THE EFFECTS OF INSTRUMENT-ASSISTED CROSS FIBER

MASSAGE ON LIGAMENT HEALING

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DEDICATION

This work is dedicated in loving memory of my grandmother, Vivian M. Worth.

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PREFACE

This research program stems from questions generated while using soft tissue manipulation techniques as a clinician. Of particular interest was instrument-assisted soft tissue mobilization (IASTM). My intrigue grew as to how this form of manual therapy resulted in the positive effects seen during the treatment of a variety of disorders involving connective tissue dysfunction, e.g. ligament sprains, tendons strain, posture imbalances, repetitive strain injuries and myofascial pain syndromes.

A specific type of IASTM, i.e. instrument-assisted cross fiber massage (IACFM), and connective tissue type, i.e. ligament, were focused on during this dissertation in order to narrow the scope of study. The primary purpose was to gain a better understanding of the tissue level effects of this treatment modality on ligament healing.

Preliminary studies in this dissertation provide support for the use of IACFM in the treatment of ligament injury. These findings are pertinent given the current health care climate of evidence-based practice and an aging population. However, it is just a beginning. It is a goal that this line of research continues on both a basic science and clinical level. Greater insight into how mechanical forces applied to the surface of the body are transduced into a beneficial response will help lead to optimal therapeutic outcomes.

ABSTRACT

Mary T. Loghmani

THE EFFECTS OF INSTRUMENT-ASSISTED CROSS FIBER MASSAGE

ON LIGAMENT HEALING

Ligament injury is one of the most prevalent musculoskeletal disorders that may lead to disability or disease, such as osteoarthritis. Conservative interventions which accelerate or augment ligament healing are needed to enhance therapeutic outcomes. The purpose of this research agenda was to investigate the tissue level effects of a type of manual therapy, cross fiber massage (CFM), in particular instrument-assisted CFM (IACFM), on ligament healing.

Bilateral knee medial collateral ligament (MCL) injuries were created using an established rodent model where one MCL received IACFM treatment and the other untreated MCL served as a within subjects control. The short and long term effects of IACFM on the biomechanical and histological properties of repairing ligaments were investigated. Tensile mechanical testing was performed to determine ligament mechanical properties. Ligament histology was examined under light microscopy and scanning electron microscopy. IACFM was found to accelerate early ligament healing (4 weeks post-injury), possibly via favorable effects on collagen formation and organization, but minimal improvement was demonstrated in later healing (12 weeks post-injury).

Regional blood flow and angiogenesis were investigated as possible mechanisms underlying the accelerated healing found in IACFM-treated ligaments. Laser Doppler perfusion imaging was used to investigate vascular function. Micro-computed tomography was used to determine vascular structural parameters. Compared to untreated contralateral injured controls, IACFM-treated injured knees demonstrated a delayed increase in blood flow and altered microvascular structure, possibly suggesting angiogenesis.

Mechanotransduction is a proposed mechanism for the beneficial effects of CFM in that application of a mechanical force was found to enhance biomechanical and histological properties as well as vascular function and structure acutely in healing ligaments. Although this thesis focused on IACFM treatment of injured knee ligaments, it is plausible for concepts to apply to other manual modalities that offer conservative alternatives to invasive procedures or pharmaceuticals in the treatment of soft tissue injuries.

Stuart J. Warden, PT, Ph.D., Chair

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LIST OF ABBREVIATIONS

RER	rough endoplasmic reticulum (8)
ROI	region of interest (53)
PBS	phosphate buffered saline (38)
PDGF-β	platelet-derived growth factor-β (83)
PGE_2	prostaglandin E_2 (67)
PGI_2	prostaglandin I_2 (67)
PU	perfusion units (21)
SEM	scanning electron microscopy (2)
STM	soft tissue mobilization (1)
TGF-β	transforming growth factor-β (6)
VEGF	vascular endothelial growth factor (83)
VEGFR	vascular endothelial growth factor receptor (83)
VOI	volume of interest (54)
V.N	vessel number (/μm) (54)
VSMC	vascular smooth muscle cell (73)
V.Sp	vessel separation (μm) (54)
V.Th	vessel thickness/diameter (μm) (54)
VV/TV	vessel volume normalized to tissue volume (%) (54)
2D	two dimensional (20)
3D	three dimensional (12)

GLOSSARY OF TERMS

<u>TERM</u>	<u>DEFINITION</u>
Brackets indicate page of first use	

Angiogenesis (2)	A process of neovascularization in which new blood vessels are formed from pre-existing vessels or by intussusception and longitudinal division to meet the demands of tissue development and repair.
Arteriogenesis (19)	Occurs when preexisting arterioles dilate and remodel through endothelial and smooth muscle cell expansion to meet increased physiological demands involving the dilation and remodeling of preexisting arterioles.
Autocrine (69)	Refers to a signaling mechanism in which a cell binds and responds to a signaling molecule (e.g. growth factor) produced by itself.
Cacodylate buffer (39)	A buffer used in electron microscopic preparations.
Compression (23)	Stress applied to materials resulting in their compaction, or decrease of volume.
Cross Fiber Massage (1)	A specific type of deep tissue massage involving the manipulation of soft tissue by applying a localized force to a soft tissue lesion. The direction of force is typically perpendicular to the structure's alignment.
Cytokine (7)	Any of a number of small secreted proteins (e.g. interleukins) that bind to cell surface receptors functioning as signaling molecules in cell-cell communication. They are involved in an immunoresponse, and growth and development. Their action may be autocrine or paracrine.
Ex vivo (63)	Meaning *out of the living*, refers to study of processes occurring outside a living organism, however, within the intact tissue.
Energy to Failure (13)	The area underneath the stress-strain curve (load-deformation curve) that reflects the toughness of the material.
Fibronectin (10)	An extracellular matrix glycoprotein that binds to membrane spanning receptor proteins called integrins and to extracellular matrix components such as collagen and fibrin.
Fibrosis (25)	The formation or development of excess fibrous connective tissue.

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Frequency (20)	Frequency is the number of occurrences of a repeating event per unit time. The basic unit of frequency is the hertz (Hz), the number of complete cycles per second.
In vitro (11)	Meaning *within the glass*, refers to the study of processes occurring outside a living organism (i.e. in culture).
In vivo (20)	Meaning *within the living*, refers to the study of processes occurring within the living organism.
Instrument-Assisted Cross Fiber Massage (1)	The use of rigid instruments to introduce cross fiber massage the body's soft tissue.
Instrument-Assisted Soft Tissue Mobilization (26)	The use of rigid instruments to augment soft tissue mobilization techniques.
Ischemic Compression (79)	Application of progressively stronger pressure usually applied by a thumb or finger on a painful trigger point to eliminate its tenderness; a.k.a. acupressure, shiatzu, myotherapy.
Laser (2)	"Laser" is the acronym for Light Amplification by Stimulated Emission of Radiation.
Massage (1)	The practice of soft tissue manipulation for anatomical, physiological and at times psychological purposes and goals.
Paracrine (69)	Refers to a signaling mechanism in which a target cell binds and responds to a signaling molecule (e.g. growth factor) produced by nearby cell(s) and reaches the target by diffusion.
Pericyte (19)	A mesenchymal-like cell, associated with the walls of small blood vessels implicated in blood flow regulation at the capillary level. As an undifferentiated cell, it serves to support these vessels, but it can differentiate into a fibroblast, smooth muscle cell, or macrophage as well if required.
Pressure (23)	Force per unit area. (Pascal)(Pa)(F/a) (N/m^2)
Shear (23)	Stress state where the stress is applied parallel or tangential to a face of the material, as opposed to normal stress when the stress is perpendicularly.
Soft Tissue Mobilization (1)	A type of manual therapy involving manipulation of the body's soft tissue.

Strain (14)	The deformation caused by the action of stress on a physical body defined as the change in length per original length. Strain is considered (+) in tension and (-) in compression.
Stress (14)	An internal distribution of force (load) per unit area that balances and reacts to external loads applied to a body. $(F/a)(N/m^2)$
Tendinosis (31)	Condition of pathologic tendon degeneration as compared to 'tendonitis,' which is a state of active tendon inflammation.
Tension (10)	Stress state leading to expansion (lengthening) of an object. (N/m^2)
Tissue volume (42)	The total VOI calculated by micro-CT analysis software.
Ultimate Force (12)	The maximum load a material can withstand before failure.
Vessel number (54)	Implies the number of traversals across a solid structure made per unit length on a linear path through a vessel region. It is calculated by micro-CT analysis software from the equation: V.N.= (VV/TV)/V.Th.
Vessel separation (54)	The thickness of the spaces as defined by binarisation within the VOI as calculated by micro-CT analysis software.
Vessel thickness (54)	Determined from an average of the local thickness at each voxel representing solid i.e vessel filled with radiopaque solution, as calculated by micro-CT software.
Vessel volume (22)	The total volume of binarised objects, i.e. vessels filled with radiopaque contrast agent, within the VOI calculated by micro-CT analysis software.
Vessel volume/tissue volume (60)	The proportion of the VOI occupied by binarised solid objects calculated by micro-CT analysis software.
Vasculogenesis (19)	'De novo' blood vessel formation. This term is usually used for fetal and neonatal growth or from bone marrow-derived enothelial progenitor cells in postnatal vessel growth.

CHAPTER ONE

Introduction

Thesis Overview

Ligament injuries are common disorders treated by clinicians. Soft tissue mobilization (STM) is a form of manual therapy frequently used in the conservative management of such musculoskeletal disorders. It is known that ligament cells are mechanosensitive to their environment. Massage is a form of STM that may have the potential to influence ligament healing since it provides a form of mechanical stimulation. This research program focuses on the effects of a type of massage, cross fiber massage (CFM), specifically instrument-assisted cross fiber massage (IACFM), on ligament healing. The findings of studies investigating the tissue level effects of IACFM on biomechanical and histological properties, and vascular function and structural parameters in injured knee ligaments in an animal model are reported in this dissertation. The dissertation outline is as follows:

Chapter One provides pertinent background information and proposes the general aims of this research agenda. It is organized into two primary parts.

