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THE EFFECT OF CHEMICAL FIXATION ON THE STRUCTURAL PROPERTIES OF HUMAN COLLATERAL KNEE LIGAMENTS

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Declaration of Authenticity and Author's Rights

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has lead to the award of a degree.

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CONTENTS

Abstract

Background: Mechanical testing of soft tissues typically requires some type of tissue preservation, such as freezing or chemical fixation. While one-time freezing has been shown to have negligible effect on the mechanical properties of connective tissues, it does not afford an antimicrobial or embalming effect. Thus, biological risks remain and long duration testing at room temperature is not possible without substantial tissue degradation. Although traditional chemical fixation methods over come such limitations, they have been shown to alter the mechanical properties of some connective tissues, particularly bone. It is unknown, however, if these fixation techniques also significantly affect the mechanical properties of soft tissues, such as ligaments. This study aims to compare the mechanical properties of fixed and non–fixed adult human collateral ligaments of the knee.

Methods: Collateral ligaments from eight fixed and dissected human cadaveric knees were tested. Specimens were fixed using conventional 10% formaldehyde or phenol-formaldehyde solutions. Bone–ligament–bone preparations were mounted in a uniaxial material testing machine with the fibres of the collateral ligaments aligned with the axis of loading. Specimens were preloaded to a nominal load while the initial length, width and thickness of the ligament were estimated using vernier callipers. Following preconditioning, specimens were extended to failure. Force and crosshead

displacement were subsequently used to estimate structural properties, such as stiffness and ultimate tensile strength. These properties were compared with those of fresh human collateral ligament tissue. Data from medial and lateral collateral ligaments were pooled and differences between fixed and non-fixed tissue evaluated using an independent samples t-test.

Results: When data for the ligaments were pooled, the mean ultimate tensile strength of fixed collateral ligaments (714.9 ± 332.7 N) did not differ significantly ($P>0.05$) from that of fresh collateral ligaments (595.2 ± 263.8 N). Similarly, while fixed specimens were approximately 9.1% stiffer than fresh specimens, there was no statistically significant difference in the structural stiffness of fixed and fresh collateral ligaments ($P>0.05$).

Discussion and Conclusions: Despite the potent cross-linking effect of conventional tissue fixatives on collagen, fixation with 10% formaldehyde and phenol−formaldehyde had negligible effect on the strength and stiffness of the collateral ligaments of the knee in this relatively small series of human specimens.

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Chapter 1 Introduction

The use of fresh cadaveric tissues in surgical training and bioengineering research is common. However, with use of fresh cadaveric tissue, the risk of diseases transmission to researchers and trainees is high and there is a relatively short window of opportunity for the study or training to take place before the tissue deteriorates. Thus, preservation methods are often used to reduced the risk of disease and maintain the ultra–structure of the tissues. This is usually done through chemical fixation (Ohman et al., 2008).

Usually chemical fixation would lead to a change in the mechanical properties of the tissue sample, such as its stiffness, thereby causing a change in the tactile sensation of the tissues. In such cases, surgical training, particularly in procedures involving incisions and stitching of fixed soft tissues, such as tendons and ligaments, would feel considerably different.

The medial and lateral collateral ligaments play an important role in maintaining the stability of the knee joint (Otake et al., 2007). The main function of the medial collateral ligament is to resist valgus movement of the knee, while the role of the lateral collateral ligament is to resist varus rotation at all positions of knee flexion (Otake et al., 2007). The medial collateral

ligament commonly injured in conjunction with the anterior cruciate ligament (Woo et al., 1992).

In medical cases where an injury to the ligament is observed, the delicate nature of such injuries usually requires well-trained surgeons with experience working with cadaveric tissues that have been well preserved thus minimizing the chances of having the surgery going contrary to expectation.

This laboratory-based study used a materials testing machine to determine the structural properties of the collateral ligaments of the human knee which had been preserved using conventional fixing techniques (conventional 10% formaldehyde and phenol-formaldehyde). These data were then compared to an existing data set, conducted on fresh human collateral ligaments and using comparable methods, to study the effect of conventional chemical fixation on ligament properties.

This dissertation starts with a brief introduction in Chapter one. Chapter two reviews the relevant anatomy of the knee and the histological anatomy of the collateral ligaments. In addition it outlines the general mechanical properties of ligaments and factors that affect these properties. Finally, conventional

methods of tissue preservation and their effect of collagen rich tissue were reviewed in this chapter. Chapter three provides a broad overview of the aims and objectives of the study and outlines specific research questions. Chapter four details the methods and the materials that were used for dissection and mechanical testing. The results of anatomical measurements and mechanical testing are presented in Chapter five. The final chapter provides a general discussion of the results, highlights the limitations of the research and draws general recommendations for further research.

Chapter 2 Literature Review

2.1 General Anatomy of the Knee joint

The knee is the largest and most complicated joint in human body (Snell, 1995), which consists of the medial and lateral condyles of the femur, the opposing condyles of the tibia, and a gliding joint between the patella and the patellar surface of the femur (Snell, 1995).

2.2 Gross Anatomy of the Collateral Ligaments

The knee joint is surrounded and supported by many ligaments. These ligaments can be divided into intra-capsular ligaments, such as the anterior and posterior cruciate ligaments, and extra-capsular ligaments including the patella and popliteal ligaments, as well as the medial (MCL) and lateral (LCL) collateral ligaments. While all of the ligaments are thought to be important in restraining joint movement, the MCL and LCL are critical for stabilising the knee (Snell, 1995).

2.2.1 Lateral Collateral Ligament (Fibular Collateral Ligament).

The LCL is a cord−like structure attached to lateral condyle of the femur superiorly and the head of fibula inferiorly (Snell, 1995). The attachment at

the femoral condyle is semicircular in shape while the fibular attachment has a fan-like appearance (Figure 1.1) (Otake at el., 2007).

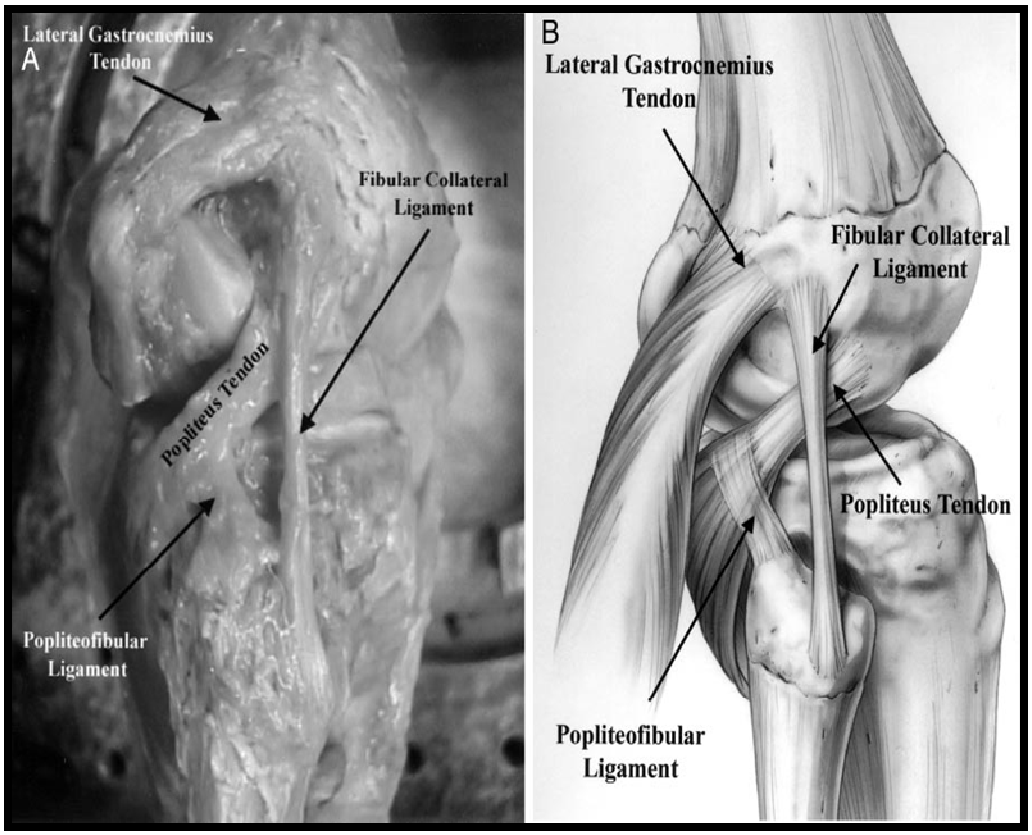

Figure 1.1 Anatomy of the lateral collateral ligament (modified from LaPrade et al., 2003).

The LCL plays an important role in maintaining stability of the knee by resisting lateral opening and limiting external rotation during knee flexion (Otake at el., 2007; Meister at el., 2000). At full extension of the knee, the length of the LCL from its most proximal and distal points of attachment ranges between 59 and 74 mm, with an average length of approximately 66 mm (Meister at el., 2000). Elliptical in shape, the smallest cross−sectional area of the LCL occurs at the middle of the ligament between its femoral and

fibular attachments, where its mean anteroposterior dimension is reportedly 3 mm to 4 mm (Meister at el., 2000). During knee flexion, the anterior bundles of LCL stretch while the posterior boundless become slack (Otake at el., 2007).

