those of the 60 minute treatments, but the changes were less dramatic. This may imply that the fluid effect deteriorated before the 180 minute time mark. The remaining effect then resulted from the enzymatic reaction of the trypsin, as well as another time-based effect that may be suggested when considering the 180 minute FBS control group.

3.4.2. FBS as an Experimental Control

Although FBS was selected as an experimental control based on previous usage by other research groups, the results of the present work suggest that at least in the short term, the fluid

effects due to increased hydration of the disc play a significant mechanical role. The injection of any fluid changes the internal pressure of the disc, in this case the AF, and may cause irreversible, mechanical damage to the collagen organization in the laminae. Any such mechanical damage could be considered actual disc degeneration rather than only an analogue, although it may be minimal compared to cases in which a person may experience LBP. An additional sham control may be needed to separate the effects of fluid and protease.

3.4.3. Environmental Effects

The present work was limited by the lack of an environmental chamber that would allow trypsin incubation and testing to occur sequentially without the intervening warm-up and cool-down times. Normal operating temperatures for trypsin use is body temperature (37° C) requiring sample incubation at that temperature [51]. Very few studies of spinal biomechanics have been conducted at body temperature, however changes in material dimensions (and thus biomechanical response) of histologically similar ligament tissue has been reported [52], indicating that testing done at body temperature would be highly relevant to the human condition.

3.4.4. Asymmetries in Sample Results

It was interesting to see slight differences between loading directions. The IVD of a bovine coccygeal spine is nearly circular and the properties were assumed to be angularly symmetric. Because coccygeal bovine vertebrae do not interact directly with the facets under the loading conditions considered in the present work, we anticipated a fully symmetric flexion-extension response. However, there were consistent differences between upper and lower stiffness and hysteresis widths, indicating that disc geometry alone is not a good indicator of

symmetry with respect to mechanical properties. Regional variation in collagen fibril organization in response to preferential loading direction (e.g, cows do not swish their tails equally in flexion versus extension) would explain these differences, although they were not examined as part of the present work.

3.4.5. Comparison to Published Data

The biomechanical changes induced by the protease action of trypsin in the AF were consistent with those observed in early to moderate disc degeneration including increased joint laxity [53] and decreased stiffness [22, 54]. Because interspecies differences in collagen and proteoglycan content in the AF complicate the applicability of the results [23, 24, 42], no quantitative comparison of these effects was attempted. However, the present work lays a strong foundation for developing a human cadaveric model of the mechanics of disc degeneration. The outlined procedure could be used to further studies on the adjacent level effects of disc degeneration *in vitro,* as well as providing valuable information regarding the performance of spinal implants in the context of degenerating discs.

4. ADDITIONAL INSIGHTS

This chapter elaborates on some interesting pieces of this research that were beyond the scope of the manuscript for publication. Herein is detailed description of the metrics that were considered and why these metrics were used over the many that may have been. A few of the lessons learned in the methodology and protocol are outlined in greater detail, and additional comments on the results and application of the results are put forward.

4.1. Separability of Observed Effects

There are three independent effects that influence the post-trypsinized mechanical behaviou of the intervertebral disc: 1) a proteolytic effect of the trypsin, 2) a transient fluid injection effect, and 3) effects from permanent localized damage due to overpressurization of the AF tissue. In the presented research, only one of the three effects was purposefully controlled—the proteolytic effect of trypsin. The other two effects were not previously described in research and thus were not anticipated. While the mechanism of mechanical degeneration of the IVD was not fully anticipated, the results nonetheless establish that this protocol was effective in inducing the desired changes in mechanical response.

The data show that the mode of loading may have emphasized distinct effects. The mechanics described in the flexion/extension results appear to have been dominated by the second two effects, as the control group was indistinguishable from the 180 minute trypsin

group. The results when loaded in axial rotation are more clearly dominated by the first, proteolytic, effect. When loaded in compression, results show a mixed contribution from all three effects.

The present experimental setup did not include sufficient control testing to fully separate the three effects. To further characterize the independent effects, a control group would need to correspond with each of the times wherein the trypsin samples were tested. An additional control of a needle only insertion would also be required.