Part A discusses the anatomy, biomechanics and physiology of a type of connective tissue, i.e. ligaments. The adaptation of connective tissues to load and the healing and repair process are also reviewed.

Part B discusses a type of massage, CFM, specifically IACFM, in the context of different manual therapy approaches and provides preliminary evidence supporting its application. Finally, the general aims of this research project are summarized.

Chapter Two describes the methodology used in the first set of studies investigating the short and long term effects of IACFM on the biomechanical and histological properties in healing knee ligaments in a rodent model. Tensile mechanical testing was performed at 4 and 12 weeks post-injury to determine ligament mechanical properties. Ligament histology was also examined under light microscopy at 4 and 12 weeks. Scanning electron microscopy (SEM) was performed to further determine ligament microscopic morphology at 4 weeks.

Chapter Three outlines the methodology for the second set of studies in this research agenda. Laser Doppler perfusion imaging (LDI) was used to investigate regional blood flow at 4 weeks post-injury. Subsequently, vascular structural parameters were determined by using micro-computed tomography (micro-CT) to explore angiogenesis as a possible underlying mechanism for IACFM effects.

Chapter Four provides a conceptual introduction to mechanotransduction as an underlying mechanism for the therapeutic effects of IACFM. Clinical implications are considered.

Chapter Five summarizes the findings of studies in this research project. Limitations and future directions are also discussed.

Part A: Ligament anatomy, biomechanics and physiology

1.1 Introduction

1.1.1 Overview

The musculoskeletal system is composed of bone, tendons, ligaments, and muscles. Connective tissue is ubiquitous and pervades the body systems. Ligaments are a type of connective tissue characterized by a dense, parallel fiber alignment. They support joint structures, attaching bone to bone. Ligaments function best under tensile load due to their fiber alignment, and serve to transfer load along their longitudinal direction (axis).[1] Ligament cells are mechanosensitive and appropriate mechanical force is critical for normal ligament development, growth, and healing and repair. On the other hand, ligaments are vulnerable to injury with loads exceeding their tensile limits. There is a need for conservative interventions to address the short- and long-term consequences of ligament injuries.

1.1.2 Ligament injury

Ligament injury (sprains) lead acutely to pain and functional limitations, and because of resultant imbalances in joint mobility and stability, can lead chronically to disability, permanent joint dysfunction, susceptibility to re-injury[2-4] and disease, such as osteoarthritis.[5] Extra-capsular and capsular ligaments heal by reparative scar versus regeneration. As a result, persistent tissue weakness and neuromuscular deficiencies may explain why a history of ligament injury is a strong risk factor for subsequent injury. In fact, patients may continue to experience significant symptoms for years following

ligament injury.[2-3, 6-7] The eventual goal of ligament healing is to fully restore its biomechanical properties so that it can appropriately guide and stabilize the joint's position and motion during static and dynamic functional activities. Accelerated tissue-level healing may allow individuals to return to function more quickly with less risk of re-injury, while augmented healing may restore the tissue to its full capacity, potentially preventing degenerative disease.

1.1.3 Epidemiology of ligament injury

Musculoskeletal conditions are the third most common reason for physician office visits, second only to respiratory and neurological system disorders,[8-9] with injury being the most costly disease annually.[10] Musculoskeletal conditions are the leading cause of disability, affecting 7% of the U.S. population on an annual basis. Unfortunately, the economic burden of musculoskeletal disease is expected to escalate over the next two decades due to an aging population.[11] Within the musculoskeletal system, ligament injuries are prevalent disorders, accounting for approximately 50% of athletic injuries, with nearly 90% of knee ligament injuries involving the anterior cruciate ligament (ACL) and medial collateral ligament (MCL).[1, 6, 12-14] Interventions for injured ligaments that facilitate early recovery (i.e. accelerate healing) and/or enhance final outcomes (i.e. augment healing) are needed.

1.1.4 Current interventions for ligament injury

Conservative treatments and surgical repair have demonstrated similar outcomes, regardless of the initial ligament damage (i.e. partial and full thickness ligament tears).[15-17] Several clinical alternatives to surgical ligament repair have been

investigated with positive effects, such as therapeutic laser,[18] direct current[19] and low-intensity ultrasound.[20-22] Other studies investigating the use of gene therapies, growth factors, biological scaffolds, and stem cell therapies have shown some promise in influencing ligament healing.[23-26] However, these techniques are costly and not readily available. Currently, there are no interventions with established clinical efficacy and acceptance in accelerating or augmenting ligament healing.[27] There remains a need to establish readily available, cost effective interventions that facilitate achievement of short and long term recovery goals from ligament injury. Manual therapies that offer conservative alternatives in the management of musculoskeletal conditions warrant further investigation towards the end goal of improving therapeutic outcomes.

1.2 Connective tissue overview

1.2.1 Connective tissue organization

Soft tissues of the body include connective tissue structures (i.e. tendons, ligaments, fascia, fibrous tissues, fat, and synovial membranes), muscles, nerves and blood vessels.[28] Beside primary connective tissue (a.k.a. connective tissue proper, ordinary connective tissue, supporting tissue), there are other specialized connective tissues: adipose (fat), bone, cartilage, myeloid and blood. Primary connective tissue originates from mesoderm, and does much more than serve to connect and provide structural support to structures/organs of the body. It surrounds other basic tissue types, i.e. muscle, nerve and endothelium, and acts as a reservoir for ion and water storage. Connective tissue functions as a physical defense barrier to infectious organisms, helping to mediate immunity, inflammation and repair. Furthermore, connective tissue

contains blood vessels and thereby supports the transport of nutrients, metabolites and waste products between tissues and the circulatory system.[28-29]

Primary connective tissue has been classified as 'dense, regular' (e.g. ligaments, tendons), 'dense, irregular' (e.g. joint and organ capsules, dermis, aponeuroses) or 'loose irregular' (a.k.a. areolar) (e.g. subcutaneous fascia, epithelial linings). In reality, there is a continuum of connective tissue fiber arrangements that exhibit the appropriate organizational structure to provide the required mechanical support. All connective tissues are composed of cells in an extracellular matrix (ECM). The ECM mostly consists of fibers in ground substance. The ground substance is composed mainly of glycosaminoglycans (GAGs), proteoglycans, structural glycoproteins and water.[28-30]

1.2.2 Connective tissue cells

Diverse cell populations are interspersed throughout connective tissue, each with different origins and functions (Table 1.1). Fibroblasts are the principal cell type in ligament. They are derived from mesenchyme and are relatively few in number, representing a small portion of total ligament volume. Fibroblast sparseness combined with low mitotic activity leads to a relatively low tissue turnover rate, and may be a factor in the inherently poor capacity for ligament healing.[31] Fibroblasts are spindle-shaped cells which align along the long axis of the ligament. They have cytoplasmic extensions, which may allow for cell-cell communication in coordinating cellular and metabolic responses in the tissue. Fibroblasts perform anabolic and catabolic functions in the synthesis and maintenance of the surrounding connective tissue matrix, compared to bone which has two cell types, i.e. osteoblasts and osteoclasts, which form and absorb bone respectively. Fibroblasts not only produce ECM components (e.g. collagen) and factors that influence growth and differentiation (e.g. TGF-β); they also produce matrix

metalloproteinases (MMPs) (e.g. collagenase) responsible for ECM degradation. MMPs

play an important role in tissue remodeling associated with various processes such as

angiogenesis, tissue repair, and disease. Of significance, fibroblasts synthesize proteins

that form ECM fibers and ground substance.[32-34]

Table 1.1 Summary of connective tissue cell types, origins and functions*

Cell Type	Origin	Function
Fibroblast, osteoblast, chondroblast	Mesenchyme	Produce fibers and ground substance of primary connective tissue, and specialized connective tissues, i.e. bone and cartilage
Osteoclast	Hematopoietic	Removes bone tissue
Adipose	Mesenchyme	Stores energy and heat
Myofibroblast	Mesenchyme	Assists with wound contracture and scar formation
Macrophage	Hematopoietic	Phagocytosis, cytokine secretion
Mast	Hematopoietic	Participates in allergic reactions, e.g. histamine release
Plasma cells	Hematopoietic	Immunological response; Produces antibodies
Lymphocyte	Hematopoietic	Immunological response
Leukocytes	Hematopoietic	Immunological response; Participates in phagocytosis

*Adapted from Junqueira, L. C. and J. Carneiro (2005). Basic Histology.[29]

1.2.3 Extracellular matrix fibers

Fibroblasts synthesize the two main types of fibrillar proteins found in connective

tissue, i.e. collagen and elastin. Collagen is the most abundant protein in the human

body and the major structural component of ligament ECM. There are 29 different types

of collagens described thus far. Two-thirds of ligament weight is water, but three-

quarters of their dry mass is made of collagen. Collagen type I predominates

(approximately 90% of ligament dry weight) followed by collagen type III (approximately

8% of ligament dry weight). Collagen type III is an immature form of collagen and is

mechanically inferior compared to collagen type I, the latter of which has the tensile

strength of steel. The collagen type I to type III ratio within a healing ligament represents

the relative maturity of collagen present. In contrast, elastin is a structural protein that

confers elastic recoil and stretching properties to the ECM.[35]

Collagen type I is organized hierarchally into fibrils, fibers, fiber bundles and fascicles that align in the direction of tensile forces.[36] Collagen synthesis begins in the fibroblast and is completed in the ECM. Amino acids (i.e. glycine 33.5%, proline 12%, hydroxyproline 10%) are used to synthesize three polypeptide α chains (two α1 and one α2 peptide chains) referred to as preprocollagen, on the polyribosomes of the cell's rough endoplasmic reticulum (RER). The amino acid sequence follows a repeating pattern. Each α chain is synthesized with a signal peptide and an extra length of terminal peptides called registration peptides, the latter of which assist in appropriate α chain positioning during procollagen assembly and prevention of intracellular aggregation of the molecules. Once the signal peptide is clipped in the lumen of the RER, the three α chains assemble into procollagen (a collagen precursor). Lysine and proline residues on the polypeptide chains are hydroxylated in the RER lumen. Hydoxylated amino acids (i.e. 3- and 4-hydoxyproline) are essential for formation of collagen cross-links. Procollagen is shipped to the Golgi apparatus where it is packaged and transported to the cell surface in vesicles and exocytosed. Once in the ECM, procollagen is converted to tropocollagen after the registration peptides are removed due to contact with extracellular substances (i.e. peptidase, GAGs). Tropocollagen, is comprised of the three polypeptide α-chains forming a triple-stranded, helical molecule (280 nm length; 1.5 nm width). The hydroxyproline residues help stabilize the tropocollagen triple helix by forming hydrogen bonds between the polypeptide chains. Tropocollagen molecules polymerize outside the cell in a quarter staggered head-to-tail formation to form fibrils (50-200nm diameter). The displacement of adjacent tropocollagen molecules in the fibril creates the characteristic periodicity of striations (64 nm wide) when stained for electron microscopy. ECM proteoglycans and structural glycoproteins play an important role in the spontaneous aggregation of the fibrils into collagen fibers.