2.2.2 Medial Collateral Ligament

In contrast to the cord-like structure of the LCL, the MCL is a flat fan-shaped ligament (Snell, 1995). Connecting the medial condyle of the femur to the medial surface of the tibia (Snell, 1995), the distal attachment of the MCL is larger than its proximal attachment (Otake at el., 2007). While Palm (1938) described the MCL as consisting of two parts; a superficial and deep part, Robinson et al (2004) suggest that the MCL consist of three separate but interdependent components; (1) the superficial MCL, (2) the deep MCL, and (3) the posterior medial capsule (Robinson et al., 2004). However, there is considerable debate as to whether the posterior oblique ligament is a separate structure or if it is a part of the superficial MCL (LaPrade et al., 2007). The main function of MCL is to resist valgus movement of the knee (Snell, 1995).

2.2.2.1 Superficial Medial Collateral Ligament

The superficial MCL is a large flat sheet, which extends from the medial epicondyle of the femur and attaches via two locations to the superior aspect of the medial surface of the tibia (Otake at el., 2007; LaPrade et al., 2007) figure1.2. The proximal femoral attachment is located in a depression approximately 2 to 5mm proximal and 3 to 6mm posterior to the medial epicondyle (LaPrade et al., 2007). LaPrade et al (2007) argue that there is no strong attachment between the superficial and underlying deep MCL. The superficial MCL has two separated distal attachments to the tibia (LaPrade et al., 2007). The proximal attachment of the superficial MCL attaches to the anterior arm of the semimembranosus tendon rather than directly attaching to bone (LaPrade et al., 2007). The broad-based distal attachment of the superficial MCL is located just anterior to the posteromedial ridge of the tibia (LaPrade et al., 2007). Laprade et al., (2007) found the average length of the superficial MCL to be between 100 and 120 mm.

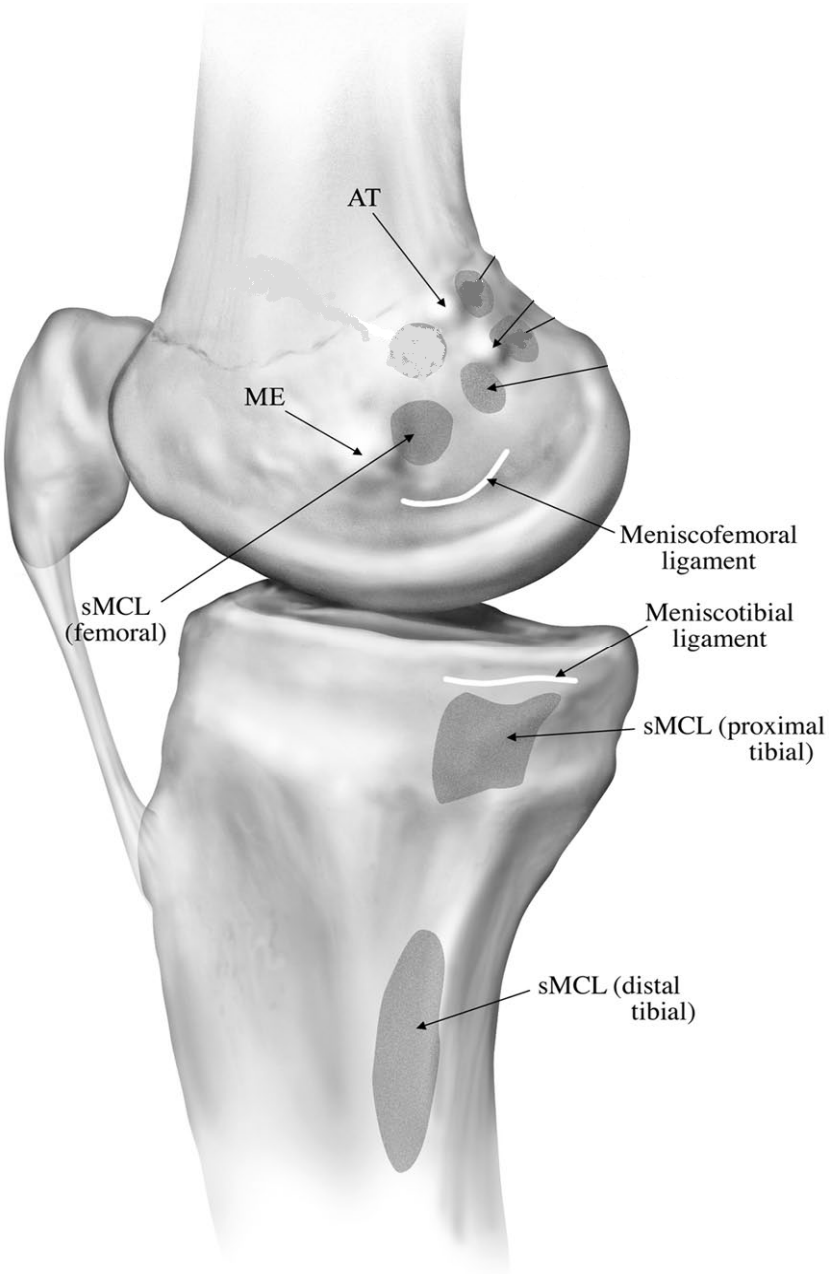

Figure 1.2 Anatomical attachment of the superficial MCL to the femur and tibia (modified from LaPrade et al 2007).

2.2.2.2 The Deep Medial Collateral Ligament

The bundles of the deep MCL originate immediately anterior and distal to the femoral attachment of the superficial MCL (Robinson et al., 2004). The deep MCL is made of distinct meniscofemoral and meniscotibal ligaments. The mensicofemoral ligament is longer than the mensicotibal ligament and is relatively short and thick. The mensicotibial ligament is attached just distal to the edge of the articular cartilage of the medial tibial plateau. (LaPrade et al., 2007).

2.2.2.3 The Posteromedial Capsule

The oblique fibres running distally and posteriorly from their attachment to the femur to the posterior aspect of the tibia were described differently .some authors described them as a part of the posterior fibres bundles of superficial capsule. Others considered them as a distinct posterior-oblique ligament, separated from the superficial MCL (Robison et al., 2004).

2.3 Histological Anatomy of Ligament

Microscopic ligaments, like tendons, consist of a dense connective tissue which is mainly comprised of extracellular matrix and cells (Silva et al., 2006). The majority of ligament cells are elongated fibroblasts located between, and parallel to, bundles of collagen fibres (Silva et al., 2006). The

extracellular matrix is comprised mainly of collagen fibres and a small amount of elastin and ground substance, which includes glycoproteins, proteoglycans, and water (Rumian et al., 2007). As much as 70% of collagen within ligament is thought to be type I collagen, with the remaining 30% representing a mix of type III, V, VI, XI, X, IV (Matyas et al. 1994). As with tendons, type I collagen in ligament is arranged hierarchically to form parallel fibrils, fibres and fascicles (Rumian et al., 2007), as shown in Figure 1.3. However, in contrast to tendon, ligaments have a greater cellularity and relatively higher quantities of glycosaminoglycans (Amiel et al., 1984). Thought to be important in the mechanical response of soft tissue (Rumian et al.,2007), proteoglycans and glycosaminoglycans are hydrophobic and play a critical role in lubrication and collagen spacing allowing collagen fibres to glide over each other (Rumian et al., 2007).

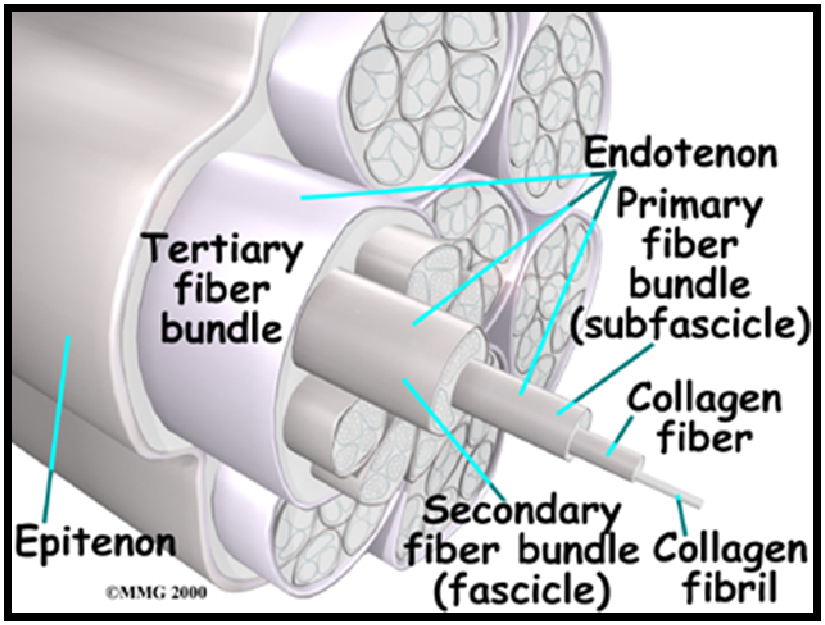

Figure1. 3. The arrangement of collagen fibres in tendons and ligaments (adapted from eOrthopod(16/8/2009)

Andriacchi et al. (1998) suggest that type I collagen plays an important role in the long term properties of the tissue matrix, and type III collagen plays a main role in early formation of the tissue matrix, foetal tissue matrix, and scar formation. The collagen fibres in ligament have a wave-like appearance, termed crimp, which allows extension at relatively low loads (Silva et al., 2006). Crimp is believed to be associated the presence of elastin in ligament (Woo et al., 1999). Representing about 5 % of the dry weight of ligament, elastin is thought to play an important role in tensile resistance and elastic recoverability at low loads (Gentleman et al., 2003).