4.2. Characterizing and Comparing Load-Displacement Curves

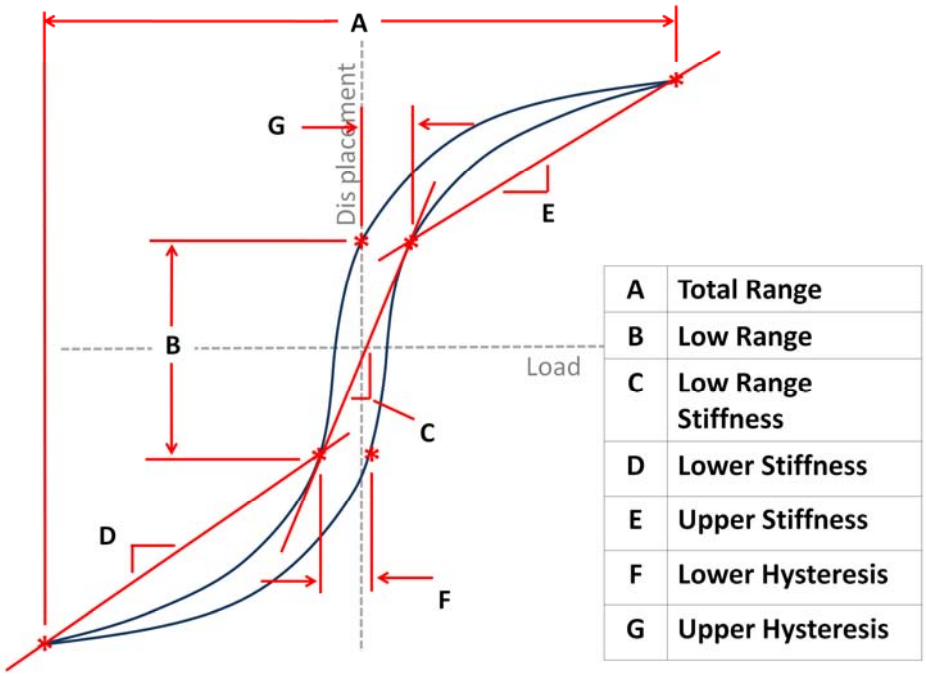

Figure 4-1. This diagram shows alternate metrics that were considered but ultimately dismissed.

A great deal of time and thought was taken to most appropriately analyze and report the recorded load-displacement curves. Figure 4-1 shows the metrics that were considered before

narrowing the selection down to the three presented in Chapter 3. Through preliminary analysis, significant changes were observed in each of the metrics except for the linear measure of hysteresis at one of the inflection points, although it did exhibit significant change at the other inflection point. However, not all of the metrics were relevant to the discussion of the results with respect to the working hypotheses.

Differences between upper and lower stiffnesses and between upper and lower hysteresis measurements led to many questions about the anisotropy of mechanical properties of the IVD. Before reporting those differences definitively, however, experimental protocol needs to more carefully control the possible influencing factors there. For this research, the protocol assumed that the nearly circular geometry would carry over into circumfrentially uniform mechanical properties. With the results attained, testing of that assumption may lead to interesting findings about the relationship between mechanical properties and the behaviors of the donor. It may be that a preferred direction of tail "swishing" is enough to change or reorganize the collagen fibers in an otherwise free-hanging IVD.

The total stiffness and low range stiffness were interesting measurements. To compare the data of this research to previously reported data, measurements of the total joint stiffness and joint laxity were necessary. The joint stiffness would be the average of the low range stiffness and the stiffness of the two high range loading segments. In the case that the two high range stiffnesses differ, or one of the two was not recorded due to calibration, stating a total stiffness might be misleading. Knowing that the tests were displacement controlled, only a total range of load experienced was reported both for ease of measurement and to not overstate what was observed, although it is effectively a measure of the total stiffness of the joint.

The low range stiffness was more certain in most cases. In a few rare cases a negative stiffness was observed suggesting that the disc was pulling itself into a loaded position. No reasonable explanation has been made for this rare occurrence. In most cases, the low range zone was increased indicating an increase in joint laxity also. The reason for looking at the range of displacement was that joint laxity as reported elsewhere has been the motion experienced before an increase in stiffness is observed. It was the range of motion rather than the change of stiffness that would best compare to other data.