During the final stages of collagen maturation, the fibrillar structure is mechanically stabilized by covalent cross-link formation between tropocollagen molecules.[37] The mature, non-reducible cross-link hydroxylysyl pyridinoline is important to improving ECM strength and tissue quality in healing ligaments.[29, 38-39] Collagen type I fiber organization, cross-links and diameter modulate tissue tensile strength and stiffness. For instance, fibril diameters in injured ligaments are smaller than in non-injured ligaments, and diameters increase as ligament mechanical properties increase during healing.[40] Ligament mechanical properties are influenced by other, non-collagenous proteins found in the ECM ground substance.[28-29]

1.2.4 Extracellular matrix ground substance

The ground substance fills connective tissue space not occupied by fibers or cells. It is formed mostly from GAGs (a.k.a. mucopolysaccharides) (approximately 0.5% of ligament dry weight) and water. GAGs are long, linear, poly-disaccharides that attach to a protein core to form proteoglycans (a.k.a mucoproteins). Despite its minor mass, GAGs play an important functional role in the matrix. The negative (acidic) charge of the GAGs repel each other, creating a voluminous bottlebrush-like structure that attracts water which causes the proteoglycans to swell and trap fluid between the collagen fibers. This hydrates the structure and contributes to its compressive strength. The huge proteoglycan molecules are electrostatically attracted to each other and the surrounding water creating a flexible, semi-fluid gel that allows rapid diffusion of water-soluble metabolites.[35, 41-42] Proper hydration of the ECM is important to cell function and viscoelastic behavior in ligaments.[41-42] Proteoglycans also influence the rate and organization of collagen fibrils during ligament healing.[32, 43]

Structural glycoproteins help to mediate the interactions of cells and other ECM constituents. They are made of protein chains bound to branched polysaccharides that function as links between the ECM and cells; binding to cells, collagen, GAGs, proteoglycans and other glycoproteins. Fibrillin and fibronectin are examples of fiber-forming glycoproteins, while laminin, entactin and tenascin are non-filamentous forms. Glycoproteins influence the mechanical properties of tissue as well. For example, fibronectin provides for stability and cell-ECM communication by binding to collagen in the ECM and integrins in cell membranes, thereby linking actin filaments of the cytoskeleton to in the ECM.[31, 36] Ligaments are primary connective tissue type structures.

1.3 Ligament anatomy and histology

Ligaments and tendons have similar fibrous connective tissue organization characterized by dense, regularly arranged fibers. In general, ligaments attach bone to bone while tendons attach muscle to bone. There are two major ligament subgroups: skeletal and suspensory. Skeletal ligaments may be extra-capsular/capsular (i.e. knee MCL) or intra-capsular (i.e. knee ACL) and can serve many functions, including stabilizing joints, maintaining skeletal alignment, guiding joint motion, providing proprioceptive input. Suspensory ligaments primarily support internal organs.[39] Tendon collagen fiber bundles are aligned parallel to each other in line of a muscle's pull. In subtle contrast, ligament fiber bundles are roughly parallel, but may have oblique or spiral arrangements; the geometry being influenced by each ligament's adaptation to its specific joint restraining function.

Ligament histology reveals a hierarchal organization of fibrils, fibers, primary fiber bundles, fascicles and whole structure. Ligaments have a crimped, waveform appearance along their long axis. The periodicity and size of the waves depends on the

specific structure and can change along the length of the ligament. The crimping pattern can be observed under light microscopy in the collagen fibril, which unfolds during initial collagen loading and allows the ligament to elongate without damage. A fiber (1-12 μm diameter) is a bundle of parallel fibrils. Fibers often appear wavy and appear to run the entire length of mature ligament.[44] A primary fiber bundle is a collection of fibers. The diameter of the fiber bundle varies with the size of the tissue structure. Groups of primary fiber bundles form fascicles. It is the alignment (arrangement) of the fascicles and their fibers that affects the mechanical response of the tissue. Fascicles are typically arranged in a parallel manner in line with the ligament long axis. The ligament proper is formed from groups of collagen fascicles.[31, 35-36] Ligaments insert into bone by a gradual transition from ligament to fibrocartilage to mineralized fibrocartilage to bone (i.e. insertional zones).[28]

1.4 Ligament biomechanics

1.4.1 Ligament biomechanics overview

Skeletal ligaments are anisotropic, i.e. being oriented to resist tension in an uni-directional manner along their long axis; and, they possess time- and history-dependent viscoelastic properties. Thus, ligaments display nonlinear mechanical behavior during *in vitro* testing under tensile loading. Reasons for this nonlinear behavior are multifactorial: a) during stretching an increasing number of ligament fibers are recruited into tension, b) the crimp pattern slowly and progressively straightens (uncrimps), and c) fiber alignment improves.[35] The nonlinear loading behavior in ligament matrix allows for some joint displacement to occur with relatively little effort, but provides increasing resistance as deformation increases. The mechanical behavior of ligaments also depends to some

degree on the environment, i.e. temperature, loading conditions such as strain-rate, and subject age and sex.[14, 35, 39]

1.4.2 Material and structural properties

As with most structures, the strength of ligament is influenced by the inherent properties of its constituents (material properties) and the way in which these constituents are arranged and interact (structural properties). In other words, the material properties of ligament are defined by its tissue-level qualities which are independent of structure or geometry. Material properties influence but cannot predict the behavior of the whole tissue, since the tissue as a whole is anisotropic and heterogeneous, and changes in its three dimensional (3D), structural geometry affect ligament mechanical properties.[39] For example, ligaments with larger, cross-sectional areas require more force to failure than smaller ligaments. Also, the longer the ligament, the more deformation required to produce a force change comparable to shorter tissue. Additionally, the orientation of the ligament relative to its joint will affect its mechanical behavior. Although mathematical models have been developed to characterize the mechanical behavior of collagenous tissues, mechanical tests are needed to determine ligament mechanical properties.[39]

1.4.3 Assessment of ligament biomechanical properties

Since ligaments resist tensile forces, the mechanical properties of ligaments are normally determined by tensile tests, often using bone-ligament-bone complexes, where the tissue is displaced to tensile failure at a pre-determined rate while the changes in force are recorded. Structural biomechanical properties are typically derived from the

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force-displacement curve (a.k.a. load-displacement, force-elongation curve) including

ultimate force (maximum load) which is the peak or apex of curve on y-axis, stiffness

(slope of linear portion of curve), and energy to failure (area under the curve). The initial,

concave portion of the curve, the "toe" region, is thought to be related to structural

changes in fibril organization from a crimped pattern to a more straightened, parallel

arrangement. In this region, initially little force (low load) is needed to elongate the

tissue. During the linear portion of the curve fibers are parallel and lose their crimped

pattern. Up to the end of this region, the structure produces an elastic response, in which

unloading restores the tissue to its original length. At the end of the linear region, small

force reductions may be observed in the curve for whole ligaments, possibly due to

sequential failure of a few greatly stretch fiber bundles. At the end of the linear region, an

endpoint is reached signifying the first major failure of fiber bundles, beyond which

additional fiber failures occur in an unpredictable manner. Complete failure occurs once

maximum load is obtained (Figure 1.1).[45]
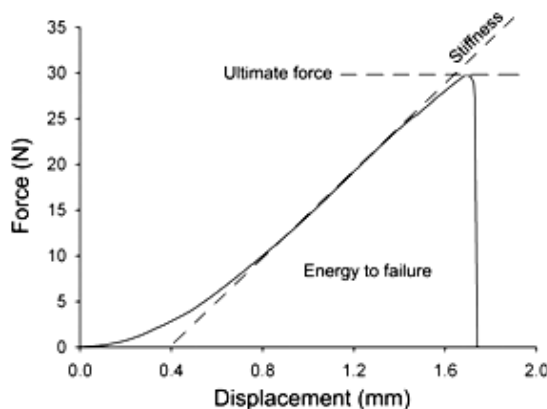
Figure 1.1. Representative force-displacement curve for a rodent knee MCL tensile mechanical test. Properties that can be derived from the curve include ultimate force (peak on the curve on the y-axis) (N), stiffness (slope of the linear portion of the curve) (N/mm) and energy absorbed prior to failure (area under the curve) (mJ).