2.3.1 Ligament Attachments or Entheses

Ligament may attach to bone by either direct or indirect insertions (Woo et al., 1999). Indirect insertions involve fibrous attachment of ligaments to bone in which the majority of collagen fibres blend with the periosteum, while deeper layers may attach via so-called Sharpey's fibres (Woo et al., 1999). Direct insertions, in contrast, involve four different morphological zones: passing from ligament midsubstance, through fibrocartilage, mineralized fibrocartilage to bone (Woo et al., 1999). In contrast to the elongated appearance of fibrocytes within the midsubstance of ligament, cells in the fibrocartilage zone are large and spherical and similar to chondrocytes in appearance in that they are surrounded by lacunae and pericellular matrix, rich in acidic proteoglycans (Woo et al., 1999; Matyas et al. 1994).

The extent of calcification within the fibrocartilaginous region of the attachment and the degree of osseous interdigitation is thought to reflect the movement and tensile strength of the enthesis (Gao and Messner, 1996; Evans et al., 1990). By incorporating calcified and uncalcified fibrocartilaginous zones, direct attachments have been suggested to provide a gradual transition from hard to soft tissue which is thought to assist in the dissipation of stress and have been shown to be mechanically stronger than

indirect or fibrous attachments (Gao et al., 1996). While the majority of ligament attachments of the knee are fibrocartilaginous, the MCL is unique in that it has both a direct (fibrocartilaginous) femoral attachment and an indirect (fibrous) attachment to the tibia (Woo et al., 1999).

2.3.2 Vascular and Neurological Anatomy

Ligament is a relatively avascular structure, with the majority of vessels located on its outer surface; the epiligament (Silva et al., 2006). Few vessels are thought to penetrate the ligament, and in doing so run parallel to and between fibrils (Silva et al., 2006). In contrast to the enthesis, ligament mid-substance has a greater density of blood vessels, arising from adjacent soft tissue. The bone-ligament attachment is avascular (Silva et al., 2006). Studies have found that the epiligament has the most nerves endings, with nerve fibres usually accompanying blood vessels along the axis of the ligament (McDougall et al., 1997).

2.4 General Mechanical Properties of the Ligament

The ligament-bone-ligament complex is primarily thought to connect and distribute load via the enthesis and midsubstance to the attachment of the

other bone (Woo et al., 1999). Mechanical properties of the ligament demonstrate time–dependent or viscoelastic properties, as demonstrated by creep and stress-relaxation tests (Robinson et al., 1995).

2.4.1 Structural Properties of Ligament

The structural properties of bone-ligament-bone specimens (e.g. femur -LCL-fibula complex, Figure1.4) are typically represented by plotting load-extension curves obtained from uniaxial tensile tests (Woo et al., 1999).

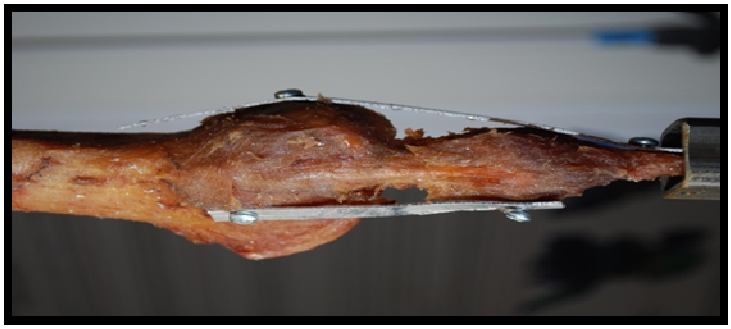

Figure1. 4. Bone-ligament-bone complex

Structural properties of the ligament such as stiffness, ultimate load, ultimate elongation and energy absorbed at failure are affected by ligament geometry, the type of bony insertion and the arrangement of collagen fibrils and their interaction with other elements of the extracellular matrix (Woo et al., 1999; Woo et al. 1976). The load-extension curves are often divided into several zones (figure 1.5).

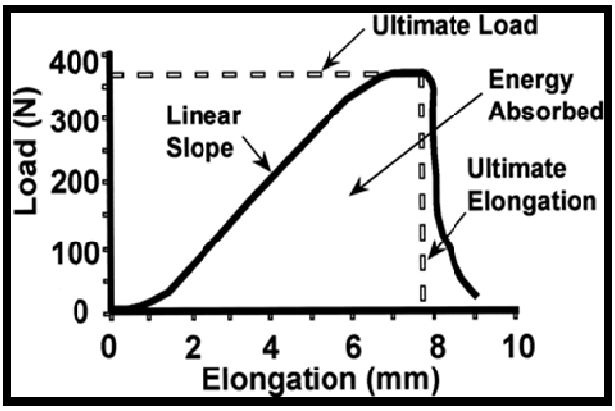

Figure 1.5. Typical load-extension curve of ligament (adapted from Woo et al., 1999)

The first zone, which is curved or nonlinear, is often referred to as the toe region and demonstrates low initial stiffness. This zone represents extension of collagen fibres from their wave-like pattern when small initial forces are applied (Woo et al., 1999). The second zone is referred to as the linear region and is thought to be related to stretching of collagen fibres. The last region of the curve is typically non-linear and is associated with progressive collagen failure and rupture (Woo et al., 1999).

2.4.2 Material Properties of Ligament

In contrast to structural properties, the material properties of ligament are independent of ligament structure and are thought to largely depend on the composition and orientation of collagen and its interaction with ground substance (Woo et al., 1999). These properties can be represented on a stress-strain curve (Woo et al., 1999). Strain is defined as the deformation per unit

length and requires precise measurement of ligament deformation via contact devices such as Hall-effect strain transducers or differential-variable–reluctance-transducers (Woo et al., 1999) or non-contact methods, such as video dimension analyser systems.

Accurate estimation of the material properties of ligaments also requires the precise determination of tissue stress. Stress is defined as load per unit across the sectional area of the ligament. The cross-sectional area of the ligament is commonly estimated using gravimetric methods in which the cross-sectional area is determined by measuring the volume of water that is displaced by the specimen and assumes a simple cross-sectional shape (Woo et al., 1999). Similarly, mechanical devices, such as Venire Callipers, have been used to take thicknesses and width measurements and subsequently estimate the cross-sectional area of the ligament with variable results (Woo et al., 1999). Non−contact devices such as optical systems and laser devices with improved accuracy have also been employed (Woo et al., 1999). While such methods are suitable for application with simple geometric structures, they give rise to considerable measurement error with more complex structures such as the MCL (Woo et al., 1999). As a consequence, this study

concentrated on evaluating the structural properties of fixed collateral ligaments.

2.4.3 Viscoelastic Properties of Ligaments

Ligaments show time–dependent viscoelastic properties, including creep, hysteresis, and stress relaxation (Woo et al., 2006). Creep is an increase in the length of ligament over time when exposed to a steady load. Stress relaxation is a decrease in load when the ligament is held in fixed elongation and hysteresis is a measure of energy dissipation with loading and unloading (Woo et al., 1999). Time-dependent properties are believed to represent a complex interaction between collagen, elastin and groundsubstance components, such as proteoglycans and water (Woo et al, 1999; Woo et al., 2006). With an increase in the number of loading and unloading cycles within the elastic region of ligament, there is a decrease in hysteresis and the loading and unloading curve become repeatable. This indicates the benefit of preconditioned cycles before any experimental loading test (Woo et al., 1999).

2.5 Factors Affecting the Structural Properties of Ligament

The mechanical properties of the ligaments can be affected by many factors. These factors can be divided into biological and experimental factors.

2.5.1 Biological Factors

The material and structural properties of ligaments are affected by several biological factors such as age, skeletal maturation, anatomic location, exercise and immobilization (Woo et al., 1999).

2.5.1.1 Effect of the Age and Skeletal Maturation

Age and skeletal maturation of the ligament have been reported to influence the mechanical properties of ligament. Woo et al., (1990) found an increase in the cross-sectional area, stiffness and ultimate load of the MCL with maturation to adulthood in rabbits, which then decreased gradually with aging (Woo et al., 1990). Similarly, Noyes and Grood (1976) found that the average stiffness and ultimate tensile strength of anterior cruciate ligaments of young adults (less than 26 years old) was almost twice that of middle aged to elderly people (over 50 years). Failure of the ligament at the osseous attachment (avulsion failure) was more common in older specimens and may

be related to a reduced bone mineral density at the site of the insertion of the ligament with senescence (Robinson et al., 1995).

2.5.1.2 Effect of Exercise and Immobilization

The effect of exercise on mechanical properties of ligament is controversial. Some studies have suggested that exercise can increase the structural properties of ligament (Woo et al., 1981), while others have found that exercise has little or no effect on the mechanical properties of ligaments (Noyes et al., 1974). Woo et al (1981) reported an increase in tensile properties and some increase in structural properties (ultimate load 38% and linear stiffness14%) of the MCL of mature swine after 21 days of exercise, while immobilization had the opposite effect on ligaments properties.

