IN-VITRO BIOMECHANICAL EVALUATION OF MULTIPLE FREEZE-THAW CYCLES ON 3D KINEMATICS OF HUMAN CADAVERIC LUMBAR SPINE

A Thesis
Presented to
The Graduate Faculty of The University of Akron

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

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August, 2010
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ABSTRACT

In-vitro biomechanical evaluations are often carried out with fresh frozen cadaveric spine specimens in order to determine the post-operative kinematics of spinal motion segments with novel spinal implants or surgical techniques. Such biomechanical studies commonly involve repeated tests with the spinal specimen in the intact state, followed by an injured state and one or more instrumented states. Previous studies have measured changes in mechanical properties of a spinal motion segment after a single occurrence of freezing, but the effect of multiple freeze–thaw cycles have not been reported.

In the current study, flexibility tests in flexion-extension (FE), lateral bending (LB), axial rotation (AR), and FE with 660 N of compressive load (FE+FL), were repeatedly carried out on a human cadaveric multi-segmented lumbosacral specimen (L3-Sacrum) on 8 separate test days, with freeze-thaw cycles between test days, and 8 sets of flexibility tests were carried out within each test day. A custom-built flexibility testing machine was used to apply 5 continuous cycles of pure bending moment up to ± 7.5 Nm at a rate of about 1°/s while ensuring that the specimen’s motion remained unconstrained. An optoelectronic camera system (NDI Optotrak Certus) was used to capture the 3-D kinematics of L3, L4, L5 and the Sacrum. The load-displacement data at the last loading cycle were analyzed to determine the range of motion (ROM) and neutral zone (NZ).
A significant difference, in the specimen’s total ROM and NZ across L3-Sacrum, was found between test days in all loading directions (all p<0.001) using one-way repeated measures ANOVA. There were no within-day variations in the ROM or NZ in FE (p=0.87, p=0.98), LB (p=0.83, p=0.86), AR (p=0.60, p=0.94), and FE+FL (p=0.69, p=0.84) as determined from the Wilcoxon Signed Rank test. The inter-segmental ROM and NZ data for L3-L4, L4-L5, and L5-Sacrum also showed significant differences between test days and negligible intra-day variations using the above mentioned statistical tests.

The current study showed that ROM and NZ of human cadaveric lumbosacral spinal segments were significantly affected by multiple freeze-thaw cycles but were not significantly different within a 12 hour test day. The results indicated that in-vitro biomechanical experiments with comparison between sequential states on a test specimen should be conducted within a test session.
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Juay Seng Tan for his continued support. He was an excellent mentor throughout the research and never refused me his time and attention. Even when my ideas and experiments did not turn out the way I expected, he managed to keep me motivated. It was always a great pleasure and privilege to work under his supervision. I also thank Dr. Bruce C. Taylor, Dr. Mary C. Verstraete and Dr. Selvon St. Clair, for taking the time and having the interest to serve on my committee.

This research is only made possible by the anonymous person who selflessly donated her body to science and participated in this study. I thank her.

My special thanks to Rick Nemer, Dale Ertley and Bill for their inspiring discussions and assistance in machining the flexibility spine test apparatus.

I would also like to thank my friends who have helped make my time at University of Akron truly fulfilling, and I will not be able to thank them all individually. I will just trust that those who have provided support and insight over the years will accept my heartfelt thanks and see something of themselves in these pages. Finally, I want to thank my family. Their love and support have helped me come this far.
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CHAPTER I

INTRODUCTION

1.1 Overview

Detailed and multifaceted in-vitro biomechanical studies of complex structures such as the human spine using cadaveric experimental models require testing durations that may be as long as a whole day. This can be attributed to the study design which requires staged specimen preparation and testing; with the spinal specimen in the intact state, followed by an injured state and one or more instrumented states. Such tests are commonly carried out sequentially within a single test day for each specimen. It is currently unclear if such sequential tests can be carried out over several days with the specimen stored back in a freezer in between test days without any damage.

If so, intact tests on batches of specimens can be carried out beforehand and the surgeon could perform surgical procedures on another day at his/her convenience. With multiple instrumented states, the surgeon would perform the next surgical procedure on all specimens after completion of tests on all specimens in the previous instrumented state. The specimens would be stored in a freezer in between test days. Biomechanical tests carried out in this manner have the advantage in minimizing time involvement for the clinical personnel participating in the study.
1.2 Significance and Objectives of the Study

The first objective of the current study was to address the effects of multiple freeze-thaw cycles on cadaveric spine kinematics and the second objective was to develop and validate an in-vitro spine testing apparatus to carry out the study.

1.2.1 Effect of Multiple Freeze-Thaw Cycles on Cadaveric Spine

Biomechanical studies on multi-segmented spinal specimens are ideally performed on freshly obtained specimens so that the tests are carried out on materials that most closely mimic the physiologic in-vivo condition. It is common practice to store the biological tissue to be used in such experiments for various periods in a frozen state (-18 °C to -20 °C) in between pre-test preparation procedures such as radiographic imaging, diagnosis of bone mineral density, dissection of surrounding soft tissues and potting in cement in preparation for biomechanical testing. This is unavoidable due to unpredictable supply of suitable human cadaveric specimens, and schedules for these aforementioned pre-test preparation activities. Freezing has been reported, in several previous studies [4, 7, 14, 42, 51, 58], to not affect the mechanical properties of human spinal specimens. However, these studies measured changes in the mechanical properties of the cadaveric specimens using a single motion segment and moreover after only a single occurrence of freezing. The effects of multiple freeze-thaw cycles on multi-segmented cadaveric spines are currently unclear.

The first main objective of this study was to determine the effects of repeated measures flexibility tests within a test day and over multiple test days with repeated freeze–thaw cycles on the biomechanical parameters, ROM and NZ, of a multi-segmented human cadaveric lumbosacral (L3-Sacrum) spine.
1.2.2 In-Vitro Flexibility Testing Apparatus

In-vitro biomechanical testing of the spine has been traditionally carried out in either a load-controlled or displacement-controlled manner. These two well-defined protocols for spine testing were proposed previously [39, 40, 43]. Each method requires certain assumptions and offers different advantages. However, their common goal is to delineate the load-displacement behavior of the motion segments in a normal or intact spine and changes in its behavior due to degeneration and subsequent stabilization, thus providing a quantitative indicator of the stiffness (or flexibility) of the motion segment being tested. This data can aid researchers and practicing surgeons alike in testing novel instrumentation and surgical techniques.

In the displacement-control method (stiffness testing protocol), the application of a known displacement (translation or rotation input) at the superior-most vertebral body of a multi-segmented spine imposes loads along the spinal segments as shown in Figure 1.1. The observed load data can be measured to obtain the desired load-displacement characteristics.

![Figure 1.1 Schematic diagram showing the principle of stiffness testing protocol in a multi-segmented spinal specimen.](image-url)
The governing equation for stiffness testing protocol is given below in Eq 1.1.

\[
\{F\} = [K] \{x\} \quad \text{---------------------------------}(1.1)
\]

where, \(\{F\}\) is the force vector, \(\{x\}\) is displacement vector, and \([K]\) is the stiffness matrix as shown below,

\[
\begin{pmatrix}
F_x \\
F_y \\
F_z \\
M_x \\
M_y \\
M_z
\end{pmatrix} =
\begin{pmatrix}
K_{11} & K_{12} & K_{13} & K_{14} & K_{15} & K_{16} \\
K_{21} & K_{22} & K_{23} & K_{24} & K_{25} & K_{26} \\
K_{31} & K_{32} & K_{33} & K_{34} & K_{35} & K_{36} \\
K_{41} & K_{42} & K_{43} & K_{44} & K_{45} & K_{46} \\
K_{51} & K_{52} & K_{53} & K_{54} & K_{55} & K_{56} \\
K_{61} & K_{62} & K_{63} & K_{64} & K_{65} & K_{66}
\end{pmatrix}
\begin{pmatrix}
x \\
y \\
z \\
R_x \\
R_y \\
R_z
\end{pmatrix}
\]

The displacement-control method offers the advantage of applying known translation and rotation inputs to the vertebrae. The motion components can be applied based on exact motions of the spine which have been quantified in-vivo using the instantaneous axis of rotation [56], thereby giving a close representation of the physiological load-displacement characteristics in an in-vitro laboratory setup. However, there are several practical difficulties in using this approach as a standardized testing protocol which are discussed below,

- The rotation inputs should be applied along the natural instantaneous axis of rotation (IAR) of the spine. If this rotation axis is not congruent with the natural axis of rotation of the specimen, then the resulting motions will be constrained, non-physiological, and can cause injury to the specimen [19].

- The IAR can only be approximated based on the curvature of the specimen. Moreover, it is not likely to remain in the same location during the entire test as the spine deforms and exhibits its non-linear behavior.
The given displacement at the superior most vertebral body imposes complex loads along the spinal segment coupling behavior of the spine. Although the reaction load can be quantified at the base of the specimen, the applied load across each individual level remains unknown.

Because of such limitations, the stiffness protocol is seldom used and the most common method used currently is the load-controlled approach or flexibility protocol. It simulates clinically relevant motions by applying a known bending moment to the spinal segment and in turn measuring the resulting three dimensional vertebral displacements.

For a three-dimensional study, each one of the six load components (three forces and three moments) are applied in a sequence and the resulting six motion components (three translations and three Euler rotations) are measured. The working equation for the flexibility testing protocol is given below in Eq 1.2.

\[
\{x\} = [C] \{F\} \ldots (1.2)
\]

where \(\{F\}\) is the force vector, \(\{x\}\) is displacement vector, and \([C]\) is the flexibility matrix. The stiffness matrix \([K]\) is the matrix inverse of \([C]\). \(\{F\}\) and \(\{x\}\) are 6 x 1 matrices representing the load and motion components along 6 DOF, and \([C]\) is the 6 x 6 symmetrical matrix with stiffness coefficients as shown below,

\[
\begin{bmatrix}
    x \\
    y \\
    z \\
    R_x \\
    R_y \\
    R_z \\
\end{bmatrix} =
\begin{bmatrix}
    C_{11} & C_{12} & C_{13} & C_{14} & C_{15} & C_{16} \\
    C_{21} & C_{22} & C_{23} & C_{24} & C_{25} & C_{26} \\
    C_{31} & C_{32} & C_{33} & C_{34} & C_{35} & C_{36} \\
    C_{41} & C_{42} & C_{43} & C_{44} & C_{45} & C_{46} \\
    C_{51} & C_{52} & C_{53} & C_{54} & C_{55} & C_{56} \\
    C_{61} & C_{62} & C_{63} & C_{64} & C_{65} & C_{66}
\end{bmatrix}
\begin{bmatrix}
    F_x \\
    F_y \\
    F_z \\
    M_x \\
    M_y \\
    M_z
\end{bmatrix}
\]
The most important advantage of the flexibility testing protocol is the application of a pure moment and uniform moment across all levels of a multi-segmented spinal specimen, regardless of the stiffness of the intact spine or changes in it as a function of injury and after the stabilization treatment. Also, the loads can be applied without imposing any constraints on the motion behavior of the spinal segments. The spine moves freely in 6 DOF, similar to in-vivo conditions, in response to the external loads exerted on spine (Figure 1.2).

Thus, the second objective of this study was to build an in-vitro spine biomechanical test machine following the principle of flexibility approach for evaluating the load-displacement characteristics of single or multi-segmented spinal specimens. The design for the test apparatus was based on a previous existing test set-up [18].

1.3 Outline and Purpose

The primary purpose of this study was to investigate the biomechanical effects of multiple cycles of freezing (-18 °C to -20 °C) and subsequent thawing at room
temperature (21 °C to 24 °C) on the three dimensional (3D) intervertebral kinematics of a human cadaveric, multi-segmented lumbosacral (L3-Sacrum) spinal specimen. The flexibility and laxity test parameters, namely range of motion (ROM) and neutral zone (NZ) respectively, of a single intact spine were measured repeatedly using the flexibility testing protocol to determine intra-day and inter-day variability as a result of the treatment effects.

The current study also involved development and validation of an in-vitro testing apparatus to simulate clinically relevant bending motions of flexion-extension (FE), lateral bending (LB), and axial rotation (AR) on a spinal specimen, with and without a compressive follower preload (FL) to account for the effect of body weight and muscle forces on the spine motion segment. This custom-built test machine was used to apply a pure bending moment across all levels in a multi-segmented spinal specimen while allowing the specimen to move freely in an unconstrained manner along all its six degrees of freedom (DOF). This mimicked the physiological in-vivo behavior of a spine in an in-vitro laboratory setup.

1.4 Specific Aims of the Study

The specific aims of the study were,

1. To evaluate the effects of multiple freeze-thaw cycles on the three-dimensional intervertebral kinematics of an intact human cadaveric lumbosacral spine in FE, AR, and LB.

2. To establish procedures for measuring and analyzing the in-vitro 3D intervertebral kinematics (ROM and NZ) of a multi-segmented spinal specimen from flexibility tests in FE, LB, and AR.
3. To design and develop an in-vitro, dynamic spine testing apparatus for conducting biomechanical tests on single and multi-segmented spinal specimens, using the flexibility testing protocol (load-controlled approach).

4. To incorporate a system of mechanisms in the flexibility testing machine for applying compressive follower preload [46] on multi-segmented spinal specimens during in-vitro tests.

1.5 Statement of Hypotheses

The current study involved repeated measures intra-day and inter-day experiments to evaluate the effects of multiple freeze-thaw cycles on the biomechanical characteristics of a cadaveric spine specimen. Two null hypotheses were proposed for evaluating the specific aim 1 of the study. Due to the qualitative nature of the specific aims 2, 3 and 4, statistical analyses were not deemed suitable and instead two research hypotheses were proposed. The statement of hypotheses are given below,

1.5.1 Null Hypotheses

1. There are no intra-day effects on the specimen’s total (L3-Sacrum) and inter-segmental (L3-4, L4-5, L5-Sacrum) ROM or NZ for each of the four loading cases in FE, LB, AR, and FE+FL when a specimen is tested repeatedly on the same day.

2. There are no inter-day effects due to freeze-thaw cycles on the specimen’s total (L3-Sacrum) and inter-segmental (L3-4, L4-5, L5-Sacrum) ROM or NZ for each of the four loading cases in FE, LB, AR, and FE+FL.
1.5.2 Alternate Hypotheses

1. There are intra-day effects on the specimen’s total (L3-Sacrum) and inter-segmental (L3-4, L4-5, L5-Sacrum) ROM or NZ for each of the four loading cases in FE, LB, AR, and FE+FL when a specimen is tested repeatedly on the same day.

2. There are inter-day effects due to freeze-thaw cycles on the specimen’s total (L3-Sacrum) and inter-segmental (L3-4, L4-5, L5-Sacrum) ROM or NZ for each of the four loading cases in FE, LB, AR, and FE+FL.

1.5.3 Research Hypotheses

1. It is possible to develop an in-vitro biomechanical test set-up capable of conducting biomechanical studies on single and multi-segmented spinal specimens as per the flexibility testing protocol.

2. It is possible to simulate muscle loads on an in-vitro spine model using the concept of compressive follower preload, thereby better evaluating its biomechanical properties.
CHAPTER II
LITERATURE REVIEW

2.1 The Human Spine

The spine is an important structure in the human body. It forms the posterior part of a person’s trunk, from the neck to the pelvis. It serves three primary biomechanical functions:

1. Allows movement between the head, trunk, and pelvis;
2. Transfers loads between the head, trunk, and pelvis; and
3. Protects the spinal cord during spinal motions and transmittal of forces.

2.1.1 Basic Anatomy and Physiology

The human spine consists of 33 vertebrae that are stacked on top of one another, separated by an intervertebral space. The vertebrae are grouped, by region, as 7 cervical (C1-C7) vertebrae, 12 thoracic (T1-T12) vertebrae, 5 lumbar (L1-L5) vertebrae, 5 fused sacral (S1-S5) vertebrae, and 4 fused coccygeal vertebrae (tail bone) as shown in Figure 2.1. The Cervical, Thoracic, and Lumbar vertebrae are considered “true vertebrae” as they maintain their independent mobility throughout life while the Sacrum and Coccyx are fused and relatively immobile.
As seen in Figure 2.1, the spinal column is usually fairly straight and symmetrical in the frontal plane. In the sagittal plane, there are four curves in the normal spine, which provide mechanical advantages such as increased flexibility, while still providing stiffness and stability. The primary curves, which develop during the fetal period, are kyphotic (convex posteriorly) in the thoracic and sacral regions. The secondary curves of the cervical and lumbar regions are lordotic (convex anteriorly) and develop after birth.