4.3. Measuring Hysteresis

One of the three characteristics used to evaluate the method set forth was hysteresis exhibited by the disc. Four different approaches were attempted to describe the hysteresis: a horizontal distance measured at the inflection points, the integral of an exponential fit, the integral of a Boltzmann fit, and a numerical integral using the trapezoid method. Each method had advantages and disadvantages when compared to the others.

4.3.1. Linear Distance

A horizontal line was the first and simplest of the approaches. The coordinates of the inflection points were contrasted with those of a data point most horizontally across the curve. This was the easiest method and results similarly to the other methods. By having a line at each inflection point gave more data on the differences between sides of the curve (i.e. flexion vs. extension or compression vs. tension). However, much information was lost throughout the remainder of the motion.

4.3.2. Area Under Exponential Fit

The next method was to fit the data to an exponential curve (Equation 4-1) and integrate the displacement (D) with respect to the load (L) as in Equation 4-2.

$$D = A + Be^{CL} \tag{4-1}$$

$$\int DdL = AL + \frac{B}{C}e^{CL} \tag{4-2}$$

Because an exponential function with a single coefficient cannot have both positive and negative values, the curve needed to be split into four distinct pieces: top-loading, top-unloading, bottom-loading, and bottom-unloading. Then each region was transformed symmetrically to be entirely in first or first and second quadrants so that no y-value (displacement) crossed 0. The benefit of this method was to have a piece-wise equation that could be understood in some generality. With only a few exceptions, the model fit the data very well as can be seen in the example in Figure 4-2. The exceptions were in the cases when the low range was large compared to the total range of displacement or when it was particularly linear and the exponential could not follow a sharp deviation.

4.3.3. Area Under Boltzmann Sigmoidal Fit

The third method for describing the hysteresis was with a sigmoidal curve fit (Equations 4-3 and 4-4) developed by the Austrian physicist Ludwig Boltzmann.

$$D = \frac{A - B}{1 + e^{\alpha(L - m_0)}} + B \tag{4-3}$$

$$\int D dL = \frac{(B - A)}{C} \ln(e^{\alpha L - \alpha m_0} + 1) + AL \tag{4-4}$$

Two equations fit the entire curve reducing the computation necessary. The shape of the sigmoid fit is aesthetically uniform and symmetric , but the fit is less precise (typical R^2 = .95 vs. R^2 = .99 for exponential model). These equations seem to have the greatest promise for describing the generalizations of the data, but it does not fit an individual data set as well to describe a single sample's hysteresis.

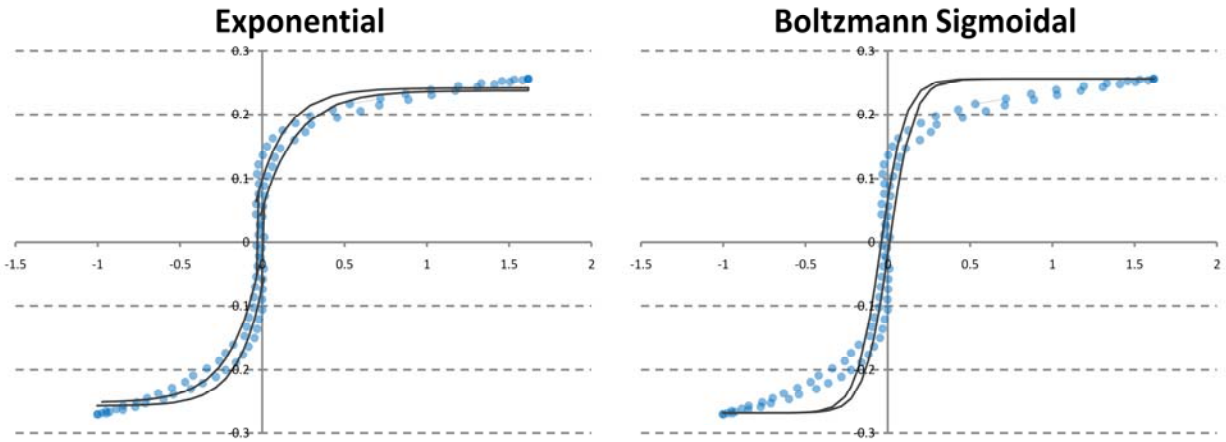

Figure 4-2. Example data show typical fit to exponential or Boltzmann Sigmoidal equations.