2.5.2 Experimental Testing Factors

2.5.2.1 Alignment and Direction of Applied Load

Alignment of the ligament and the direction of the applied external load are important for accurate assessment of mechanical properties of ligament (Woo et al., 1999). Several studies have suggested that the orientation of the femur to the tibia and the direction of the axial load in relation to the ligament axis

are critical factors in determination of the mechanical properties of the ligament (Lyon et al., 1989; Rogers et al., 1990; Woo et al., 1991). It was noted that the stiffness of the anterior cruciate ligament was higher when it was loaded along the ligament axis than when the loads were applied along the tibial axis (Momersteeg, et al., 1995). Momersteege et al., (1995) reported that changing the angle of the femoral insertion site during the mechanical tensile test of anterior cruciate ligament by as much as 5 degrees, significantly decreased the stiffness of the knee ligament by 43 N/mm.

2.5.2.2 Effect of Strain Rate

It is well known that ligaments demonstrate time–dependent behaviour, and, as such, their properties are influenced by the rate of strain or loading. For instance, Kennedy et al (1976), in evaluating the mechanical properties of the MCL and cruciate ligaments of the knee, found that higher rates of extension increased the load to failure of the ligaments. However, Woo et al., (1990) reported that the ultimate tensile strength of rabbit MCLs was only 40% higher when strain rates increased from 0.01%/s to 200%/s. Thus, the ultimate strength of ligament appears to be relatively insensitive to strain rate, given the effect is small relative to the increase in the strain rate (20,000 fold).

The effect of strain rate on the cause of ligament injury is not very clear. Some studies found that the majority of ligament injury during sport happened at strain rates as high as 500% per second. The site of injury at the bone-ligament-bone complex, however, appears to be related to the type of insertion more than the strain rate (Woo et al., 1999).

2.5.2.3 Effect of Temperature and Hydration

The water content of tissue can have a significant effect on its mechanical properties. The stiffness of ligament is increased by dehydration and is decreased by increasing the water content of the tissue. Betsh and Baer (1980) found that the stiffness of rat tail tendon increased as result of dehydration.

Although Rigby et al (1959) found that a temperature range of between 0-37°C had no effect on the biomechanical properties of rat tendon. Apter et al (1972) found that the elastic modules and stiffness of the ligaments decreased with an increase in temperature. Similarly, Woo et al (1987) tested canine knee ligaments in a saline bath at temperatures ranging between 2°C and 37°C, and noted that there was an inverse relationship between the temperature and stiffness of the ligament. Thus, ambient temperature would

appear to be an important consideration in testing the mechanical properties of ligament.

2.5.2.4 Tissue Preservation Techniques

The use of cadaveric tissues in surgical practice and in bioengineering research has experienced a lot of growth in the last few years. Fresh cadaveric bodies are thought to have similar mechanical properties to that of living humans and are, therefore, commonly used in surgical practice or bioengineering research (Moon et al, 2006). The risk of disease and the often extended duration of use has brought about the need to preserve these tissues (Ohman et al., 2008). There are several preservation techniques, including freezing and chemical fixation, the main purpose of which is to retain the original mechanical properties of the tissue (colour, odour, texture, and flexibly) (Majewski et al., 2003).

2.5.3 Freezing

Numerous studies have suggested that one-time freezing of ligament has little or no affect on their mechanical properties (Dorlot et al., 1980; Noyes and Grood, 1976; Woo et al., 1986). More recently, Moon et al (2006) found

that freezing, thawing and refreezing had no significant effect on the mechanical properties (stiffness, ultimate load, elongation at failure and strain energy) of ligaments. This study was unique from previous studies where the specimens were frozen and thawed twice before the test, and suggest that specimens may be frozen twice with negligible effect on their structural properties.

In contrast to tissue fixation, however, freezing does not have antimicrobial or embalming effects. Thus, the risk of disease transmission still remains. Moreover fresh frozen tissue is not suitable for lengthy tests at room temperature because of deterioration of the tissues (Ohman et al., 2008).

2.5.4 Chemical Fixation

The use of conventional chemical fixation techniques such as formaldehyde and phenol-formaldehyde has overcome certain disadvantages of the fresh frozen technique, such as the minimization of the risk of transmitted diseases (Ohman et al., 2008). Chemically fixed tissues are also more suitable for lengthy tests at room temperature.

Formaldehyde as a chemical fixative was discovered by the German physician Blum (1865-1959) in 1893 (Fox et al., 1985). The chemistry of formaldehyde is complex; in aqueous solution it undergoes two reactions, the first involves the formation of Methylene glycol, which preserves the tissue. The second involves a reaction with oxygen to form formic acid. Excessive formation of formic acid results in pigmentation of the tissue (Fox et al., 1985). Formaldehyde is thought to react rapidly with different functional groups of biological macromolecules resulting in the formation of cross−links between proteins, glycoproteins, nucleic acids and polysaccharides (Fox et al., 1985).

Methylene glycol can form cross−links with a number of amino acids such as lysine, cysteine, glutamine, tyrosine, arginine and histidine. Reaction with primary amines creates more methyl groups (Fox et al., 1985), and the subsequent reaction acts to preserve tissues by forming protein cross−links which inhibit enzymatic degradation and kill bacteria (O'Leary et al., 2009).

In theory, intra and intermolecular cross-linking associated with conventional fixation methods would alter the mechanical properties of soft tissues (Fox et al., 1985). Cross−linking of collagen is known to increase the strength and stiffness of various soft tissues, including skin and tendon

(Reddy et al., 2003; Reihsner et al., 2000). While few studies have evaluated the effect of embalming on mechanical properties of the collateral ligaments, Goh et al., (1989) investigated the effect of embalming on the mechanical properties of feline femoral bone fixed in 10% formalin solution for 3 and 21 days. The authors found that ultimate load and stiffness did not change during torsional testing, while the energy absorbed at failure significantly decreased. Similarly, Ohman et al., (2008) reported that low concentrations (4%) of formalin had no effect on the compressive stiffness, yield force and ultimate strength of human cortical bone over a short period of fixation (48 hr). However, with long term (8weeks) fixation there was a significant decrement in Young's modulus (-24%) and concomitant increase in ultimate strain (+53%). Thus, while collagen cross−linking has been shown to increase the strength and stiffness of soft tissues (Reddy et al., 2003; Reihsner et al., 2000), there is minimal evidence that the cross−linking induced by fixatives, such as formaldehyde, affect the structural properties of connective tissues, albeit in bone. There is a need, therefore, to establish the effect of conventional chemical fixation on the structural properties of soft tissues, such as ligament.

In summary, some biological factors, such as age and dehydration, have more pronounced effect on the mechanical properties of ligament than other

biological factors such as exercise and immobilization. Similarly, experimental factors, such as alignment of specimens and the direction of external loading are likely to have a greater effect on the mechanical properties of ligament than experimental factors such as strain rate and temperature.

Chapter 3 Study Rationale

3.1 Aims

To compare the structural properties (stiffness, ultimate load, ultimate extension, strain energy, yield) of fixed and fresh adult human collateral ligaments of the knee.

3.2 Hypotheses

Three hypotheses were tested:

1. Anatomical measurements in terms of length, thicknesses and width will be similar between fixed and fresh human collateral ligament.
2. Fixed human collateral ligament will fail at its midsubstance under tensile load.
3. The structural properties in terms of stiffness, ultimate load, ultimate extension, and strain energy of fixed human collateral ligament will be higher than the structural properties of fresh human collateral ligament.

Chapter 4 Materials and Methods

4.1 Specimens

Eight fixed human cadaver knees were sourced from the anatomy department of the University of Glasgow. Two knees were excluded; with one showing evidence of knee ligament surgery and the other incurring damage during dissection. The remaining six knees showed no gross evidence of previous surgery or underlying disease. The knees were less than three years from the date of death of the donor and were preserved using two different fixation techniques; a 10% formaldehyde-based technique and a phenol-formaldehyde-based method (Table 4.1). Two of the knees were fixed using the formaldehyde-based technique and the other four were fixed using the phenol-formaldehyde-based technique. Four knees were harvested from female donors, while the other two were obtained from male donors. The age of the donors ranged between 83 and 93 years with a median age of 84.5 years.

Table4.1 Fixation and donor characteristics.

Specimen	**Side**	**Age (**years)	**sex**	**Fixation method**	**Time since death**
1	Left	83	female	Formalin-based	3 years
2	Right	83	female	Formalin-based	3 years
3	Right	85	female	Phenol-based	2 years
4	Left	84	female	Phenol-based	2 years
5	Right	93	male	Phenol-based	2 years
6	Right	93	male	Phenol-based	2 years

4.2 Specimen Preparation

Dissection of the specimens started by removing the skin and subcutaneous tissues. This exposed the underlying fascia and muscles. The anterior aspect of the knee was exposed by reflection and dissection of the quadriceps muscle. The quadriceps muscle was then removed by an incision through the patellar ligament at its attachment to the tibial tuberosity.