To account for structural variances, the load-deformation curve may be adjusted by dividing the force by the original cross-sectional area (tensile stress), and deformation by initial length (tensile strain). The resulting stress-strain curve provides mechanical (material) parameters of the collagen that are independent of tissue dimensions. However, stress-strain curves are often not used experimentally in bone-ligament-bone preparations due to challenges in the means to assess the cross-sectional area and length of ligaments during dynamic tensile testing in a practical and precise manner.[39, 46]

1.4.4 Models of ligament injuries used for biomechanical assessment

A ligament sprain is defined as an acute injury to a ligament. Clinically, more than 85% are sub-failure injuries (Grade I and II are sub-failure injuries; Grade III is a complete failure injury). Ideally, a ligament injury model would re-create the diffuse and extensive nature of a clinical sprain; however, these types of injuries are difficult to consistently reproduce and quantify experimentally.[47] Ligament injuries have been created by mid-substance scalpel cuts or wire rupture/pulls. The latter method causes more extensive damage, creating larger gap injuries.[6] This is relevant, in that injury size affects healing, i.e. larger injuries show inferior structural properties at all healing intervals.[48] Ligament transection injuries are commonly used for complete ligament disruption.[49] Although surgical transection is not a perfect simulation of clinical injuries, it allows for controllable, reproducible injuries in size, pattern, and location.[50] Mid-substance ligament injuries are usually created since injury location has also been found to affect healing with injuries at either bony end tending to heal more slowly.[51] The prevalence of MCL injuries and its propensity to spontaneously heal without surgical repair makes it a useful experimental model of ligament healing.[1, 7]

<u>1.5 Ligament healing and repair process</u>

Injured ligaments go through overlapping phases of healing: inflammation, repair and remodeling.[52] Invariably, each stage entails a complex sequence of physiological and cellular events. The inflammatory phase is marked by vasodilation, increased vascular permeability, hemorrhage and clot formation. It begins immediately upon injury and lasts around 72 hours. The ECM in the injured area is highly disorganized with numerous inflammatory cells (e.g. phagocytes) infiltrating the area. The repair phase lasts from 72 hours until approximately 6 weeks in ligaments and is characterized by fibroblast proliferation, activation and matrix production. During this stage, fibroblasts appear plump and more numerous and disorganized ECM forms a scar that bridges between the torn fiber ends. However, the scar matrix gradually becomes organized and shifts from collagen type III to type I.[14, 53] The remodeling phase is marked by improved collagen fiber alignment and increased collagen matrix maturation which can continue for years.[14, 53-55]

The MCL heals spontaneously. In fact, long term results indicate surgical intervention results are not superior to conservative management of MCL healing in animals[7, 21, 56-57] or humans.[58-60] In contrast, ACL injuries show very poor recovery of function and typically require surgical repair.[1, 6-7, 55, 57, 61-62] Subsequently, functional outcomes of MCL injuries are superior to ACL tears.[63] Nonetheless, the MCL heals with grossly visible scar comparable to wound healing, that has altered ultra-structure and biochemical composition from normal.

Ligament healing is affected by a number of factors; however, it requires adequate blood flow into the region for the transport of cells and metabolites.[14, 55, 63] The greater capacity of the MCL to heal compared to other knee ligaments, may relate to its vascular anatomy.[53]

1.6 Ligament vascularity

1.6.1 Ligament vascular anatomy

Uninjured ligaments and tendons are typically hypovascular compared to other tissue, hence their whitish color. Ligaments contain sparse but distinctly organized microvascular distribution patterns. Blood vessels in ligaments play more than a nutritive role. They help maintain water content, excavate plasma components during inflammation, and regulate fluid and electrolyte balance in the ECM. Furthermore, the abundant association of nerves with blood vessels in ligaments may have several implications including proprioception, neuromodulation of blood flow under various condition such as growth and development, joint motion, inflammation and repair, maintenance of tissue metabolic demands, and local temperature regulation.[41]

Gross dissection has demonstrated predictable patterns of blood supply to the different ligaments of the knee.[64] In general, the key sources of blood supply to the MCL and lateral collateral ligament (LCL) are the superior and inferior geniculate arteries, while the middle geniculate artery is the primary source for the ACL.[41, 64] The MCL has a relatively greater vascular supply, followed by the LCL, then the ACL.[41, 64] Blood supply patterns may be related to the different healing capacities of the knee ligaments. For example, the ACL has a very limited number of vessels in its central portion which helps to explain its comparatively deficient healing ability.[42]

Ligaments have an epiligamentous covering defined as any surrounding adherent connective tissue removed simultaneously with the ligament but grossly distinguishable from the ligament tissue proper. It is a more vascular layer on the ligament surface housing a vascular plexus containing a relative abundance of branching and anastomotic blood vessels.[41, 65] Generally, the epiligamentous tissue

merges with the periosteum at the ligament attachment sites where it is also thicker and has a denser distribution of vessels. This covering contains more sensory and proprioceptive nerve endings than the ligament proper.[66] Nerves tend to travel in close proximity with blood vessels and thus, also lie closer to the insertion sites. Tendons have a similar covering, suggesting loose connective tissue adjacent to ligaments and tendons contain an important source of vessels that eventually penetrate these structures and give rise to intra-ligament vessels.[4, 41] The MCL epiligament is variable in thickness but covers the entire surface of the ligament at the microscopic level, while its joint surface aspect is continuous with the synovial membrane. It is well vascularized with some vessels penetrating the ligament's mid-substance, as compared to the ACL epiligament which is less vascular with few vessels penetrating the ligament proper.[36]

Intra-ligamentous vessels are typically distributed sparsely within the ligament proper. In fact, one study using microspheres found vessels in the epiligamentous tissue surrounding ligaments receive approximately 75% of blood flow compared to only 25% going to the ligament proper.[42] The vessels within ligaments appear to run in an organized manner parallel with the collagen fiber bundles of the densely organized ECM. Large areas of tissue within the ligament may remain devoid of vessels resulting in avascular areas that rely on diffusional pathways for oxygen and nutrients.[41]

1.6.2 Ligament vascular physiology

Injured ligaments demonstrate altered vascular dynamics that may be related to the healing response. Blood flow increases dramatically following injury during early ligament healing and stabilizes to near normal levels between 6-17 weeks. Blood flow and vascular volume increase with injury in both the ACL and MCL, but the responses are significantly amplified in the MCL and may be a prime factor in its superior healing

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potential. [42, 53, 63] Nonetheless, a functional blood supply via an adequate tissue blood

vessel network is critical for function in all ligaments. An appropriate therapeutic stimulus

during all phases ligament healing would neither promote excessive blood vessel

formation (i.e. angiogenesis) associated with a chronic inflammatory state or provide an

inadequate stimulus that leads to a poor supply (ischemia), a small blood vessel

network, or interruption of nascent blood vessels and granulation buds. [67] In summary,

increased tissue blood flow and angiogenesis plays an important role in all phases of

ligament healing.

1.6.2.1 Blood flow

Blood flow is defined as the movement of blood in the cardiovascular system.

The homeostatic health and function of tissues relies upon adequate perfusion of

capillary beds, for nutrient and oxygen delivery from arterial blood and waste removal by

the venous system. Furthermore, blood flow affects, in part, how tissues remodel and

repair in adaptive response to changing stresses. Blood flow to articular connective

tissue is comparatively much less than to other organ tissues, but adequate supply

remains essential for normal tissue function. Ligament injury is considered a potent

stimulus for increasing blood flow to and neo-vascularization of this tissue type. [42]

1.6.2.2 Angiogenesis

Angiogenesis is a process of neovascularization in which new blood vessels are

formed from preexisting ones to meet the demands of tissue development and repair.

Increased angiogenesis occurs with inflammation and wound healing. [4] The process of

vascular growth can occur via the sprouting of new capillaries from pre-existing vessels

or by intussusception and longitudinal division (angiogenesis), or dilation and remodeling of preexisting arterioles (arteriogenesis). The term arteriogenesis is used to describe the process of arteries acquiring smooth muscle and viscoelastic and vasomotor characteristics, or to refer to collateral growth from preexisting arteries.[68-69] Angiogenesis should be distinguished from vasculogenesis, or 'de novo' blood vessel formation, which is typically associated with the initial events during embryonic, fetal and neonatal vessel development involving primary vessel formation.[68, 70-71]

Angiogenesis in adult pathological conditions involves a complex sequence of events that signals and permits endothelial cells proliferation and migration which assemble, form cords and acquire lumens. Effective growth, development and long term survival of endothelium into a three-dimensional blood vessel network that meets the local functional demands requires a process that is tightly regulated by genetic, environmental and angiogenic factors, and multiple integrins affecting a variety of cell types and tissues.[68, 72] Eventually, peri-endothelial cells (pericytes in small vessels and smooth muscle cells in larger vessels) migrate the length of sprouting vessels as they elongate and surround the nascent blood vessels. Peri-endothelial cells are markers for more mature blood vessels, and help to stabilize the vessels by inhibiting endothelial cell proliferation and migration.

1.6.3 Assessment of vascular properties in ligament

1.6.3.1 Assessment of blood flow

Blood flow can be determined by a variety of techniques, e.g. microangiogram,[73] radio-active or colored microspheres,[42] contrast-enhanced ultrasound,[74] magnetic resonance angiography;[75] all of which may require invasive procedures. Several

methods use optical techniques that rely on the Doppler shift effect, including laser Doppler flowmetry (LDF) and laser Doppler perfusion imaging (LDI), used for *in vivo* studies of tissue perfusion. The Doppler shift, named after Johann Christian Doppler in 1843, is defined as the change in frequency and wavelength of a wave that is perceived by an observer moving relative to the source of the waves.[76]

LDF is useful in monitoring temporal variations in blood flow and dynamic responses to a stimulus at a specific site. However, this method is not useful for quantitative diagnostics measures since it involves single point measurements (typically ≤ 1 mm^3) which assess only a small fraction of the entire tissue microvasculature, and requires direct tissue contact. Consequently, LDF findings are affected by the heterogeneity of the tissue, and are highly sensitive to probe location placement resulting in measurements that cannot be used for comparison studies.[77] Comparatively, LDI does not require tissue contact since it uses a laser beam to scan several points across a tissue surface.

