Mechanical Measurement of Progressive Damage in Sucrose-Treated Medial Collateral Ligaments

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ABSTRACT

The knee is the most complex joint in the human body. It consists of a system of muscle, bone, and ligaments that endures repetitive loading during daily and athletic activities. When this loading is excessive, damage to the knee occurs leading to a decreased quality of life.The medial collateral ligament (MCL) is one of the 4 major ligaments known to be commonly injured in the knee. The risk of injury to the knee joint increases with the elderly and individuals who experience chronic dehydration. For this reason, the focus of this study is to compare different mechanical quantitites that can be used to analyze damage to the MCL.

In this study, a novel mechanical testing protocol is used to progressively induce damage in dehydrated rat MCLs by performing tensile tests. This involves stretching the ligaments along their longitudinal axes to consecutive and increasing displacements starting at a 0.4 mm dispalcement and in increments of 0.2 mm until complete failure occurs. The load and change in length that the ligament experiences are measured at each displacement. Three different methods were evaluated to determine subfailure and damage propagation in rat MCLs: changes in tangent stiffness and chord stiffness, and changes in the load value at the 0.4 mm displacement for each load-displacement curve. The findings of this study indicate that the tangent stiffness and load at the 0.4 mm displacement provide information of the early onset of damage propagation. The decrease in chord stiffness of the ligament does not indicate damage progression in the ligament, but rather is the sign of the imminent failure of the MCL.This study provides insightful data into understanding the subfailure damage in the MCL.

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Chapter 1: Introduction and Background

1.1 Clinical Motivation

The knee is the largest joint in the human body. It acts as a synovial hinge that allows flexion and extension as well as medial and lateral rotation. The knee consists of a system of bone, muscle, tendons, and ligaments that help support the majority of the body's weight. This articulation can withstand large (~ 7-x body weight) forces (Taylor et al., 2004) and transfer compressive loads. However, it must also resist large torques due to forces that twist the lower extremity. At full extension, the knee is capable of resisting 90 N-m of valgus and 120 N-m of tibial axial torque (Hull, 1997). The knee joint consists of three bones: the tibia, femur, and patella. This joint has medial, lateral, and patellofemoral compartments. The lateral and medial meniscus tissue within the knee serves as fibro-cartilage support. Finally, the knee has 2 articulations: the patellofemoral joint and the tibiofemoral joint.

Muscle activity progressively increases as the knees are flexed and decreases as the knee are extended (Escamilla, 2001). The knee's structure allows it to resist repetitive loading and resist deformation in activities such as walking (Holden et al., 1993). The knee plays an essential role in carrying body weight in multiple directions (i.e. running and jumping). Excessive stretching of the ligament can result in gross joint instability. Knee joint instability can lead to altered joint kinematics, altered load distribution, and increased susceptibility to injury of musculoskeletal tissue. Four main ligaments contribute to the knee complex: the medial collateral ligament (MCL), the lateral collateral ligament (LCL), the anterior cruciate ligament (ACL), and the posterior cruciate ligament (PCL). Ligaments, and their mechanical properties, are of interest in this particular study.

1.2 Ligament Composition and Structure

Ligaments consist mainly of collagen. Their smallest basic structural unit is the collagen fibril, with diameters ranging from 10 to 500 nm depending on the age, location, and species from which the tendon/ ligament is sampled (Dyer & Enna, 1976). The hierarchical structure of a typical tendon was described by Kastelic et. al. (1978) as collagen molecules laid down into fibrils, bundles of fibrils forming fibres, and fibre bundles surrounded by endotenon to form fascicles which group together to form the tendon. Tendons and ligaments have similar structure. *Figure 1.1* details a schematic that represents the hierarchal structure of the ligament. Type I collagen is the major fibrillar collagen of the knee ligaments, and types III and V collagen are quantitatively minor components (Amiel et al., 1984, Niyibiza et al., 1995, Watanabe et al., 1994). Type I collagen is abundant and found in several parts of the human body, i.e. dermis, bone, tendon, and ligament. Type III collagen content increases with age or after ligament injury (Bland et al., 1996). Type V collagen is normally found in tissue that contains Type I collagen.

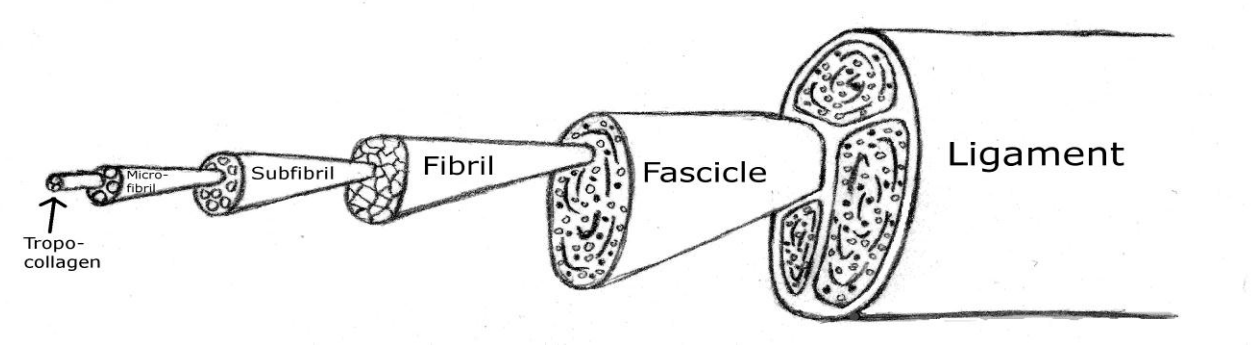

Figure 1.1: Schematic representation of ligament hierarchical structure

Fascicles exhibit a planar zigzag or crimp, and the stretching out of crimped fibrils is thought to account for the ‘toe’ region of the tendon/ ligament stress-strain curve, as described by Butler et al. (1978). The number of fiber bundles in a single fascicle and the number of fascicles varies in a tendon or ligament, and often within the same ligament (Jozsa & Kannus, 1997). The

main function of skeletal ligaments is to guide normal joint motion and restrict abnormal joint movement. The composition of each ligament is approximately similar. The major cell type is the fibroblast. The fibroblasts are interspersed in the parallel bundles of collagen. Fibroblasts are the cells responsible for synthesizing the extracellular matrix (ECM) and collagen (Chan et al., 2007). Previous studies have demonstrated that during early phases of ligament healing, type III collagen is highly elevated relative to type I collagen (Inoue et al., 1990), which is believed to produce small collagen fibrils (Amiel et al., 1987). Healed ligaments with smaller collagen fibrils are mechanically weaker than ligaments with normal collagen fibrils (Doilon et al., 1992).

The ground substance of a ligament is the gel-like mixture of proteins, proteoglycans (PGs), glycosaminoglycans (GAGs) and water that surrounds the ordered collagen fibrils. The ground substance is the main component responsible for holding the water within the ligament. Proteoglycans provide the structural constituent and are responsible for the highly viscous character of the ground substance. Proteoglycans consists of proteins (~5%) and polysaccharide chains (~95%) covalently linked to each other. Common proteoglycans found in ligaments are decorin and byglycan. GAGs are long, repeating disaccharides that link to protein cores to create the proteoglycans (Raman et al., 2005). GAGs are negatively charged due to their sulfate groups and results in their property of large osmotic pressures that allow the retention of water. This characteristic leads to the resistance of deformation and relatively high compressive modulus (Kiani et al., 2002). Common classes of GAGs include chondroitin sulfate and dermatan sulfate, a derivative of chondroitin sulfate. Chondroiton sulfate is a repeating disaccharide unit consisting of an acidic sugar-like molecule and a sulfated amino sugar, excluding hyaluronic acid (Raman et al., 2005). Apart from the cellular content, which contributes to only 1% to 3% of the dry

weight, the remaining mass of the extracellular matrix (ECM) consists of lipids, inorganic components, and non-collagen proteins such as elastin and various glycoproteins.

In normal ligaments, 80% of the proteoglycan is decorin, with the remainder biglycan and a large proteoglycan thought to be similar or related to versican (Hey et al., 1990, Campbell et al., 1996). Biglycan and decorin are found mainly between the collagen fiber bundles in the collateral ligaments, whereas in the cruciate ligaments, these proteoglycans are largely cell associated (Vogel et al., 1993; Benjamin & Ralphs, 1998). Elastin contributes only to a small proportion to the ligament's makeup, generally 1% to 2%. Elastin is composed of mainly hydrophobic amino acids, with a high proportion of glycine and proline and serves as a very stable and insoluble protein (Uitto, 1979).

1.3 Medial Collateral Ligament

The MCL is a commonly injured body tissue in athletes due to rapid impact trauma. *Figure 1.2* displays the right knee with the location of the MCL designated. The MCL is vital in the knee's maintenance of joint stability. Ninety percent of knee ligament injuries involve the ACLs and MCLs (Miyasaka et al., 1991). Specifically, the MCL is involved in approximately 40% of all severe knee injuries (Miyasaka et al., 1991), while approximately 50% of partial MCL tears and 80% of complete MCL tears occur in conjunction with injury to other knee ligaments (Fetto et al., 1978). The MCL complex is composed of the superficial MCL, the deep MCL, and the posterior oblique ligament. The superficial MCL is the focus of this study, known to be the primary stabilizer to valgus forces. This ligament was chosen to investigate due to its location for dissection as well as being able to load the MCL collagen fibers uniaxially. The posterior oblique ligament provides static resistance to valgus loads as the knee moves into full extension. The

deep MCL is a major secondary restraint to anterior translation (Wheeles, 2012).These ligaments are similarly injured in people involved in automotive crashes. If the MCL is torn, the loss of its functionality affects the surrounding ligaments and can create cumulative damage if not treated correctly and in a timely manner (Almarza et al., 2007). Many of the injuries will heal with conservative treatment, which is no surgery. However, knowing when to resume full physical activity is still under debate among orthopedic surgeons, as healing ligament mechanical properties are much different from normal ligament mechanical properties.