The LCL was isolated by using the same methods described previously by Espregurira-Mendes and Da Silva (2006); Laprade and Hamilton (1997). Briefly, the LCL was isolated by dissecting the skin and subcutaneous tissue. A longitudinal incision was made along the fibres of the Iliotibial band, which was reflected both anteriorly and posteriorly to expose the more superior aspect of the LCL and aponeurotic attachments of the long and short heads of the Biceps femoris muscles (Sneath, 1995). Once the LCL was identified, the Iliotibial was transected and reflected proximally and distally

to expose the femoral attachment of LCL. The lateral aponeurosis of the long head of the Biceps femoris muscle was separated and dissected from the lateral and posterior aspect of the LCL to identify the margins of the LCL. The distal part of LCL was covered by the anterior arm of the long head of the Biceps Femoris muscle. An incision was subsequently made between the anterior arm of the long head of the Biceps femoris muscle and its lateral aponeurosis to access the superior aspect of the Biceps femoris bursa, whose floor is formed by the LCL. The anterior arm of the Biceps femoris muscle was divided and retracted distally to display the fibular attachment of the LCL. At this stage the entire length of the LCL was exposed and isolated.

After isolation of the LCL, the medial side of the knee was dissected to expose and isolate the MCL. Dissection of the medial knee commenced by identifying, dissecting and distally reflecting the Sartorius, Gracilis and Semitendinosus muscles from their tibial attachment, thereby exposing the distal part of the MCL. The overlying connective tissues were carefully removed to expose the proximal part of the MCL and its attachment to the femoral condyle. An incision was made through the Semimembranosus tendon to remove the Semimembranosus muscle. The capsule was separated from the inferior borders of the MCL by making an incision parallel to the ligament fibres. The oblique fibres of the ligament, which attach to the

medial meniscus, were left intact. The MCL was isolated from the posterior capsule by making an incision in the region of posterior oblique ligament using the methods described by Barett (1954).

In order to maintain some stability to the knees before the test, the cruciate ligaments, menisci, posterior capsule and the oblique popliteal ligament were kept intact. All other soft tissues around the femur, tibia and fibula were removed.

4.3 Gross Anatomical Measurements

Following dissection, anatomical measurements of the MCL and LCL were taken with the knees in full extension using a Vernier Calliper (Mitutoyo Digimatic Vernieie, Radio spares; Resolution, 10 μm). In accordance with Otake et al (2007), the length of the middle fibres of the MCL and LCL were measured from the most proximal aspect of the femoral attachment to the most distal aspect of the tibial and fibular attachment, respectively. The mediolateral thickness and the anteroposterior width of the ligaments were measured at the joint line (Silva et al., 2006). To allow comparisons to previous research (Wilson, 2009), the distance of the femoral attachment of each ligament was recorded relative to the most anterior, posterior, and

inferior margins of the respective condyle (Otake et al., 2007). Each measurement was taken three times and the average calculated.

4.4 Mechanical Tests of Structural Properties

Mechanical tests of the ligaments were conducted over three days using mechanical, uniaxial materials testing machine (Instron 5800R, Instron High Wycombe, UK) fitted with a 2 kN load cell.

4.4.1 Specimen Preparation for Mechanical Testing

To ensure minimal knee joint movement and subsequent ligament loading during specimen preparation, four metal swathes were rigidly fixed across the knee joint using screws. Two swathes were screwed into the femur and the tibia providing protection for the MCL, while the LCL was protected by two swathes which attached the fibula and the femur. Once the joints were fixed, medial and lateral bone-ligament-bone specimens were prepared by dividing the femur and tibia with a band saw. The cruciate ligaments and posterior joint capsule were subsequently dissected and removed. The entire lateral meniscus was also removed. However, given that fibres of the MCL are contiguous with the medial meniscus, the medial meniscus was freed from its anterior and posterior tibial attachments, leaving the meniscus

attached to the MCL. All trabecular bone was removed from the sectioned ends of the femur and tibia to aid in potting the bone. The ends of the medial femur and fibula were subsequently potted in square steel tubes using two-component styrene-based filler (ISOPON Metalik, Willingorough, UK). Potted specimens were pinned within custom grips. A stainless steel pin, 3 mm in diameter, was used to securing the potted fibula, while a 6 mm diameter pin was used for securing femoral and tibial components.

4.4.2 Load to Failure Protocol

Following calibration of the uniaxial load cell, the bone-ligament-bone complex was mounted within the material testing machine with the longitudinal fibres of the ligament visually aligned with the axis loading (Figure4.1). The metal swathes were unscrewed and removed and the specimen was preloaded to a nominal no-load condition of 2 N. The initial length and thickness of the ligament were subsequently measured to the nearest 0.1 mm using a set of Vernier Callipers (Mitutoyo Digimatic Vernieie, Radio Spares; Resolution, 10 μm). Specimens were then subjected to 10 preconditioning cycles at 2%/s at a frequency of 0.5 Hz (Woo et al., 1992). Following preconditioning, the specimen was distracted to failure at a strain rate of 500mm/min. Specimen failure was visually classified into four types;

1. Midsubstance failure – in which the ligament failed at its midsubstance
2. Insertion failure – in which the ligament failed at the attachment without avulsion of the bone.
3. Bony avulsion – in which the ligament failed at the attachment and incorporated bone from the attachment site.
4. Bone failure – in which the bone failed at a location other than the attachment site. Force–displacement data from tests involving bone failure were excluded from further analysis.

Tensile force and crosshead displacement were sampled at 100Hz and recorded to the nearest 0.1 N and 0.1mm, respectively. Force and displacement data were subsequently used to calculate the principal structural properties of the ligaments, including the ultimate tensile strength and stiffness.

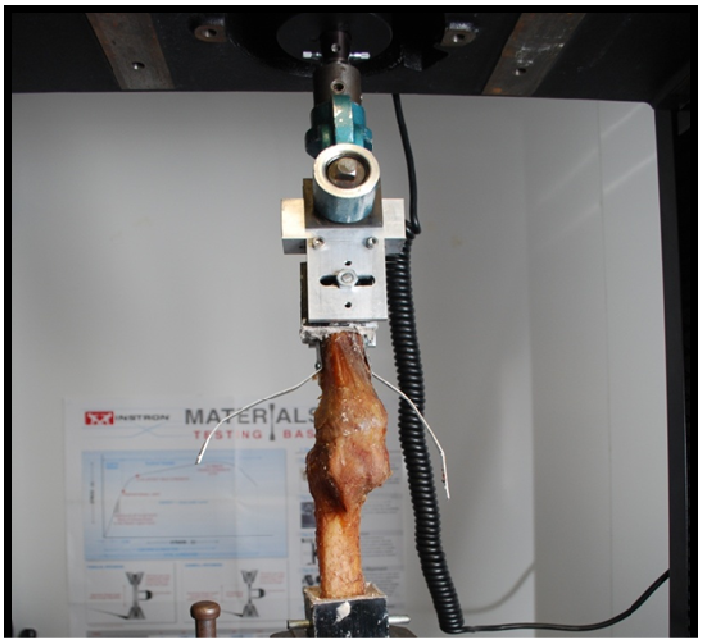

Figure 4.1 Illustration of grips and alignment of a typical specimen in material testing machine

4.5 Data Reduction and Statistical Analysis

Force–displacement data were plotted. Ultimate load was defined as the peak force occurring during loading, while ultimate elongation was defined as the displacement of the ligament at peak force. Ligament stiffness was calculated by finding the gradient of the linear section of the load–extension curve (Robinson et al., 1995) using custom Matlab software (MathWorks Inc, Natick, Massachusetts, USA).

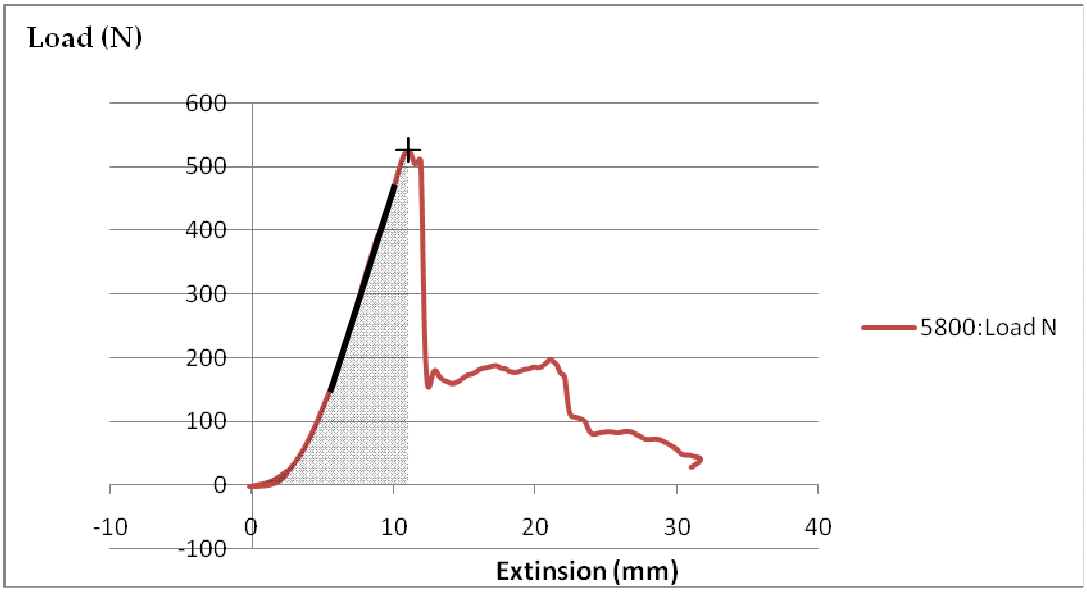

Figure 4.2. Typical load–extension curve for the medial collateral ligament. Stiffness was calculated as the gradient of the linear section of the load–extension curve and is represented by the bolded black line on the curve, the ultimate load was calculated as the peak force recorded during the test and the yield point represented the first time point were a negative gradient was recorded. In the illustration, ultimate load and yield coincide and are depicted by a '+' sign. The energy density was defined as the area under the load-extension curve to peak load and is illustrated by the shaded area.