LDI has been used to assess blood flow in a variety of tissues and conditions,[4, 77-82] including ligament perfusion, [42, 77] and the influence of blood flow on tissue healing.[79] LDI findings have been correlated to the gold-standard of determining blood flow, i.e. spectrometry to calculate the number of microspheres in a sample.[42, 77] LDI allows for continuous, near real-time monitoring of blood flow to a specified region of tissue (typical capillary diameter 10 μm; velocity spectrum measurement between 0.01–1.0 mm/s). Measures are limited to the tissue surface (1–1.5 mm depth) unless modified for high resolution in which case the tissue sample depth may reach 2-3 mm. Each LDI measurement point is used to generate a two-dimensional (2D), color-coded map directly related to tissue blood flow. LDI provides a relative estimate of microvascular perfusion. Its signal cannot generate absolute values since a single calibration factor cannot be determined due to the different optical factors of various tissues. The term

'flux' is commonly used to describe blood flow measures. Flux is a quantity proportional to the average speed of blood cells times the cell concentration (blood volume), and is expressed as arbitrary 'perfusion units' (PU).[42, 77] Averaging of several neighboring pixels permits comparison between subjects since it helps to compensate for tissue heterogeneities.[80]

Disadvantages of the LDI method may include low image resolutions and possibly long scan times during which the subject has to remain still. LDI is not effective in monitoring high frequency flow fluctuations since it depends on sequential versus simultaneously captured image points, nor is it effective in extremely low flow situations. Nonetheless, LDI offers the ability to study regional variations in tissue blood flow with measures that are highly reproducible.

1.6.3.2 Assessment of angiogenesis

LDI, histochemical and angiography methods are inadequate to visualize, quantify and characterize vascular development.[83] Various vascular filling techniques and imaging methods have been used to investigate new blood vessel growth and microvasculature parameters. Radio-opaque imaging was first reported by Salmon in 1936 who perfused a lead oxide gelatin mixture into small radicles of the vascular tree in human cadavers for imaging.[84]

Perfusion methods have an advantage over immunohistological labeling methods in that the former can measure anatomical changes in vascular volume irrespective of the pathophysiological status of the tissue being examined. The latter assumes the tissue being labeled is the same in a pathological condition as in the normal state, which may not be a correct assumption.[70] Several different contrast mediums have been used for perfusion methods to quantitatively describe fine vascular patterns in tissues.[70-71, 85-90]

One study using India-ink compared the longitudinal orientation and total vessel volume in normal and injured rabbit MCLs and found increased vascularity with more chaotic angular distribution of blood vessel segments in ligament scar tissue compared to normal ligament tissue.[85] Many perfusion methods allow acceptable 2D visualization of microvascular channels in tissues, but smaller superficial vessels can obscure deeper vessels making accurate, quantitative information hard to obtain.[73]

Recently developed micro-CT based quantitative methods allow 3D imaging for microvascular parameter analysis. These methods typically involve a radiopaque silicone rubber containing a suspension of lead chromate, e.g. Microfil®, that is perfused into the specimen. Samples are then imaged using a micro-CT scanner.[91-92] Micro-CT evaluation has been used in a variety of investigations including those determining neo-vascularization.[89, 92-93,94,88] One study on tumor blood vessel formation resolved vessels filled with Microfil® smaller than 22 μm in diameter.[93] In another study, vessels 8 μm in diameter were resolved using high-resolution micro-CT.[83] Micro-CT methods overcome many of the limitations of other imaging methods since it allows 3D imaging and provides a means to obtain quantitative information on volumetric parameters and architecture of fine blood vessels without tissue disruption.

Micro-CT methods are based on the assumptions that the lead chromate is mixed homogeneously with the silicone rubber and the vasculature is completely filled, but not over-distended.[65, 70, 89, 95] Precise estimates of vessel diameter may be difficult to obtain due to non-uniformity of the vessel contrast;[89, 95] however, micro-CT results have been correlated with histological methods for vessel volume analysis.[92] Micro-CT cannot be done *in vivo*, nor does it give information on the actual tissue; it simply fills the structure;[96] however, micro-CT imaging allows unbiased comparisons between the tissues of interest.

1.7 Response of connective tissue to load

1.7.1 Tissue adaptation to load

Tissues must be able to adequately respond to external mechanical forces in order to adapt to changes in load. This adaptive response is accomplished in part through cooperation of mechanical and growth factor signaling. Connective tissue sustains mechanical stress and requires it for its growth and maintenance. Remarkably, connective tissue cells i.e. fibroblasts, are able to distinguish the mode (i.e. tension, compression, shear), frequency, magnitude, direction and duration of applied mechanical forces and translate this information into specific tissue adaption response. For example, a tendon fibroblast exists primarily under tensile force, but if it is subjected to compressive forces, it may form fibrocartilage or sesamoid bone in that specific location of pressure (e.g. sesamoid bones embedded in the flexor hallucis brevis tendon).[97]

The effects of tissue immobilization (inadequate load) and remobilization (appropriate overload) on the musculoskeletal system have been well documented.[57, 98-103] Appropriate mechanical loading has been shown to enhance effector cell response. The ECM functions to transmit physical forces acting on the body while protecting embedded cells from adverse effects from excessive mechanical force. For example, fibroblasts sense strains (deformations) in the ECM caused by mechanical stresses and translate this stimulus into an adaptive response (i.e. increase or decrease in ECM production). Conversely, inappropriate load levels (i.e. overuse or disuse) impair growth and survival signals.

1.7.2 Effects of inadequate load (disuse)

Models of disuse include forced bed rest[104-105] and limb immobilization (i.e. casting),[106-107] space flight,[108-109] and spinal cord injury/paralysis[110] and animal hindlimb unloading;[49, 111] however, joint immobilization has been used most commonly as a model of disuse in ligaments and tendons.[32, 112-113] The effects of immobilization on connective tissue include a permanent loss of GAGs and water leading to a loss of critical inter-fiber distance, smaller collagen fiber bundles and increased formation of reducible collagen cross-links indicative of immature collagen. New collagen fibrils may form cross-links between existing collagen fibers. Cross-links can limit mobility between fibers which may interfere with restoration of full, pain-free motion.[114-115] New collagen displays a disorganized arrangement and its breakdown has been shown to exceed synthesis with eventual net loss of collagen and eventual structure weakening observed. Increased MMP expression in immobilized connective may be related to accelerated collagen breakdown.[34] Immobilized ligaments show reductions in ultimate load to failure, stiffness and energy absorption. The insertional sites of ligaments, tendons and joint capsules demonstrate weakening at soft tissue-bone junctions. Decreased connective tissue extensibility, limited joint range of motion and contracture may ensue.[116]

1.7.3 Effects of excessive overload (overuse)

Excessive tissue loading results in high tissue strains leading to excess forces acting on fibroblasts embedded in the tissue. This can cause inflammation, degeneration or cell death (i.e. apoptosis) which can disrupt the repair tissue and delay or prevent healing. The effect is magnified if the ECM is already damaged or injured.[117] Excessive

tissue loading over time can lead to fibrosis. Fibrosis is defined as the excessive accumulation of collagen, e.g. excess scar formation in wound healing.[29]

1.7.4 Effects of overload (re-mobilization)

The deleterious effects of immobilization can be reversed to some degree by re-mobilization of the soft tissue. Connective tissue mobilization facilitates GAG production, which maintains inter-fiber distance and lubrication. Mobilization increases gene transcription for ECM proteins, helps maintain the balance between collagen synthesis and degradation, improves the matrix organization and increases the strength and stiffness of the tissue.[97, 118-119] Extensibility can be improved over time due to the viscoelastic nature of connective tissue. Besides restoring mobility, mobilization activates fibroblast and myofibroblast alignment in the direction of stress, and increases cellular activity and proliferation, i.e. in fibroblasts, macrophages, mast cells.[119-121] Increases in blood flow, which can result in a subsequent increase in skin temperature, may be related to a histamine response from mast cell degranulation. Appropriate tissue loading reportedly reduces muscle tone, releases fascial restrictions and adhesions, separates fibers and breaks down collagen cross-links.[30, 122] Methods to re-mobilize soft tissue may include exercise, modalities and manual therapies.

1.8 Introduction

Manual therapy involves the application of specifically directed forces to the body to induce physiological and/or structural tissue changes. Manual therapy may be subcategorized into joint mobilization or STM techniques, the former being directed at moving joint surfaces to restore joint motion, the latter being directed at manipulating the body's supple tissue to improve soft tissue mobility.[115] STM can be administered manually by hand, or with rigid instruments to facilitate the delivery of the massaging force, i.e. instrument-assisted soft tissue mobilization (IASTM). Massage is an ancient form of STM that encompasses several approaches.[123] CFM, also referred to as transverse friction or deep friction massage, is a specific type of deep tissue massage. Instruments may be used to augment its delivery, i.e. IACFM.

1.9 Massage

1.9.1 Massage overview

Massage is a form of manual therapy. The earliest writings about massage date back to 2,000 B.C. in ancient cultures such as in Chinese, Greek and Roman societies. Hippocrates advised rubbing as a treatment for stiffness.[123-124] Massage is defined as a mechanical manipulation of body tissues with rhythmical pressure and stroking intended to promote health and well-being.[124] There are currently several massage approaches, e.g. classic Swedish massage, Rolfing, acupressure, neuromuscular, myofascial release, and IASTM.[27, 123, 125] Depending on the clinician experience and desired

outcome, techniques are selected and frequently used in the conservative management of a variety of conditions; from general to targeted treatment purposes, e.g. sports preparation, relaxation, low back pain,[126] and neck pain[127] to name a few.[27, 123, 126, 128-129] Long-term trends show a growing popularity in complementary and alternative medicine (CAM) over the past several decades, especially among the younger population (post-baby boomers).[130] There were an estimated 93.1 million visits to manual therapists in the US in 2007.[131] Proponents claim many benefits of massage related to mobilizing/re-mobilizing the soft tissue, e.g. increased soft tissue pliability, increased skin and muscle blood flow, decreased pain and muscle spasm, and relaxation. Although there is preliminary evidence suggesting massage has beneficial effects, the related research remains limited both in quantity and quality.[123]

1.9.2 Evidence supporting the use of massage

One of the challenges in establishing the efficacy of massage therapy is the variety of techniques and potential uses. Some studies indicate massage therapy may induce both local and systemic changes. Different mechanisms have been suggested for the positive effects of massage, e.g. increased parasympathetic activity, reduced neuromuscular excitability, enhanced vascular function, but these are not fully understood.[123, 127-128] The frequent use of CAM in the management of musculoskeletal symptoms (i.e. back, neck, shoulder problems), accounts for about 60% of massage applications,[27] suggesting a degree of clinical efficacy; however, this needs to be explored and demonstrated scientifically. To establish its efficacy, research needs to focus on more frequently used massage applications and techniques. One of the massage therapy approaches most often employed for the management of

musculoskeletal symptoms is deep tissue massage, credited for approximately 65% of all massage visits.[27]

1.10 Cross fiber massage

1.10.1 Cross fiber massage overview

CFM is a specific type of deep tissue massage used by clinicians to treat musculoskeletal impairments. In modern times, Dr James Cyriax is credited with the technique;[132] although, it was first described by Hippocrates.[132-133] It is predominantly used with injury, overuse or degenerative conditions in muscles, tendons and ligaments.[134-138] The technique involves moving the skin and subcutaneous tissues over deeper tissue lesions so as to exert localized, controlled mechanical forces with the intent to induce physiological (e.g. hyperemia) and structural tissue changes, reduce pain and prevent adhesions. It differs from other massage techniques in that there is little motion between the therapist's contact and the patient's skin to prevent bruising while mobilizing deeper tissues. The direction of the CFM manipulating forces is usually applied perpendicular to the underlying collagen substructure. Therefore, it is well suited for connective tissues with parallel fiber arrangements, i.e. ligaments and tendons. Since reparative cells (fibroblasts) are mechanosensitive and respond to external loading of their environment, it is theorized that CFM stimulates fibroblasts collagen production and scar formation, without stretching or tearing the healing fibers.[139-140,141-142] Rigid devices are often used to augment CFM implementation (i.e. IACFM).