4.3.4. Numerical Integration

Finally, for the purpose of this research, having an equation was less important than understanding the hysteresis. To that end, numerical integration of the data was used to calculate the area between loading and unloading curves with minimal error. The calculations were

simple and able to follow every peculiarity of each data set. This method however does little to understand the load-displacement curve in more general terms.

4.4. Sample Fixation

Among the lessons learned, one of the first was fixating the FSUs for mechanical testing. Bondo® has been an industry standard for potting samples, however, the compound is not enough by itself. Most of the experiments were run successfully, but on a few occasions the sample would pull out of the potting while loaded in tension, or it would bend out while loaded in flexion and extension. It seems that the oils and soft tissue which were not removed would prevent a union of the Bondo® with the vertebral body. Fixation therefore relied more on larger geometric interplays than on friction to hold the vertebrae and thus the FSU in place.

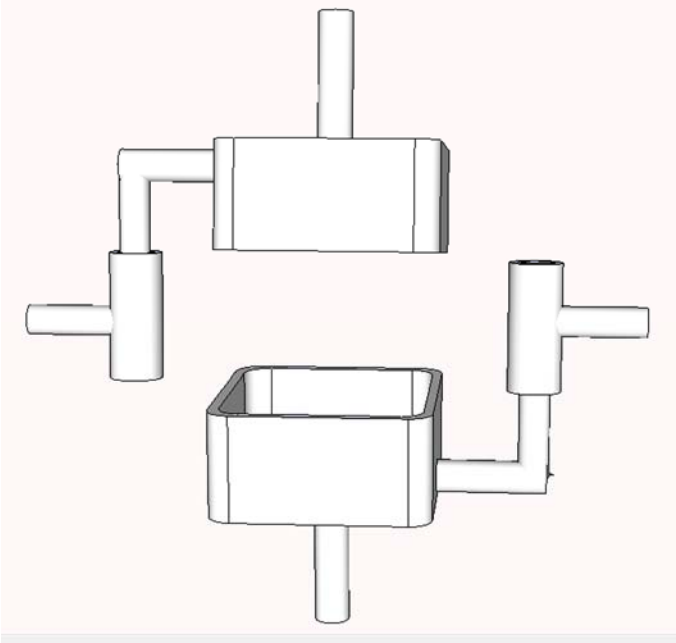

Figure 4-3. Bi-axial test fixtures were designed and used to test samples on a uniaxial machine.

Other researchers have relied on screws passing through the Bondo and into the vertebrae. It was decided in this case that it would be sufficient to leave as much of the bony processes as would still fit in the fixtures. For some of the largest vertebrae artificial geometries were cut into the vertebral body to allow the Bondo® something to hold to.

Another unique element of this research was the fixtures used in the testing. Upon deciding the modes of loading that would be required, work went to designing original bi-axial fixtures (Figure 4-3) that would allow testing in two axes on a uniaxial machine: compression and axial rotation on one axis and then a moment applied about a perpendicular axis through the center of the disc for flexion/extension. The second axis could be adjusted along a slider arm in the case of longer or shorter specimens.

4.5. Uncalibrated Testing

Some of the tests were not well calibrated to load symmetrically across the low range zone as was exhibited by a long tail on one side of the low range but very little on the other side (Figure 4-4). Each FSU was potted in the custom fixtures with slight, unintentional bias in any of six translational directions or 3 axes of rotation. Then the fixtures were clamped into the servohydraulic press, with some similar bias. No effort was made to center the loading of the test around the low range which could have been discovered through some preliminary testing. Because the sample was removed during incubation, a different set of bias would be introduced upon retesting.