In the human knee, the MCL is approximately 80 mm long and runs from the medial femoral epicondyle distally and anteriorly to the posteromedial margin of the metaphysis of the tibia (Woo et al., 2006). At 25° of knee flexion, the MCL provides 78% of the restraining force against valgus injury. With extension, it plays a decreasing role, providing 57% of the restraining force at 5° (Pressman et al., 2003). The anterior fibers of the ligament tighten during knee flexion (Pressman et al., 2003). In the femoral insertion of the MCL, fibers attach directly into the bone and the transition of ligament to bone occurs in four zones: ligament, fibrocartilage, mineralized fibrocartilage and bone (Woo et al., 1987). The tibial insertion of the MCL is an indirect insertion in which superficial fibers are attached to periosteum while the deeper fibers are directly attached to the bone at acute angles (Woo et al., 1987). Advances in the study of the mechanical properties of the ligaments are needed in order

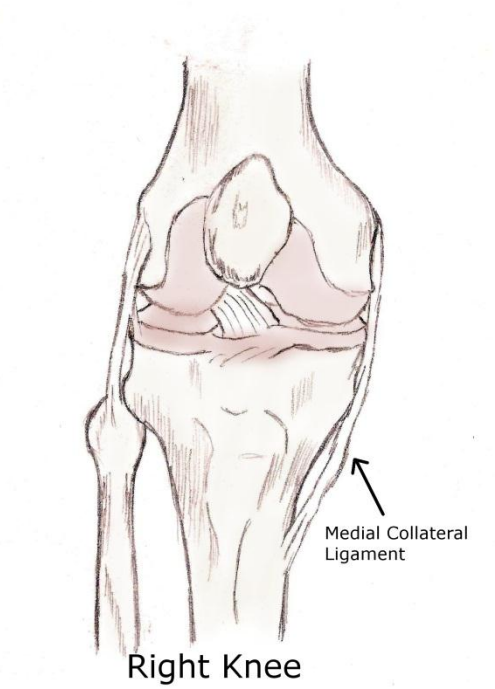

Figure 1.2: Location of MCL on Right Knee

to ultimately prevent and treat injury. A thorough analysis of damage criteria and mechanisms in the MCL has the potential to lead to a more efficient ligament grafting design.

1.4 Ligament Injury

Injuries to ligaments have been categorized into three types of sprains. Grade I sprains are mild stretches with no discontinuity of the ligament and no clinically detectable increase in joint laxity. Grade II sprains are moderate stretches of the ligaments with some torn fibers. Grade III sprains are severe and consist of a complete or nearly complete ligament disruption and result in significant joint laxity (Andriachi et al., 1987). Severe sprains involving complete disruption of the ligament and resulting in significant joint laxity constitute less than 15% of all ligament sprains. More than 85% of the sprains consist of subfailure damage.

In order to prevent injury, athletes often stretch within a certain optimal range of force and time. Exceeding this range may be detrimental to the laxity of the ligament leading to potential damage. After the MCL has been injured special care must be taken to maintain normal range of motion in order to ensure healthy recovery of the ligament. Thus, it is imperative that the damage properties of collagenous tissue be investigated.

Microtrauma or subfailure injury in tendon and ligament may occur either as a result of overuse or as a single traumatic event (Buckwalter et al., 1994). Partial tears to the ACL often lead to reduced levels of activity and performance (Freunsgaard et al., 1989). Subfailure injury has also been associated with increased laxity in the affected ligament that can lead to degenerative joint disease and osteoarthritis (Daniel et al., 1994).

The MCL, in many cases of subfailure injury, can heal unassisted. The MCL heals in 3 overlapping phases: inflammation, proliferation, and remodeling (Frank et al., 1983). The

formation of granulation tissue with the invasion of white blood cells to the damaged area occurs shortly after inflammation. Fibroblast proliferation and matrix synthesis mark the onset of the proliferation phase. Histological examination illustrates that fibroblasts become the dominant cell 3 weeks after ligament damage. The remodeling phase begins at approximately the sixth week when the fibroblasts decrease in number and size and their nuclei align along the long axis of the ligament (Frank et al., 1983, Woo et al., 1983). The replacement of the damaged tissue continues to mature for at least 48 weeks, but the mechanical properties do not return to the values assumed prior to injury. When damage to the MCL is severe to the point of complete failure and natural healing is not possible, ligament grafting becomes an option.

While the majority of ligament reconstructions yield good short-term clinical results, 20–25% of patients experience complications including instability that could progressively damage other knee structures (Aglietti et al., 1997). Xenograft tissues that do not rely on cellular activity to perform their necessary function are ideal material grafts and offer benefits as repair aids to damaged ligaments. Cellular activity is thought to be minimal in tendons and ligaments and their function is due mainly to their dependence on the properties and arrangement of the comprising collagen fibers (Milthorpe, 1994). Tissues from cadavers or allografts have proven to exhibit the necessary mechanical strength and promotion of cell and tissue growth. Both allografts and xenografts share the risk of transfer of harmful diseases, bacterial infection, and complications due to the host's immune system (Cooper et al., 2005, Laurencin et al., 1999, Freeman et al., 2007). The optimum choice for grafting surgery is autografting. Autografts possess the necessary amount of initial mechanical strength and promote new cell growth without the risk of infection or a patient's immunological response (Freeman et al., 2008). Characterizing the mechanical behavior of ligaments is important in tissue engineering. It provides essential information that

can be used to generate ligamentous tissue that has material and mechanical properties comparable to the original body tissue.

1.5 Hydration Effects

Ligaments can be described as hydrated fiber-reinforced matrix, in which collagen fibers provide mechanical stability to the ground substance. The mechanical properties of ligaments are greatly influenced by the hydration levels. Being a major component of the structure, a change in hydration of the tissue leads to a difference in its elastic and viscoelastic behavior. Injury to a joint often results in inflammation producing swelling of the affected soft tissues (Frank et al., 1983). Ligaments may be subject to changes in water content because of injury (Frank et al., 1983) or as a result of treatments such as ligament reconstruction (Sabiston et al., 1990) and joint immobilization (Akeson et al., 1987). Some surgeries (e.g., ligament grafting) and many types of ligament experiments in vitro involve irrigation with saline solutions that have the potential to alter tissue properties (Thornton et al., 2001).

Dehydration and nutritional deficiencies can lead to dry and stiff ligaments as well as hardened fascia. Therefore, dehydration may lead to chronic joint pain and stiffness. Dehydration of the knee joint and its constituents during exposure to air has been visually observed in the form of surface undulations during surgery (Moshurchak & Ghadially, 1978; Ghadially et al., 1983). In addition, the collagen fiber diameter and the water content in ligaments have been shown to decrease with aging (Natali et al., 2008).

The complex interactions of collagen with elastin, proteoglycans, ground substance, and water result in the time- and history-dependent viscoelastic behaviors of ligaments. In response to various tensile loading protocols, ligaments exhibit hysteresis (i.e. internal energy dissipation),

creep, and stress relaxation (Woo et al., 2006). Significant changes in connective-tissue water content have been observed following exposure to air (8 min) or after 3 min of immersion in distilled water, Ringer's solution, human plasma, or Macrodex (Tkaczuk, 1968). Testing has shown that the rabbit ACL swells after immersion in "physiological" saline, inducing larger deformations at failure and higher failure energies (Viidik & Lewin, 1966). On the other hand, dehydration in air causes tendons and ligaments to be stronger and stiffer than their moist equivalents and causes a decrease or elimination in the ligament's toe region of the stress-strain curve (Eldon, 1964; Galante, 1967; Betsch & Baer et al., 1980; Haut & DeCou et al., 1984).

Investigators have seen difference in water content in ligaments comparing MCLs immersed in a phosphate buffered solution (PBS) with a 74% initial water content against MCLs immersed in a 25% sucrose PBS solution with 50% initial water content (Chimich et al, 1992). The majority of the structure of MCLs is composed of water due to its ground substance. The 25% sucrose PBS solution has been shown to cause a physiologically relevant dehydration level for the MCL. This study aims to investigate the role of the ground substance in damage initiation and progression by using dehydrated MCLs.

1.6 Role of Sucrose in Soft Tissue

As stated previously, the viscoelastic response of soft tissues is likely affected by their two most abundant components: water and collagen (Frank et al., 1985). Previous studies have documented that increases and decreases in water content lead to changes in the mechanical behavior of ligaments. Sucrose is an organic compound that has been used to decrease water content in tissues within the body in modern medicine. For example, intravenous administration of hypertonic solutions of sucrose has become popular within recent years for the reduction of

increased intercranial pressure. Sucrose reduced cerebrospinal fluid pressure without a secondary increase of pressure such as the one that follows with the administration of dextrose or saline solutions (Bullock et al., 1935).

Sucrose has been found to be an osmotic dehydrator. Placing a tissue in a hypertonic solution of sucrose forces the water to move from the inside of such tissue to the outside. For strips of patellar tendons placed in distilled water, the amount of load relaxation and the rate of load relaxation increased as the water content increased (Atkinson et al., 1999). Similarly, rabbit MCLs with higher water contents (PBS and 2% sucrose PBS solutions) exhibited significantly greater cyclic load relaxation when compared to ligaments with lower water contents (10% and 25% sucrose PBS solutions) (Chimich et al., 1992). Stress relaxation tests of rat tail tendons revealed a decrease in diameter of the collagen fiber bundles with dehydration that has been attributed to the fluid escaping the tissue (Lanir et al., 1988).

Through the years, different solutions have been used to change the amount of water within the knee ligaments. In-vivo, ligaments and tendons are subjected to time-varying loadings that are often cyclic as in walking or running (Hoffman et al., 2005). Hypotonic solutions of distilled water have been found to lead to faster stress relaxation rates of the ligaments as opposed to hypertonic solutions of 25% sucrose (Haut & Haut, 1997).