The yield point was defined as the first point at which an increase in length was not associated with an increase in force (Grood and Noyes, 1976) and the strain energy was defined as the area under the load–extension curve (Butler et al., 1986).

The anatomical measurements and structural properties of the ligaments were compared to corresponding data collected on 9 fresh specimens using comparable methods (Wilson, 2009) Appendix B. Descriptive statistics were used to summarise both anatomical and structural properties of fresh and fixed specimens. Given the small sample size, inferential statistics were not

used to compare the effects of fixation on the structural properties of individual ligaments. Rather, where appropriate, data for the MCL and LCL were pooled and comparisons between fixed and fresh material were made using independent student t−tests. An alpha level of 0.05 was used for all tests of significance.

Chapter 5 Results

5.1 Anatomical Measurements of the Ligament

The mean length, width and thickness of the fixed and fresh ligaments are summarised in Table 5.1.

Table 5.1. *Mean and standard deviation () of anatomical measurements for the medial (MCL) and lateral (LCL) collateral ligaments of fixed and fresh† specimens.*

	MCL			**LCL**		
	Fixed	**Fresh**	**Difference (%)**	**Fixed**	**Fresh**	**Difference (%)**
N	6	10		6	10	
Length (mm)	110.8	112.1	0.6%	58.6	69.9	8.8% *
	(5.3)	(5.9)		(1.8)	(6.4)	
Width (mm)	33.6	32.1	2.3%	5.1	4.7	8.5%
	(0.8)	(3.1)		(4.0)	(1.1)	
Thickness (mm)	2.6	2.1	23.8%	2.8	2.6	7.6%
	(0.5)	(0.6)		(0.3)	(0.3)	

† Data for fresh specimens were obtained from Wilson (2009)
** Indicates a statistically significant difference between fixed and fresh specimens ($P<0.05$)*

The average length of fixed MCL was 110 ± 5.3 mm, which was slightly shorter than the average length of the fresh MCL 112.1 ± 5.3 mm.

While there was no significant difference in the width and thickness of fixed and fresh LCLs, fresh specimens were found to be significantly longer (8.8%) than their fixed counterparts ($t_{11} = -5.2$; $P< 0.05$).

The location of the femoral attachment sites of the MCL and LCL relative to the most anterior, inferior and posterior aspects of the femoral condyles are demonstrated in Table 5.2. The femoral location of fixed MCLs was more inferior and posterior than that of fresh MCLs. There was no significant difference in the location of the femoral attachment of fixed and fresh LCL.

Table 5.2 *Mean and standard deviation () of the femoral attachment sites of fixed and fresh medial (MCL) and lateral (LCL) collateral ligaments*

	MCL			**LCL**		
	Fixed	**Fresh**	**Difference (%)**	**Fixed**	**Fresh**	**Difference (%)**
n	6	10		6	10	
Anterior (mm)	*33.6*	*31.9*	5.3%	41.9	*45.6*	8.1%
	(*4.9*)	(*5.7*)		(*6.8*)	(4.5)	
Inferior (mm)	*33.3*	*37.6*	11.4%*	29.5	*28.8*	2.4%
	(*2.8*)	(*2.9*)		(2.2)	(6.8)	
Posterior (mm)	*22.6*	*28.5*	20.7%*	22.1	*22.8*	3.1%
	(*1.5*)	(*2.9*)		(1.1)	(4.4)	

† Data for fresh specimens were obtained from Wilson (2009) .
** Indicates a statistically significant difference between fixed and fresh specimens ($P < 0.05$)*

5.2 Modes of Failure

Table 5.3 summarises the mode of ligament failure observed during testing. Three of the fixed LCLs (50%) failed via bone avulsion at the fibula attachment, while two others failed at the fibula insertion without obvious bony involvement. Similarly, three of the six fixed MCLs (50%) failed via

avulsion of the femoral attachment while two others failed at the femoral insertion but without bony involvement. While one fixed LCL failed via a true midsubstance break, no midsubstance failures were observed for fixed MCL specimens. One of the fixed bone–MCL–bone specimens (specimen 7) failed via fracture of the femoral shaft, while two bone–LCL–bone preparations (specimens 6 & 7) failed via a fracture of the fibula shaft. In addition, data from one MCL specimen (specimen 12) was not recorded due to technical difficulties. Consequently, data pertaining to the structural properties of these four ligaments were excluded from further statistical analysis.

5.3 Structural Properties

The structural properties of the fixed collateral ligaments are shown in Table 5.3. The ultimate tensile strength of the four fixed LCL specimens ranged from 273 N to 585 N, while those of the MCL were higher than the LCLs and ranged from 778 N to 1321 N. Similarly, the MCL was generally stiffer than the LCL with a stiffness ranging between 71 N/mm and 131 N/mm. The MCL was also the tougher of the two fixed specimens, with a strain energy density 1.5 to 6 times that of the LCL, and ranging from 4.5 to 6.5 joules.

Table 5.3. *Structural properties and modes of failure for all test specimens**.

Sample	Side	Ligament	Failure Type	Ultimate Load (N)	Ultimate Displacement (mm)	Strain Energy Density (J)	Stiffness (N/mm)	Yield (N)	Displacement at Yield (mm)
1	left	LCL	Bony avulsion - fibula	585.8	7.2	1.9	95.3	585.8	7.2
2	right	LCL	Bony avulsion - fibula	272.8	8.3	1.0	48.0	251.3	7.2
3	left	LCL	Midsubstance failure	523.5	11.1	2.2	67.5	522.5	10.9
4	right	LCL	Insertion failure - fibula	472.1	15.9	3.2	59.0	403.7	8.2
5	right	MCL	Bony avulsion - femur	777.7	16.3	6.5	71.8	688.8	11.3
6	right	MCL	Bony avulsion - femur	941.8	14.3	5.6	83.9	941.8	14.3
7	right	MCL	Insertion failure- femur	824.5	15.1	4.5	74.3	824.4	15.1
8	right	MCL	Insertion - femur	1321.0	13.5	6.5	131.9	1321.0	13.6
9	left	LCL	Bone failure - fibula	205.9	7.5	0.7	39.5	193.4	5.4
10	right	LCL	Bone failure - fibula	499.8	8.9	1.7	74.5	499.8	9.0
11	left	MCL	Bone failure - femur	709.0	11.7	2.8	84.3	709.0	11.6
12	left	MCL	Bony avulsion - femur	N/A	N/A	N/A	N/A	N/A	N/A

* Note that data for ligaments 9 to 12 were excluded from further analysis due to bone failure and technical difficulties.

When data for the LCL and MCL were pooled and compared to pooled data from fresh specimens, the mean ultimate tensile strength of fixed collateral ligaments (714.9 ± 332.7 N) did not differ significantly ($P > 0.05$) from that of fresh collateral ligaments (595.2 ± 263.8 N). Similarly, while fixed specimens were approximately 29% stiffer than fresh specimens (Table 5.4), there was no statistically significant difference in the structural stiffness of fixed and fresh collateral ligaments ($P > 0.05$). As demonstrated in Table 5.4 there were also no statistically significant differences in displacement at failure, yield force, or strain energy density between fixed and fresh specimens ($P > 0.05$).

Table 5.4. Mean and standard deviation () of the structural properties of fixed and fresh† collateral ligament when data for the lateral and medial collateral ligament were pooled.

	Fixed	Fresh†	% Difference	P
n	8	18		
Ultimate Strength (N)	714.9	595.2	20.1%	0.380
	(332.7)	(263.8)		
Ultimate Displacement (mm)	12.7	12.7	0.0%	0.962
	(3.5)	(5.4)		
Stiffness (N/mm)	79 N	61.3	28.9%	0.863
	(25.8)	(13)		
Strain Energy (J)	3.9	3.7	5.4%	0.099
	(2.2)	(3.3)		
Yield (N)	692.4	508.2	26.6	0.198
	(336.6)	(265.4)		
Displacement at Yield (mm)	11	10.7	2.8%	0.885
	(3.2)	(4.8)		

† Data for fresh specimens obtained from Wilson (2009)

** Indicates a statistically significant difference between fixed and fresh specimens ($P < 0.05$)*

Chapter 6 Discussion

The aim of this study was to evaluate the structural properties of fixed collateral ligaments of the knee. Given the influence of ligament size on structural properties, gross anatomical measurements of specimens were undertaken prior to mechanical testing. The findings from the collateral ligaments of eight fixed knees were specifically compared to those of 10 fresh knees previously obtained by Wilson (2009), in which comparable methods were used.