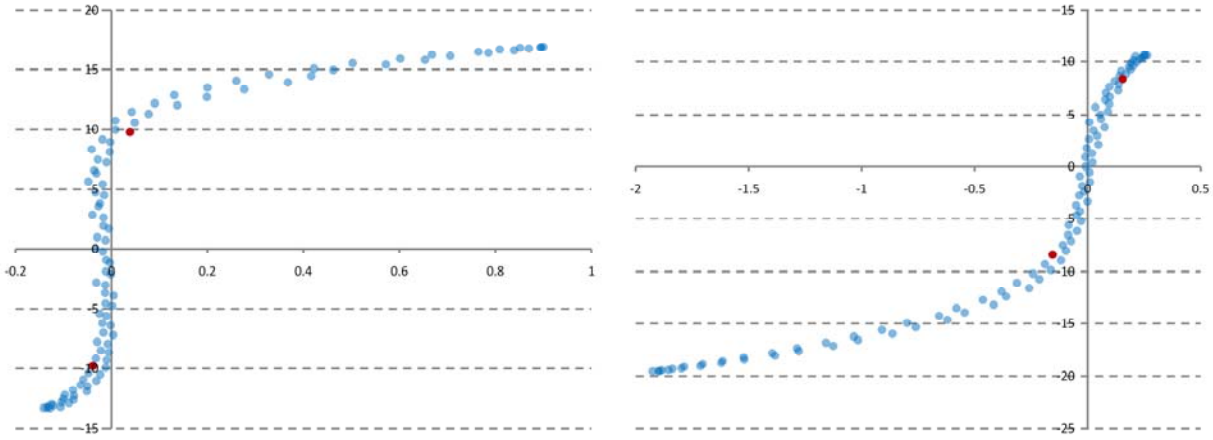

Figure 4-4. Example data sets show where loading was not centered about the low range.

4.6. Testing environment and Incubation

Although consistent with other published research, the testing environment and incubation was not ideal. Testing at room temperature with the sample being heated and and the cooled between tests introduces possible error due to differing rates of heat transfer. Petroleum jelly and saline solution were used generously, but it was observed that in incubation a few samples began to sweat or release moisture as droplets. For these reasons, a constant, physiologic temperature with 100% humidity (not a bath) would be ideal for the testing environment.

5. SUMMARY

This chapter summarizes the research presented in this thesis, its motivation, and results. It also proposes a few pertinent paths in which this work may be continued.

5.1. Research

Because of the immense costs associated with LBP in the U.S. and throughout the world and because of the lack of satisfactory treatment, much understanding of disc degeneration remains to be gained. A method to induce mechanical degeneration in a cadaver has potential to expedite the study of the degenerate environment and treatment methods. Enzymatically degenerating a disc induces the same general changes in biomechanical response as has been observed in naturally degenerate discs. Trypsin-treated FSUs exhibited the decreased stiffness and increased joint laxity that previously has been linked to initial stages of disc degeneration.

5.2. Future Work

To further expand on the work presented here, studies may bring the same method to human cadaveric specimens and examine further the effects of dosage and time. A few changes to the protocol will be necessary and others would be interesting. A preliminary protocol for the human testing is included in Appendix A and is being implemented by other investigators in the BYU Applied Biomechanics Laboratory. The posterior elements on a human spine complicate

the injection process, as will the non-circularity of the IVD. The bovine coccygeal IVD is smaller than a human lumbar IVD, so a larger dosage of protease may be necessary, however, because the chemical composition is different and the swelling pressure of the human disc tends to be less, further investigation may be required to effect the desired results.

An effect was observed in the results of this research suggesting any fluid injection would affect the mechanics of the disc. To this end a different or second control group with either no injection or a needle only injection would be important so as to better differentiate the source of the observed results.

In this thesis, average changes were used but the variation between samples was large. A sensitivity analysis would be interesting to see what factors introduce the greatest variation and where more care ought to be taken to control the effects so that the method may more consistently reach the intended results.

The method presented in this thesis should lay a foundation for greater understanding and relevancy for medical device design and regulation. One of the limitations in testing medical devices is the environment into which it is being placed. Prevalently observed is adjacent disc degeneration when any procedure is performed to a painful disc. This method will allow research to be done at varying levels of degeneration not only of the IVD in focus, but also of the other tissue. Cadavers cannot always be found in the state that a researcher wishes, but this will allow designers to better understand what is most likely to happen in a prescribed situation.

A predictive mathematical model will be beneficial in moving forward with implementing this process in pre-clinical testing for medical devices and procedures. This bovine cadaveric study has set a strong foundation, however, differences in physiology and biochemical composition would severely limit the applicability of a model based on these results

to human cadaver IVDs. Ideally, a human model of the protocol will incorporate the three effects described in Chapter 4 of this thesis (proteolytic effect of trypsin, transient fluid effect, and permanent localized damage due to overpressurizing the AF tissue) and their significant interactions. As previously discussed, control studies must be carefully constructed to separate the influence of each effect on the mechanical behavior of the cadaveric IVD. Statistical analysis, including least squares regression, could then be used to correlate the composite effects. Inverting the equation, a prescribed process could be obtained by entering the desired biomechanical change associated with one or more levels of degeneration.