1.10.2 Instrument-assisted cross fiber massage

Instruments have been used to augment the manual delivery of massage techniques for millennia.[143] Hand held rigid instruments used to augment manual therapies may be comprised of common devices (i.e. stones, pencils, soup spoons), or specially designed tools (i.e. contoured stainless steel instruments). IACFM is a type of CFM that employs hand-held instruments made out of solid materials to administer localized mechanical forces to soft tissue structures (Figure 1.2). IACFM is currently used by several clinics and athletic organizations.[125] IAFCM may provide benefits over the traditional means of manually applying the CFM force by hand only, in that IACFM simultaneously offers mechanical leverage to the clinician along with precise application and depth of penetration of the treatment force during soft tissue examination and treatment.[144] Other purported advantages include enhanced detection and treatment of soft tissue dysfunctions, especially when using instruments with uniquely designed treatment edges, contoured shapes and materials (i.e. lucite, acrylic, ceramics or stainless steel).[144-145] Secondary benefits of IACFM are improved therapist joint comfort and reduced fatigue; perhaps due to improved wrist and hand ergonomics.[146]

1.10.3 Evidence supporting the use of cross fiber massage approaches

CFM has been used with mixed results for a multitude of conditions, including tendonitis, ligament sprains, and bursitis. CFM has been shown to immediately improve muscle function as indicated by an increase in force production;[147-148] however, its longer-term effects on soft tissue dysfunction have not been firmly established. For example, CFM has not shown consistent benefit in the management of pain or functional deficits in infrapatellar tendinopathy[149] or iliotibial band friction syndrome.[150] A recent

Figure 1.2. Cross fiber massage approaches. Cross fiber massage is depicted being delivered to the knee MCL in a human subject manually A) by hand and B) augmented by use of a rigid device made of stainless steel, i.e. instrument-assisted cross fiber massage (IACFM). Arrows indicate the direction of movement forces perpendicular to ligament fiber alignment.

study demonstrated decreased motor neuron pool excitability in forearm flexor muscles treated with CFM.[151] It has been suggested that CFM introduces a controlled amount of microtrauma into the tissue that triggers the healing cascade; however, this has not been substantiated in ligament.[144-145] Conclusions regarding the effectiveness of CFM is limited by the small sample sizes of the previous randomized controlled trials.[123, 150, 152]

Clinical pilot studies using IACFM have demonstrated positive outcomes for diagnoses such as carpal tunnel syndrome,[153] chronic ankle pain,[154] plantar fasciitis, achilles and supraspinatus tendinosis.[155] For patellar tendinopathy, IACFM had better efficacy than standard CFM using the fingers.[156] Initial animal model studies found IACFM applied to chemically induced rat Achilles tendon injuries increased fibroblast proliferation and activation, especially at higher therapeutic pressures, which may be related to improved healing.[157-158]

Preliminary evidence suggests CFM approaches may benefit tissue healing and repair, but its efficacy needs to be further established. The ultimate outcome of any healing process in load-bearing tissue, such as a ligament, is the restoration of mechanical properties. To assess tissue strength, an animal model is necessary since it requires destructive mechanical testing; as does the assessment of microvascular structural parameters associated with healing. In order to help answer the important clinical question regarding the benefits of CFM, a precise form of CFM, IACFM, will be used to investigate its tissue level effects in healing ligaments using an established rodent model.

1.11 Summary and general aims

Ligament injuries are common clinical disorders resulting in functional limitations and disability. A number of interventions exist for this condition; however, a limited

number have been shown to accelerate or augment ligament healing. STM techniques, such as CFM, offer conservative, manual therapy alternatives to invasive procedures and pharmaceuticals in promoting ligament healing. The purpose of this dissertation is to investigate the effects of a discrete CFM approach, specifically IACFM, on ligament healing. An established rodent model for knee ligament injury will be used in all studies. A basic understanding of the tissue-level effects of this form of manual therapy is needed to support clinical decision-making leading to optimal therapeutic outcomes.

The general aims of this dissertation are to investigate the:

1. Short and long-term effects of IACFM on biomechanical and histological properties during knee ligament healing.
2. Effects of IACFM on regional blood flow and angiogenesis during early knee ligament healing.

CHAPTER TWO

The Short and Long Term Effects of Instrument-Assisted Cross Fiber Massage on

Biomechanical and Histological Properties in Healing Ligaments

2.1 Introduction

CFM exerts localized mechanical forces through the skin and subcutaneous tissues to deeper connective tissues. As the reparative cells (fibroblasts) responsible for producing collagen and forming a scar following ligament injury are mechanosensitive,[141-142] it is possible that IACFM, a type of CFM described in Chapter One, facilitates matrix production and the restoration of tissue-level mechanical properties.

Based on preliminary evidence demonstrating the potential efficacy of IACFM and its mechanical mechanism of action, the aim of this set of studies were to examine the short- and long-term effects of IACFM on tissue-level healing of knee MCL injuries in an established animal model. It was hypothesized that IACFM would accelerate early and augment long-term ligament healing. The primary variables of interest were ligament mechanical properties since the ultimate outcome of any healing process in a load-bearing tissue, such as ligament, is the restoration of its mechanical properties. The secondary variable of interest was ligament morphology as this may help to explain differences in tissue mechanical properties.

2.2 Methods

2.2.1 Animals

Fifty-eight six-month-old, virgin, female, Sprague-Dawley rats (280-300 grams) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) and acclimated for a minimum of seven days prior to experimentation. Animals had *ad libitum* access to standard rat chow and water at all times, and were housed two per standard sized cage (16"L x 7.75"W x 8"H). All procedures were approved *a priori* by an Institutional Animal Care and Use Committee.

2.2.2 Ligament injury

Fifty-one animals underwent surgery on entry to the study to create bilateral knee MCL injuries ('injured' animals) as previously described.[22] The remaining seven animals served as age-matched, ligament-intact cage controls and were not operated on ('control' animals). Following a pre-operative subcutaneous dose of buprenorphine hydrochloride analgesia (0.05 mg/kg; Buprenex[®]—Reckitt & Colman Pharmaceuticals Ltd., Richmond, VA), surgical anesthesia was achieved using a mixture of ketamine (60-80 mg/kg; Ketaset[®]—Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (7.5 mg/kg; Sedazine[®]—Fort Dodge Animal Health, Fort Dodge, IA) introduced intraperitoneally. Using a sterile technique, a 5 mm longitudinal incision was made over one knee medial joint line, and the MCL sharply transected at the joint line using a size 11 scalpel blade. This resulted in complete disruption of the MCL at its mid-substance, transverse to the underlying collagen fiber alignment. No ligament material was removed, and the ligament ends were juxtaposed but not sutured prior to closing of the

skin incision with a single subcuticular absorbable suture. The procedure was repeated on the contralateral side to create bilateral injuries. All animals demonstrated normal, symmetrical hindlimb use upon recovery from surgery and were allowed normal cage activity (without access to exercise wheels) for the duration of the study.

2.2.3 IACFM intervention

IACFM was introduced using a rigid tool fabricated from stainless steel (GT6—Graston Technique®, TherapyCare Resources, Indianapolis, IN). The 'GT6' instrument was used since it is designed to apply force through its tip to small structures, such as finger collateral ligaments in humans (i.e. rat knee-sized ligaments) (Figure 2.1). IACFM was initiated one week post-operatively (post-injury) to allow the initial inflammatory response/phase of ligament healing to complete based on healing and repair timelines. Delaying the initial introduction of IACFM is consistent with its clinical use following an acute injury.[155] IACFM was administered with the animals under isoflurane anesthesia (3% at 1.5 l/min for initial knock-down in a plastic container, and 1.5% at 1.5 l/min via a face mask for maintenance of anesthesia). Approximately 250-300 g of instrument downward force was applied during treatment. This force was equivalent to that previously used in demonstrating IACFM benefits on rat Achilles tendon healing,[158] and was determined by using the massage instrument on a force plate with kinesthetically similar pressure as would be used clinically to treat a comparably sized ligament at an equivalent tissue depth (i.e. collateral ligament of a human interphalangeal joint). Thirty-one injured animals were treated three times per week for three weeks (total treatments = 9), while the other 20 injured animals were treated three times per week for 10 weeks (total treatments = 30). The number of treatments in the later animals is more than would typically be introduced in a clinical setting; however, were implemented to maximize the

potential of finding any long-term benefit of IACFM. IAFCM was applied to the left MCL in injured animals for one minute per session (IACFM-treated). This treatment duration was based on the recommended clinical use of IACFM for the treatment of isolated tissue lesions[159] and evidence from previous preclinical studies demonstrating the efficacy of short duration IACFM interventions.[157-158] The contralateral, injured MCL in these animals served as an internal control and did not receive IACFM (non-treated). The seven control animals were not treated with IACFM.