6.1 Anatomy

The average length of the middle fibres of the fixed MCL in the current study (110.8 ± 5.3 mm; range, 105.2 to 118 .7 mm) was comparable to those of fresh MCL (112.1 ± 5.9mm; range, 102.0 to 121.1 mm) reported by Wilson (2009), and Laprade et al (2007) (range, 82.7 to 112.7 mm). Similarly, there was no significant difference observed in the average thickness or width of the fixed collateral ligaments when compared to fresh collateral ligaments.

In contrast, the average length of the fixed LCL (56.9 ± 1.8 mm) was significantly shorter than the average length of fresh LCL (69.9 ± 4.7 mm)

reported by Wilson (2009) and LaPrade et al (2003) (range, 62.6 to 73.5 mm). While fixation of liver tissue with formaldehyde solution has been shown to produce tissue shrinkage and result in reduced specimen length in animal models (Fox et al., 1985), the discrepancy in LCL length between studies is most likely due to the difficulty in defining the end points of the attachment of the collateral ligaments, as their insertion to the bone covers a large area and is often ill-defined (Woo et al., 1976). For instance, Otake et al. (2007) reported the mean length of the LCL to be 54 mm, approximately 20 mm shorter than the current study, when measured from the centre of the proximal femoral condyle to the centre of the distal attachment to the fibula. Moreover, there was no significant difference between the average thickness and widths of the fixed and fresh collateral ligaments, suggesting that systematic shrinkage with fixation did not take place.

Specimen age and sex have been reported to influence the size and shape of knee ligaments (Otake at el., 2007), making comparisons between studies difficult. Specimens in the current study were from relatively old donors (aged between 83 and 93 years) and had a greater proportion of females (female: male, 2:1) than those of Wilson (2009) (80 ± 11 years and female: male, 5:4).

6.2 Mode of Failure

In the current study, seven out of twelve of the fixed specimens failed by bony avulsion at the attachment site, which was also the predominant mode of the failure in fresh spacimens. For fixed MCLs, failure exclusively involved the attachment to the femoral condyle, with two out of four specimens failing by bony avulsion. Failure of the lateral collateral ligament, in contrast, predominantly involved the fibular attachment (1 insertional and 2 avulsion failures).

While ligament misalignment with the direction of loading has been shown to result in a preponderance of attachment site failures, the findings are consistent with the majority of studies, in which failure of the medial collateral ligament has primarily involved the femoral attachment (Lee et al., 1996; Gardiner et al., 2001). In a novel technique in which the entire medial collateral ligament was coated with a photoelastic film, Kadwada et al. (1999) observed that the greatest strain concentration involving the femoral attachment during mechanical tensile testing , which in contrast to its tibial insertion, is known to be fibrocartilagenous in nature (Woo, 1999). Moreover, Noyes and Grood (1976) observed that, in specimens obtained

from older donors, ligament failure was more likely to involve the bony attachment. Robinson et al. (2005) made similar observations and proposed that the effect reflected a decrease in the density of the bone at the site of the ligament attachment with aging. The preponderance of avulsion failures in the current study, therefore, may reflect the relatively advanced age of donors, stress concentration at the MCL or the effect of fixation on bone rather than the effects of fixation on the ligament itself.

6.3 Structural Properties

There is considerable variance in the structural properties of the collateral ligaments reported within the literature. As outlined in Section 2.5, numerous biological and experimental factors have been shown to influence the structural properties (stiffness, ultimate load, ultimate extension and strain energy) of ligament.

6.3.1 Medial Collateral Ligament

In the current study, the mean ultimate strength of the MCL of fixed specimens (966.2 ± 246.4 N) was on average 21% higher than that of fresh MCL (789.8 ± 209.2 N) reported by Wilson (2009) and approximately two

times greater than those reported within the literature (Table 6.1). Similarly, the mean stiffness (90.5 ± 28 N/mm, range 71.8-131.9 N/mm) of fixed MCL was 45% greater than that of fresh MCL (62.4 ± 13.7 N/mm) tested by Wilson (2009) and are consistent with the upper limits reported for fresh specimens within the literature (Table 6.1).

6.3.2 Lateral Collateral Ligament

In current study, the mean ultimate strength of LCL of fixed specimens (463.6 ± 135.4N) was on average 21% higher than that of fresh LCL reported by Wilson (2009) and 40 % higher than the mean ultimate strength of LCL reported by Sugita et al. (2001) (Table 6.2). The mean stiffness (67.2 ± 20.2N/mm, range 47.9- 95.3 N/mm) of fixed LCL was 16% higher than that of fresh (58.0 ± 12.2 N/mm) recorded by Wilson but within the range of values cited within the literature (Table 6.2).

Table 6.1. Structural properties of fresh, human medial collateral ligament reported within the literature compared to fixed specimens in the current study.

Study	Specimen	n	Specimen Age	Displacement Rate	Preconditioning Cycles	Ultimate Load (N)	Stiffness (N/mm)
Trent et al (1976)	Bone-ligament-bone	4	29 - 55	500	5	516	70.6
Kennedy et al (1976)	Soft tissue preparation	10	-	125	-	468 ± 33	-
Marionozzi et al (1983)	Bone-ligament-bone	5	55 - 90	100	-	465 ± 190	60 ± 22
Class et al (1987)	-	10	-	60%s^{-1}		-	94 ± 21
Momersteeg et al (1994)	Bone-ligament-bone	5	63 - 81	-	-	-	134 ± 1
Bull et al (1998)	Bone-ligament-bone	2	-	200 (50%s^{-1})	-	-	44.3
Robinson (2005)	Bone-ligament-bone	8	77 ± 5.3	1000	10	534 ± 85	80 ± 8
Wilson (2009)	Bone-ligament-bone	10	81 ± 11	500	10	799 ± 209	62 ± 14
Current Study	Bone-ligament-bone	4	87 ± 5	500	10	966 ± 246	91 ± 28

Table 6.2 Structural properties of fresh, human lateral collateral ligament reported within the literature compared to fixed specimens in the current study.

Study	Specimen	n	Specimen Age	Displacement rate	Preconditioning cycles	Ultimate Load (N)	Stiffness (N/mm)
Class et al (1987)	-	10	-	200 ($50\%s^{-1}$)	-	-	47 ± 13
Momersteeg et al (1994)	Bone-ligament-bone	5	63 - 81	-	-	-	114 ± 29
Sugita et al (2001)	Bone-ligament-bone	10	70	200	None	309	58
Wilson (2009)	Bone-ligament-bone	10	81	500	10	384 ± 101	58 ± 12
Current Study	Bone-ligament-bone	4	87	500	10	464 ± 135	67 ±20

6.3.3 Fixed Ligaments (pooled data)

As the number of specimens was relatively small, the mechanical properties of the MCL (n=4) and LCL (n=4) of fixed specimens were pooled and compared to those of 18 fresh collateral ligaments (9 MCL and 9 LCL). Although the ultimate strength and stiffness of the fixed collateral ligaments was 20% and 29% higher than that of fresh collateral ligaments, the difference was not statistically significant. Similarly, there was no statistically significant difference in yield force, displacement at ultimate load or strain energy between fixed and fresh specimens. Although the sample size of the current study was relatively small, the findings are consistent with those reported for cortical bone, in which short term fixation with formaldehyde had a negligible effect on its compressive stiffness, yield force or ultimate strength. However, the results contrast those in which collagen cross−linking via glucose derivatives has been shown to increase the strength and stiffness of soft tissues (Reddy et al., 2003; Reihsner et al., 2000). It is possible, therefore, that chemical fixatives such as formaldehyde, may result in a different cross-linking pattern of collagen than that typically induced with glucose for evaluating the effects of diabetes.

6.4 Limitations

This study has several limitations, which may be broadly categorised to those associated with dissection, mechanical testing and the research methods.

6.4.1 Dissection

The MCL is continuous with the posterior knee joint capsule. Thus isolation of MCL fibres presents difficulties. While high powered magnification loops may have assisted in dissection, every effort was taken to ensure that all visible fibres associated with the MCL were dissected free of the capsule. Similarly, a small number of fibres of the superficial MCL were observed to attach directly to the underlying semimembranosus tendon. These fibres were dissected to ensure axial loading of the bone-ligament-bone specimens. While it is anticipated that dissection of these scant fibres would have no appreciable effect on the measured structural properties of the ligament as a whole, the possibility of premature failure secondary to dissection of ligament fibres cannot be discounted.

It is well recognised that anatomical measurements of ligament length, width and thickness are technically difficult to perform. While non-contact methods for determination of cross sectional area are available, accurate measurement of length was difficult given the nature and area of the attachment of the ligaments to bone. Measurements of ligament thickness and width were conducted using the methods outlined by Otake et al. (2007), which allowed for direct comparison between anatomical measurements of fresh collateral ligaments and fixed collateral ligaments. In the current study, all measurements were conducted in triplicate. As demonstrated in Appendix A, the average standard deviation of repeated measurements was generally less than 1 mm, except for ligament length which was approximately 2mm (~2%). There was also considerable anatomical variation noted between specimens highlighting the future need to obtain a larger sample size.