REFERENCES

1 Hughes JE. 2007. NCQA launches back pain recognition program. (February 8, 2011 2011; http://www.aaos.org/news/bulletin/jun07/clinical7.asp)

2 Battie MC, Videman T, Levalahti E, et al. 2008. Genetic and environmental effects on disc degeneration by phenotype and spinal level: a multivariate twin study. Spine 33: 2801-2808.

3 Battie MC, Videman T, Parent E. 2004. Lumbar disc degeneration: epidemiology and genetic influences. Spine 29: 2679-2690.

4 Videman T, Battie MC, Gill K, et al. 1995. Magnetic resonance imaging findings and their relationships in the thoracic and lumbar spine. Insights into the etiopathogenesis of spinal degeneration. Spine 20: 928-935.

5 Videman T, Sarna S, Battie MC, et al. 1995. The long-term effects of physical loading and exercise lifestyles on back-related symptoms, disability, and spinal pathology among men. Spine 20: 699-709.

6 Battie MC, Videman T, Gill K, et al. 1991. 1991 Volvo Award in clinical sciences. Smoking and lumbar intervertebral disc degeneration: an MRI study of identical twins. Spine 16: 1015-1021.

7 Boden SD, Davis DO, Dina TS, et al. 1990. Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am 72: 403-408.

8 Jensen MC, Brant-Zawadzki MN, Obuchowski N, et al. 1994. Magnetic resonance imaging of the lumbar spine in people without back pain. N Engl J Med 331: 69-73.

9 Sessions J, Bishop T, Bowden A, Sessions A. 2009. Mechanical Characterization of a Bovine Tail Analog for Enzymatic Disc Degeneration. ORS 55th annual meeting. Las Vegas.

10 Moore KL, Dalley AF, Agur AMR, Moore ME. 2006. Clinically Oriented Anatomy. Baltimore, MD: Lippincott Williams & Wilkins.

11 Jiang H, Raso JV, Moreau MJ, et al. 1994. Quantitative morphology of the lateral ligaments of the spine. Assessment of their importance in maintaining lateral stability. Spine (Phila Pa 1976) 19: 2676-2682.

12 Watanabe H, Yamada Y, Kimata K. 1998. Roles of aggrecan, a large chondroitin sulfate proteoglycan, in cartilage structure and function. J Biochem 124: 687-693.

13 Adams MA and Roughley PJ. 2006. What is intervertebral disc degeneration, and what causes it? Spine 31: 2151-2161.

14 Osti OL, Vernon-Roberts B, Moore R, Fraser RD. 1992. Annular tears and disc degeneration in the lumbar spine. A post-mortem study of 135 discs. J Bone Joint Surg Br 74: 678-682.

15 Hilton RC and Ball J. 1984. Vertebral rim lesions in the dorsolumbar spine. Ann Rheum Dis 43: 302-307.

16 Thompson JP, Pearce RH, Schechter MT, et al. 1990. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine 15: 411-415.

17 Auerbach JD, Johannessen W, Borthakur A, et al. 2006. In vivo quantification of human lumbar disc degeneration using T(1rho)-weighted magnetic resonance imaging. Eur Spine J 15 Suppl 3: S338-344.

18 Kingma I, van Dieen JH, Nicolay K, et al. 2000. Monitoring water content in deforming intervertebral disc tissue by finite element analysis of MRI data. Magn Reson Med 44: 650-654.

19 Pfirrmann CW, Metzdorf A, Zanetti M, et al. 2001. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine 26: 1873-1878.

20 Krismer M, Haid C, Behensky H, et al. 2000. Motion in lumbar functional spine units during side bending and axial rotation moments depending on the degree of degeneration. Spine 25: 2020-2027.

21 Tanaka N, An HS, Lim TH, et al. 2001. The relationship between disc degeneration and flexibility of the lumbar spine. Spine 1: 47-56.