Between the vertebrae are the intervertebral discs and the facet joints which allow, yet limit motion between vertebrae. The upright posture and stability of the spinal column is supported and controlled by numerous ligaments and muscles. The vertebrae, intervertebral discs, facet joints and ligaments possess unique characteristics, important biomechanical functions and properties in relation to the motion, load transfer and protection roles of the spine.
Each vertebra (except C1) consists anteriorly of a vertebral body and posteriorly of a neural arch from which the posterior elements arise (Figure 2.2).

![Figure 2.2 Schematic diagrams of a typical lumbar vertebra viewed from superior, lateral, and posterior directions showing the important structural elements.](image)

The vertebral body supports the majority of the load through the spinal column. The vertebral bodies are composed mainly of cancellous bone with a thin cortical shell and are kidney shaped when viewed superiorly. The posterior elements are mainly made up of cortical bone, with some cancellous bone within. The neural arch surrounds the spinal cord and spinal nerve roots that run through the vertebral column. It consists of two pedicles that project posteriorly from the vertebral body and a lamina that extends from each pedicle towards the midline. Extending posteriorly from the lamina is the spinous process, which is the bony surface that one can palpate along the midline of the back. Projecting laterally on each side from the pedicle-lamina junction are the transverse process. The spinous and transverse processes provide points for ligament and muscle attachments and act as levers to accentuate the action of muscle forces into bending moments.
Each vertebra also consists of four articular processes called facets, which are masses of bone that extend inferiorly and superiorly from the laminae. The medial surface of the superior facets and the lateral surface of the inferior facets articulate with corresponding articular processes from the levels above and below to form the facet joints (also known as Zygapophysial joints) as shown in Figure 2.3.

![Facet Joint](image)

Figure 2.3 Schematic diagram of the facet joint in lumbar spine.

The articular facets, as a pair, are true synovial (diarthrodial) joints. They possess articular cartilage and a synovial space, are enclosed by a fibrous capsule and are mobile. Their function is to control certain motion, mainly by resisting forward displacement and rotation and to allow motion along other directions. They transfer a small proportion of the total load through the vertebral column in extension and axial rotation and about 20% of the total load through each level in axial compression.

The space between adjacent vertebral bodies is maintained by round, spongy pads of cartilaginous tissue called the intervertebral discs. The intervertebral discs act much like shock absorbers during dynamic and impact activities while transferring loads as the body moves. The intervertebral discs are composed of a fibrous outer ring known as the annulus fibrosus and a gelatinous center called the nucleus pulposus (Figure 2.4).
The nucleus pulposus behaves like an incompressible fluid material. Under compression, a uniform hydrostatic pressure is created in the nucleus of the healthy disc, which in turn exerts a radial stress onto the annulus fibrosus. One important role of the annulus is thus to enclose and contain the nucleus during load and motion. Thus, it is designed to have a lamellar structure, containing fibres that are oriented approximately between 20° to 30° with the horizontal plane, with alternating directions between consecutive lamellae, making it strong in resisting the hydrostatic pressure of the nucleus pulposus. At the interfaces between the vertebral body and the superior and inferior intervertebral discs are the vertebral endplates. The endplate in the adult consists of an osseous layer integrated with the vertebral body, and a thin cartilaginous layer in contact with the intervertebral disc. The endplate is not vascularized and it prevents blood flow into the intervertebral disc. The principal functions of the intervertebral disc are to enable movement between the vertebral bodies and to transmit load between adjacent vertebrae. The disc is a viscoelastic and anisotropic structure, and as such, its mechanical properties and behavior are time and direction dependent. The disc exhibits a non-linear stiffness and provides greater resistance to displacement as the load magnitude increases. Therefore, the disc allows flexibility at low loads and stability at high loads.
The main role of spinal ligaments is to enhance stability by restricting excessive motion, i.e. they resist tensile forces and stabilize the spinal column. The seven primary spinal ligaments are the anterior longitudinal ligament (ALL), posterior longitudinal ligament (PLL), ligamentum flavum (LF), inter-transverse ligament (ITL), inter-spinous ligament (ISL), supra-spinous ligament (SSL) and capsular ligament (CL) (Figure 2.5).

![Figure 2.5 Schematic diagram of the sagittal view of a lumbar spine segment showing the spinal ligaments.](image)

The anterior longitudinal ligament (ALL) is a strong fibrous band that covers and connects the antero-lateral aspect of the vertebral bodies and intervertebral discs and helps prevent hyperextension of the column. The posterior longitudinal ligament (PLL) lies within the vertebral canal along the posterior aspect of the vertebral bodies and discs. It helps prevent hyper flexion of the spinal column. The ligamentum flavum (LF) is short and thick and bilaterally connects the laminae of adjacent vertebrae. The LF aids to restore the flexed spine to its neutral position. The inter-spinous and supra-spinous ligaments (ISL, SSL) are the other two posterior ligaments. Inter-spinous ligaments lie between spinous processes while the supra-spinous ligament connects on the posterior surface and across multiple spinous processes.
Lastly, the facet capsular ligament (CL) connects adjacent articular processes and prevents excessive motion of the facet joint. Several other ligaments are present in the thoracic spine which connects the costovertebral joints between the ribs and the vertebra.

Two adjoining vertebrae and their connecting soft tissues (the seven spinal ligaments and the intervertebral disc) form a functional spine unit (FSU). A FSU is the most basic motion segment in the spinal column (Figure 2.6).

![Diagram of a functional spinal unit (FSU)](image)

Figure 2.6: Schematic diagram of a functional spinal unit (FSU) viewed from the side, showing a pair of vertebrae, the intervertebral disc (cross-section), and a facet joint (the other facet joint is blocked from view).

Thus, the spinal column can be considered to be made up of a series of functional spinal units (FSUs). The intervertebral disc and the pair of facet joints together form the three joint complex in a FSU. The facet joints connect the FSU posteriorly and together with the intervertebral disc transmit loads and allow motion. A higher amount of compressive load is transmitted through the intervertebral disc in forward flexion, while more load is transmitted through the facet joints during extension. One important role of the facet joints is thus to prevent excessive motion by acting as a bone-on-bone mechanical stop between the articular processes.
The vertebral column protects the spinal cord as it descends from the brain. The spinal cord passes through the spinal canal and exits as spinal roots through the intervertebral foramen (Figure 2.7). Protection of the spinal cord is provided anteriorly by the vertebral body and intervertebral disc, and posteriorly by the spinous process and laminae. Laterally, the spinal cord is protected by the transverse process and the pedicle. The posterior longitudinal ligament lies anterior and the ligamentum flavum lies posterior to the spinal cord, and they both help to provide further protection to the cord.

![Figure 2.7 Schematic diagram showing the passage of spinal cord and its nerve roots through the vertebral canal and the intervertebral foramen.](image)

2.1.2 Basic Biomechanics

As previously mentioned, the three fundamental biomechanical functions of the spine are 1) to allow motion between levels, 2) to transfer load across levels, and 3) to protect the spinal cord and spinal roots [65]. An unstable spine could become incompetent in performing one or more of these functions. The cervical, thoracic, lumbar and sacral regions of the spine perform these functions to different degrees. The lumbar region, for example, is subjected to larger loads than the thoracic and cervical spine, and provides most of the motion in flexion-extension of the trunk [65].
In contrast, the cervical region protects the central nervous system that connects the brain to the body and limbs, withstands a much smaller load as compared to the thoracic and lumbar spine, which in most instances is equal to the weight of the head, and provides a wider range of motion (ROM) for the head and neck.

Motion between vertebral levels from the second cervical vertebra to the sacrum is made possible by the three-joint complex at each level, made up of the intervertebral disc and the pair of facet joints that connect adjacent vertebral bodies. Rotational motions are the predominant modes of motion across these FSUs. Translational motion in the normal spine exists as part of the coupled motions during rotational motion. For example, a mean forward translation of the lumbar FSU of between 2.3 to 3.3 mm was measured during full flexion-extension [54], together with 2° of coupled axial rotation and 3° of coupled lateral bending [47, 48]. The ROM at each FSU differs between regions of the spine and also between individual levels in each region. The lumbar spine, for example, has a ROM of about 12° to 17° in flexion and extension, while the thoracic spine has about 4° to 12° [65]. In axial rotation, the lumbar spine has a ROM of about 1° to 2° unilaterally while the thoracic spine has between 2° to 9°. The ROMs in lateral bending are about the same for FSUs in the thoracic and the lumbar spine, and range between 5° to 8°.

While motion across individual FSU is limited, the cumulative rotational ROM of the spinal column allows for substantial motion of the trunk and head. Load transfer across spinal levels mainly passes as compressive forces through the facet joints and intervertebral disc, onto the adjacent vertebrae. Surrounding ligaments and both agonist and antagonist muscles provide counteracting forces to stabilize the spine and prevent excessive motion. The external forces necessary to create motion are
provided by the agonist muscles. Activation of the agonist muscle creates motion towards it while concurrent relaxation of the antagonist muscle allows the agonist to effect movement. The antagonist muscles work in coordination with the agonist muscles to achieve mechanical stability [6].

2.2 Biomechanical Testing of Spine

Flexibility testing of spine motion segments (FSUs) forms the foundation for evaluating the biomechanical responses of the spine. The relationship between the loads and the displacements provides a quantitative indicator of the flexibility (or stiffness) of the motion segment. For instance, the deviation of the stiffness of a spine motion segment from a normal range generally indicates an aberrant condition. Increased flexibility may indicate ligamentous disruption after injury. Decreased flexibility (high stiffness) may suggest an advanced degenerative process. The assessment of biomechanical integrity of the spine in each of these situations is based on the quantitative measurement of the load-displacement response. Thus, an accurate measurement of the spine’s load-displacement response is important.

For decades, researchers have sought to characterize the mechanical environment of the spine using in-vivo techniques. Most in-vivo studies of the spine are generally restricted to the sagittal plane motion as it is relatively easier to study the measurements using lateral radiographs taken with the spine in full flexion and extension [8, 50]. Some three-dimensional in-vivo studies have used biplanar radiography [33, 47, 61]. Although such in-vivo measurements provide important functional information, they have substantial shortcomings. For instance, the loads that are applied to the spine are unknown, and the accuracy of the measurement system is generally poor. More accurate systems for in-vivo measurements of motion
in spine have also been reported in literature, like the use of roentgenstereophotogrammetry [57], or a more recent approach using sensor-embedded implants placed in between the interbody spaces [24]. But these methods are invasive, which stands as a major drawback for their use as a standard testing protocol. Also, the time-dependent, viscoelastic properties of the tissues comprising the human spine affects the mechanical response and stiffness of the FSU which depends on the test conditions used during load-displacement measurements and on previous biomechanical conditions. In summary, although in-vivo based displacement data of the spine gives its true behavior and can be easily replicated, in-vivo based load measurement data is invasive and complex to monitor. For reasons such as this, the exact description of the mechanical response of the spine presents a challenge to researchers and clinicians. The most direct approach for limiting the effect of these complicating factors is to simulate the in-vivo displacement conditions during laboratory tests. This approach provides a more objective assessment of the biomechanical effects on spine as the involved variables are entirely under the control of the researcher.

The load-displacement conditions can be simulated by applying sets of forces and moments (load-controlled testing) or sets of translations and rotations (displacement-controlled testing) to the in-vitro spinal specimen. During such an experiment, half of these load-displacement parameters may be specified independently, and the remaining must be measured to quantify the response of the material or the spine motion segment. The main objective of each method is to apply a combination of loads or displacements in the laboratory that represent the in-vivo conditions. A multitude of in-vitro studies have been performed on cadaveric spinal segments in an effort to describe its mechanical properties [1, 15, 25, 32, 37, 40, 61].
Such tests, however, require suitable experimental devices to impose physiologically reasonable loads under standardized conditions. Most reported in-vitro experiments are conducted with special devices mounted in available material testing machines [62, 66] as shown in Figure 2.8. Under such test conditions, for sake of convenience, several assumptions regarding muscle forces, axial preload, etc. are made under which either load application, or motion, or both are often constrained. In addition, each loading condition often requires a different experimental set-up since the loads can only be applied separately, and not in combination. All these lead to potential errors in comparing data between experiments. In spite of these difficulties, in-vitro biomechanical studies can however provide the load-displacement characteristics of the spine in its natural and surgical states which are critical to further improve clinical practices.

Figure 2.8 The BioPuls multi-axial spine testing system based on the ‘free-end model’ approach to testing of spinal segments developed by Instron®.

In this study we report a new dynamic spine testing apparatus to determine continuous, three dimensional, load-displacement characteristics of single and multi-segmented spinal specimens. The apparatus was based on a previous design of similar
machine developed by a research group in the University of British Columbia, Vancouver, British Columbia, Canada [18]. It applies pure bending moments to the cranial most vertebra of the specimen in each of the three orthogonal directions to simulate clinically relevant physiological motions in flexion-extension, lateral bending, and axial rotation. The caudal vertebra remains fixed on the testing platform. A spline actuator loading arm allows linear translations of the specimen while transmitting the bending moment, thus allowing it to move freely in all of its degrees of freedom. We also report a mechanism to apply a compressive preload on the spinal specimen during in-vitro tests in flexion-extension to simulate the effect of upper body weight and musculature. This technique was developed by Patwardhan et al [46].

2.3 In-Vitro Cadaveric Experiments

The experimental testing and design considerations for in-vitro studies in general is discussed below,

2.3.1 General Test Protocols

The most relevant in-vitro results, as compared to human in-vivo behavior, stem from fresh frozen human cadaveric testing, although due to limited availability in some instances, testing on other species such as porcine, ovine, or bovine is acceptable to address certain research questions [67, 68]. Specimens are excluded from biomechanical studies if there is evidence, radiographic or macroscopic, of injury and tumors. The fresh unembalmed specimens are harvested and stored at minus 20 °C until use. Before testing they are thawed at room temperature (21 °C to 22 °C), which has been shown to have negligible effect on the behavior of the intervertebral disc and bone [42].
In preparation for in-vitro biomechanical tests, surrounding musculature is carefully dissected away to have only the ligamentous and bony structures intact. The superior and inferior vertebrae are potted in a suitable polymeric or low-melting-point alloy in order to allow the specimen to be mounted onto the testing machine with a well-defined and reliable positioning load. One end remains fixed to the base while the loads or displacements are applied to the other free end [43]. The duration of testing should not exceed 10-12 hours, since exposure to room temperature for a period longer than this can lead to changes in the properties of the specimen [69]. Efforts are made to protect the specimen from drying by conducting tests in a humidity chamber (100 % relative humidity), or periodically spraying the specimen with 0.9 % saline solution.

2.3.2 Assumptions and Limitations

Significant problems are associated with the use of such in-vitro models. These are related to many factors, ranging from stripping of excess musculature from the specimen, age of the spine being tested, anatomical fit of the non-human specimen tested to the human clinical scenario, etc. These are but a few issues associated with biomechanical testing that result in the introduction of assumptions into the experiment which might lead to a significant potential of error regarding the interpretation of the results and their inferences into the clinical domain where they are actually intended to be applied. In-vitro biomechanical tests also do not take into consideration the long-term bone remodeling that occurs in-vivo and thus it only simulates the immediate post-operative conditions.
2.3.3 Effect of Freezing on Cadaveric Specimens

Detailed and multifaceted biomechanical studies of complex structures, such as the spine, require repeated tests with the spinal specimen in the intact state, followed by an injured state and one or more instrumented states. The testing durations can thus be prolonged and often span several days. It is a common practice to store biological tissue to be used for such experiments for various periods in a frozen state. Freezing at -20 °C is the simplest and least expensive method for storing spinal specimens compared to other modes such as cryo-preservation and lyophilization [14]. The desired mode of storage for biological tissues differs depending on whether it concerns an allografting program for use as transplants or their use in an experiment, such as biomechanical testing of vertebral specimens on a test bench.