6.4.2 Mechanical Testing

Alignment of the ligament to the direction of the applied load is important in uniaxial testing. The collateral ligaments have a complex geometry, which makes uniform loading of all the ligament fibres difficult, if not impossible. The fan-shaped structure of the MCL, in particular, prevented uniform loading. Consequently, load was applied along the central axis of the

ligament corresponding to its thickest cross sectional area. Every attempt was made to visually align the ligaments within the material testing machine. It is recognised, however, that even a relatively large misalignment (5°) would result in a reduction of stiffness of about of 10 N/mm (Momersteeg et al 1995b). Thus, while the effect of misalignment likely increased measurement variability, it was unlikely to have a substantial impact on the findings of the current study in which fixed collateral ligaments were found to be 18 N/mm stiffer on average than fresh specimens.

6.4.3 Research Design and Specimens

The major limitation of the current study was associated with the number of specimens evaluated. While the sample size is comparable to some published studies (Table 6.1) the size of the sample was too small to conduct meaningful inferential statistics. Consequently, data for the MCL and LCL were pooled to evaluate the overall effect of fixation on the properties of the collateral ligaments. It is recognised, however, that fixation may have a differential effect on the ligament properties. Moreover, the specimens were fixed using two different techniques (conventional 10% formaldehyde and phenol-formaldehyde). Phenol aids penetration of formaldehyde and protein precipitation and, as such, would have a greater potential to influence

ligament properties. Given the sample size, the current study was unable to address this issue. Thus, further research involving a larger number of samples would be necessary to evaluate the effect of fixation on the MCL and LCL.

Finally, minimal information was available regarding the past medical history of the donors. Thus, while all specimens were visually screened for evidence of musculoskeletal pathology, the presence of underlying disease that had the potential to affect connective tissue properties could not be discounted. For instance, osteoporotic changes may account for the bone failure observed for both the MCL and LCL of specimen seven.

6.5 Conclusions

In conclusion, this study evaluated the effect of chemical fixation by 10% formaldehyde on the structural properties of the MCL and LCL of six human knees. The results were statistically compared with previously collected data from nine fresh human knees which were obtained using comparable methods (Wilson, 2009). The findings indicate that despite an increase in ultimate strength, yield and stiffness of fixed specimens to the order of 20-30%, there was no statistically significant difference in structural properties of the ligaments. While the results are consistent with those reported for

short term fixation in cortical bone, the findings are based on a relatively small sample size. Further research, employing a larger sample size, therefore, would seem warranted.

6.6 Future Research

Further research should be carried out in this area to determine the exact effect of chemical fixation on the mechanical properties of the collateral ligament of the knees. Future research should employ larger sample sizes, different fixation methods and if possible measure the material properties of these collateral ligaments in order to gain a greater understanding of the effects of chemical fixation on soft tissue properties.

Appendix A: Anatomical Measurements of fixed Collateral Ligaments

Table 1. All anatomical measurements for the medial collateral ligament of each specimen

Specimen	Length (mm)	Width (mm)	Thickness (mm)	Inferior (mm)	Anterior (mm)	Posterior (mm)
1	111.6 (1.6)	33.8 (0.7)	3.1 (0.7)	33.3 (1.1)	30.8 (0.8)	23.2 (1.1)
2	118.7 (3.0)	31.2 (0.1)	2.9 (0.4)	36.5 (1.1)	31.5 0.4	21.5 (0.6)
3	109.6 (2.5)	34.6 (1.2)	2.2 (0.8)	29.7 (1.0)	32.9 (0.8)	20.8 (0.6)
4	105.2 (0.9)	34.3 (1.2)	2.3 (O.3)	32.5 (1.5)	30.7 (1.2)	22.7 (0.5)
5	104.7 (2.4)	32.8 (1.3)	2.9 (0.9)	30.1 (2.8)	32.0 (3.9)	23.6 (1.8)
6	114. 7 (3.1)	34.7 (0.6)	2.03 (0.4)	35 (0.7)	43.5 (1.7)	23.6 (0.7)

Table 2. All anatomical measurements for the lateral collateral ligament of each specimen

Specimen	Length (mm)	Width (mm)	Thicknesses (mm)	Inferior (mm)	Anterior (mm)	Posterior (mm)
1	59.4 (0.8)	5.6 (0.5)	3.3 (0.1)	28.5 (1.8)	41.4 (1.6)	21.1 (1.8)
2	59.2 (1.5)	5.2 (0.7)	3.2 (0.3)	30.4 (0.7)	39.8 (0.6)	21.6 (0.2)
3	59.5 (0.8)	4.7 (0.2)	2.8 (0.05)	28.2 (1.8)	40.9 (0.3)	21.5 (0.5)
4	55 (0.6)	4.5 (0.4)	2.5 (0.0)	27.0 (1.3)	36.8 (0.5)	21.3 (1.2)
5	(58.8) (1.3)	5.4 (0.5)	2.5 (0.1)	29.9 (1.0)	40.1 (1.9)	23.3 (2.1)
6	59.9 (0.3)	5.2 (0.4)	(2.7) (0.1)	33.2 (6.0)	55.3 (0.7)	23.6 (0.5)

Appendix B: Anatomical Measurements of Fresh Collateral Ligaments

Table 1. All anatomical measurements for the medial collateral ligament of fresh specimens reported by Wilson (2009).

Specimen	Length (mm)	Width (mm)	Thickness (mm)	Inferior (mm)	Anterior (mm)	Posterior (mm)
1	117.5	34.0	2.5	40.0	29.5	29.0
2	113.0	29.0	2.5	39.0	27.5	29.0
3	109.5	36.5	3.0	38.0	28.5	33.0
4	113.0	27.5	2.0	36.0	28.0	33.0
5	115.0	28.0	1.0	38.0	34.5	25.0
6	114.0	33.0	1.5	35.5	35.5	29.5
7	103.0	32.0	1.5	34.5	42.0	28.5
8	113.0	34.0	2.0	39.0	25.0	27.5
9	102.0	31.5	2.5	33.0	39.5	24.5

Table 2 All anatomical measurements for the lateral collateral ligament of fresh specimens reported by Wilson (2009)

Specimen	Length (mm)	Width (mm)	Thickness (mm)	Inferior (mm)	Anterior (mm)	Posterior (mm)
1	69.0	4.0	2.5	29.5	51.0	22.5
2	69.0	4.0	2.5	22.5	48.5	20.0
3	68.0	7.0	3.0	27.5	42.5	25.0
4	57.0	4.0	2.5	22.0	44.0	16.0
5	71.5	4.0	2.0	37.0	48.0	31.5
6	73.5	6.0	2.5	27.0	38.0	22.0
7	79.5	4.0	2.5	43.5	42.0	24.5
8	72.0	5.0	2.5	30.0	42.0	27.0
9	63.0	5.0	2.5	25.0	42.5	19.0

Appendix C: Structural properties and modes of failure of fresh collateral ligaments

Table 1. *Structural properties and modes of failure for all medial collateral ligaments of fresh specimens reported by Wilson (2009).*

Sample	Side	Ligament	Failure Type	Ultimate Load (N)	Ultimate Displacement (mm)	Strain Energy Density (J)	Stiffness (N/mm)	Yield (N)	Displacement at Yield (mm)
1	left	MCL	Bony avulsion-femur	618.8	15.0	3.8	41.9	361.3	10.1
2	left	MCL	Bony avulsion-femur	1128.5	21.1	10.6	63.2	1128.5	21.1
3	left	MCL	Bony avulsion-femur	924.7	17.6	7.9	63.5	902.9	16.9
4	left	MCL	Midsubstance	547.9	10.4	2.4	66.8	547.9	10.4
5	left	MCL	Bony avulsion-femur	826.2	20.4	6.0	60.2	826.2	20.4
6	left	MCL	Bony avulsion-femur	792.5	13.0	3.2	95.8	792.5	13.0
7	right	MCL	Bony avulsion-Tibia	1076.4	24.6	11.9	61.4	132.8	6.7
8	left	MCL	Bony avulsion-femur	632.5	14.5	4.0	52.8	632.5	14.5
9	left	MCL	Bony avulsion-femur	641.8	12.8	3.6	62.5	570.2	11.05

.

Table 2. *Structural properties and modes of failure for lateral collateral ligaments of fresh specimens reported by Wilson (2009).*

Sample	Side	Ligament	Failure Type	Ultimate Load (N)	Ultimate Displacement (mm)	Strain Energy Density (J)	Stiffness (N/mm)	Yield (N)	Displacement at Yield (mm)
1	left	LCL	Midsubstance	288.1	7.5	0.9	47.3	288.1	7.5
2	left	LCL	Midsubstance	459.8	10.8	2.4	57.5	389.5	7.9
3	left	LCL	Bony avulsion-fibula	431.9	10.4	1.9	50.7	431.9	10.4
4	left	LCL	Bony avulsion-fibula	189.1	5.6	0.4	40.9	189.1	5.6
5	left	LCL	Midsubstance	456.3	10.7	2.03	63.1	454.3	9.9
6	left	LCL	Midsubstance	548.9	9.3	2.02	74.4	548.9	9.3
7	right	LCL	Bony avulsion-fibula	394.2	8.7	1.5	63.1	278.3	5.9
8	left	LCL	Bony avulsion- fibula	383.8	6.7	0.1	77.9	383.8	6.7
9	left	LCL	Bony avulsion- fibula	372.8	8.4	1.4	60.2	288.5	5.9

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