22 Brown MD, Holmes DC, Heiner AD. 2002. Measurement of cadaver lumbar spine motion segment stiffness. Spine 27: 918-922.

23 Alini M, Eisenstein SM, Ito K, et al. 2008. Are animal models useful for studying human disc disorders/degeneration? Eur Spine J 17: 2-19.

24 Lotz JC. 2004. Animal models of intervertebral disc degeneration: lessons learned. Spine 29: 2742-2750.

25 Adams MA, Freeman BJ, Morrison HP, et al. 2000. Mechanical initiation of intervertebral disc degeneration. Spine 25: 1625-1636.

26 Buckwalter JA. 1995. Aging and degeneration of the human intervertebral disc. Spine 20: 1307-1314.

27 Johannessen W, Auerbach JD, Wheaton AJ, et al. 2006. Assessment of human disc degeneration and proteoglycan content using T1rho-weighted magnetic resonance imaging. Spine 31: 1253-1257.

28 Marchand F and Ahmed AM. 1990. Investigation of the laminate structure of lumbar disc anulus fibrosus. Spine 15: 402-410.

29 Natarajan RN, Williams JR, Andersson GB. 2004. Recent advances in analytical modeling of lumbar disc degeneration. Spine 29: 2733-2741.

30 Guerin HA and Elliott DM. 2006. Degeneration affects the fiber reorientation of human annulus fibrosus under tensile load. J Biomech 39: 1410-1418.

31 Peng B, Hao J, Hou S, et al. 2006. Possible pathogenesis of painful intervertebral disc degeneration. Spine (Phila Pa 1976) 31: 560-566.

32 Coppes MH, Marani E, Thomeer RT, Groen GJ. 1997. Innervation of "painful" lumbar discs. Spine 22: 2342-2349; discussion 2349-2350.

33 Freemont AJ, Peacock TE, Goupille P, et al. 1997. Nerve ingrowth into diseased intervertebral disc in chronic back pain. Lancet 350: 178-181.

34 Adams MA. 2004. Biomechanics of back pain. Acupunct Med 22: 178-188.

35 Brinckmann P. 1986. Injury of the annulus fibrosus and disc protrusions. An in vitro investigation on human lumbar discs. Spine (Phila Pa 1976) 11: 149-153.

36 Hamai A, Hashimoto N, Mochizuki H, et al. 1997. Two distinct chondroitin sulfate ABC lyases. An endoeliminase yielding tetrasaccharides and an exoeliminase preferentially acting on oligosaccharides. J Biol Chem 272: 9123-9130.

37 Smith L and Brown JE. 1967. Treatment of lumbar intervertebral disc lesions by direct injection of chymopapain. J Bone Joint Surg Br 49: 502-519.

38 Moneret-Vautrin DA, Feldmann L, Kanny G, et al. 1994. Incidence and risk factors for latent sensitization to chymopapain: predictive skin-prick tests in 700 candidates for chemonucleolysis. Clin Exp Allergy 24: 471-476.

39 Moss J and Roizen MF. 1985. Adverse reactions to chemonucleolysis: anesthetic considerations. Int Anesthesiol Clin 23: 119-132.

40 Tsay YG, Jones R, Calenoff E, et al. 1984. Chymopapain-induced hypersensitivity following chemonucleolysis. Spine (Phila Pa 1976) 9: 769-771.

41 Mwale F, Demers CN, Michalek AJ, et al. 2008. Evaluation of quantitative magnetic resonance imaging, biochemical and mechanical properties of trypsin-treated intervertebral discs under physiological compression loading. J Magn Reson Imaging 27: 563-573.

42 Roberts S, Menage J, Sivan S, Urban JP. 2008. Bovine explant model of degeneration of the intervertebral disc. BMC Musculoskelet Disord 9: 24.

43 An HS, Anderson PA, Haughton VM, et al. 2004. Introduction: disc degeneration: summary. Spine 29: 2677-2678.

44 Cs-Szabo G, Ragasa-San Juan D, Turumella V, et al. 2002. Changes in mRNA and protein levels of proteoglycans of the anulus fibrosus and nucleus pulposus during intervertebral disc degeneration. Spine 27: 2212-2219.

45 Zhao CQ, Wang LM, Jiang LS, Dai LY. 2007. The cell biology of intervertebral disc aging and degeneration. Ageing Res Rev 6: 247-261.