1.7 Previous Experimental Methods

The tensile mechanical behavior of the MCL has been thoroughly investigated. Three main regions can be detected in the load-displacement data collected by performing a tensile test along the longitudinal direction of the ligaments. The initial non-linear portion of the load-displacement curve has been termed the toe-region. This nonlinearity has been associated with

the uncrimping of the collagen fibers and fibrils within the ligament. The following section is the linear region determined by the straight collagen fibers and fibrils that are stretched. The final region of the tensile behavior is the damage region, in which the collagen fibers and fibrils begin to tear until the ultimate rupture of the MCL. The changes in fiber organization during loading and the 3 regions mentioned above are shown in *Figure 1.3*.

The influence of short-term prednisolone treatment, an anti-inflammatory hormone to reduce swelling, on the mechanical properties of skin and muscle tendon has shown an increase in stiffness values (Oxlund et al., 1981). Changing the testing environment changes the outcome of mechanical tests in connective tissue. Investigators determined the effect of ligament water content on ligament mechanical behavior by altering the test environment (Chimich et al., 1992). Experimenters have documented the changes in the load-deformation curve until the failure point, after the ligament had been subjected to an 80% subfailure stretch (Panjabi et al., 1996).

Ligaments have been subjected to load relaxation experiments prior to being stretched to failure with the objective of quantifying the effect of tissue hydration on the structural properties of human patellar tendons (Haut & Haut, 1997). Studies have quantified the onset of structural damage in the MCL as characterized by nonrecoverable change in tissue length after a subfailure stretch, and quantified regions of cellular damage in ligament as a function of subfailure ligament strain (Provenzano et al., 2002).

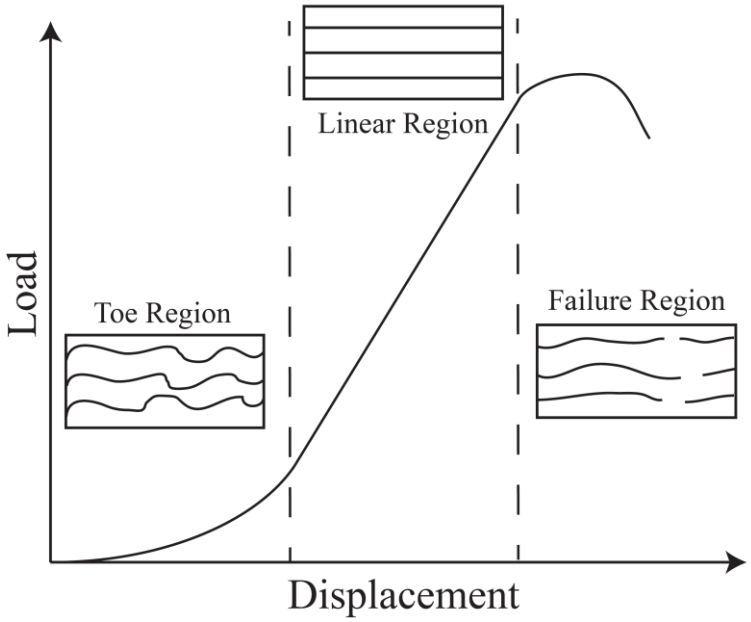

Figure 1.3: The behavior of collagen fibers/ fibrils during loading of the MCL. Initially, the collagen fibers are crimped. In the toe region, the fibers begin to uncrimp, continuing into the linear region in which the fibers straighten. Finally, damage occurs when the collagen fibers begin to break. When the majority of the fibers break, the ligament ultimately fails.

There have been many cases of ex-vivo and in-vitro experiments on the MCL with comparatively few in-vivo testing measures (Huijing et al., 2001, Andarawis-Puri et al., 2012). Human cadaveric testing has been useful in discovering the previously unknown mechanical and material properties of the knee ligament. As technology continues to advance, more non-invasive methods can be developed to test in-vivo the human knee to ultimately determine conditions that will help to prevent injury. Experimentation of ligament mechanics is often technically difficult, costly, and prone to error. The mechanical properties within ligaments are nonhomogeneous, although in many tests they are assumed homogenous. Research with conclusive evidence requires large numbers of animals or significant amounts of human tissue (Weiss et al., 2001). As mentioned, research has been conducted to understand how the MCL is damaged. By comparing different methods of quantifying damage in the MCL, one can evaluate which method among those compared indicate damage progression in the tissue.

1.8 Research Goals

While impact trauma is a great threat to ligaments, the laxity of these tissues can cause severe damage to their structure and alter their function. Mechanical quantities will be determined in this study to characterize damage mechanisms in ligaments. This preliminary study into the subfailure mechanics of MCLs could ultimately lead to advances in the biomechanics field.

For purposes of this project, murine ligaments will be used. Their MCL structure is physiologically similar to that of humans (Cook, 1965, Chiasson, 1988). Rats are relatively inexpensive and readily available for the use of experimentation. Testing dehydrated MCLs can give some insight into the role of the ground substance in concern to damage progression.

In summary, there is a great need for further the knee's ligaments. MCL injuries are occurring too frequently and reduce the quality of life of many people. This study aims to compare new and previous methods of quantifying damage in the ligament to better understand which method can better describe damage progression in the MCL. By successfully completing this study, clinical progress can be made towards preventing and treating knee injuries. This chapter provided some introductory and background information into the MCL, how injuries to this tissue occur, and research proposed about the tissue's mechanical properties. The following chapter describes the experimental testing method, the results obtained, and a discussion of these findings.

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Chapter 2: Analysis of Damage in MCLs

2.1 Introduction

Researchers have investigated the mechanical properties of the ligament for several decades in order to learn how to prevent injuries and improve the recovery from injury. Overstretching and partial tears in the ligament associated with grade I and grade II sprains can lead to cumulative damage in the ligament. The relatively miniscule tears in the ligament can ultimately lead to complete tissue failure.

There has been insignificant research on the determination of mechanical methods that can be employed to quantify the mechanical characteristics of MCLs that are associated with damage propagation. The aim of this study is to compare different mechanical quantities that can be used to measure damage in MCLs. To begin, a preliminary paired study was performed to investigate the changes in mechanical behavior by altering the ligament water content. Here, one of the rat's MCL was taken as a control and immersed in a PBS solution while the contralateral MCL of the same rat was taken as the experimental and immersed in a 25% sucrose PBS solution. Toward this end, tensile tests were performed on 5 pairs of rat MCLs, comparing the mechanical behavior of the rat's ligaments. Each MCL was subjected to consecutively increasing displacements until complete rupture. Once the preliminary paired study determined the effect of dehydration in the ligaments, the project transitioned into the damage evolution protocol testing 30 individual dehydrated MCLs and measuring the different mechanical properties to quantify damage. The same experimental protocol performed in the preliminary paired study was used for this damage evolution study. The specimens were kept in the hypertonic PBS solution with 25% sucrose for this study in order to reduce excessive hydration. Load-displacement data were

collected and analyzed to determine different quantities that have been used to quantify damage. This study represents the first investigation of the comparison of different mechanical parameters used to quantify damage in dehydrated MCLs. The results of this study can offer new guidelines to future researchers interested in characterizing damage initiation and propagation in MCLs.

2.2 Materials and Methods

The study was conducted in accordance with applicable laws, regulations, guidelines, and policies such as the U.S. Animal Welfare Act, Public Health Service Policy, U.S. Government Principles, and the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee at Virginia Tech approved the conduct of this experimental study. Five Harlan Sprague Dawley male rats (376.60 ±16.80 g, body mass) were used for the preliminary paired study. Seventeen Harlan Sprague Dawley male rats (232.50 ± 8.30 g, body mass) were used in the damage evolution experiments. The animals were obtained from KemPharm Inc. after euthanization by carbon dioxide gas and stored in a freezer at -6° F.

The specimens were thawed by placing the rats in a plastic bag and placing the bag in a heated water bath for 1 hour prior to dissection. A surgical scalpel (size 10) was used to expose the MCL by removing the surrounding skin, muscle, and fascia tissues. The hind limbs were amputated distal to the hip joint and the tibia was amputated from the ankle joint. This extracted component of the hind limb is termed the femur-MCL-tibia complex (FMTC). The average length of the MCL in these rats was 10 ±0.8 mm. A stereomicroscope (Zeiss Stereoscope Stemi 2000C) was used to further inspect the MCL to remove the fine tissue layers surrounding the ligament. During the course of dissection, the tissue was kept hydrated by spraying the sample with a phosphate buffered solution (PBS) (pH 6.8, Sigma-Aldrich). The dissected FMTCs were wrapped in gauze, bagged in durable plastic, and stored in PBS solution at -6° F until required for testing. This method of storage does not affect the mechanical properties of the ligament, but instead provides a larger window in which the tests can be performed (Woo et al., 1986). Four FMTCs of the damage evolution study were damaged during dissection and, hence, were excluded from the study.

Prior to mechanical testing, the FMTCs were thawed in a heated bath while in their plastic bags for 15 minutes. For the preliminary paired study, one of the rat's MCLs was taken as a control and immersed in a PBS solution. The rat's contralateral MCL was taken as the experimental and immersed in a 25% sucrose PBS solution. Each FMTC was placed in their designated solution for 1 hour before testing. This time was chosen based on previous studies. Investigators have shown that this time period is sufficient for water content equilibrium between the ligament and the solution by using a tritiated water tracer (Chimich et al., 1992). For the damage evolution study, each of those FMTCs were immersed in the 25% sucrose PBS solution for 1 hour before testing. By visually inspecting each ligament, the ligaments appeared more translucent after being placed in the 25% sucrose PBS solution (See *Figure 2.1*). Special care was taken to ensure that the entire ligament and its insertion sites were submerged in the solution to avoid prolonged air exposure .

After immersing the ligaments in their respective solutions, the specimens were bonded at the tibial and femoral ends to a plastic (polyethylene terephthalate copolymer) grid using an ethyl cyanoacrylate epoxy (EZ Quilting, Antioch, Tennessee, 2008). The epoxy was allowed to set and the sample was kept hydrated. The femur was placed in a position that corresponded to the anatomic position of 70° knee flexion. A typical prepared FMTC can be seen in *Figure 2.2*.