The widespread acceptance of freezing as a storage method has evolved from several investigations where researchers questioned the pertinence of data collected from previously frozen tissue. In general, these studies demonstrated that the viscoelastic (time-dependent) properties of trabecular [26] and cortical [55] bone, ligament [70], tendon [28, 59], muscle [64], and articular cartilage [23] are not significantly altered by storage at -20 °C. More specific to the intervertebral disc (IVD), Hirsch and Galante [21] demonstrated that the elastic tensile stiffness of the annulus fibrosus is not altered by freezing, an observation in agreement with that of Hickey and Hukins [20], who used x-ray diffraction to demonstrate that freezing does not alter the arrangement of annular collagen fibrils. In addition, Nachemson [36] reported that freezing appears to have no affect on the hydrostatic pressure of the nucleus developed during short duration compressive loading but results in swelling
due to increased disc hydration over long term periods. The elastic stiffness of the intact IVD in compression [5, 58], flexion, torsion, and lateral bending [41] is maintained when stored in a freezer (-18 °C). It does not significantly alter the mechanical properties.

In contrast to the above, several investigators have reported subtle, freezing-induced changes in the time-dependent properties of various tissues. For example, Linde et al. [26] non-destructively tested cubes of human trabecular bone in compression and noted that freezing caused a 34 % increase in the energy dissipated during their loading cycle. Woo et al. [70] subjected the rabbit medial collateral ligament to cyclic tensile deformation and reported that freezing resulted in a 30-60 % decrease in the energy dissipated, but the difference was lost after 10 successive cycles of loading. More recent studies on porcine spines have shown that long term freezing does appear to increase the ultimate compressive load and energy absorbed to failure [5]. Additionally, compressive creep behaviors were altered by freezing [4]. Thus, the validity of mechanical properties measured from previously frozen material is often questioned. The argument being that the ice crystal growth and osmotic changes during the freezing and thawing process disrupts the soft tissues which in turn altered the mechanical integrity of the structure being studied.

In summary, the effects of freezing on the biomechanical properties of spinal specimens have been reported in several previous studies [4, 7, 14, 41, 51, 58]. However, most of them used a single FSU or just the IVD to test their hypotheses. Also, the studies measured changes in the mechanical properties after a single occurrence of freezing. But, as mentioned before, since most biomechanical studies involving whole body joints follow multi-phased study design and complex
Experimental protocols and preparations, the specimens are subjected to one or more freeze-thaw cycles. This exposes them to a variety of ambient conditions that may affect its load-displacement characteristics. The effect of multiple freeze–thaw cycles on the mechanical properties of the multisegmented spinal specimens remains unknown.

In the current study we address this practical problem, relevant to the biomechanical studies of whole body joints. We chose a multi-segmented human spinal specimen from the lumbosacral region (L3-S1) as our specimen. It includes different types of tissues, hard bone, intervertebral disc, various spinal ligaments, and articulating synovial joints including hyaline cartilage. Some of these are located closer to the outer surface and, therefore, may be affected more by the environment of moisture, freezing, and thawing. We expect that the results from this study would be useful for researchers in designing better experimentation protocols for carrying out multi-staged biomechanical studies.
CHAPTER III

FLEXIBILITY SPINE TESTING MACHINE

3.1 Overview

As discussed in Chapter II, new implants and new surgical approaches should be tested for primary stability through standardized in-vitro laboratory tests before being accepted for clinical use, in order to decide the most appropriate approach. Because of the complexity of simulating in-vivo motion with in-vitro testing, there are still no standard biomechanical tests for evaluating spinal structures. Based on generally accepted methods described in earlier studies [16, 43], most research groups have adopted flexibility testing as their standard. Flexibility testing is based on simplified, uniform loading conditions, which would allow the results of various research groups working on spine biomechanics to be comparable.

This chapter discusses a new, in-vitro, biomechanical testing apparatus to determine the continuous, three-dimensional, load-displacement characteristics of single or multi-segmented spinal specimens. This design for a spine test machine uses the flexibility testing protocol. It is capable of applying a pure bending moment about the three orthogonal directions individually with no constraints on the resulting motion of the specimen. The loads can be applied at user-chosen rates with continuous recording of the applied load and 3D displacements. Thus, this testing device provides a tool for many kinds of stability tests for basic research in the spine.
3.2 Performance Capabilities of the Flexibility Spine Testing Machine

The design for the test frame was adapted from an existing machine in the Division of Orthopaedic Engineering Research, University of British Columbia, Vancouver, British Columbia, Canada [18]. The Flexibility Spine Tester has the following performance capabilities:

1. The test frame is adaptable for fixation and testing of single or multi-segmented specimens from all regions of a spine; namely cervical, thoracic, and lumbosacral.

2. The loading actuator is capable of applying a physiological bending moment to the specimen while allowing linear translations and rotations so as to accommodate for the 6 degrees of freedom and coupled motion behavior of the spine observed in-vivo (Figure 3.1).

3. The machine is capable of carrying out flexibility tests in three orthogonal directions. The corresponding loading directions in flexion/extension, lateral bending and axial rotation are interchangeable without manipulation of the specimen (Figure 3.2).

![Coupled motion schematic](image)

Figure 3.1 Schematic showing coupled motion characteristics in a lumbar spine. In lateral bending there is coupled axial rotation.
4. The machine is capable of continuous rotation in both positive and negative directions (forward–backward or left–right) up to a predetermined maximum and minimum moments. The servo mechanism on the machine is capable of applying torques up to ± 10 Nm at a rated speed of 3500 RPM. 1 RPM is 360°/s.

5. The test machine incorporates a compressive follower load mechanism to simulate body weight and musculature on the in-vitro spinal specimen using a pulley and lever mechanism to apply a static load (Figure 3.3). The compressive load follows along the COR at each level in the multi-segmented specimen.
6. The test machine is synchronized with an optoelectronic 3D motion analysis system (Figure 3.4) to measure the linear and angular displacements of the vertebral bodies corresponding to the applied load in real time. The infra-red camera system (Optotrak CERTUS, Northern Digital Inc., Waterloo, Ontario, Canada) has built-in software functionality to calculate relative kinematic transformations of rigid bodies and save data in spreadsheet format for further analysis.

Figure 3.4 An optoelectronic camera system with accuracy of ±0.1 mm and ±0.13 degrees was used to capture the 3D kinematics of the specimen during flexibility tests.
7. The spine testing machine is operated through a graphical user interface (GUI) to control the entire application (Figure 3.5). It allows for user input parameters such as maximum and minimum loads, rate of loading, sampling rate, and file save properties. It also provides real time display of the load-displacement characteristics of the specimen during tests as well as the ability to save data in spreadsheet format for further analysis.

![Figure 3.5 Screenshot of the graphical user interface developed using LabVIEW v8.5 for operating the flexibility spine testing machine.](image)

3.3 Design and Development

The details of the flexibility spine testing machine including the loading actuator mechanism, servomechanism, motion control logic, data acquisition system, 3D motion analysis system, and other mechanical support structures are described here.
3.3.1 Loading Actuator Mechanism

The loading actuator for the spine test machine is comprised of a high-torque, low-angular backlash linear ball spline (Model: LBF15UU, THK America Inc., Schaumburg, IL). The linear ball spline allowed for linear translation of the loading arm while it transmitted the servo generated moment (Section 3.3.2) across from one end to the other. This allowed for unconstrained translation of the specimen in line with the axis of the applied moment. Within the ball spline, a single spline nut provided rigidity with minimal angular backlash (Figure 3.6).

![Figure 3.6 Schematic of the LBF model linear ball spline from THK America Inc.](image)

The actuator arm also contained two universal joints (Model: UJ-SS 100010, 100026, Belden Inc., Broadview, IL) to allow for unconstrained rotations in other directions while transmitting the rotary motion to the specimen. They are attached to both ends of the ball spline. One U-joint connected the spline shaft to the gearhead of a servomotor while a second U-joint was connected via a hollow aluminum tube to the sliding nut on the linear ball spline (Figure 3.7).

![Figure 3.7 Articulating arm unit showing the U-joint - Linear ball spline - U-joint linkage](image)
In summary, the loading actuator was used to apply a pure and uniform bending moment at the cranial end of the specimen while accommodating for linear translations and resulting rotations along all three directions in the multi-segmented specimen as depicted in Figure 3.8.

![Figure 3.8 Schematic of the loading actuator positions to simulate physiological bending motions on a specimen along a 3D coordinate system while allowing it to move unconstrained in other directions.](image)

3.3.2 Servomotor System

A brushless AC servomotor (Model: AKM22E-VBNPC, Danaher Motion Inc., Radford, VA) with an in-line, low-backlash planetary gearbox with 25:1 reduction ratio (Figure 3.9) was used to generate the desired torque across the actuator arm to the cranial-most vertebra of the specimen. The AC servomotor was powered by a serial servo drive module (Model: S20360-VTS, Danaher Motion Inc., Radford, VA) capable of velocity and torque feedback to the controller (Figure 3.10).
3.3.3 Motion Control System

The servomotor was controlled by a computer based motion control card (PXI 7350, National Instruments, Austin, TX) and LabVIEW (Version 8.5) software. The motion control card has a dual-processor for embedded real-time control and high speed communications while enabling motion functions from the host PC. It has the capability for a combination of servo and stepper motor controllers up to four independent axes of motion. The current application used servo axes to control the brushless servo motor in a closed-loop mode. The axes used quadrature encoders for feedback control and provided analog command outputs with an industry-standard...
range of \(\pm 10\) V. The interface between the motion controller card and the servomotor system was provided through a Universal Motion Interface unit (UMI-7764, NI, Austin, TX) (Figure 3.11). An overview of the motion control system for the flexibility spine testing machine between the host PC (PXI Chassis), with a motion control card, universal motion interface unit, and compact servo drive to the servomotor is shown in Figure 3.12.

Figure 3.11 NI Universal Motion Interface unit (UMI-7764) for connecting NI motion control hardware to a third party servo drive.

Figure 3.12 Schematic showing the motion control system for the flexibility spine tester application.
3.3.4 Data Acquisition (DAQ) System

The DAQ system measured the applied and observed loads across the multi-segmented spinal specimen during flexibility tests. It comprised of a PXI-slot-compatible multi-series DAQ card, PXI 6251 (NI, Austin, TX). It served three primary functions: to acquire, analyze, and display the data obtained from the reaction torque sensor. The PXI 6251 DAQ system is a high speed multi-function data acquisition unit with 16 analog inputs at 16-bits and 1.25 MS/s (1 MS/s scanning), 2 analog outputs at 16-bits and 2.8 MS/s (2µs full-scale reading), and 24 TTL/CMOS digital I/O lines, 7 programmable input ranges (±100 mV to ±10 V) per channel, two 32-bit 80 MHz counter/timers. In all, it provides high resolution and fast sampling rates.

An I/O signal connector block (SCC-68) from NI was used to connect the transducers to the DAQ device (Figure 3.13). The SCC is designed for small measurement systems with few channels of each signal type, or for portable applications. The SCC systems also offer comprehensive and flexible signal connectivity options.

Figure 3.13 SCC-68 I/O connector block with 4 SCC signal conditioning slots for M-series plug-in DAQ devices.
A torque sensor (Model: TRT-100, Transducer Techniques Inc., Temecula, CA) was used to monitor the servo generated moment applied to the specimen (Figure 3.14). It was mounted in between the actuator arm and the specimen, on the upper fixture block as shown in Figure 3.15.

Figure 3.14 TRT-100 general purpose reaction torque sensor from Transducer Technique Inc. It is rated for measuring ± 100 Lbf-in (± 11.3 Nm).

Figure 3.15 Schematic showing the reaction torque sensor (TRT-100) mounted in between the loading actuator and the upper fixture block of the specimen.

The TRT-100 is rated to measure a maximum load of 11.3 Nm (100 Lbf-in) in either direction, with a rated output of 2 mV/V. In addition, it is also a TEDS IEEE 1451.4 compliant sensor. TEDS, or Transducer Electronic Data Sheet, is a set of electronic data in a standardized format defined within the IEEE 1451.4 standard that is stored in an EEPROM on the transducer itself. This data specifies the type of sensor present, describes its interface, and gives technical information such as sensitivity, bridge type, excitation, etc. to a Digital Panel Mount meter (DPM3) as shown in Figure 3.16.
Once a TEDS Sensor has been detected by the smart load cell meter, it displays a front panel TEDS indicator light, reads the EEPROM and stores the information in memory and performs an automatic configuration. The automatic system configuration function performs all steps needed to calibrate the sensor and the load cell meter. This includes the configured precision of 32 bits, 19 bits or 11 bits and the configured excitation voltage.

The raw voltage signal from the reaction torque sensor is conditioned in the DPM3 and provided as input to the DAQ system via the SCC-68 (Figure 3.17) which in turn is used as a feedback signal for velocity control of the motion control hardware mentioned in Section 3.3.3. Using the feedback signal, the motion hardware controlled the servomotor to flex and extend the specimen in a continuous cyclic motion. During motion the applied moment gets transmitted through the specimen to the caudal end where a 6-axes force/torque sensor (Model: Delta Net F/T, ATI Industrial automation, Apex, NC) was rigidly fixed to measure the ground reaction forces as shown in Figure 3.18.
Figure 3.17 Schematic showing signal connections from the reaction torque sensor to the DAQ system for establishing the feedback control loop.

Figure 3.18 Schematic showing a lumbar spine model mounted in between the reaction torque sensor and the six-axis network force transducer.

The Delta 6-axis force and torque transducer simultaneously measured the forces $F_x$, $F_y$, $F_z$ and moments $M_x$, $M_y$, $M_z$ about all three directions. The Delta is connected to a Net F/T system which communicates via Ethernet/IP, CAN Bus, Ethernet, and is compatible with DeviceNet. In our application we used it as a server on Ethernet/IP network thus making it easy to set up and monitor the device in real-time using the LabVIEW TCP/IP communication module. A schematic showing the
Net F/T components is given below in Figure 3.19. The Net F/T system sends the six-axis force data over Ethernet/IP using user defined protocol (UDP). This method of fast data collection is called Raw Data Transfer (RDT). RDT provides an easy method to get the forces, torques, and status codes of the Net F/T system.

Figure 3.19 Schematic of the ATI Net F/T system for measuring the six axes forces at the caudal end of the specimen.

3.3.5 LabVIEW Control Algorithm and Graphical User Interface (GUI)

The flexibility spine tester application was controlled using a custom-developed code written in LabVIEW v 8.5 (National Instruments, Austin, TX). The labview program controlled the servomotor through a NI motion control card and universal motion interface card. The feedback signal (applied moment) was obtained from the reaction torque sensor mounted at the end of the loading actuator and through a signal connector block and multi-function DAQ card. A schematic of the control block diagram is shown in Figure 3.20. A graphical user interface (GUI) was developed using LabVIEW to provide user control for the flexibility spine tester application. A screenshot of the GUI is shown in Figure 3.21.
Figure 3.20 Schematic of the control block diagram for servo motor using torque feedback.

Figure 3.21 A screenshot of the graphical user interface for the spine tester application developed using LabVIEW v8.5.

The user interface had a tabbed structure for better organization as well as ease to navigate. The main window contained controls which the user could input sampling rate, speed of loading, upper and lower torque set points, and number of loading cycles. It also had the START and STOP controls for the application. The other windows displayed real-time values of the system parameters such as reaction torques, motor axes data, and Net F/T force data.
3.3.6 Optoelectronic Camera System

An optoelectronic camera system (Optotrak Certus, Northern Digital Inc., Waterloo, Ontario, Canada) was used to measure the linear and angular displacements of each rigid body within a multi-segmented spinal specimen during flexibility tests. The camera system tracked the three dimensional (3D) positions of infra-red light emitting diodes (IREDs) within its field of view (Figure 3.22). 

![Figure 3.22 Optotrak camera system positioned approximately 2 meters in front of the test specimen to capture 3D kinematics of the spinal vertebral bodies during a flexibility test.](image)

The optoelectronic camera system measured the three-dimensional position of each IRED marker to within 0.1 mm in plane (x, y-axes) and 0.15 mm out of plane (z-axis). It has an accuracy of ±0.1 mm and ±0.13 degrees in measuring the 3D rigid body motion, as stated by the manufacturer. In order to determine the position of a body in three-dimensional space, the coordinates of at least three non-collinear points on that body are required. In the current study, attached to each vertebral level was a set of marker array with 4 IREDs as shown in Figure 3.23. The extra redundant point
increased the measurement accuracy. The marker arrays faced the Optottrak system and were rigidly attached to Kirschner wires using hex stand-offs and small screws as shown in Figure 3.24.

Figure 3.23 A “marker array” consisted of 4 IR-LEDs attached onto a Plexiglas substrate.