2.2.4 Assessment time points and specimen preparation

Animals were euthanized post-injury at either four (all animals treated for nine sessions [N=31] and two control animals) or 12 (all animals treated for 30 sessions [N=20] and five control animals) weeks. Animals euthanized at four weeks had both hindlimbs harvested and prepared for mechanical testing (N=18 injured animals), scanning electron microscopy (SEM) (N=11 injured and N=2 control animals) or histological assessment (N=2 injured animals). Animals euthanized at 12 weeks had both hindlimbs removed and prepared for mechanical testing (N=17 injured; N=4 control animals) or histological assessment (N=3 injured; N=1 control animals).

2.2.5 Mechanical testing

Ligament mechanical properties were assessed as previously described.[160-161] Hindlimbs destined for mechanical testing were initially stored at –80°C with the knee tissues intact. Postmortem storage of ligaments by freezing does not influence their mechanical properties.[162] On the day of mechanical testing, the hindlimbs were allowed

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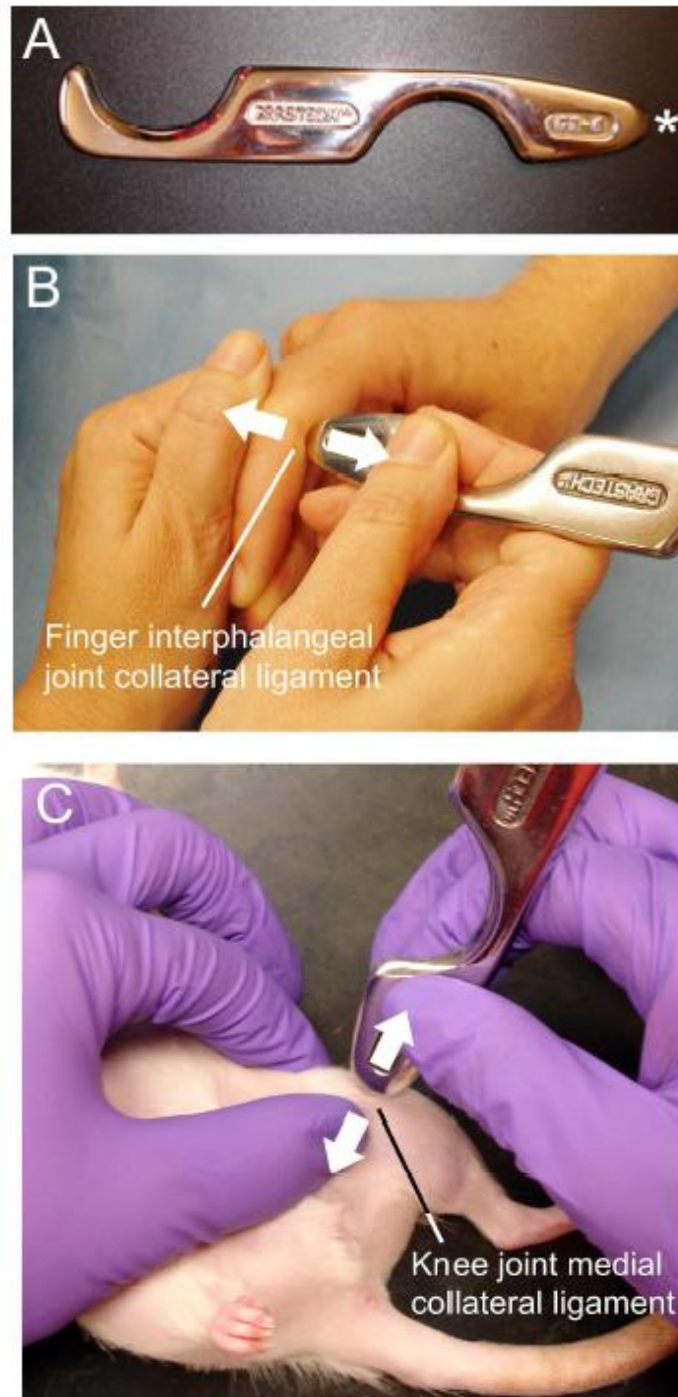
Figure 2.1. IACFM intervention. A) The rigid Graston Technique® GT6 tool fabricated from stainless steel has a tapered tip (*) which permits treatment of small structures. IACFM of a: B) human finger, and; C) similarly-sized rodent knee joint medial collateral ligament using the GT6 tool. Arrows indicate the direction of movement/force application perpendicular to the collagen substructure of the ligament.

to thaw to room temperature in phosphate-buffered saline (PBS). Femoral-MCL-tibial (FMT) complexes were prepared, by dissecting clear extraneous tissue (including the joint capsule and adherent medial meniscus) while keeping the MCL and its insertion sites hydrated with PBS. The femoral and tibial insertions of the MCL were left intact, and the proximal tibia growth plate was removed to permit more space within the knee joint during testing. MCL thickness and width were measured optically at the knee joint line, and MCL area estimated using an elliptical geometry.[49] Each FMT complex was placed in a customized testing jig with the knee joint positioned in 70° flexion for MCL testing. This position appears to load all ligament fibers simultaneously.[49] The femoral and tibial portions were embedded in Wood's low melting-point metal (Bismuth alloy LMA-117—Small Parts, Inc., Miami Lakes, FL) for fixation. The jig was coupled to an electromagnetic material testing device (TestBench 200 N ELF LM-1—EnduraTEC Systems Group, Bose Corp., Minnetonka, MN) equipped with a 50 N load cell (Figure 2.2). This system possesses a force and displacement resolution of 0.01 N and 0.001 mm, respectively. A preload of 0.05 N was applied and the ligaments were preconditioned by cyclically loading at 1 Hz for 10 cycles to 1% strain to reduce the effect of deep freezing on low-load mechanical properties by normalizing area of hysteresis.[162] The ligaments were unloaded and allowed to recover for 1.5 minutes while being kept moist with PBS. Following tissue recovery, ligaments were again preloaded (0.05 N) and pulled to tensile failure in displacement control at a rate of 0.8 mm/s (~10%/s). Force and displacement data were collected at 100 Hz, and the mechanical properties of ultimate force (N), stiffness (N/mm) and energy to failure (mJ) obtained from the force-displacement curves.
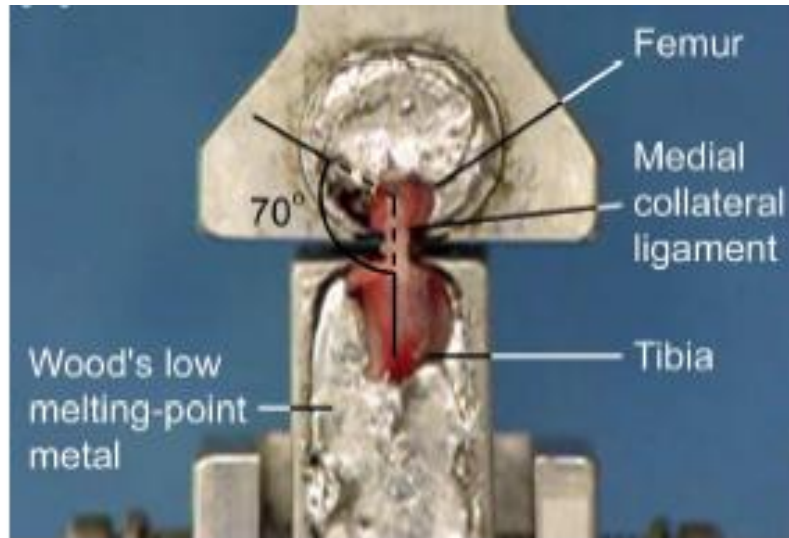
Figure 2.2.   Tensile mechanical testing of rat knee medial collateral ligaments. Representative image of the setup for testing. Femur-MCL-tibia complexes were placed in fixtures that supported ('cupped') the distal femur and proximal tibia to prevent bone slippage, and fixed in place using Wood's low melting-point metal. The knee joint was positioned in 70° of flexion for testing.

2.2.6 Scanning electron microscopy

Immediately after harvest, specimens for SEM were placed in a custom limb frame that held the knee positioned in 70° flexion. The MCL was exposed and drip fixed for one hour with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Services, Hatfield, PA). After drip fixation, the MCLs were removed using a micro-surgical scalpel with the femoral insertion marked by an angled cut. Any adherent tissue was removed under a dissecting microscope. Ligaments were then rinsed twice in buffered solution, and dehydrated by immersing for 15 minutes each in fresh solutions of 70%, 95% and 100% ethyl alcohol. They were subsequently immersed in liquid nitrogen, placed on pre-cooled microscope slides and fractured under a dissecting microscope in the sagittal plane from the femoral to tibial end using one-half of a pre-cooled double-edged stainless steel razor blade (Electron Microscopy Sciences, Hatfield, PA). The

ligament samples were then critical point dried (Samdri model 780A—Tousimis

Research Corp., Rockville, MA), mounted on 10 mm SEM specimen mount blocks using

non-conductive adhesive tabs (Ted Pella, Inc., Redding, CA), and surrounded by

colloidal silver paste (Electron Microscopy Sciences, Hatfield, PA). After drying overnight

in a vacuum dessicator with dissecant, the samples were sputter coated with gold-

palladium (Polaron, Energy Beam Sciences, East Gramby, CT) for 1.75 minutes, and

stored in a vacuum dessicator with desiccant until imaged. The samples were imaged on

a scanning electron microscope (JSM-6390LV—JEOL Ltd., Peabody, MA) using a 5kV

accelerating voltage and working distance of 11 mm. The ligaments were aligned at low

magnification (approximately X25) by orienting the femoral end of the ligament to the top

of the screen, and the residual and scar regions were identified. The morphology of

collagen fibrils and fibers for each ligament in the residual and scar tissue regions were

examined at magnifications of X250 to X11000, and digitally imaged.