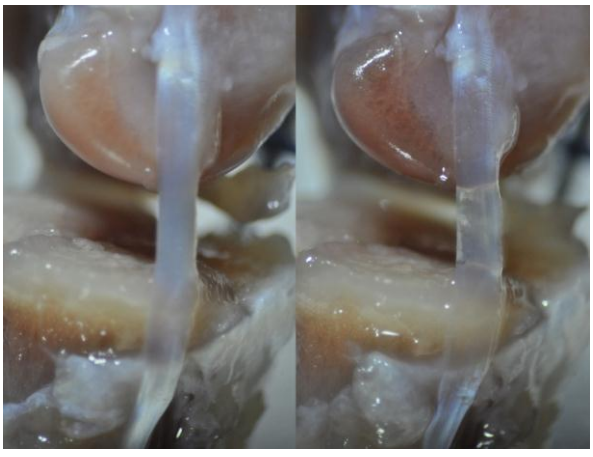

Figure 2.1: The left picture shows a normally hydrated ligament and the right picture shows the more translucent, dehydrated ligament after immersion in the 25% sucrose PBS solution.

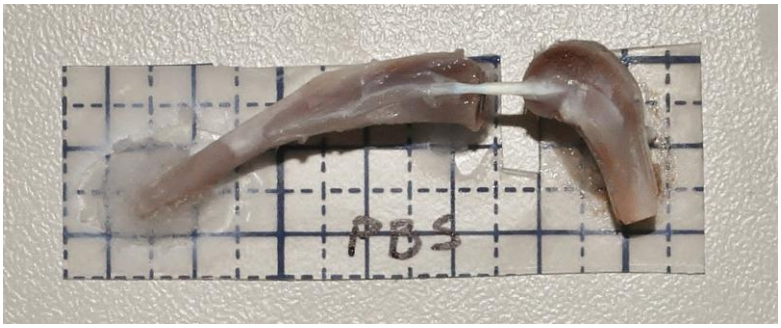

Figure 2.2: A typical femur-MCL-tibia complex (FMTC) that is used for experimentation.

Hose barbs were used for mounting the specimens on the tensile testing machine. Dental bone cement powder (Stoelting Company, Chicago, Illinois, 1886-2013) filled the hose barbs and the FMTC was placed into the hose barb. The cement powder was solidified using the accompanying cement liquid. This reaction was allowed to set for 15 minutes before any testing occurred. This hose barb was designated as the tibial clamp. The tibial end of the hose barb was gripped by the upper mechanic grips that accompanied the tensile testing machine. The MCL's longitudinal axis was aligned with the load axis of the machine. The lower mechanical grip of the testing system gripped a second hose barb designated as the femur clamp. Cement powder filled the second hose barb. Then, the crosshead of the machine was lowered to place the femur end of the FMTC inside of the femur clamp. Cement liquid was used to set the FMTC in place for fifteen minutes. Care was taken to avoid the cement powder from covering the femoral and tibial insertions of the MCL since the exothermic reaction of the dental cement can affect the properties of MCL. Throughout the cement setting process, the designated testing solutions was used to irrigate the ligament to maintain hydration. Once cement's reaction had set, the grid plastic was cut to allow movement in the FMTC during testing. After the specimens were mounted to the testing system, an immersion bath was used. For the preliminary paired study, the control FMTCs were placed in a PBS solution bath while the experimental FMTCs were

immersed in the 25% Sucrose PBS solution bath. For the damage evolution study, every FMTC was immersed in the 25% Sucrose PBS solution for the testing bath. PBS is isotonic and isosmotic for ligaments whereas sucrose is organic, physically compatible with the ligament, and osmotically active (Chimich et al., 1992). The complete test set-up can be seen in *Figure 2.3*.

Displacement-controlled tests were conducted on FMTCs using a tensile testing machine. The testing system used in these experiments was the Instron ElectroPuls E1000 with a static 50 N load cell (accuracy: 0.25% of indicated load or 0.025% of load cell rated output, whichever is greater, resolution: 0.01 N). A linear variable differential transformer accompanying the machine measured the displacement (accuracy: 0.5% of indicated displacement, resolution: 0.01 mm). The experimental protocol is shown in the schematic in *Figure 2.4*. To begin, the ligaments were preloaded to 0.1 N. The displacement value corresponding to this load was set as the starting zero displacement point. This value was chosen to eliminate slack in the MCL and give each MCL a comparable starting point. Next, the ligaments were preconditioned using 10 Havertriangle cycles with a peak amplitude of 0.3 mm at 1.0 Hz. Preconditioning all of the samples ensured that the ligaments had a similar loading history prior to the actual experimental protocol. After preconditioning, the load on the specimens was set to the 0.1 N preload. A recovery period of 10 minutes was chosen between preconditioning and the beginning of testing.

Each MCL was then stretched to a series of incremental displacement values, d_k (k=1, 2 ,3 ,…) with d_1= 0.4 mm, d_2= 0.6 mm, d_3= 0.8 mm, d_4= 1.0 mm, and onward where d_{k+1}-d_k= 0.2 mm at a displacement rate of 1.0 mm/sec until failure occurred. After each progressive stretch to a designated displacement, the MCL was unloaded and allowed to recover for 10 minutes before the subsequent displacement occurred. The load cell recorded the load data at a sampling rate of 10 Hz during testing. To test the change in tensile behavior due to differences in ligament water

content, the preliminary paired tests were performed using this experimental protocol. Load-displacement data were recorded for each incremental displacement. The results obtained for the 5 rat MCL pairs were compared. Following the preliminary paired study, the damage evolution study used the same experimental protocol for each of the 30 dehydrated FMTCs.

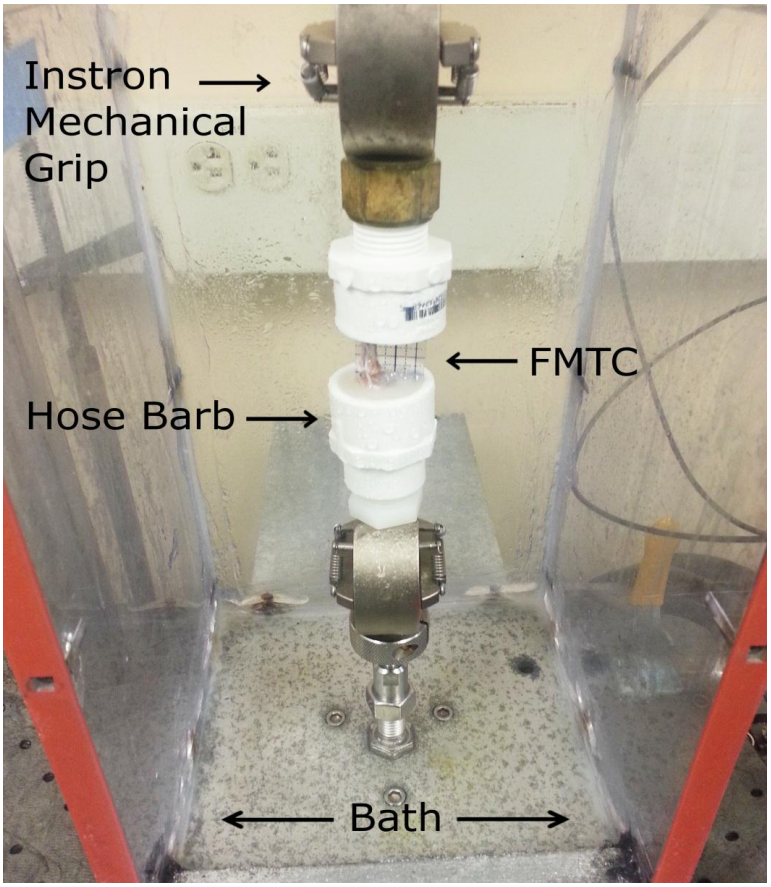

Figure 2.3: The experimental setup. The FMTC, hose barbs, bath, and the Instron grips are labeled inside the figure.

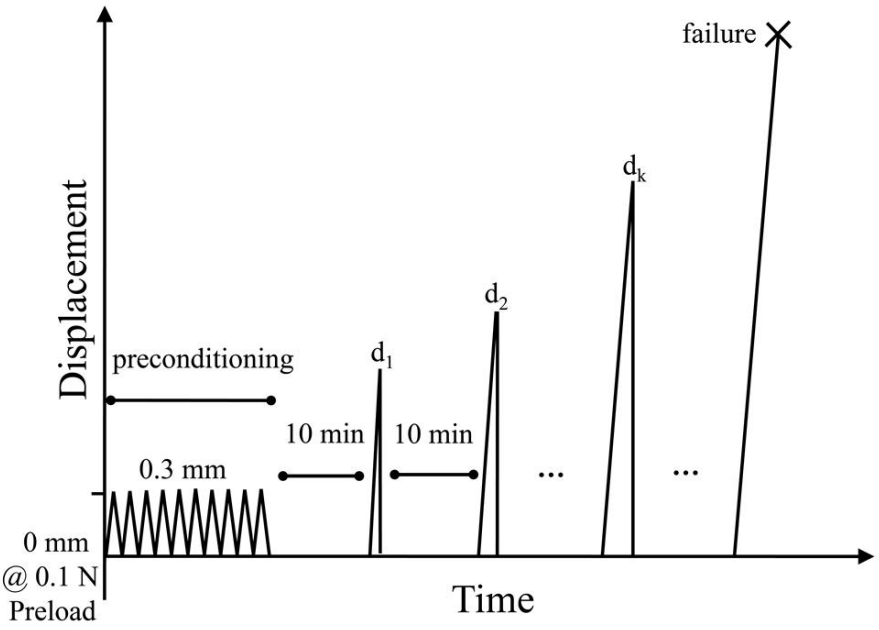

Figure 2.4: The testing protocol for each tested FMTC. The ligaments are preloaded and preconditioned initially. The ligaments are returned to the preload position and allowed to recover for 10 minutes. The ligaments are stretched to a displacement d_1 at a displacement rate of 0.1 mm/sec, and then returned to the preload position. The process is continued by stretching the ligaments to higher displacement values at the same displacement rates and then returned to original position until specimen failure.