Figure 3.24 Schematic showing the specimen fixed in the test machine with IRED marker arrays rigidly attached to individual vertebral levels.

The camera system also incorporated a pre-calibrated digitizing tool (Figure 3.25) for mapping additional points of interest in the experimental setup. This helped in creating an outline of the vertebral level being studied by mapping prominent bony landmarks such as the vertebral body, pedicles and spinous process.
3.3.7 Mechanical Aspects of the Flexibility Spine Testing Machine

A. Main Structural Frame

The entire structural framework of the flexibility spine tester was built using solid aluminum extrusions (80/20 Inc., Columbia City, IN) as shown in Figure 3.26.

This made the design modular for future modifications and at the same time very stable. The frame was designed to hold the actuator arm in each of the three orthogonal test directions. These three motion axes aligned at the center of a test platform as shown in Figure 3.27. The specimen was rigidly fixed to the test platform.
at this position such that the local axes of the specimen were aligned with the motion axes of the test frame. A latch fixed the servomotor onto the loading frame while four screws fixed the upper fixture block to a plate screwed to the dental stone potting of the cranial vertebra of the specimen. After one axis of motion was tested, the motor and articulating arm unit was moved to the next motion axis to apply loads in flexion extension, lateral bending, or axial rotation. The test frame was rigidly anchored at the back using deadweights and to maintain a low center of gravity. Additional structural support was provided from the front in form of angled feet which ensured that the structure withstood the weight of the experiment.

Figure 3.27 Schematic of the structural frame design for the flexibility spine testing machine. The adjustable platform can be raised or lowered depending on the specimen’s length. The dotted line represents imaginary extensions of the loading arm that meet concurrently at the center.
The jigs and fixtures used in the test setup were designed and modeled using PTC ProEngineer 3D CAD software. A few examples are shown below in Figures 3.28 and 3.29. The fixtures were machined at the workshop facilities in Department of Biomedical Engineering and Department of Mechanical Engineering at University of Akron.

Figure 3.28 A ProE assembly model of the specimen plate holding the cranial vertebra to a block mounting the torque cell and attached to the loading actuator.

Figure 3.29 A ProE assembly model of the fixture for holding the servomotor to the 80/20 test frame.

B. Counterbalance Mechanism

A 500 g weight on a threaded rod counterbalanced the static moment caused by the weight of the actuator arm as shown in Figure 3.30.

Figure 3.30 A weight was attached to the upper fixture block to counterbalance the static moment caused by the actuator arm.
The weight of the articulating arm, upper specimen fixture, and dental stone potting placed above the specimen was offsetted by a counterweight (approximately 2000 g) which was suspended by a cord over a pulley (Figure 3.31). The pulley was hung from a custom-built XY table that allowed the counterweight to follow the x-y translations of the cranial end of the spinal specimen.

Figure 3.31 Counterbalance mechanism for compensating the added weight of the actuator arm, upper specimen fixture, and dental stone block on the specimen. A custom-built xy table above allowed the counterbalance to follow passively as the specimen moves in its 6 DOF.

C. Follower Compressive Preload

All the tests in flexion-extension were conducted with and without the presence of a compressive follower preload on the specimen. A pair of follower loads simulated the effect of body weight and musculature on the spinal segment during in-vitro tests [46]. A schematic showing the principle of follower load in a lumbosacral spine segment is shown in Figure 3.32.
Under the compressive follower load, the resultant internal load on the spine remains tangential to the spinal curve, passing through the center of rotation of each vertebral segment thus allowing the spinal segment to retain its natural curvature and support large compressive loads. The magnitude of the follower preload was chosen to be approximately 660 N since it falls within the range of loads that the lumbar spine segment is usually subjected to within our body [69]. The magnitude of the follower compressive pre-load applied also depends upon the region of spine being tested as well as upon the physical activity being simulated during the in-vitro test.

Custom stainless steel frames were attached non-invasively to the vertebral bodies of L4 and L5. The follower load frames were attached bilaterally around the pedicles and compressed against the anterior aspect of the vertebral body as shown in Figure 3.33.

Figure 3.33: Follower load frame attachment to the L2 vertebral body on a spine model.
The path of the follower preload was optimized in the neutral position to minimize rotation on the mid-sagittal plane upon application of the static preload. The follower preload should be applied at the center of rotation of each segment. The path of the applied compressive load was fine-tuned by adjusting the components on the frames to alter the anterior-posterior position of the cable at each level as shown in Figure 3.34.

![Figure 3.34 A right lateral view of the follower load path in a lumbosacral specimen undergoing flexion moment.](image)

The load was applied bilaterally by cables running along the specimen. Deadweights (11.34 kg) were hung at the ends of these cables. A pulley mechanism amplified the force caused by this deadweight (2 x 11.34 kg) by a factor of approximately 3 (68 kg) (Figure 3.36). This simulated a total weight of approximately 70 kg (average upper body weight of a person) on the specimen (Figure 3.35). This amount of compressive follower preload was chosen since it covers a significant portion of the physiologic range experienced by spine during most daily activities [69].
Figure 3.35: A compressive follower preload of ~ 660 N distributed on either side of a lumbosacral specimen

Figure 3.36 Schematic of the pulley arrangement at the base of the test frame to add a compressive follower load of 75 lbs (333.63 N) on either side of the specimen.

3.4 Performance Evaluation of the Flexibility Spine Testing Machine

The flexibility spine test apparatus was validated in terms of its compliance in applying a pure moment to the specimen using a series of performance tests.

3.4.1 Compliance Testing

The initial testing comprised of verifying proper application and measurement of loads by the two force transducers mounted at either end of the specimen. This was
performed by applying a known moment to a very stiff model (such as, rigid block of aluminum) mounted in between the force transducers. The loads measured on the 6-axes load cell was used as the standard for comparison. The sensitivity of the ATI Net F/T model was 66 N/V in $F_x$, $F_y$ and 198 N/V in $F_z$. The torque sensitivity was specified at 6 Nm/V. Its accuracy was 32 counts/N for the forces and 533.34 counts/Nm for the torques. The applied versus the measured torque data about the principal axis of rotation for the loading actuator were compared in all three orthogonal directions (Figure 3.37 A, B, and C). The differences in the maximum applied and resultant torque were used to carry out an uncertainty analysis of the measuring apparatus (Table 3.1).

![Figure 3.37 (A)](image1.png)

**Figure 3.37 (A)** Comparison of applied bending torque versus the measured torques for a compliance test in FE (rotation about y-axis). The load application was at a constant rate of 1°/s for 3 continuous cycles up to 10 Nm.

![Figure 3.37 (B)](image2.png)

**Figure 3.37 (B)** Comparison of applied bending torque versus the measured torques for a compliance test in LB (rotation about x-axis). The load application was at a constant rate of 1°/s for 3 continuous cycles up to 10 Nm.
Figure 3.37 (C) Comparison of applied bending torque versus the measured torques for a compliance test in AR (rotation about z-axis). The load application was at a constant rate of 1°/s for 3 continuous cycles up to 10 Nm.

Table 3.1 Measurement Uncertainty Analysis for the Flexibility Spine Testing Apparatus. The Table shows % Error in Between the Applied and Measured Torques from the Compliance Tests in each of the Three Orthogonal Loading Directions.

<table>
<thead>
<tr>
<th>Load Cycle</th>
<th>Flexion-Extension (FE)</th>
<th>Lateral Bending (LB)</th>
<th>Axial Rotation (AR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Applied Torque (Nm)</td>
<td>Measured Torque (Nm)</td>
<td>Error (%)</td>
</tr>
<tr>
<td>1</td>
<td>Max 10.1</td>
<td>-10.0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Min -10.1</td>
<td>10.1</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>Max 10.1</td>
<td>-10.0</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>Min -10.1</td>
<td>10.1</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>Max 10.1</td>
<td>-10.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Min -10.1</td>
<td>10.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Mean</td>
<td>1.5</td>
<td>2.1</td>
<td>3.5</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.4</td>
<td>1.1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

As seen in Figure 3.37 and Table 3.1, the applied torque was verified by the 6-axes force transducer mounted at the bottom of the rigid block in all the three loading cases. The mean and standard deviation in error of 1.5 – 3.5 % was within the manufacturer specified sensitivity ranges for the force transducer. This ensured that the motion control logic written using torque feedback could handle application of loads within the ranges of the designed servomechanism. A slight time delay was noticed in between the applied and measured data as seen in Figure 3.37. This was
possibly due to the fixture attachments between the model and the force transducers. However, as seen the time delay remained consistent across several test cases thus validating the measurement uncertainty in test apparatus.

3.4.2 Pure Bending Moment

Another main criterion in evaluating performance of the flexibility spine tester was its ability to apply a pure, uniform bending moment across all vertebral levels in a multi-segmented spinal specimen, while allowing it to move freely in the other five degrees of freedom. This was verified by comparing data from the reaction torque sensor and the six-axis load cell mounted at the cranial and caudal vertebrae of the specimen. The results for a test case in lateral bending (simulating a bending moment about x-axis, $\pm M_x$) are shown in Figure 3.38.

![Graph](image)

Figure 3.38 Output of the reaction torque sensor and ATI 6-axis force transducer showing the five load cycles of applied moment at the cranial vertebra of the specimen and the transmitted moment at the fixed caudal level.

As shown above, a bending moment of $\pm 7.5$ Nm was successfully applied for 5 cycles to the cranial vertebra (L3) of the specimen. Since, the specimen was rigidly fixed to the test frame at its caudal end, the bending moment was transmitted through
the subsequent vertebral levels (L4, L5, and Sacrum) and resulted in left and right lateral bending of the specimen. The loads measured by the 6-axis force transducer reflect the transmitted moments at the caudal vertebra (Sacrum) of the specimen. We see that the applied bending moment ($\pm M_y$) remained pure and uniform across all the four levels of the multi-segmented spinal specimen. We also observe a pattern in the moments recorded along the other two axes of the force transducer, namely $\pm M_y$ and $\pm M_z$. However their magnitude is minimal and negligible (approx. 10% of the applied moment along y-axis which is also the predominant motion that is occurring). They were thus, within tolerable limits as defined in the design criterion for the flexibility spine tester. The 6-axis force transducer also measured the force components along the three orthogonal axes that resulted due to the applied bending moment (Figure 3.39).

![Graph showing Applied Torque (TRT-100) vs. Transmitted forces (ATI 6-axis F/T)](image)

Figure 3.39 Output of the reaction torque sensor and ATI 6-axis force transducer showing the five load cycles of applied moment at the cranial vertebra of the specimen and the transmitted forces at the fixed caudal level.

As seen in Figure 3.39, in addition to the ground reaction force ($F_z$) there were also shear forces ($F_x$ and $F_y$) acting on the specimen. But observations during actual experiments showed that this pattern was largely attributed to the misalignment of the
specimen in the center of the force transducer and the loading axes of the test frame, as well as the attachment of the force transducer to the fixtures holding the specimen. The magnitude of these forces, however, remained within tolerable limits. Similar results were observed for flexibility tests along the other two loading directions, namely flexion-extension ($\pm M_y$) and axial rotation ($\pm M_z$). This verified the performance of the spine tester to conduct in-vitro biomechanical tests on single, or multi-segmented spinal specimens using the flexibility protocol.

3.4.3 Coupled Motion Behavior

Another criterion for evaluating the performance of the flexibility spine tester was its ability to allow the spinal specimen to move in its natural coupled motion paths that occur in-vivo. For instance, a bending moment about an axis simulating flexion-extension inadvertently results in a rotation about other axes that causes lateral bending as well. Another example of coupled motion in spine is lateral bending coupled with axial rotation. This was verified using the three-dimensional kinematic data obtained from the optoelectronic camera system.

The three dimensional rotational data for each vertebral level for a test case in flexion-extension is shown in Figure 3.40 (A,B, and C). As seen the rotation of all the vertebral bodies about y-axis (flexion-extension) was mainly coupled with a rotation about x-axis (lateral bending). There was some axial rotation about z-axis as well, but the peak-to-peak remained fairly low. The translational data for the vertebral bodies for the same test case is shown in Figure 3.41 (A,B, and C) which remained consistent with the rotational data.
Figure 3.40 (A) Absolute angular displacements about y-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment about y-axis (± M_y) simulating FE.

Figure 3.40 (B) Absolute angular displacements about x-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment about y-axis (± M_y) simulating FE.

Figure 3.40 (C) Absolute angular displacements about z-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment about y-axis (± M_y) simulating FE.
Figure 3.4 (A) Absolute linear displacements along y-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment in FE.

Figure 3.4 (B) Absolute linear displacements along x-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment in FE.

Figure 3.4 (C) Absolute linear displacements along z-axis for each vertebral level in a lumbosacral spinal specimen subjected to a pure bending moment in FE.
As seen in Figure 3.41, a main rotation about y-axis resulted in a linear translation of the vertebral bodies along the z-axis (Figure 3.41 C) while the coupled motion caused a translation along x-axis (Figure 3.41 B). Figure 3.41 (A) shows translation component along y-axis which is seen to be fairly minimal as expected. These results indicated that the design with a linear ball spline actuator arm in allowing unrestrained motion of the spinal specimen along all the remaining five degrees of freedom while still applying a pure bending moment along the main loading axis was achieved.
CHAPTER IV

METHODOLOGY

This chapter describes in detail the experimental methods used for conducting the current biomechanical study to evaluate the effects of multiple freeze and thaw cycles on the three dimensional intervertebral kinematics of a human cadaveric lumbosacral spine.

4.1 Study Design

The study was designed to analyze the specimen’s overall and intervertebral range of motion (ROM) and laxity / neutral zone (NZ) over a period of eight days with eight sets of repeated flexibility tests in each of the four loading cases, namely FE, LB, AR, and FE+FL with intra-day and inter-day comparison.

The specimen was tested at a loading rate of about 1°/s up to a torque limit of ±7.5 Nm for 5 continuous loading cycles to simulate physiological bending movement and to account for the viscoelastic properties of the specimen. The testing order in FE, LB, AR, and FE+FL was randomized to reduce possible variability due to test sequence. These four test cases were repeated for a total of eight sets to obtain a total of 32 load-displacement curves within a day. These tests were repeated after the specimen was frozen at -20 °C for at least 24-48 hours and thawed at room temperature (21 °C to 24 °C) for 16-24 hours. The specimen was thawed,
tested and refrozen for a total of eight post-thaw tests. Care was taken to keep the specimen moist using 0.9 % saline solution throughout each test day. During storage the specimen was enclosed in a double sealed thick (2µm) thick plastic bag.

4.2 Specimen Selection and Preparation

A single fresh-frozen, intact cadaveric lumbar spine specimen composed of L3 to Sacrum was obtained from a 70 year old female donor through a local organ donation program. The specimen was carefully inspected for any signs of structural damage. Inclusion criteria consisted of absence of fracture to the spinal column or any bony diseases. Bone mineral density from full body DXA scan was 0.96 g/cm² with a T-Score of -0.5 which suggested that the bone quality was normal as per WHO classification. The protocol for handling cadaveric material followed the guidelines of the Centers for Disease Control and our institute’s safety regulations.

The specimen was prepared for testing by removing extraneous soft tissue, leaving the intervertebral discs, facet joints, and spinal ligaments intact as shown in Figure 4.1. It was wrapped in a saline soaked towel and kept frozen in a thick double sealed plastic bag at -20 °C until testing.

Figure 4.1 A L3-S1 specimen dissected of musculature and unwanted soft tissue structures while preserving the spinal ligaments and intervertebral discs.
The most cranial (L3) and caudal (S1) vertebral levels were embedded using dental stone in order to secure the specimen in the test apparatus. First, the specimen was oriented such that the L4-L5 disc space was horizontal, while L5-S1 had its approximate lordotic angle. Wood screws were partially inserted into the vertebral bodies in order to obtain additional mechanical advantage (anchoring) into the potting material. Second, dental stone powder was mixed with water to make a thick paste and poured into a mold around the vertebral body. Next, the cement was allowed to cure for 30 minutes to 1 hour after which the procedure was repeated for the other end. The sequence of steps for potting is shown in Figure 4.2. The completed specimen was wrapped in a paper towel soaked with saline and refrozen at -20 °C in a double sealed, 2 µm thick plastic bag until test day as shown in Figure 4.3.

![Figure 4.2 Potting of L3-S1 specimen with dental stone in preparation for biomechanical tests.](image1)

Figure 4.2 Potting of L3-S1 specimen with dental stone in preparation for biomechanical tests.