46 Cavanaugh JM, Ozaktay AC, Yamashita T, et al. 1997. Mechanisms of low back pain: a neurophysiologic and neuroanatomic study. Clin Orthop Relat Res: 166-180.

47 Cheh G, Bridwell KH, Lenke LG, et al. 2007. Adjacent segment disease followinglumbar/thoracolumbar fusion with pedicle screw instrumentation: a minimum 5-year follow-up. Spine 32: 2253-2257.

48 Hilibrand AS and Robbins M. 2004. Adjacent segment degeneration and adjacent segment disease: the consequences of spinal fusion? Spine J 4: 190S-194S.

49 Schulte TL, Leistra F, Bullmann V, et al. 2007. Disc height reduction in adjacent segments and clinical outcome 10 years after lumbar 360 degrees fusion. Eur Spine J 16: 2152-2158.

50 Elliott DM, Yerramalli CS, Beckstein JC, et al. 2008. The effect of relative needle diameter in puncture and sham injection animal models of degeneration. Spine 33: 588-596.

51 Sriram P, Kalogerakis N, Behie LA. 1996. Experimental determination of the rate of autolysis of trypsin at 37 degrees C. Biotechnology techniques 10: 601-606.

52 Bass CR, Planchak CJ, Salzar RS, et al. 2007. The temperature-dependent viscoelasticity of porcine lumbar spine ligaments. Spine 32: E436-442.

53 Iatridis JC, Setton LA, Weidenbaum M, Mow VC. 1997. The viscoelastic behavior of the non-degenerate human lumbar nucleus pulposus in shear. J Biomech 30: 1005-1013.

54 Schmidt TA, An HS, Lim TH, et al. 1998. The stiffness of lumbar spinal motion segments with a high-intensity zone in the anulus fibrosus. Spine 23: 2167-2173.

APPENDIX A

Preliminary Human IVD Trypsinization Protocol

Dissection/Preparation

Four human lumbar spines will be dissected until only bone and IVDs remain. Facet joints will also be removed. The spine will be cut with a band saw into single functional spinal units (FSU) consisting of a single IVD and bone on the superior and inferior ends. The FSU will be potted on both ends into fixtures using Bondo®. Special care must be taken not to damage the IVD in dissection or in the potting process. Petroleum jelly and saline spray will be used as needed to maintain hydration of the specimen. Specimens will then be heated to 37ºC (Physiologic operating temperature) in 100% humidity environmental chamber.

Mechanical Testing

The mechanical response of each potted specimen will be tested in flexion/extension, lateral bending, and axial rotation with a 400 N compressive follower load.

Flexion/Extension – The specimen will be cycled from a neutral position ±8º. The resultant torque will be recorded.

Lateral Bending – The specimen will be cycled from a neutral position ±6º. The resultant torque will be recorded.

Axial Rotation – The specimen will be cycled from a neutral position ±1º. The resultant torque will be recorded.

Injection and Incubation

The AF of 14 FSUs will be injected with 25μL of trypsin or FBS in eight equally spaced locations around the disc (a total of 200μL) with a 28ga needle (Figure 3). Seven FSUs will be used as a control with no injection. The specimens will then be tested and then held at 37ºC for 1/2 hr and then tested again. The incubation time and testing will be repeated three more times so that a total of five tests over 3 hours are recovered for each specimen. (Table 1)

Table A. Chronology of the testing procedures

Test Procedure:
1. Warm to 37ºC
2. Intact mechanical test
3. Inject trypsin, FBS, or none
4. Mechanical Test (0hr)
5. Wait 1/2hr
6. Mechanical Test (1/2hr)
7. Wait 1/2hr
8. Mechanical Test (1hr)
9. Wait 1hr
10. Mechanical Test (2hr)
11. Wait 1hr
12. Mechanical Test (3hr)

Solutions to Anticipated Difficulties

Screws will be used to help fixate the vertebral bodies in the fixtures and Bondo. A box will be built with all of the testing equipment where greater temperature and humidity control can be maintained throughout the testing time. The box will also reduce the actual test time which will be a significant portion of the total incubation time. A compressive follower load on the FSU bending and rotation testing may be removed from the procedure if the coupled loading system proves unwieldy.