Three different mechanical quantities were used to quantify when damage had progressed in the ligament in the damage evolution study. These were the tangent stiffness, the chord stiffness, and the load at the 0.4 mm displacement computed for the different consecutive stretches. Tangent stiffness, measured in N/mm, is defined as the change in load with respect to the change in displacement in the linear region of the load-displacement curve. Chord stiffness, measured in N/mm, is defined as the difference between the maximum load and the minimum load, where L_{min}=0.1 N, divided by the difference between the displacement at the maximum load and minimum load. Zec et al. have used the chord stiffness in characterizing fatigue behavior in MCLs (Zec et al., 2010). A schematic presenting the tangent and chord stiffness values is depicted in *Figure 2.5*.

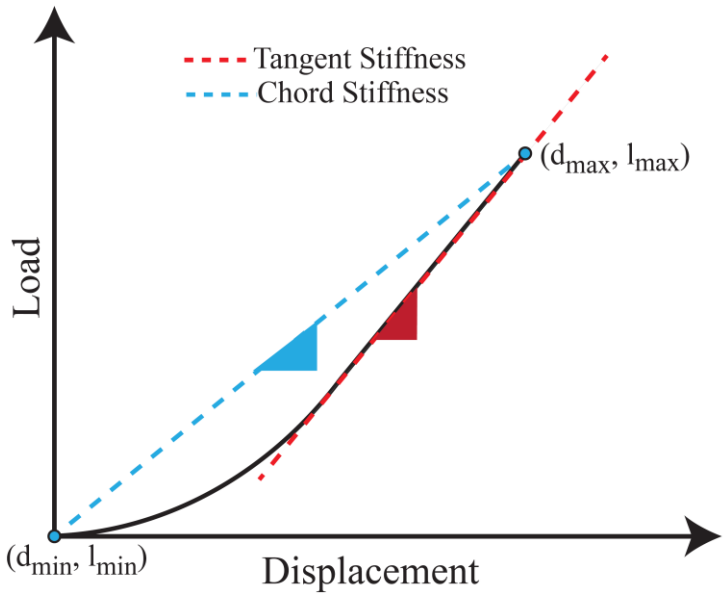

Figure 2.5: This schematic shows a typical load-displacement curve from one of the stretches in the FMTCs. This schematic indicates how the slopes were calculated to determine the tangent and chord stiffness. The tangent stiffness is the slope from the linear region of the load-displacement curve. The chord stiffness is the slope from the maximum displacement and load value to the resting displacement and load value of the FMTC.

In order to measure changes in the toe region of the load-displacement curve after each displacement was applied to each FMTC, the load at 0.4 mm displacement was recorded. The load values computed at the 0.4 mm displacement for each consecutive displacement were denoted as $l_{0.4}$, $l_{0.6}$,$l_{0.8}$, and so on. The subscript indicates the maximum displacement applied to obtain the load-displacement curve considered. The differences between load values at 0.4 mm displacements were computed for each tested FMTC and are here referred to as load increments. These load increments are schematically presented in *Figure 2.6*. In order to determine whether there was a significant change between the load increments at two consecutive displacements, a Wilcoxon Signed Rank test was conducted. Statistical analysis was done using JMP software (JMP Pro, Version 10.0, SAS Institute Inc., Cary, NC, 1989-2012). This statistical test is advantageous since the tested parameter is not required to have a normal distribution.

The 3 main assumptions made in using the Wilcoxon Signed Rank test are 1) a simple random sampling for the FMTCs, 2) dependent sampling, and 3) symmetric distribution of the differences about the median value. In this statistical analysis, the p-value designates if there is evidence to reject or not reject the null hypothesis. The null hypothesis here is that the differences between the load increments at 0.4 mm displacements are symmetric about zero. The alternative hypothesis is that the differences between the load increments are not symmetric about zero. Therefore, if the null hypothesis is not rejected, then there is no significant change in the load increments. The α-value represents the level of significance for this statistical test and, for this study, α=0.05, a value commonly used in statistical analysis (Blaesild et al., 2003). The null hypothesis will be rejected if the p-value is less than the α-value.

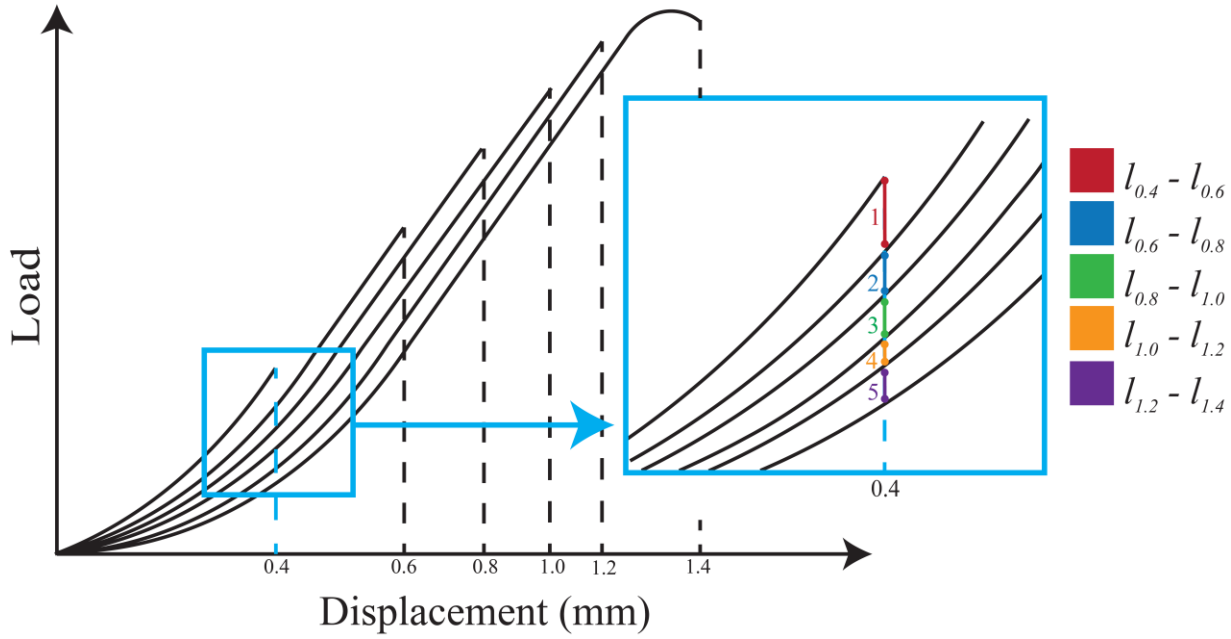

Figure 2.6: Load-displacement curves obtained by stretching one FMTC to incremental displacements. The right hand side of the schematic shows an increased view of the load-displacement curves of a single FMTC at the 0.4 mm point. The colored lines designate the different load increments, numbered 1-5. The toe region is analyzed by determining whether there is a significant difference between the load increments of consecutive displacements.

2.3 Results

In the preliminary paired study in which the ligament water content was changed, the load-displacement curves for the contralateral control PBS treated rat MCL and the PBS-sucrose treated MCL obtained are displayed in *Figure 2.7*, each plot showing a different maximum displacement. The main difference between the control MCL and the MCL immersed in the 25% sucrose solution is the loss or reduction of the toe region for the 25% sucrose treated MCLs. Larger loads are also sustained by the MCLs immersed in the 25% sucrose solution.

The typical load versus displacement data collected on each FMTC for the damage evolution study are shown in *Figure 2.8*. Each load-displacement curve was obtained by stretching the ligament to a displacement d_k as indicated in the legend and using the 0.1 N preload as the reference configuration. By qualitatively examining the curves, one can observe that as the value of d_k increases, the toe region of the curve elongates. These changes in the tensile behavior of the ligament have been associated with the initiation and propagation of damage (Guo, 2011).