![Figure 4.3 Specimen during freeze storage at -20 °C. It was wrapped in saline soaked paper towel and double sealed in a 2 µm thick plastic bag.](image2)

Figure 4.3 Specimen during freeze storage at -20 °C. It was wrapped in saline soaked paper towel and double sealed in a 2 µm thick plastic bag.
4.3 Test Setup

The following sections describe the pre-test preparations for the freeze-thaw flexibility study.

4.3.1 Mechanical Fixtures and Attachments

The specimen was allowed to thaw at room temperature (21 °C-24 °C) for 16 to 24 hours prior to testing. Pre-test preparations included attachment of follower load (FL) fixtures to L4, L5 vertebral bodies and infrared emitting diode (IRED) markers to individual vertebrae. The FL jig was secured with a pair of flexible wires looped around the pedicles and tightened with a screw anteriorly as shown in Figure 4.4.

![Figure 4.4 Attachment of the follower load apparatus on L4 and L5 vertebral bodies.](image)

The follower load fixture attached to the specimen was positioned to guide the cable along the natural lordotic curvature of the specimen. A compressive load applied through the follower load cables will result in only compression in each motion segment, without any flexion or extension.

Marker arrays, including four IREDs mounted on a Plexiglas substrate, were rigidly attached to the individual vertebral levels at L3, L4, L5 and S1 with the help of hex stand-offs mounted onto Kirschner wires. A power drill was used for inserting the
K-wires through the laminae into the vertebral bodies from the posterior direction as shown in Figure 4.5. Thereafter, the prepared specimen was rigidly fixed onto the test frame with the anatomical planes of the specimen aligned to the loading axes of the test frame using a laser positioning tool as shown in Figure 4.6. The completed test setup is shown in Figure 4.7.

Figure 4.5 Insertion of the Kirscher wires into the vertebral bodies for attachment of Optotrak marker arrays.

Figure 4.6 Alignment of the specimen with respect to the loading axes of the test frame using a pair of laser position tools.

Figure 4.7 Schematic showing the specimen mounted on the test frame and attached to the loading actuator with counterbalance and FL apparatus secured onto it.
4.3.2 Optoelectronic Camera Setup

The IRED marker arrays attached to each vertebra were characterized into rigid bodies using the optoelectronic camera system. The default global coordinate system of the camera was transformed to a local coordinate system near the experiment. The new coordinate system was defined using a fixed marker array that was aligned with the specimen and the loading axes of the test frame using a laser positioning tool (Figure 4.8). The axes of rotation were as follows; y-axis simulating FE, x-axis simulating LB, and z-axis simulating AR as shown in Figure 4.9.

Figure 4.8 Alignment of the optoelectronic camera’s fixed local coordinate system with respect to the specimen and loading actuator using a laser positioning tool.

Figure 4.9 Rigid body characterization of IRED marker arrays at individual vertebral levels with respect to the fixed local coordinate system.
The 3D kinematic data (3 rotations: R_z, R_y, and R_x and 3 translations: T_x, T_y, and T_z) of the individual rigid bodies were calculated with respect to the fixed origin (Figure 4.9). The built-in software of the camera system provided the functionality for calculating relative transformations of any two rigid bodies individually in real-time to provide the kinematic data for adjacent vertebral levels, namely the relative motion between L3-L4, L4-L5, and L5-S1. Also, imaginary pseudo-markers were ‘attached’ to prominent bony landmarks of interest (Figure 4.9) to create an anatomical map for each vertebral body. The imaginary markers were created using a calibrated digitizing tool provided with the camera system. The use of these imaginary markers provided additional data for use in advanced kinematic analysis using position vectors to calculate center of rotation, helical axis of motion, etc. The fully constructed digital image of the specimen as viewed using the optoelectronic camera software is shown in Figure 4.10.

Figure 4.10 A screenshot showing the initial 3D positions of all IRED markers (real and imaginary) attached to the specimen as captured by the camera system.
4.4 Test Protocol

Biomechanical tests were carried out to measure the flexibility of the specimen in FE, LB, and AR. All the tests were performed under load control at a loading rate of about 1°/s up to a bending moment of ±7.5 Nm for 5 continuous cycles. The specimen was tested in FE with and without 660 N of compressive preload. All motions started and finished in the unloaded neutral position. The load magnitudes were consistent with the current standards in spine biomechanical testing [69] and were chosen to restrict the motion within the physiologic range of spine, so as not to damage the specimen. The specimen was allowed to move in an unconstrained manner in the remaining five degrees of freedom. The motion across each individual vertebra was measured by the optoelectronic motion analysis system (Optotrak Certus, Northern Digital Inc., Waterloo, Ontario, Canada). The total time of the freeze-thaw procedures and the total duration that the specimen was in ambient conditions was recorded (Figure 4.11). A total of 256 flexibility tests were carried out in this study over a period of eight test days with eight repeated test sets within each day in all the four loading cases.

<table>
<thead>
<tr>
<th>Test day</th>
<th>Freeze-Thaw Procedures</th>
<th>Test Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>2</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>3</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>4</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>5</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>6</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>7</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
<tr>
<td>8</td>
<td>16 Days, 11 hrs</td>
<td>8.5 hrs</td>
</tr>
</tbody>
</table>

Figure 4.11 Schematic of the total time of freeze-thaw procedures and test durations of the specimen for the eight test days. The blue line indicates the freezing duration, the green line is representative of the thawing time, and the red line is the actual test duration.
4.5 Data Analysis

The applied load versus displacement data were obtained for analysis using National Instruments hardware with LabVIEW software and NDI Optotrak 3D-position camera systems respectively. Data were sampled at 20 Hz and saved in a format compatible with Microsoft Excel. Due to the unique nature of each of the three testing modalities in flexion/extension, lateral bending, and axial rotation, individual templates were created in order to import the test data and calculate both the ROM and NZ and generate the hysteresis plots for visual data interpretation. All analyzed data were then saved to the specimen’s unique directory on the PC for future use.

The 3D-motion, resulting from each load application, was presented by its six motion components: x, y, and z (translations of the specimen along x, y, and z-axes) and R_x, R_y, R_z (rotations of the specimen about x, y, and z-axes). The mechanical behavior of the specimen was documented in the form of flexibility curves by plotting on the horizontal axis the applied moment in Newton meters, and on the vertical axis the resulting rotation components of the specimen in degrees as shown in Figure 4.12. This method of presentation was adopted as the load is the independent variable. One of the three rotations corresponds to the main motion, and the others are a result of coupled motions inherent to spine.

The moment and kinematics data, both sampled at 20 Hz, were matched based on maximum and minimum peaks. The first four cycles served to precondition the specimen [43, 69] and minimize the viscoelastic effects (time-dependent material properties) in the specimen. The load-displacement characteristics for the last loading cycle in each flexibility test were analyzed and the ROM and NZ variables compared.
4.5.1 Range of Motion (ROM)

Only the range of motion (ROM) for the primary axis of rotation was considered for analysis. For FE, rotations about the y-axis was considered and coupled rotations about x and z-axes were ignored (Figure 4.13). The red, blue, and green traces correspond to flexion/extension, lateral bending and torsion respectively. It can be seen from the figure that during a flexion/extension test, the magnitude of the flexion/extension angle is greater than the magnitude of the other two angles. This was as expected, and any nonzero values of the off-axis angular displacement data were due to coupled motion of the specimen and misalignment of the local coordinate system with the anatomical axes. Similarly, only rotations about the x-axis were considered for LB and only rotations about the z-axis were considered for AR.

The ROM was calculated separately for both directions of rotation. The positive ROM was the difference between the maximum angular displacement and the neutral point while negative ROM was the difference between the neutral point and minimum angular rotation.
Figure 4.13 A graph showing the angular displacement data of the IRED marker array fixed to the cranial vertebra (L3) of the spinal specimen. The primary axis of rotation about y-axis simulates flexion-extension with some coupled rotations about x and z axes.

4.5.2 Neutral Zone (NZ)

The NZ is a measurement of the laxity in the spinal specimen. It describes the magnitude of motion over which the joint moves essentially freely without applied loading. It was calculated as the maximum rotation of the specimen at zero Nm of applied load as shown in Figure 4.12. The intercepts of the flexibility curve with the abscissa represents twice the neutral zone. The NZ characterizes the laxity of the specimen as a measurement of its mechanical resistance and acts as an indicator of the specimen’s stability.

4.6 Statistical Methods

Standard statistical techniques were used to determine the sample size for the current study and for analyzing the data obtained from the in-vitro biomechanical testing of the multi-segmented cadaveric lumbosacral spine.
4.6.1 Sample Size Determination

Sample size required for this study was determined using the following equation:

\[
    n \geq 2 \cdot \left( \frac{\sigma}{\delta} \right)^2 \left( t_{e(\nu)} + t_{2|1-P|} \right)^2
\]

\[\text{Eq 4.1}\]

where, \( n \) : sample size, \( \sigma \) : true standard deviation, \( \delta \) : smallest true difference to be detected, \( \nu \) : degrees of freedom, \( \alpha \) : significance level, \( P \) : power of the test, \( t \) : two-tailed t-table value [60]. The number of repeated measures was calculated to be eight with an assumed true standard deviation of 15 %, the smallest true difference to be detected was proposed to be 30 %, the power of the test assumed to be 85 % and a significance level of 0.05 was assumed in order to determine the sample size.

4.6.2 Experimental Model

The statistical model assumed for this study was the one-factor repeated measures analysis of variance (ANOVA). The main effect was defined as the test day. It evaluated the significant differences in ROM and NZ of the specimen in between test days (Inter-day variations). Due to the small sample size, a non-parametric statistical technique; Wilcoxon Signed-Rank test was used to analyze the data obtained for each test day (Intra-day variations). If there were any significant differences in the results, a post-hoc Student-Newman Kewl (SNK) tests was conducted to detect which of the test data were significantly different than the rest. All statistical analyses were performed using SAS software (SAS Institute, Cary, NC).
5.1 Overview

The results discussed in this chapter are obtained from the flexibility biomechanical testing of a single, intact human lumbosacral spine motion segment. A multi-segmented (L3-S1) cadaveric spinal specimen was tested in a repeated measures experimental design (n=8) to evaluate the effects of frozen storage (-20 °C) and subsequent thawing at room temperature (21 °C) on its in-vitro kinematic behavior. The results investigated the effects of long-term freezing and exposure to ambient temperature on the test performance of human cadaveric biomechanical specimens.

A one-way repeated measures analysis of variance (R-ANOVA) compared the inter-day variations in the range of motion (ROM) and neutral zone (NZ) of the specimen. It yielded a p-value less than 0.0001 for the whole specimen (L3-S1) as well as for the inter-segmental levels (L3-L4, L4-L5, and L5-S1) in all four flexibility loading cases of flexion/extension (FE), lateral bending (LB), axial rotation (AR), and FE with a follower compressive preload (FE+FL). These results indicated a significant effect of multiple freeze-thaw cycles on the in-vitro biomechanics of the specimen. Similarly, results from the intra-day repeated measures flexibility tests were evaluated for any significant changes using non parametric Wilcoxon Signed rank test. The p-value was found to be greater than the accepted level of significance.
in all the test cases, for the whole specimen and the inter-segmental levels, which suggested that there were no significant within-day variations in the kinematic behavior of the spinal specimen due to the effects of long exposure to the ambient temperature and testing conditions within each test day.

5.2 Load-Displacement Characteristics

By the methods outlined in Chapter 4, the angular displacement data for the individual vertebral levels as well as their relative transformations with respect to each other in response to the applied bending moment for all the four loading cases (FE, LB, AR, and FE+FL) were analyzed. Figure 5.1 shows a typical plot of the observed load-displacement characteristics for the whole specimen when subjected to 5 continuous load cycles at 1°/s up to ± 7.5 Nm.

![Flexibility curves for L3-S1 (Entire test)](image)

Figure 5.1 Overall flexibility curves of a typical lumbosacral spine segment subjected to a pure bending moment of ±7.5 Nm at 1°/s for 5 continuous cycles in FE, LB, AR, and FE+FL.

As shown in Figure 5.1, the flexibility curve yielded an S-shaped curve with hysteresis as a result of the viscoelastic properties inherent to human biological tissue. In order to minimize the effects of these time-dependent and loading rate-dependent material deformations in the specimen, the specimen was tested for 5 cycles and ROM
and NZ were analyzed at the last (5th) loading cycle in all test cases. The typical flexibility curves for the whole specimen and the intervertebral levels in the 5th loading cycle are shown in Figures 5.2 to 5.5.

Figure 5.2 Overall and inter-segmental flexibility curves of a typical lumbosacral spine segment for the last (5th) flexion-extension loading cycle.

Figure 5.3 Overall and inter-segmental flexibility curves of a typical lumbosacral spine segment for the last (5th) lateral bending loading cycle.
Figure 5.4 Overall and inter-segmental flexibility curves of a typical lumbosacral spine segment for the last (5th) axial rotation loading cycle.

Figure 5.5 Overall and inter-segmental flexibility curves of a typical lumbosacral spine segment for the last (5th) flexion-extension loading cycle with compressive follower preload.

The results shown in Figures 5.2 to 5.5 were obtained from the first repeated measures flexibility test set on Test day 1. The study design yielded a total of 32 flexibility curves (8 test sets, each in four loading cases, FE, LB, AR, and FE+FL) for each test day. A total of 8 post-thaw test days provided 256 flexibility curves to be analyzed.
5.3 Range of Motion (ROM)

The intra-day and inter-day comparison of the experimental data for the specimen’s total and inter-segmental ROM from the repeated measures freeze-thaw study are presented in this section.

5.3.1 Intra-Day Variations due to Repeated Flexibility Tests

The observed ROM value from the intra-day repeated measures flexibility tests did not show any considerable changes in between test sets for FE (p=0.87), LB (p=0.83), AR (p=0.59), and FE+FL (p=0.66). The ROM values remained consistent and repeatable. A typical case for the last test day (8th post-thaw test) is shown below in Figure 5.6. The other test days were also observed to follow a similar pattern as shown in Figures 5.7 to 5.10.

![Graph showing ROM at L3-S1 Intra-day results (Test day 8)](image)

Figure 5.6 Absolute range of motion across L3-S1 for an intact human lumbosacral spine under pure moment in FE, LB, AR, and FE+FL over the eight repeated flexibility test sets for Test day 8. This evaluated the effects of long exposure periods at room temperature and ambient moisture conditions.
Figure 5.7 Absolute ROM at L3-S1 in FE for the eight repeated test sets within a day for the eight test days.

Figure 5.8 Absolute ROM at L3-S1 in LB for the eight repeated test sets within a day for the eight test days.

Figure 5.9 Absolute ROM at L3-S1 in AR for the eight repeated test sets within a day for the eight test days.
Figure 5.10 Absolute ROM at L3-S1 in FE+FL for the eight repeated test sets within a day and for the eight test days.

As shown in Figures 5.7 to 5.10, the data corresponding to each of the test days remained fairly consistent which suggested that the within-test day results did not show any significant variations. A similar pattern was observed for the inter-segmental ROMs as well (Figures A.1 to A.3).

There were certain fluctuations in the observed ROM in AR and FE+FL on some test days (Figures 5.9 and 5.10). They were observed only for test days wherein the specimen was frozen-thawed for 4-5 cycles already, which would mainly suggest the effect of multiple freeze-thaw cycles. The significant variations within certain test days data suggested that there might have been an injury to the specimen due to over-stressing following repeated flexibility testing. But visual inspections during the actual experiments did not show any significant damage to the specimen other than change in color or smell.

To factor out the variation in the specimen’s biomechanical behavior due to intra-day testing, a Wilcoxon Signed Rank statistical test was used. This test was used on account of the low sample size and repeated measures on the same subject. Thus, the data could not be assumed to be normally distributed. In order to draw a
conclusion about the preliminary null hypotheses, $H_0$: The intra-day repeated measures flexibility testing on cadaveric spine does not effect the biomechanical properties of the specimen, a non-parametric statistical ranked test was performed. The results showed no significant difference between the intra-day tests and similar trends were observed for inter-segmental ROM. A summary of the p-values is shown in Table 5.1.

Table 5.1 Summary of the p-values from Wilcoxon Signed Rank Tests for the Specimen’s Total and Inter-Segmental ROM in all Four Loading Cases.

<table>
<thead>
<tr>
<th></th>
<th>FE</th>
<th>LB</th>
<th>AR</th>
<th>FE+FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3-L4</td>
<td>0.8030</td>
<td>0.3960</td>
<td>0.8226</td>
<td>0.3493</td>
</tr>
<tr>
<td>L4-L5</td>
<td>0.9711</td>
<td>0.9095</td>
<td>0.8148</td>
<td>0.4439</td>
</tr>
<tr>
<td>L5-S1</td>
<td>0.7954</td>
<td>0.9925</td>
<td>0.9561</td>
<td>0.8717</td>
</tr>
<tr>
<td>L3-S1</td>
<td>0.8723</td>
<td>0.8279</td>
<td>0.6091</td>
<td>0.6918</td>
</tr>
</tbody>
</table>

Since the p-value was greater than the accepted level of significance for the study ($\alpha=0.05$), it was concluded that in-vitro kinematical behavior of the specimen did not vary significantly due to the effects of repeated measures flexibility tests on the in-vitro specimen within a test day. Thus, the specimen’s performance was said to be consistent and repeatable as long as the ambient temperature and moisture conditions were maintained within the test durations.

5.3.2 Inter-Day Variations due to Multiple Freeze-Thaw Cycles

As there were no significant difference in the intra-day ROM, results from the eight tests within each day were pooled and used for inter-day comparison. The inter-day mean and standard deviation of ROM as a result of the repeated freeze-thaw effect are presented in Table 5.2.
Table 5.2 Mean ± S.D. of the Intersegmental and Total Range of Motion (ROM) in Degrees for an Intact Lumbosacral Spine Subjected to Eight Repeated Measures Freeze-Thaw Tests in all Four Loading Cases.

<table>
<thead>
<tr>
<th>Test day</th>
<th>Test case</th>
<th>Intervertebral Joint</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L3-L4</td>
</tr>
<tr>
<td>1</td>
<td>FE</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>FE</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>12.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>3</td>
<td>FE</td>
<td>7.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>11.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>3.4 ± 2.0</td>
</tr>
<tr>
<td>4</td>
<td>FE</td>
<td>8.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>14.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>FE</td>
<td>10.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>13.5 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>6.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>4.8 ± 2.0</td>
</tr>
<tr>
<td>6</td>
<td>FE</td>
<td>10.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>12.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>7</td>
<td>FE</td>
<td>11.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>4.3 ± 2.9</td>
</tr>
<tr>
<td>8</td>
<td>FE</td>
<td>10.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>16.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>3.2 ± 1.6</td>
</tr>
</tbody>
</table>
As shown in Table 5.2, the mean ROM across the total specimen (L3-S1) on the first test day were higher in FE (23.3°) followed by LB (17.8°), and AR (9.8°). Application of the follower compressive preload in FE decreased the ROM to 15.5° in FE+FL. The subsequent test days also followed a similar pattern in the observed results as shown in Figure 5.11. The graphical summary of Table 5.2 is given in Appendix A (Figure A.7).

The ROM magnitude was higher in L5-S1 followed by L3-L4 and L4-L5 in FE (9.1°, 7.8°, 5.9°); FE+FL (7.1°, 5.9°, 7.4°), and in AR (3.6°, 3.9°, 2.4°). In LB, ROM was the highest in L3-L4, followed by L5-S1 and L4-L5 (3.9°, 3.6°, 2.4°) respectively.

![Figure 5.11 Mean ±S.D ROM for the whole specimen (L3-Sacrum) in each of the four loading cases FE, LB, AR, and FE+FL showing the trend in inter-day variations of the specimen.](image)

The specimen ROM, overall and for the relative inter-segmental levels, showed a gradual increase in direct relation to the number of times it was subjected to the freeze-thaw test protocol. The average overall ROM on Test day 8 was observed to be 39.3° in FE, 43.7° in LB, 16.0° in AR, and 13.9° in FE+FL. As compared against
the results for test day 1, the percentage increase in FE, LB, and AR on test day 8 was more than 50%. The values for FE+FL remained consistent throughout the study which suggested that the application of a compressive follower preload allowed the specimen to withstand higher magnitudes of load while preventing any significant injuries to the specimen over time.

A one-way repeated measures ANOVA (R-ANOVA), with each post-thaw test day as the treatment factor and ROM as the dependent variable, evaluated the p-value (corresponding to type I error in the experiment) to be less than 0.0001 for all four loading cases, FE, LB, AR, and FE+FL. The R-ANOVA for the inter-segmental levels also resulted in p-values less than 0.0001. The detailed SAS program and outputs for the overall ROM are included in Appendix B. A summary of the p-values obtained from the statistical analysis is given below in Table 5.3.

Table 5.3 Summary of the p-values from One-Way Repeated Measures ANOVA for the Total and Inter-Segmental ROM in all Four Loading Directions.

<table>
<thead>
<tr>
<th></th>
<th>FE</th>
<th>LB</th>
<th>AR</th>
<th>FE+FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3-L4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L4-L5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L5-S1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L3-S1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Since the p-value was less than the accepted level of significance for the study (α=0.05), it was concluded that the in-vitro kinematic biomechanical behavior of the test specimen varied significantly due to the effects of subsequent freeze-thaw sessions. A post-hoc Student-Neuman-Keul (SNK) test provided a grouping of the test days that were significantly different (Figure 5.12).
Figure 5.12 Post-hoc SNK grouping for the overall ROM in each of the four loading cases. Means with the same symbol or under the same line are not significantly different from each other.
As seen in Figure 5.12, the post-hoc test showed that in FE, test days 4 to 8 were significantly different from the earlier tests. In AR, significant differences were found on test day 6 while in LB significant difference occurred on test day 2. A similar grouping was observed for the inter-segmental data as well. These results generally showed that there were no significant changes in the specimen’s biomechanical properties within the first few freeze-thaw sessions, however there was a significant increase in its ROM from the subsequent freeze cycles. The results from the current study suggested that the degradative changes in a cadaveric specimen that are initiated post-mortem keep progressing and gradually increases through the number of freezing cycles it is subjected to. The consistent data between any two subsequent freezing sessions such as test days 1, 2 or 3, 4 or 5, 6 or 7, 8 suggested that a biomechanical specimen may be subjected to a single freeze-thaw cycle and this result is consistent with previous studies.

5.4 Neutral zone (NZ)

The intra-day and inter-day comparison of the experimental data for the total and inter-segmental NZ from the repeated measures freeze-thaw study are presented in this section.

5.4.1 Intra-Day Variations due to Repeated Flexibility Tests

The observed NZ value from the intra-day repeated measures flexibility tests did not show any considerable changes in between the test sets within a test day in FE (p=0.98), LB (p=0.87), AR (p=0.75), and FE+FL (p=0.88). The values remained consistent and repeatable. A typical case for the last test day (8th post-thaw test) is shown below in Figure 5.13. The other test days also followed similar patterns as shown in Figures 5.14 to 5.17.
Figure 5.13 Absolute neutral zone across L3-S1 for an intact human lumbosacral spine under pure moment in FE, LB, AR, and FE+FL over the eight repeated flexibility test sets for Test day 8.

Figure 5.14 Absolute NZ at L3-S1 in FE for the eight repeated test sets within a day and for the eight test days.

Figure 5.15 Absolute NZ at L3-S1 in LB for the eight repeated test sets within a day and for the eight test days.
Consistent with the ROM results, the data for NZ corresponding to each of the test days remained fairly consistent which suggested that the within-test day NZ results did not show any significant variations. There were certain fluctuations in the observed NZ in AR and FE+FL on some test days (Figures 5.16 and 5.17), which suggested that there might have been an injury to the specimen due to over-stressing it beyond the physiological ranges, thus affecting its kinematic behavior. The same observation was made on ROM results, but during experimental observations there was no such visible damage to the specimen other than slight change in some of its structural properties such as smell, color, etc.
The results from the statistical analysis for intra-day NZ test data suggested that there were no significant changes in the specimen within the repeated flexibility sets. Similar to the statistical evaluation of ROM as mentioned in Section 5.4.1, a non-parametric Wilcoxon signed rank test determined the intra-day variations in NZ. A summary of the total and inter-segmental p-values is shown in Table 5.4.

Table 5.4 Summary of the p-values from Wilcoxon Signed Rank Tests for Overall and Inter-Segmental NZ in all Four Loading Cases.

<table>
<thead>
<tr>
<th></th>
<th>FE</th>
<th>LB</th>
<th>AR</th>
<th>FE+FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3-L4</td>
<td>0.9358</td>
<td>0.4850</td>
<td>0.9072</td>
<td>0.6179</td>
</tr>
<tr>
<td>L4-L5</td>
<td>0.9953</td>
<td>0.9911</td>
<td>0.9577</td>
<td>0.4230</td>
</tr>
<tr>
<td>L5-S1</td>
<td>0.9781</td>
<td>0.7790</td>
<td>0.9790</td>
<td>0.9634</td>
</tr>
<tr>
<td>L3-S1</td>
<td>0.9822</td>
<td>0.8604</td>
<td>0.9387</td>
<td>0.8809</td>
</tr>
</tbody>
</table>

Since the p-value was greater than the accepted level of significance for the study (α=0.05), it was concluded that in-vitro kinematic behavior of the specimen did not vary significantly due to the effects of repeated measures flexibility tests on the in-vitro specimen within a test day. The specimen performance remained consistent and repeatable as long as the ambient temperature and moisture conditions were maintained.

5.4.2 Inter-Day Variations due to Multiple Freeze-Thaw Cycles

As there were no significant difference in the intra-day NZ, results from the eight tests within each day were pooled and used for inter-day comparison. The inter-day NZ for the whole specimen (L3-Sacrum) and the inter-segmental levels was found to be significantly different for FE, LB, AR, and FE+FL (all p < 0.0001). Mean NZ for all the eight test days are presented in Table 5.5. The first day repeated measures flexibility testing of the specimen yielded a mean NZ across L3-S1 of 1.8° in FE, 1.3° in LB, and 0.4° in AR.
Table 5.5 Mean ± S.D. of the Interverbral and Total Neutral Zone (NZ) in Degrees for an Intact Lumbosacral Spine Subjected to Repeated Measures Flexibility Tests in all Four Loading Cases.

<table>
<thead>
<tr>
<th>Test day</th>
<th>Test case</th>
<th>Intervertebral Joint</th>
<th>L3-L4</th>
<th>L4-L5</th>
<th>L5-S1</th>
<th>L3-S1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FE</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>FE</td>
<td>0.7 ± 0.4</td>
<td>0.7 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>2.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>FE</td>
<td>0.4 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>0.9 ± 0.3</td>
<td>0.6 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>2.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.6 ± 0.3</td>
<td>0.4 ± 0.4</td>
<td>0.5 ± 0.2</td>
<td>1.3 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FE</td>
<td>0.8 ± 0.4</td>
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<td>1.0 ± 0.2</td>
<td>2.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>1.1 ± 0.3</td>
<td>3.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
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<td>0.6 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>FE</td>
<td>0.7 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>3.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>0.9 ± 0.6</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>4.9 ± 1.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.7 ± 0.6</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.7 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.4 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>FE</td>
<td>0.7 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>3.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>1.5 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>5.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>2.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>FE</td>
<td>1.1 ± 0.2</td>
<td>2.8 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>6.3 ± 0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>1.2 ± 0.2</td>
<td>3.2 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>5.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.5 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>0.7 ± 0.2</td>
<td>3.5 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>FE</td>
<td>1.1 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>6.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LB</td>
<td>1.5 ± 0.3</td>
<td>4.0 ± 0.6</td>
<td>1.9 ± 0.5</td>
<td>5.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>0.9 ± 0.4</td>
<td>2.0 ± 0.5</td>
<td>0.5 ± 0.3</td>
<td>2.9 ± 1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FE+FL</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>
As shown in Table 5.5, the NZ across the inter-segmental levels, L3-L4, L4-L5, and L5-S1 were about the same in FE (0.5°, 0.6°, 0.6°), LB (0.3°, 0.5°, 0.5°), AR (0.2°, 0.2°, 0.1°) and FE+FL (0.4°, 0.3°, 0.5°) on test day 1.

The specimen NZ, overall and for the relative intervertebral levels, showed a gradual increase in direct relation to the number of times it was subjected to the freeze-thaw test protocol (Figure 5.18). The average overall L3-S1 NZ on Test day 8 was observed to be 6.2° in FE, 5.8° in LB, 2.9° in AR, and 1.2° in FE+FL. As seen, the percentage increase in FE, LB, and AR was more than 100 %. The NZ was previously determined to be a measure of injury to the spine [45], as it represents the level of instability in the spine. The values for FE+FL remained consistent throughout the study which suggested that the application of a compressive follower preload allowed the specimen to withstand higher magnitudes of load while preventing any significant injuries to the specimen over time. Similar trends were observed for the NZ at the intervertebral levels as well. (Figures A.4 to A.6).

Figure 5.18 Mean ±S.D NZ for the whole specimen (L3-Sacrum) in each of the four loading cases FE, LB, AR, and FE+FL showing the trend in inter-day variations of the specimen.
A one-way repeated measures ANOVA (R-ANOVA), with each post-thaw day as the treatment factor and NZ as the dependent variable, evaluated the p-value to be less than 0.0001 for all four loading cases, FE, LB, AR, and FE+FL. The R-ANOVA for the intervertebral levels also resulted in p-values less than 0.0001. The detailed SAS program and the outputs for the overall stiffness of the specimen are included in the Appendix B. A summary of the p-values obtained from the statistical analysis is given below in Table 5.6.

Table 5.6 Summary of the p-values from One-Way Repeated Measures ANOVA for the Total and Inter-Segmental NZ in all Four Loading Directions.

<table>
<thead>
<tr>
<th></th>
<th>FE</th>
<th>LB</th>
<th>AR</th>
<th>FE+FL</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3-L4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L4-L5</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L5-S1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>L3-S1</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Since the p-value was less than the accepted level of significance for the study (α=0.05), it was concluded that the in-vitro kinematic biomechanical behavior of the specimen varied significantly due to the effects of subsequent freeze-thaw sessions. A post-hoc Student-Neuman-Keul (SNK) test provided the grouping of the test days that were significantly different than the remaining as shown in Figure 5.19.
Figure 5.19 Post-hoc SNK grouping for the overall NZ in each of the four loading cases. Means with the same symbol or under the same line are not significantly different from each other.
As seen in Figure 5.19, the post-hoc test showed that the first three test days did not result in significantly higher NZ. A similar grouping was observed for the inter-segmental data as well (Figure A.8). These results showed that there were no significant changes in the specimen’s biomechanical properties within the first few freeze-thaw sessions, however there was a significant increase in its ROM from the subsequent freeze cycles. The consistent data between any two subsequent freezing sessions such as test days 1, 2 or 3, 4 or 5, 6 or 7, 8 indicated that a biomechanical specimen may be tested under the freeze-thaw and exposure to ambient conditions followed in the current study for up to 3 test days without significant deviation in the NZ values.
CHAPTER VI
DISCUSSION AND CONCLUSIONS

6.1 Overview

The purpose of this study was to investigate the effects of multiple inter-day freeze-thaw cycles and long test periods (intra-day repeated measures tests) on the in-vitro, three-dimensional intervertebral kinematics of an intact human cadaveric lumbosacral spine. The specimen was tested under load control, at a constant rotation rate of 1°/s, in a custom-built test apparatus in three orthogonal loading directions simulating physiological bending motions: flexion-extension (FE), lateral bending (LB), axial rotation (AR), as well as FE in presence of follower compressive preload (FE+FL). The load-displacement characteristics were evaluated for the total and intersegmental range of motion (ROM) and neutral zone (NZ) indicating specimen flexibility or relative laxity.

The inter-day results from this study clearly indicated an increase in ROM and NZ of the specimen over the eight freeze-thaw cycles it was subjected to. The results from the intra-day repeated measures flexibility tests indicated no significant increase in any of the test parameters as a response to the applied load. The results from the current study suggested that in-vitro biomechanical studies with repeated tests on spinal specimens, starting with the intact state, followed by an injured and one or more
instrumented states, carried out to compare the post-operative kinematics of the spinal motion segments, should be ideally conducted within a single test day in order to get consistent results that would eventually translate into making reliable conclusions about the study hypotheses being tested. As the current results also found that one freeze-thaw cycle did not significantly affect the ROM and up to two freeze-thaw cycles did not significantly affect the NZ, in-vitro biomechanical studies could be carried out over up to 3 test days (2 freeze-thaw cycles) if absolutely necessary.