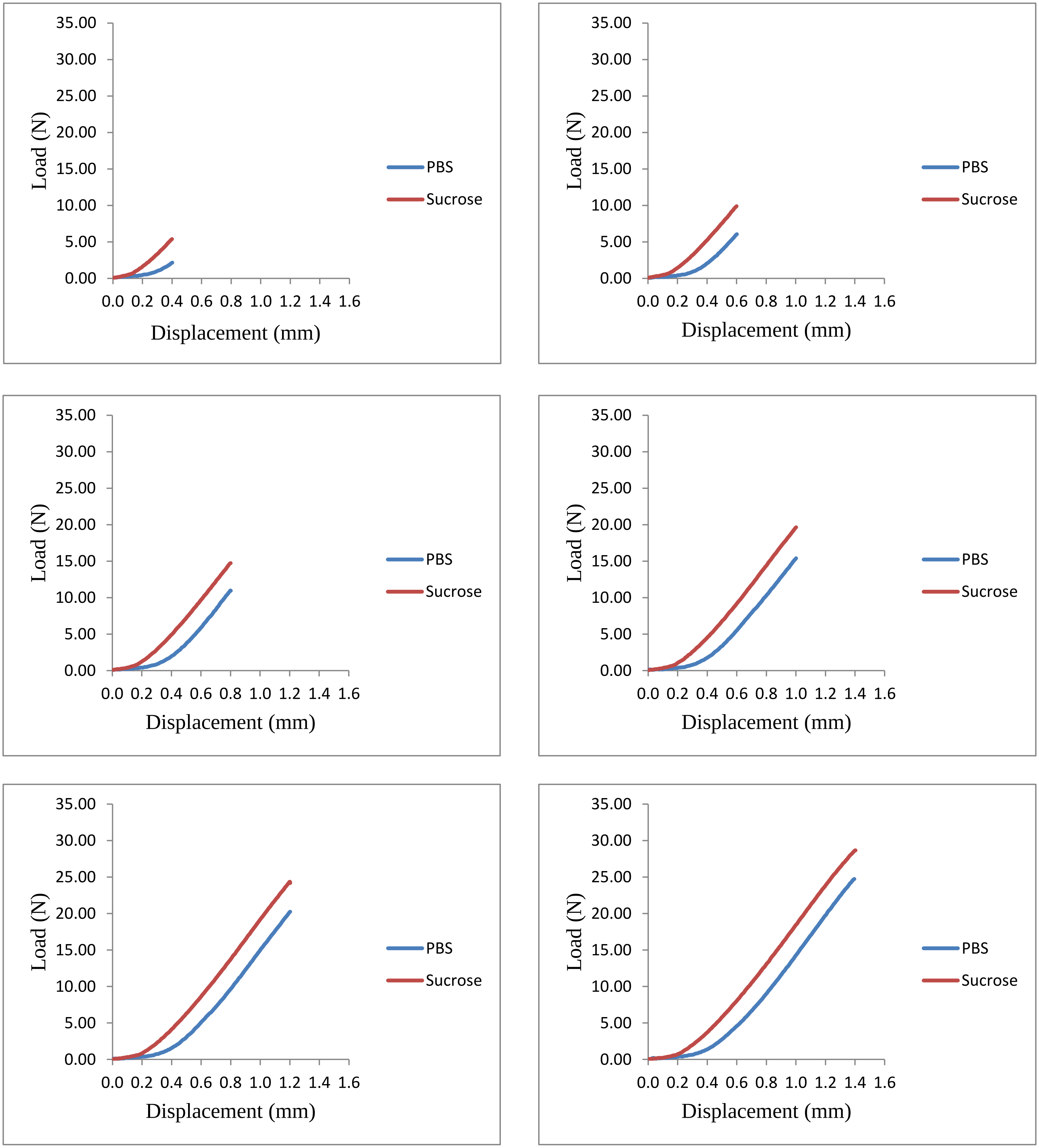

Figure 2.7: Tensile behavior at different displacements of PBS-treated and PBS-sucrose treated MCLs from one rat MCL pair.

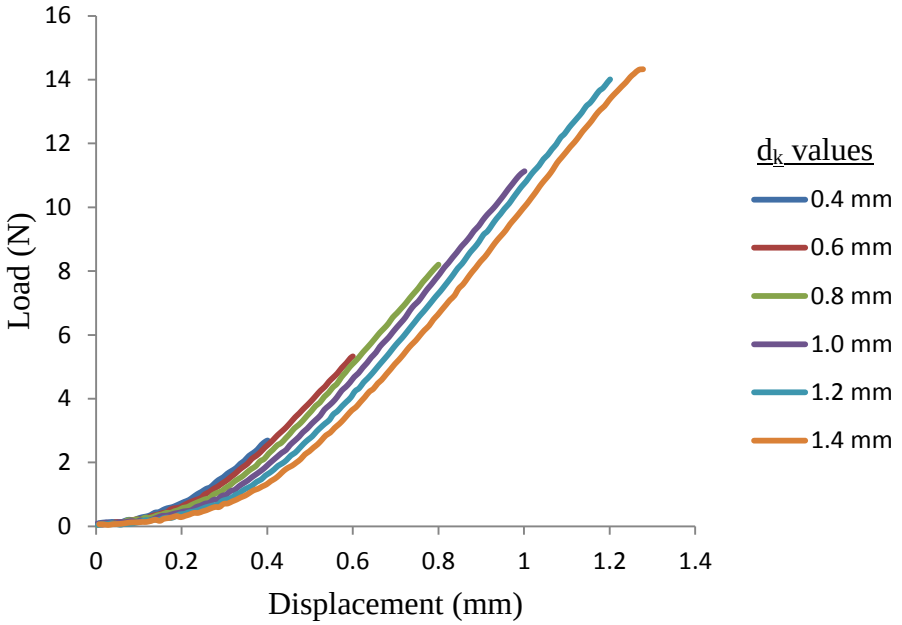

Figure 2.8: The load-displacement curves of one of the tested FMTCs.

After the load-displacement curves were obtained for all of the FMTCs, the tangent stiffness values were computed from their linear regions. Specifically, a linear regression equation was fitted to the linear portion of the load-displacement curve. The slope of such line determines the tangent stiffness. The R^2 correlation value for each linear fit was greater than 0.99. A drop of at least 0.1 N/mm indicated a decrease in stiffness value for the tested FMTCs. The experimental results have shown that for 27 out of 30 (90%) tested FMTCs the tangent stiffness decreased after the 0.8 mm displacement. The tangent stiffness decreased after the 0.6 mm displacement for 2 out of the 30 (6.7%) tested FMTCs. One out of the thirty (3.3%) tested FMTCs had a tangent stiffness that decreased after the 1.0 mm displacement. Similarly, the chord stiffness values were obtained from each load-displacement curve as described in the methods section. One out of the thirty (3.3 %) FMTCs showed a decrease in chord stiffness after

the 0.6 mm displacement, 9 out of the 30 (30%) FMTCs showed a decrease after the 0.8 mm displacement, 16 out of 30 (53.3%) FMTCs exhibit a decrease after the 1.0 mm displacement, and 4 out of 30 (13.4%) FMTCs show a decrease after the 1.2 mm displacement. The changes in tangent and chord stiffness due to the application of progressive displacements are plotted in *Figure 2.9* for 6 representative specimens.

The graphs in *Figure 2.10* show the change in load increment value for 6 representative tested FMTCs. The consecutive load increments' magnitude changes as the displacement value increases for the tested specimens. In order to run the Wilcoxon test, the symmetry of the load increment data about their median value had to be determined. The histograms of the differences between load increments at 0.4 mm displacement showed a median value that was centered in the distribution of the differences in the load increment values (See *Figure 2.11*). The other 2 assumptions required to use the Wilcoxon Signed Rank test, having a simple random sampling and dependent samples, were also satisfied due to the testing protocol used. The p-values were calculated comparing the differences between the load increments at 0.4 mm displacements.

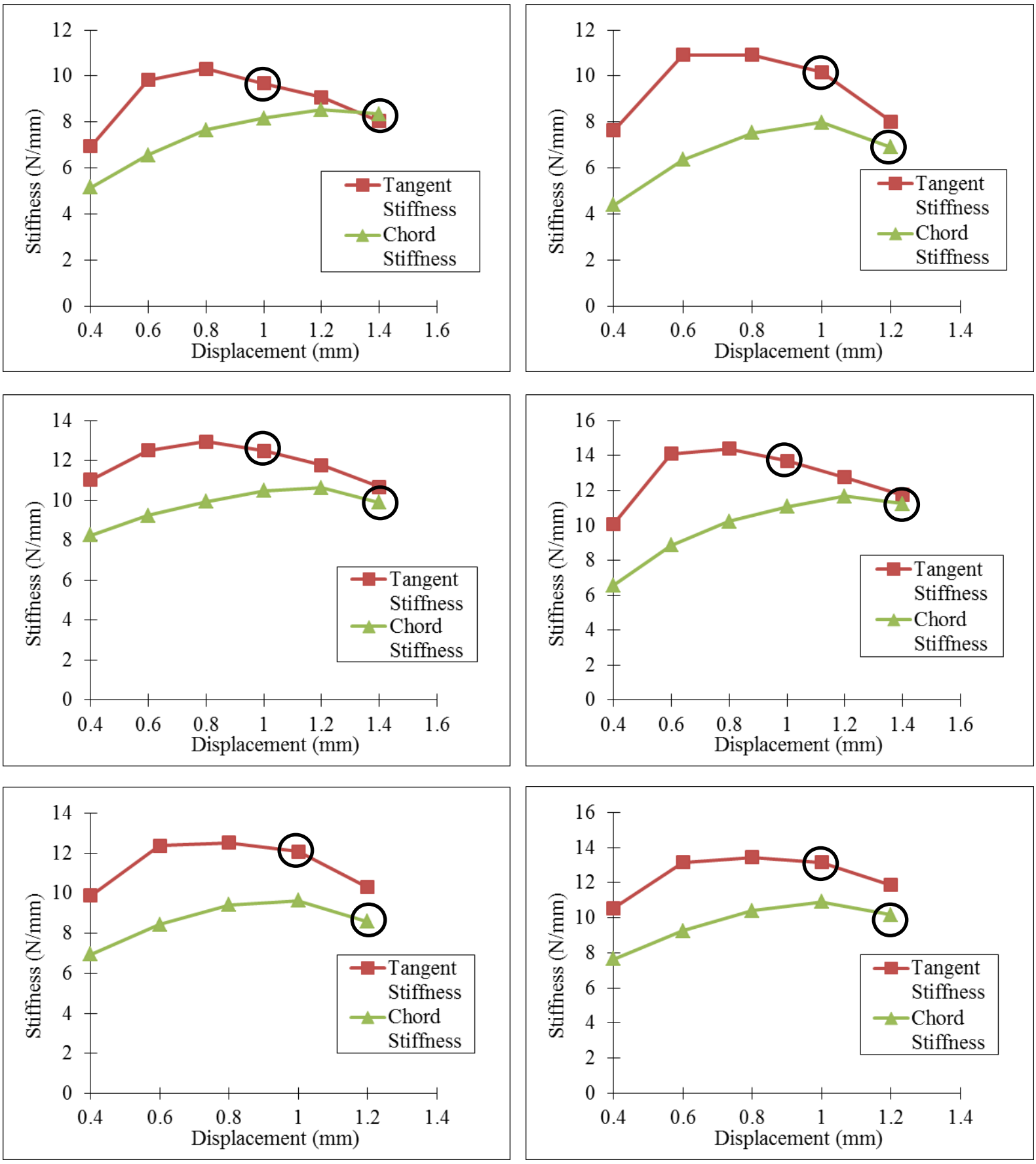

Figure 2.9: Tangent and chord stiffness values for each of the consecutive displacements for six of the tested FMTCs. The majority of the decreases in tangent stiffness (90%) occur after the 0.8 mm displacement. In contrast, the decrease in chord stiffness mainly occurs (76.7%) only before impending failure of the ligament. The black circles around the displacement, stiffness values indicate the first decrease in tangent and chord stiffness.