6.2 Assumptions and Limitations

While the findings of the study yielded important information pertaining to the biomechanical effects of repeated measures flexibility tests with multiple freeze-thaw cycles on the 3D kinematical behavior of a human cadaveric spinal specimens, there were several limitations and assumptions in the study. Suggestions are made to improve the testing machine in future works.

1. The sample size used for conducting the experiment was limited to a single specimen. This was mainly due to the difficulty in obtaining cadaveric tissue for biomechanical research; both in terms of logistics (constant supply) as well as cost of a specimen (approximately $2000). However, the study was designed on the basis of a repeated measures experimental model to provide valid data that could identify any significant trends and could be extrapolated to an experiment with larger “n”.

2. The flexibility spine tester was designed to apply a pure, uniform moment across all levels in a multi-segmented spine specimen. But as seen from the results of the applied versus transmitted torque (Section 3.4.2) there were torque and force
components, though low in magnitude, along the axes other than the principal axes of rotation. These small off-axis loads had minimal effects on the results of the current study.

3. Though care was taken for proper alignment of the specimen on the test frame (using laser position tools) such that it was in line with respect to the optotrak apparatus and the loading arms in the three orthogonal directions, there could have been a slight difference from one test to other. However, the design of the spline actuator with U-joints ensured transmission of the applied torque onto the specimen which would negate the effects of misalignment.

4. The passive counterbalance mechanism, using a set of pulleys and dead-weights, that helped to nullify the effect of actuator arm mass on the specimen in turn resulted in addition of off-axis force components in the load-displacement data resulting in noise as the specimen moved continuously through a complete load cycle. An active system would be ideal. The off-axis loads and noise were however small in magnitude and did not significantly affect the current results.

5. The follower load system implemented in the current study used custom-built fixtures to guide a cable along approximated center of rotation (COR) of the specimen and simulated muscle loads using a pair of dead-weights hung bilaterally (Figure 3.35). This static compressive FL system did not account for changes in the location of the COR of the specimen during motion which would change the muscle loads as well.
Thus, a dynamic system with the compressive loads as a function of spinal motion would be a better representation. This technique of static FL is currently accepted as ‘state-of-the-art’ in in-vitro spine biomechanical testing.

6. The input (LabVIEW system) control for the spine testing machine and the output (Optotrak CERTUS) system that measures the 3D displacement data were manually synchronized. This involved a certain amount of post-processing for each test. An external digital trigger signal could be activated from the LabVIEW control program as the application starts to perform auto-synchronization between these systems. Intra-observer error is typically by less than 2 time steps in synchronizing these data collected at 20 Hz. The resulting difference in ROM and NZ is typically about 0.05°.

7. From the perspective of statistical analysis, the underlying assumptions were that there was equal variance between the conditions and that the data were normally distributed [60]. A repeated measures ANOVA (R-ANOVA) analysis was advantageous compared to conventional ANOVA since individuals are treated as blocks with the assumption that the within-subject variation is lower than the between-subject variation in order to increase the precision of the experiment.

6.3 Discussion

The following sections discuss the current study from the point of view of cellular level physiological effects due to freeze-thaw mechanisms and the assumptions of standard in-vitro testing apparatus on the biomechanical characterization of human cadaveric tissue.
6.3.1 Biomechanical Relevance of Freezing Cadaveric Specimens

Biomechanical testing on human biological tissue offers a practical means for evaluating different surgical techniques before clinical utilization. The use of unembalmed, fresh frozen cadaveric specimens is widely accepted in biomechanical research. It is a common practice to store these specimens for various periods in a frozen state due to an irregular supply of material, especially with human cadaveric specimens. It also provides a convenient time frame for the researcher to conduct detailed and multifaceted studies of complex anatomical structures that require the specimens to go through multiple stages of preparation and testing. Currently, most studies addressing these topics are often performed with the assumption that the mechanical properties of fresh frozen specimens reflect the in-vivo conditions and remain consistent over time.

In theory, freezing has been shown to produce a dysfunction of cell metabolism (modification of intra-cellular pH and release of proteolytic enzymes) either directly due to formation of intra-cellular and/or extra-cellular ice or indirectly due to cytolysis [30]. Rubinsky et al. [52] reported that, during freezing, ice preferentially forms and propagates along the vascular system. Thermal expansion associated with this ice formation produced a four-fold increase in the volume of the vascular space, resulting in tearing of vascular membranes and the endothelium. In another instance, Palmoski and Brandt [38] noted in a cartilage study that freezing increased the proportion of smaller-sized proteoglycans, diminished the proportion of proteoglycans that could be isolated in large aggregates associated with hyaluronic acid and decreased the hydrodynamic size of disaggregated proteoglycans. They attributed this proteoglycans degradation to the release of enzymes from the chondrocytes killed during repeated freeze-thawing of the
tissue. These freezing induced changes in proteoglycans are similar to those observed in aged discs that have diminished capacity to swell [3, 70].

Other damage mechanisms, such as cell death, may also affect mechanical behavior indirectly. Studies on plants and animals demonstrated that when tissue was frozen, either intracellular (rapid cooling) or extracellular (slow cooling) ice formation may kill cells [9]. When these cells die, they release degradative enzymes that are normally sequestered from the extracellular matrix which may have deleterious effects on the proteoglycans and therefore on the osmotic pressure [4]. The value of the osmotic pressure directly governs the biomechanical behavior of the disc and hence the intervertebral kinematics in a spinal motion segment.

The enzymatic degradation of connective tissue after cell death is not limited only to frozen tissue and could also occur in other situations where the environmental conditions are not appropriate for maintaining cell viability. For example, this process may be responsible for alterations in biomechanical behavior noted for disc, muscle [10], and bone [26] in the first hours of death, when these tissues are maintained at room temperature. Keller et al., [22] demonstrated that after death there is a significant decrease in the creep rate, increase in stiffness, and increase in viscosity of porcine spine. These results were attributed to extradiscal factors, especially the cessation of the cardiovascular and respiratory systems, although the specific mechanism of this interaction was not described.

Freezing at very low temperature (-80 °C to -106 °C) would seem to be a feasible alternative. It has the advantage of increasing the tolerance of the cadaveric specimens,
but requires the use of cryoprotectors (dimethyl-sulfoxide) and special conditions for freezing and thawing to limit cell death as much as possible [27]. Though use of cryoprotectors is not necessary in simple freezing at -20 °C, the rapid rate of freezing or thawing could damage the tissues.

Several studies in the past have tried to address this issue related to biomechanical time-tolerance of frozen specimens. Bone is one such tissue that has been most extensively studied. The data in the literature regarding its frozen use are consistent and in essence there are no biomechanical modifications after freezing at low temperatures (below -20 °C) [49]. As for ligaments and menisci, according to McCarthy et al., cryopreservation is a better mode of storage than simple freezing [29]. Other studies showed that simple freezing at -20 °C does not damage the elasticity of the tendons and ligaments [28].

However, data for the intervertebral disc are rare. All the studies are consistent in affirming that the intervertebral disc undergoes morphologic and biomechanical degradation in fresh air or air that is warm and dry. Storage of the disc by immersion in aqueous saline solutions also impairs its biomechanical properties (decrease in rigidity after 5 hours immersion) [21]. Flynn et al. [11] compared the behavior of the L2-L3 intervertebral disc in terms of rigidity in flexion/extension and lateral flexion after 2 weeks of simple freezing at -18 °C and after lyophilization. They noted a significantly greater decrease in rigidity after lyophilization than after freezing. Bass et al., in 1997, [4] carried out a biomechanical study on 16 porcine vertebral segments with comparison of mechanical behavior of 8 fresh spines versus 8 spines after 3 weeks of freezing at -20 °C.
and concluded that there is an irreversible degeneration of the biomechanical properties of the intervertebral disc after freezing.

The current study demonstrated that in-vitro biomechanical tests of human cadaveric lumbosacral spine that have been freeze-thawed multiple times do not exhibit repeatable and consistent biomechanical behavior. The repeated measures flexibility tests over the eight test days showed a change in the biomechanical characteristics of the specimen in terms of its ROM and NZ. The specimen exhibited larger displacements during creep loading in each loading direction, which the previous studies suggested is principally due to increased permeability of the soft tissue structures such as the intervertebral disc. Equally important, the divergence of the behavior of the frozen specimens after repeated cycles suggested that the structural properties have been altered permanently which was also observed visually in the specimen in terms of change in its color, smell, moisture content, etc. The precise nature of any freezing induced damage however remains to be identified.

Our findings contrast with some of the previous studies dealing with similar topics. For example, Panjabi et al. [42] demonstrated that there were no significant differences in a human thoracic FSU even after a 14-day test period. But other investigators found a similar linear increase in ROM up to 25% and of NZ up to 50% after 48 hours of room temperature exposure. Thus, an acceptable time limit of exposure to the ambient conditions and temperature settings should be considered for ordinary spine biomechanical experiments. Furthermore, it is advisable, especially in the case of comparative implant testing, to complete all treatment cases; intact, injury, and instrumented for a specimen within the test session to avoid skewing the results toward
one device or another due to different exposure times. Considering that long exposure and repeated measures might cause injury to the specimen, researchers could factor that in and design their studies with a more stable construct towards the end of a series of tests.

6.3.2 Flexibility Spine Testing Apparatus

This study also presented a novel test apparatus for conducting biomechanical studies on the spine using the flexibility protocol. The flexibility approach presented here asserts current trends of incorporating realistic in-vivo loading conditions using in-vitro laboratory setup. The load-controlled testing allowed for applying pure, uniform bending moments across all intervertebral levels in a multi-segmented spinal specimen while allowing it to move freely about other axes thereby accommodating for the naturally occurring coupled motion behavior in the spine. The use of an optoelectronic camera system for measuring the three-dimensional position data of the vertebrae provided an easy method for analyzing the intervertebral kinematics and obtaining the desired load-displacement characteristics. The test machine was also shown to be capable of testing multi-segmented specimens from different regions of the spine (Figure 6.1), one of the design criteria for the flexibility spine test machine as mentioned in Section 3.2.
Figure 6.1 A human cervical spine (C2-T1) being tested in the flexibility spine tester for evaluating the in-vitro kinematical performance of open door laminoplasty surgical technique for treatment of spinal stenosis.

The current study also reported a technique for applying a compressive load to a multisegmented spine specimen along a follower load path, i.e. the path that approximated the tangent to the curve of the lumbar spine passing through the centers of rotation of the lumbar segments [46]. The follower load path described in this study provided an explanation of how the whole lumbar spine can be lordotic and yet resist large compressive loads of up to 3 to 5 times body weight in vivo. The resultant load of multiple muscles to stabilize and move the spine is mainly compressive load and bending moment with small shear load. Follower load is known to stabilize the spine even with high compressive loads. The consistency in the data over multiple freeze-thaw test days suggested that in-vitro simulation of muscle forces using follower load in FE on a multi-segmented spinal specimen helped stabilize the spine and supports its utilization during in-vitro experimentation for more accurate load-displacement characteristics.
From the perspective of the biomechanical testing apparatus, the advent of such computer-controlled spine testing machines allowed more thorough and efficient testing of spinal specimens than was possible with manual loading techniques. Experiments with these dynamic machines can be carried out over extended load cycles and under variable rates. The angular-deformation rate has been observed to affect the stiffness of the spinal specimens because of the viscoelastic properties [35]. The specimens may thus be tested over several hours at a room temperature of 20 °C to 23 °C under various moisture conditions. Such tests can be very sensitive to the ambient conditions under which they are carried out, as demonstrated by Hirsch and Galante [21], although the influence of these conditions has been described with widely varying results. For instance, the data of Panjabi et al. [42] suggested that spine specimens may be tested for up to 14 days when they are refrigerated at 4 °C between tests with insignificant changes in their range of motion.

Therefore, though the problems of ideal testing environment for biological tissues have been addressed in the past [2, 69], there seems to be a lack of available data on the daily changes in the mechanical properties of the biological structures due to prolonged test periods and intermittent freeze-thaw sessions. Moreover, as most previous biomechanical studies were carried out on a single FSU, the published literature on freeze-thaw effects were on single FSUs. In recent years, adjacent level effects are of concern and many biomechanical studies are carried out on multi-segmented spines. No prior study addressed the effects of multiple freeze-thaw cycles on a whole spine segment. The current study tries to address this gap in the published literature.
6.4 Analysis of Test Results

The kinematic behavior of the intact lumbosacral spine segment was found to be significantly affected by multiple freeze-thaw cycles and long exposure to ambient conditions in the current study. The ROM and NZ values for the total specimen (L3-S1), as well as across the intersegmental levels (L3-L4, L4-L5, and L5-S1), followed a gradual increasing trend in between test days (Figure 5.11 and Figure 5.18) for all the loading directions namely, FE, LB, and AR, except for in FE+FL where the data was seen to be consistent. This might suggest and support previous work done by Patwardhan et al. in 1999 that FL application in in-vitro testing in fact helps to stabilize the specimen over long durations of testing without causing much damage to it. The intra-day data, on the other hand, were not significantly different within each test day as was seen from the day-wise trends showing the repeatability of the test specimen within the eight repeated test sets within a day in each of the four loading cases (Figures 5.7 to 5.10 and Figures 5.14 to 5.17).

It is acknowledged that the factors responsible for the inter-day differences in the specimen’s kinematic behavior may not be isolated to the effects of multiple freeze-thaw cycles. Variability due to other factors were however less significant on the observed as discussed below.

1. The specimen was tested repeatedly over the study duration and thus a major contributing factor that could have affected the data was specimen fatigue due to over-stress beyond its physiological load and motion ranges. The applied load and
ROM data from the current study were compared with the values in the literature [12, 13, 34, 44, 53, 72] and the physiologic range of motions (Tables 6.1 and 6.2).

Table 6.1 ROM Comparison for an Intact Lumbosacral Spinal Specimen between the Current Study and Previous Studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Mean ROM in L3-L4 (Standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Applied moment</td>
</tr>
<tr>
<td></td>
<td>(Nm)</td>
</tr>
<tr>
<td>Panjabi et al. [44]</td>
<td>7.5</td>
</tr>
<tr>
<td>Fujiwara et al. [13]</td>
<td>6.6</td>
</tr>
<tr>
<td>Mimura et al. [34]</td>
<td>10</td>
</tr>
<tr>
<td>Schmoelz et al. [53]</td>
<td>10</td>
</tr>
<tr>
<td>Yamamoto et al. [72]</td>
<td>10</td>
</tr>
<tr>
<td>Freudiger et al. [12]</td>
<td>18.3</td>
</tr>
<tr>
<td>Present Study</td>
<td>7.5</td>
</tr>
<tr>
<td>Physiological range [65]</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Table 6.2 Intersegmental Mean ROM Comparison as a % of Physiological ROM for an Intact Lumbosacral Spine Specimen within Each Test Day in all Three Loading Directions.

<table>
<thead>
<tr>
<th>Vertebral Level</th>
<th>Flexion-Extension (FE)</th>
<th>Lateral Bending (LB)</th>
<th>Axial Rotation (AR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute ROM (°)</td>
<td>% of Physiologic ROM</td>
<td>Absolute ROM (°)</td>
</tr>
<tr>
<td></td>
<td>Max</td>
<td>Mean</td>
<td>%</td>
</tr>
<tr>
<td>L1-L4</td>
<td>11.4</td>
<td>9.5</td>
<td>76</td>
</tr>
<tr>
<td>L4-L5</td>
<td>13.1</td>
<td>10.0</td>
<td>77</td>
</tr>
<tr>
<td>L5-S1</td>
<td>15.6</td>
<td>11.3</td>
<td>78</td>
</tr>
</tbody>
</table>

As seen in Table 6.1, the load magnitude (applied moment) chosen for the current study was lower than the expected levels physiologically. It was also within the ranges that have been used in most of the other earlier studies. The within-day repeated measures flexibility tests yielded a resultant mean ROM (Table 6.2) that
remained within the accepted physiological ranges [65] and also comparable to the data from existing literature, except in AR. Thus the specimen could have been overstressed in certain test cases that could have lead to micro-damage. However, the statistical analysis of the intra-day data (Section 5.4.1) did not yield any significant differences which suggested that damage due to overload or strain were not sufficient to cause changes to the specimen’s ROM and NZ.