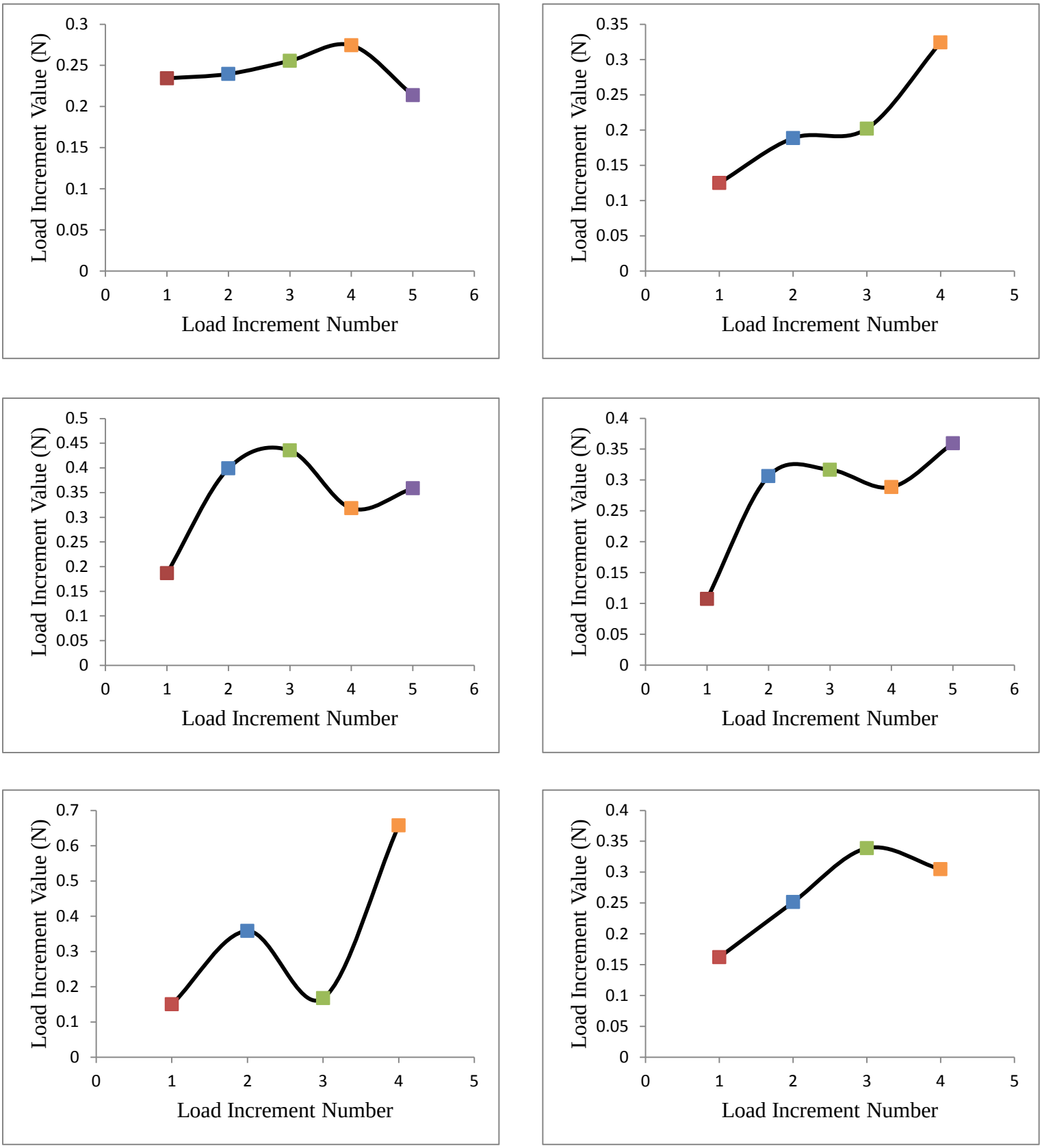

Figure 2.10: Load increment values for 6 of the tested FMTCs. These graphs for the tested FMTCs show the differences in load increment value as the displacements increase. The colors of the symbols denoting load increments are matched with the schematic in Figure 2.6 presented in the methods section. Thus, the red symbol denotes the load increment $l_{0.4} - l_{0.6}$, the blue symbol denotes the load increment $l_{0.6} - l_{0.8}$, the green symbol denotes the load increment $l_{0.8} - l_{1.0}$, and so on.

The p-values and the designated outcomes following the rejection of the null hypothesis if p-value <0.05 obtained for each of the load increment comparisons are shown in Table 2.1. One must note that the specimens failed at different displacements: 30 FMTC samples sustained the stretch to the 1.0 mm displacement. Only 19 of the 30 FMTCs sustained the 1.2 mm displacement, and out of those 19 FMTCs, 9 sustained the 1.4 mm displacement before failing.

Table 2.1: Wilcoxon Signed Rank Test Results

Load Increment Comparison	P-Value	Outcome
$l_{0.4} - l_{0.6}$ and $l_{0.6} - l_{0.8}$	<0.0001	Significant Difference
$l_{0.6} - l_{0.8}$ and $l_{0.8} - l_{1.0}$	0.0003	Significant Difference
$l_{0.8} - l_{1.0}$ and $l_{1.0} - l_{1.2}$	0.0141	Significant Difference
$l_{1.0} - l_{1.2}$ and $l_{1.2} - l_{1.4}$	0.2031	Not a Significant Difference

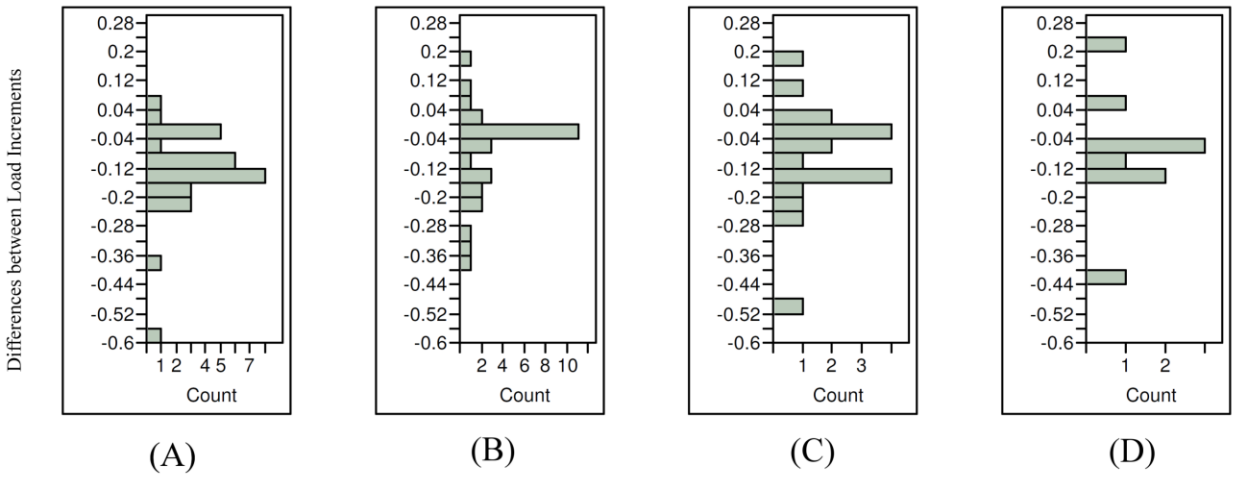

Figure 2.11: The histograms for the paired differences between the load increments at 0.4 mm displacement of the load-displacement curves for all of the FMTCs. (A) differences in load increments $\boldsymbol{l_{0.4} - l_{0.6}}$ and $\boldsymbol{l_{0.6} - l_{0.8}}$. (B) differences in the between load increments $\boldsymbol{l_{0.6} - l_{0.8}}$ and $\boldsymbol{l_{0.8} - l_{1.0}}$. (C) differences between the load increments $\boldsymbol{l_{0.8} - l_{1.0}}$ and $\boldsymbol{l_{1.0} - l_{1.2}}$. (D) differences between load increments $\boldsymbol{l_{1.0} - l_{1.2}}$ and $\boldsymbol{l_{1.2} - l_{1.4}}$. The medians of these differences values are (A) -0.134, (B) -0.0371, (C) -0.0786, and (D) -0.0711. The data are approximately distributed about the respective medians therefore the assumption of symmetry about the median was met by the data.

Every FMTC complex failed at the tibial insertion of the MCL. As mentioned in Chapter 1 of this study, the fibers at the femoral insertion of the MCL are into the bone while the fibers at the tibial insertion are attached to the periosteum and into the bone at acute angles. The morphology of the ligament is thus associated with the failure location of the FMTCs (Wei & Messner, 1996). This suggests that the tibial insertion is weaker than its femoral counterpart, causing the failures to occur at this location.

2.4 Discussion

This study represents the first attempt to compare different mechanical quantities that can be used to characterize subfailure damage in the MCL. Prior investigations examining the mechanical properties of ligaments using stress relaxation or fatigue testing techniques have found that elongation in the toe region of the load-displacement (stress-strain) curve is associated with structural damage occurring within the ligament (Pollock et al., 2000, Wren et al., 2003, Fung et al., 2009). Decreases in tangent stiffness (modulus) have been associated with damage in other studies (Wang et al., 1994, Thornton G. et al., 2007, Schechtman H. et al., 2002, Quinn K et al., 2010). Fatigue behavior in MCLs has been studied by monitoring changes in chord stiffness (Zec et al., 2010).