2. The measurement accuracy (Section 3.4.1) of the spine testing machine could have the reliability of the collected data. However as seen in Table 3.1, the % error of the resultant force of 1.5 % - 3.5 % was within the specified sensitivity provided by the manufacturer. This small % error suggested that the flexibility spine testing machine was consistent in applying a repeatable load to the specimen over the study duration thus minimizing the test apparatus related errors.

3. The freeze-thaw protocol followed for evaluating the specimen in the current study was designed to represent the actual time frame for conducting a biomechanical experiment with a cadaveric specimen; 1-2 days of freezing time, followed by 16-24 hours of thawing time and 8-10 hours of actual test durations. While duration of freeze storage was previously found to not affect the biomechanical properties of cadaveric tissues [42], the variations in thaw and test durations could cumulatively affect subsequent test day results. As seen in Figure 4.11, except for one instance where the freeze duration in between test days was extended, the thaw and test durations were followed strictly.
These factors were considered to not significantly affect the biomechanical response of a specimen in-vitro and thus the observed results on the effects of multiple freeze-thaw cycles in the current study stands. The hypothesis of freezing induced micro-damage to the tissue is supported by experimental data in this study.

6.5 Conclusions

A significant difference in ROM and NZ was found between test days in all loading directions following multiple freeze-thaw cycles. There were however no within-day variations in the ROM or NZ.

6.5.1 Study of Hypotheses

Based on the in-vitro kinematic experimental results from mechanical testing of the multi-segmented lumbosacral spine using the flexibility spine tester and the statistical analysis of the test parameters, the first null hypothesis (H₀₁) was accepted (p>0.05). No intra-day effects on the ROM and NZ of the whole specimen (L₃-Sacrum) and the inter-segmental (L₃-L₄, L₄-L₅, L₅-Sacrum) levels were found for each of the four loading cases in FE, LB, AR, and FE+FL when the specimen was tested repeatedly on the same day while maintaining the moisture and ambient temperature conditions. The second null hypothesis (H₀₂) was rejected (p<0.0001). There were significant inter-day effects due to freeze-thaw cycles on the overall (L₃-Sacrum) and inter-segmental (L₃-L₄, L₄-L₅, L₅-Sacrum) ROM and NZ for each of the four loading cases.

Similarly, both research hypotheses that were proposed were accepted. For the first research hypothesis, it was determined that it was possible to construct a test frame
to conduct load controlled testing of single/multi-segmented spinal specimens by applying a pure, uniform bending moment across all its vertebral levels, yet accommodating for linear translations and rotations along remaining degrees of freedom. For the second research hypothesis tested, it was determined that it was possible to apply a compressive pre-load to the spine that followed along the center of rotation at each vertebral level in the multi-segmented specimen. This follower preload applied a pure compression to the specimen without imposing any additional bending moments.

6.5.2 Recommendations

The current study determined that experimental studies involving cadaveric tissue and requiring multiple stages of testing should be completed within a specified time period (10-12 hours). To remain unaffected by ambient testing conditions, the specimen should be kept relatively moist. The number of freeze-thaw sessions that it is subjected to must be kept minimal to avoid specimen degradation. In experimental practice, the aim is to maintain the anatomy and physiology as near as possible to those of fresh specimens. Freezing at a temperature of -20 °C is thus an appropriate method of storage for cadaveric spinal specimens if the conditions of freezing and thawing are maintained strictly so as to avoid damaging the tissues. Low rate of freezing and thawing, and thawing in a moist environment are desirable. This mode of storage allows biomechanical researchers to obtain and store anatomic specimens without introducing any experimental bias.
REFERENCES


APPENDICES
APPENDIX A

INTERSEGMENTAL FLEXIBILITY CURVES

Inter-day comparison of the intersegmental (L3-L4, L4-L5, and L5-S1) ROM and NZ in all four loading cases, namely flexion-extension (FE), lateral bending (LB), axial rotation (AR), and flexion-extension with a follower compressive preload of 660 N (FE+FL) are shown in Figures A.1 to A.3 and Figures A.4 to A.6 respectively. The mean and standard deviation of ROM and NZ for the whole specimen (L3-S1) and for the intersegmental levels are shown in Figures A.7 and A.8 respectively.
Figure A.1 Absolute ROM at L3-L4 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
**Figure A.2** Absolute ROM at L4-L5 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
Figure A.3 Absolute ROM at L5-S1 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
Figure A.4 Absolute NZ at L3-L4 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
Figure A.5 Absolute NZ at L4-L5 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
Figure A.6 Absolute NZ at L5-S1 in all four loading cases for the repeated test sets within a day and a comparison in between the eight post-thaw test days.
Figure A.7 Mean ± S.D. ROM across the intersegmental levels for an intact human lumbosacral spine under pure moment in FE, LB, AR, and FE+FL over a period of eight test days with subsequent freezing at -20°C and thawing at room temperature in between.
Figure A.8 Mean ± S.D. NZ across the intersegmental levels for an intact human lumbosacral spine under pure moment in FE, LB, AR, and FE+FL over a period of eight test days with subsequent freezing at -20°C and thawing at room temperature in between.
APPENDIX B

SAS STATISTICAL CODE AND OUTPUT

The general structure of the SAS program for all test cases is common. A sample program for ROM at L3-S1 in FE is shown below.

/* Name of the dataset with level of interest and loading direction */
data L3S1FE;
input day$ set $ rom @;
/*Input variables: Dependent and Independent*/
datalines;
/*Actual ROM or NZ data: Day-, and set-wise */
D1 S1 20.1
D1 S2 22.2
D1 S3 24.4
D1 S4 23.4
:
D8 S5 39.4
D8 S6 40.3
D8 S7 40.2
D8 S8 40.4
;
proc MIXED data=L3S1FE;
   /* Proc MIXED procedure */
   class day set;
   /* Independent variables */
   model rom = day;
   /* Dependent variable */
   repeated set/type=cs group=day;
   /* Repeated measures on set */
   lsmeans day/ pdiff cl adjust=TUKEY;
run;

proc GLM data=L3S1FE;
   /* Proc MIXED procedure */
   class day;
   /* Independent variables */
   model rom=day;
   /* Dependent variable */
   means day/SNK;
   /* SNK Grouping of S.D. factors */
run;

proc NPAR1WAY data=L3S1FE Wilcoxon;
   /* Non-Param Signed rank test */
   class set;
   /* Independent variable */
   var rom;
   /* Dependent variable */
run;
Output:

L3-S1_ROM_FE

The Mixed Procedure

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>414.53</td>
<td>&lt;.0001</td>
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</table>

The GLM Procedure

Student-Newman-Keuls Test for rom

NOTE: This test controls the Type I experiment wise error rate under the complete null hypothesis but not under partial null hypotheses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error Degrees of Freedom</td>
<td>56</td>
</tr>
<tr>
<td>Error Mean Square</td>
<td>2.382567</td>
</tr>
</tbody>
</table>

# of Means

<table>
<thead>
<tr>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>1.5460981</td>
<td>1.8581089</td>
<td>2.0436238</td>
<td>2.1753655</td>
<td>2.2772582</td>
<td>2.3601088</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40.8000</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>A</td>
<td>39.3000</td>
<td>8</td>
<td>D8</td>
</tr>
<tr>
<td>B</td>
<td>36.5750</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>C</td>
<td>32.3500</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>D</td>
<td>30.2750</td>
<td>8</td>
<td>D4</td>
</tr>
<tr>
<td>E</td>
<td>24.5750</td>
<td>8</td>
<td>D2</td>
</tr>
<tr>
<td>E</td>
<td>23.3375</td>
<td>8</td>
<td>D1</td>
</tr>
<tr>
<td>E</td>
<td>23.3000</td>
<td>8</td>
<td>D3</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_FE)

Average scores were used for ties.

Kruskal-Wallis Test

| Chi-Square | 3.1345 |
| DF         | 7     |
| Pr > Chi-Square | 0.8723 |
The Mixed Procedure

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>540.74</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure

Student-Newman-Keuls Test for rom

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error Degrees of Freedom</td>
<td>56</td>
</tr>
<tr>
<td>Error Mean Square</td>
<td>14.86547</td>
</tr>
</tbody>
</table>

# of Means 2 3 4 5 6 7 8
Range 3.8619247 4.6412815 5.1046704 5.4337416 5.6882545 5.895203 6.069238

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>43.763</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>A</td>
<td>43.750</td>
<td>8</td>
<td>D8</td>
</tr>
<tr>
<td>B</td>
<td>38.813</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>B</td>
<td>38.475</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>C</td>
<td>30.775</td>
<td>8</td>
<td>D4</td>
</tr>
<tr>
<td>D</td>
<td>25.150</td>
<td>8</td>
<td>D2</td>
</tr>
<tr>
<td>D</td>
<td>23.100</td>
<td>8</td>
<td>D3</td>
</tr>
<tr>
<td>E</td>
<td>17.813</td>
<td>8</td>
<td>D1</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_LB)

Average scores were used for ties.

Kruskal-Wallis Test

| Chi-Square | 3.5689 |
| DF         | 7     |
| Pr > Chi-Square | 0.8279 |
The Mixed Procedure

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>138.17</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure

Student-Newman-Keuls Test for rom

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error Degrees of Freedom</td>
<td>56</td>
</tr>
<tr>
<td>Error Mean Square</td>
<td>8.407946</td>
</tr>
</tbody>
</table>

# of Means  | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Range     | 2.9044203 | 3.4905477 | 3.8390465 | 4.0865295 | 4.2779399 | 4.4335788 | 4.5644645 |

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21.813</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>B</td>
<td>17.875</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>B</td>
<td>16.000</td>
<td>8</td>
<td>D8</td>
</tr>
<tr>
<td>C</td>
<td>12.313</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>C</td>
<td>11.888</td>
<td>8</td>
<td>D3</td>
</tr>
<tr>
<td>C</td>
<td>10.688</td>
<td>8</td>
<td>D4</td>
</tr>
<tr>
<td>C</td>
<td>10.200</td>
<td>8</td>
<td>D2</td>
</tr>
<tr>
<td>C</td>
<td>9.825</td>
<td>8</td>
<td>D1</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_AR)

Average scores were used for ties.

Kruskal-Wallis Test

| Chi-Square | 5.4179 |
| DF         | 7     |
| Pr > Chi-Square | 0.6091 |
The Mixed Procedure

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num</th>
<th>Den</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>16.49</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure

Student-Newman-Keuls Test for rom

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha                        0.05
Error Degrees of Freedom     56
Error Mean Square             5.225982

# of Means 2 3 4 5 6 7 8
Range     2.2898048 2.7518995 3.0266511 3.2217633 3.3726686 3.4953721 3.5985606

Means with the same letter are not significantly different.

SNK Grouping     Mean   N  day
A                  16.463 8  D4
A                  15.550 8  D1
B                  14.138 8  D5
B                  13.900 8  D6
B                  13.900 8  D7
B                  13.863 8  D8
B                  12.663 8  D2
C                  10.850 8  D3

The NPAR1WAY Procedure (L3-S1_FE+FL)

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square     4.7386
DF             7
Pr > Chi-Square 0.6918
L3-S1_NZ_FE

The Mixed Procedure (L3-S1_FE)

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
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<tbody>
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<td>day</td>
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<td>56</td>
<td>169.89</td>
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</table>

The GLM Procedure (L3-S1_FE)

Student-Newman-Keuls Test for nz

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha                        0.05
Error Degrees of Freedom      56
Error Mean Square             0.399464

# of Means  2  3  4  5  6  7  8
Range       0.6330725 0.7608299 0.8367917 0.8907353 0.9324567 0.9663811 0.9949101

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.3000</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>B</td>
<td>3.5875</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>B</td>
<td>3.1000</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>B</td>
<td>2.9625</td>
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<td>D4</td>
</tr>
<tr>
<td>C</td>
<td>2.1875</td>
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<td>D2</td>
</tr>
<tr>
<td>C</td>
<td>1.8625</td>
<td>8</td>
<td>D3</td>
</tr>
<tr>
<td>C</td>
<td>1.8250</td>
<td>8</td>
<td>D1</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_FE)

Average scores were used for ties.

Kruskal-Wallis Test

<table>
<thead>
<tr>
<th>Chi-Square</th>
<th>DF</th>
<th>Pr &gt; Chi-Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5030</td>
<td>7</td>
<td>0.9822</td>
</tr>
</tbody>
</table>
The Mixed Procedure (L3-S1_LB)

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>183.73</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure (L3-S1_LB)

Student-Newman-Keuls Test for nz

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error Degrees of Freedom</td>
<td>56</td>
</tr>
<tr>
<td>Error Mean Square</td>
<td>0.96904</td>
</tr>
</tbody>
</table>

# of Means | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>0.9860192</td>
<td>1.1850031</td>
<td>1.3033147</td>
<td>1.3873325</td>
<td>1.4523143</td>
<td>1.505152</td>
<td>1.5495863</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.9375</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>A</td>
<td>5.8375</td>
<td>8</td>
<td>D8</td>
</tr>
<tr>
<td>A</td>
<td>5.6000</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>A</td>
<td>4.8875</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>B</td>
<td>3.0750</td>
<td>8</td>
<td>D4</td>
</tr>
<tr>
<td>C</td>
<td>2.2250</td>
<td>8</td>
<td>D3</td>
</tr>
<tr>
<td>C</td>
<td>1.9250</td>
<td>8</td>
<td>D2</td>
</tr>
<tr>
<td>C</td>
<td>1.3250</td>
<td>8</td>
<td>D1</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_LB)

Average scores were used for ties.

Kruskal-Wallis Test

| Chi-Square | 3.2559 |
| DF         | 7      |
| Pr > Chi-Square | 0.8604 |
L3-S1_NZ_AR

The Mixed Procedure (L3-S1_AR)

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>81.65</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure (L3-S1_AR)

Student-Newman-Keuls Test for nz

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

Alpha                        0.05
Error Degrees of Freedom       56
Error Mean Square        0.513058

# of Means 2 3 4 5 6 7 8
Range   0.7174601 0.8622473 0.9483347 1.0094688 1.0567517 1.0951982 1.12753

Means with the same letter are not significantly different.

SNK Grouping Mean N day
A     3.5250 8 D7
A     2.8875 8 D8
A     2.4750 8 D6
B     1.6625 8 D5
B     1.3375 8 D3
B     0.9500 8 D4
C     0.4750 8 D1
C     0.4000 8 D2

The NPAR1WAY Procedure (L3-S1_AR)

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square 2.3391
DF 7
Pr > Chi-Square 0.9387
The Mixed Procedure (L3-S1_FE+FL)

Type 3 Tests of Fixed Effects

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>day</td>
<td>7</td>
<td>56</td>
<td>21.03</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The GLM Procedure (L3-S1_FE+FL)

Student-Newman-Keuls Test for nz

NOTE: This test controls the Type I experimentwise error rate under the complete null hypothesis but not under partial null hypotheses.

<table>
<thead>
<tr>
<th>Alpha</th>
<th>0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Error Degrees of Freedom</td>
<td>56</td>
</tr>
<tr>
<td>Error Mean Square</td>
<td>0.053973</td>
</tr>
</tbody>
</table>

# of Means  | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
Range       | 0.2327039 | 0.2796648 | 0.3075867 | 0.3274152 | 0.3427511 | 0.355221 | 0.3657076 |

Means with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>SNK Grouping</th>
<th>Mean</th>
<th>N</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.5500</td>
<td>8</td>
<td>D5</td>
</tr>
<tr>
<td>B</td>
<td>1.4500</td>
<td>8</td>
<td>D4</td>
</tr>
<tr>
<td>C</td>
<td>1.3125</td>
<td>8</td>
<td>D7</td>
</tr>
<tr>
<td>D</td>
<td>1.2375</td>
<td>8</td>
<td>D1</td>
</tr>
<tr>
<td>E</td>
<td>1.1875</td>
<td>8</td>
<td>D6</td>
</tr>
<tr>
<td>F</td>
<td>1.1875</td>
<td>8</td>
<td>D8</td>
</tr>
<tr>
<td>G</td>
<td>1.0875</td>
<td>8</td>
<td>D3</td>
</tr>
<tr>
<td>H</td>
<td>0.7875</td>
<td>8</td>
<td>D2</td>
</tr>
</tbody>
</table>

The NPAR1WAY Procedure (L3-S1_FE+FL)

Kruskal-Wallis Test

| Chi-Square | 3.4914 |
| DF         | 7     |
| Pr > Chi-Square | 0.8361 |