Changes in ligament length during tensile tests were computed from the clamp displacements. The entire femur-MCL-tibia complexes were tested to determine the damage progression in the MCLs. The femur and tibia were not displaced much due to their relatively higher stiffness compared to the ligaments. Previous studies have found the elastic modulus of cortical bone to be at least twenty times stronger than that of a ligament (Reed & Brown et al., 2001, Rho et al., 1993). Polymethylmethacrylate (PMMA), the material that constitutes the cement powder used to clamp the FMTCs, in tension is typically two to three times stronger than

that of a rabbit MCL (Kindt-Larsen et al., 1995; Vallittu 1998; Hansen & Steen, 1992) and, thus, is not displaced while the ligament is stretched. In this preliminary experiment, the displacement of the clamps of the tensile testing machine was assumed primarily to be equal to the displacement experienced by the ligament.

The comparison among the three different mechanical quantities analyzed in this study suggest that there is an agreement between the decrease in tangent stiffness and the elongation of the toe region of the load-displacement curve. The main difference between the change in tangent stiffness and chord stiffness was the displacement at which the stiffness values decreased. The majority of the tested FMTCs exhibited a decrease in tangent stiffness at different displacements before the displacement leading to complete failure. In comparison, the majority of the FMTC tested (76.7%) had a decrease in chord stiffness at the displacements that precede the one leading to compete failure. Specifically, the majority of the specimens had a decrease in tangent stiffness occurring after the 0.8 mm displacement and failed at the 1.2 or 1.4 mm displacement. This suggests that the changes in tangent stiffness are an indicator of subfailure damage. The decrease in chord stiffness seems to be an indicator of imminent failure of the tissue rather than an indicator of damage. For that reason, monitoring the changes in chord stiffness value should not be used as an indicator for damage progression in MCLs.

The statistical results for the decreases in the load increment values at 0.4 mm indicated that damage in the MCL occurs at lower displacement values than those determined by using the tangent and chord stiffness parameters. The measurements of the elongation of the toe region showed that damage is already present at the 0.6 mm displacement. It appears that damage continued to occur at each incremental displacement. Our findings indicated that the changes in $l_{1.0} - l_{1.2}$ and $l_{1.2} - l_{1.4}$ were not significantly different. It must be noted that only nine of the

FMTCs were able to sustain the 1.4 mm displacement. Given the small sample size of ligaments that reached 1.4 mm displacement, more experiments need to be carried out to determine whether the toe region remained unaltered immediately before failure.

The changes in the tangent stiffness and the elongation of the toe region seem to indicate that damage occurs after the first few displacements are applied. The measurements of the elongation of the toe region indicate that damage initiates at the 0.6 mm displacement. The tangent stiffness calculation indicates that damage begins at the 0.8 mm displacement. The changes in these displacement values can be attributed to different structural changes that occur in the ligament. The elongation of the toe region has been associated with the uncrimping and permanent deformation of the collagen fibrils/fibers, while the decrease in tangent stiffness has been attributed to the breakage of the collagen fibrils/fibers (Viidik, 1968, Silver et al., 2003). The use of the chord stiffness does not seem to be appropriate to determine damage propagation in the ligament. It is rather a measure of ultimate failure. Conversely, the decreases in tangent stiffness and the change in the load increments value at the 0.4 mm displacement present comparable results in reference to progressive damage. Thus, our findings suggest that changes in tangent stiffness and load increments at 0.4 mm displacement occur at lower values of the displacements for the experimental protocol used in our study.

This study can offer some insight on the damage progression in collagenous tissues that is due to changes in the ligament's ground substance. Dehydrating the MCLs removed the water from within the tissue, primarily collected in the ground substance of the ligament. Decreasing the ligament water content suggests that the toe region of the MCL's mechanical behavior is reduced. The investigators hypothesize that as the MCL is stretched, collagen fibers slide past one another in the hydrated ground substance of the ligament. When you have a reduction of the

water within the ligaments, the MCL becomes stiffer and more collagen fibers are stretched sooner than when the MCL is hydrated, causing earlier damage and failure to occur.

The experimental procedure used in the preliminary paired study comparing the control and experimental MCLs has shown the changes in the ligament's mechanical behavior due to dehydration. The loss of water in the ground substance of the ligament causes, most probably, more collagen fibers and fibrils to elongate. It is also likely to cause the uncrimping of collagen fibers to occur prematurely as opposed to their hydrated counterparts. The loss of water increases the stiffness of the ligament so that the tissue reaches higher loads at the same displacement. The dehydrated MCLs, however, cannot be stretched as far as the hydrated samples before failure occurs. The results seem to indicate that the loss of water content in the ligament mainly affects the toe-region of their load-displacement curve. Alterations to the ligament's microstructure can have an effect on its load-bearing capacity. Individuals that experience chronic dehydration and the elderly can be especially susceptible to joint and ligament damage due to the loss of ligament water content. In summary, this study focused on analyzing the progression of damage in dehydrated MCLs by comparing different methods used by previous investigators. This chapter has described the methods and results of this experimental study. The final chapter discusses the overall conclusions drawn from the results of this experimental study and future work in this area.

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Chapter 3: Future Work and Conclusions

3.1 Future Work

As the field of biomechanics advances, the prospect of testing in-vivo biological tissue becomes more realistic. By testing how the MCL behaves mechanically within the body, one can eliminate the many artifacts created by in-vitro settings when analyzing damage progression. In-vivo testing of the ligaments will lead to a better understanding of the more physiologically relevant subfailure mechanisms. Some suggestions that could improve the proposed experimental study are presented hereafter.

The type of osmotic solution that is used to alter hydration of the ligaments can be modified. For example, by using either a distilled water solution or a 2% to 10% sucrose solution the water content within the ligaments can be changed as shown by other investigators (Chimich et al., 1992). The ligaments can be dehydrated by using an alcohol or solvent solution such as petroleum ether. Finally, the comparison between completely dehydrated and highly hydrated specimens can be made to better quantify the role of the ground substance on damage progression.

Investigators in tissue mechanics recognize that fresh tissue is more physiologically relevant over stored frozen tissue. Due to the time restraints and multiple usage of the experimental equipment by other investigators, we could not test the MCLs immediately after euthanizing the rats. However, it is well known that the mechanical properties of the ligaments are not affected by the freezing procedure (Woo et al., 1986). Another limitation to this study is the use of a heated bath to thaw the specimens quickly. The thawing environment should not have a meaningful effect on the structure of the MCLs as the bath was not in direct contact with

the tissue. A recommendation would be to thaw the specimens in room temperature or a refrigerated setting. The mechanical properties of the MCL are largely determined by the structure of the tissue and not the cellular activity within the ligament that may change due to freezing methods (Milthorpe, 1994). The tests also occurred in a bath at room temperature of approximately 21 °C. In order to simulate a more physiologically relevant environment, a temperature-controlled bath of body temperature should be used.

Human tissue samples are preferred over the murine tissue used in this study. For this research rat MCLs were used due to the lack of readily available human MCLs. However, by testing cadaveric human ligamentous tissue, a mechanical characterization that is more useful to the development of ligament grafting and reconstruction surgery can be obtained. The use of murine, leporine, canine, porcine animal models have nevertheless helped to quantify the characteristics of the MCL. Indeed, the structure of the MCLs for these animals is similar to humans.

In this preliminary study, we only analyzed load-displacement data of the MCLs. A future investigation needs to be conducted to examine the stress-strain data using the experimental protocol used in this study. A video capturing system can be employed to measure strain in the ligament. An analysis of the damage propagation can then be carried out to calculate the tangent modulus and chord modulus from the stress-strain curve of the MCL. Furthermore, the stress increments at a given strain can be obtained from the stress-strain curves collected by stretching the MCLs to consecutively increasing displacements. The load-displacement data from the MCLs provides information on the tissue's mechanical behavior, but does not take into account the different dimensions of the ligaments, such as the differences in cross sectional area and length. By considering the stress-strain data, the mechanical quantities that characterize

damage in the MCLs can be computed irrespectively of their size. The findings obtained by comparing the analysis of stress-strain versus load-displacement data can offer some insight about the damage mechanisms.

Scanning Electron Microscopy (SEM) can be used to evaluate qualitatively the structural damage that occurs at each incremental stretch in the ligament. By observing the microstructure of the ligaments associated with the changes in tangent and chord stiffness and in load increments at 0.4 mm displacement, one can gain a better understanding of damage and failure that occur during the proposed testing protocol.

One limitation of this study was the use of only one loading rate and one flexion angle for every tested MCL. Investigators who have used a higher loading rate during mechanical testing have found that failure of the ligament occurs more often in the mid-substance of the tissue rather than the tibial insertion (Yiannakopoulos et al., 2005; Panjabi & Courtney, 2001). In addition, varying the flexion angle of the knee can determine if this angle affects the failure rate of the MCL. The effects of strain rate and angle flexion were, however, not the focus of this project.

3.2 Project Conclusions

The MCL is a frequently injured ligament in the knee and several studies have investigated the ultimate properties of the tissue. Little research has been devoted to establish methods that can be used to quantify mechanically induced damage. The overall goal of this project was to compare different methods for analyzing and categorizing damage in the MCL. This study has found that damage propagation in the MCL can be determined by monitoring the decreases in tangent stiffness as well as the elongation of the toe region. The changes in chord

stiffness value have been used to monitor fatigue behavior in MCLs. However, based on the results of this investigation, this mechanical quantity does not prove to be an indicator or good measure of damage initiation or damage progression in MCLs. The results have also shown that subfailure damage induced by the application of stretches of incremental values occurs at lower stretches in osmotically dehydrated ligaments. Dehydration of soft tissues has been also associated with aging. For this reason, this study may also help in understanding the effect of aging on the damage mechanisms of ligaments. In summary, this study has found conclusive evidence that changes in tangent stiffness and the change in load at 0.4 mm displacement are effective in determining subfailure damage initiation and propagation in rat MCLs subjected to displacement-controlled uniaxial tests along their longitudinal direction.

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