2.2.7 Histology

Ligaments for histology were fixed under tension in 4% paraformaldehyde at 4°C

for 48 hours. They were subsequently dehydrated in graded alcohols, washed with two

changes of xylene, and infiltrated and embedded in paraffin using a Shandon automatic

tissue processor (Thermo Electron Corp, Waltham,. MA). Sagittal plane thin (4 µm)

sections were cut using a rotary microtome (Reichert-Jung Model 2050; Reichert-Jung,

Heidelberg, Germany), mounted onto microscope slides, and stained with Harris'

hematoxylin and eosin (H&E) on a linear stainer (Shandon Linistain GLX—Thermo

Electron Corp, Waltham, MA). Three sections per specimen were qualitatively assessed

for collagen fiber alignment and cellularity under light microscopy using a Nikon

Optiophot 2 microscope (Nikon, Inc., Garden City, NY).

2.2.8 Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 16.0—SPSS Inc., Chicago, IL). All comparisons were two-tailed with a level of significance set at 0.05. Unpaired t-tests were performed to assess time (four vs. 12 weeks post-injury) and group (injured vs. control animals) effects on body mass. The Kolmogorov–Smirnov test normality test was used to determine a normal distribution of the data. IACFM effects were principally determined using paired t-tests to compare IACFM- and contralateral non-treated MCLs. Paired t-test results were confirmed by calculating mean percent differences between IACFM- and non-treated MCLs ([IACFM-treated – non-treated] / non-treated x 100), which were analyzed using single sample t-tests with a population mean of 0%.

2.3 Results

2.3.1 Animal characteristics

There were no operative or post-operative complications. Animals assessed at 12 weeks post-injury were significantly heavier than those assessed at four weeks (291.4 ± 13.2 g vs. 313.5 ± 22.6 g; $p<0.05$). There were no differences in weight between injured and control animals ($p=0.76$).

2.3.2 Ligament macroscopic morphology

All surgically-induced ligament defects were bridged with scar tissue at the time of harvest. At four weeks post-injury, the injured region was clearly distinguishable from the

uninjured ligament tissue by the presence of a thickened, somewhat translucent, pinkish scar. In comparison, ligaments at 12 weeks post-injury had difficult to see whitish scars that were relatively indistinguishable from the uninjured tissue, and the thickness of the scar region was continuous with that of the uninjured portions of the ligament. There were no grossly observable differences between IACFM- and non-treated ligaments at either four or 12 weeks post-injury; however, non-treated ligaments often had more adhesions and granular tissue, and were more difficult to harvest than IACFM-treated ligaments. Cross-sectional area of IACFM- and non-treated ligaments did not differ significantly at either four (5.46 ± 1.01 mm^2 vs. 5.16 ± 1.55 mm^2; p=.45) or 12 (3.80 ± 1.02 mm^2 vs. 4.09 ± 0.79 mm^2; p=0.29) weeks post-injury.

2.3.3 Ligament mechanical properties

At four weeks post-injury, IACFM-treated ligaments could resist 6.4 N (95% confidence interval [CI], 1.6 N to 11.2 N; p=0.01) greater force than contralateral non-treated ligaments (Figure 2.3A). This was reflected by IACFM-treated ligaments having 43.1% (95% CI, 8.2% to 78.0%; p=0.02) greater mean difference in tensile strength than non-treated ligaments. Similarly, IACFM-treated ligaments had 4.9 N/mm (2.4 N/mm to 7.4 N/mm; p=0.001) (Figure 2.3B) and 5.8 mJ (95% CI, 0.7 mJ to 10.9 mJ; p<.05) (Figure 2.3C) greater stiffness and energy to failure at four weeks post-injury than non-treated ligaments, respectively. This was reflected by IACFM-treated ligaments being 39.7% (95% CI, 15.9% to 63.5%; p<.01) stiffer and being able to absorb 57.1% (95% CI, 3.4% to 110.9%; p=0.04) greater energy before failure than non-treated ligaments.

At 12 weeks post-injury, IACFM-treated ligaments had 2.6 N/mm (95% CI, 0.2 N/mm to 5.0 N/mm; p<.05) greater stiffness than non-treated ligaments, resulting in the former being 15.4% (95% CI, 0.1%-30.7%; p<.05) stiffer (Figure 2.4B). However, there

were no differences at 12 weeks post-injury between IACFM- and non-treated ligaments in ultimate force (1.1 N; 95% CI, -2.6 N to 4.7 N; p=0.54) (Figure 2.4A) or energy to failure (-0.6 mJ; 95% CI, -6.7 mJ to 5.5 mJ; p=0.84) (Figure 2.4C). Mechanical properties of ligaments in injured animals at both four and 12 weeks post-injury remained inferior to intact, non-injured ligaments from control animals (p<0.05).
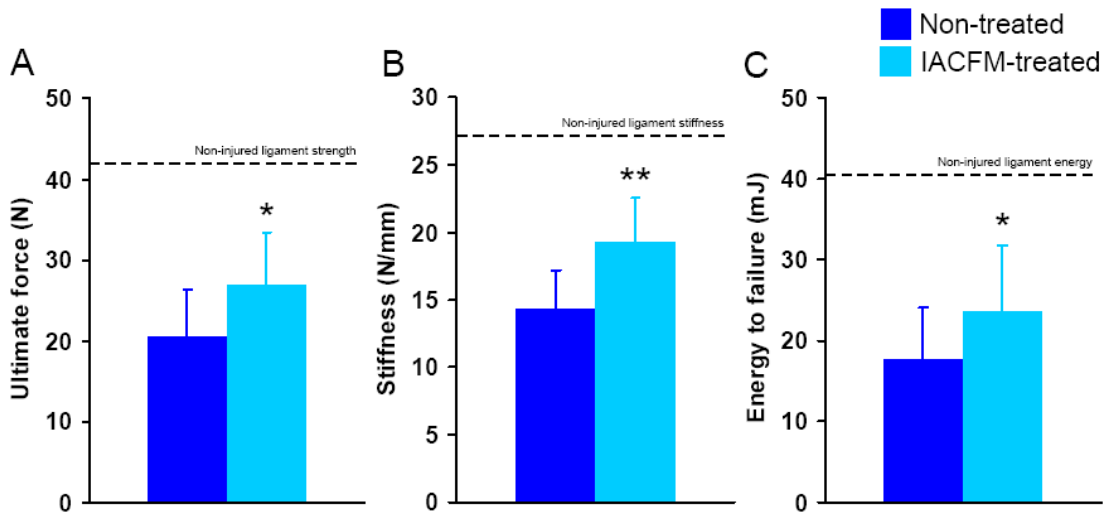
Figure 2.3. Effect of nine sessions (1 min/session) of IACFM on ligament mechanical properties assessed at four weeks following injury. IACFM-treated ligaments had greater: (A) ultimate force; (B) stiffness, and; (C) energy to ultimate force than contralateral, non-treated ligaments. *indicates p<0.05 and **indicates p<0.01, as determined via single sample t-tests on the mean percent differences with a population mean of 0%. Error bars indicate standard deviation.
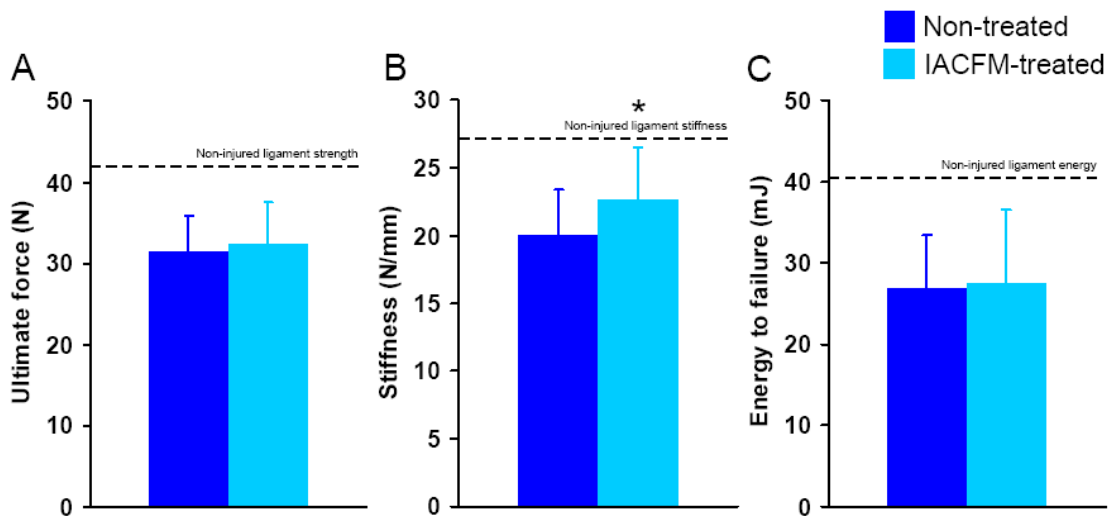
Figure 2.4. Effect of 30 sessions (1 min/session) of IACFM on ligament mechanical properties assessed at 12 weeks following injury. IACFM-treated ligaments had: (A) equivalent ultimate force; (B) enhanced stiffness, and; (C) equivalent energy to failure than contralateral, non-treated ligaments. *indicates $p<0.05$, as determined via single sample t-test on the mean percent difference with a population mean of 0%. Error bars indicate standard deviation.

2.3.4 Ligament microscopic morphology

Light microscopy of non-injured ligaments from control animals revealed a uniform appearance of tightly-packed, well-aligned collagen fibrils with interspersed fibroblasts aligned parallel to the fibrils (Figure 2.5A). In contrast, ligaments from injured animals appeared to have scar morphology with ECM disorganization and hypercellularity, particularly at four weeks post-injury (Figure 2.5B-E). The scar region of IACFM-treated ligaments at four weeks post-injury also appeared to have greater cellularity with collagen fiber bundles appearing to be orientated more along the longitudinal axis of the ligament than observed in contralateral non-treated ligaments (Figure 2.5B, C). At 12 weeks post-injury, there were limited histological differences between IACFM- and non-treated ligaments (Figure 2.5D, E).

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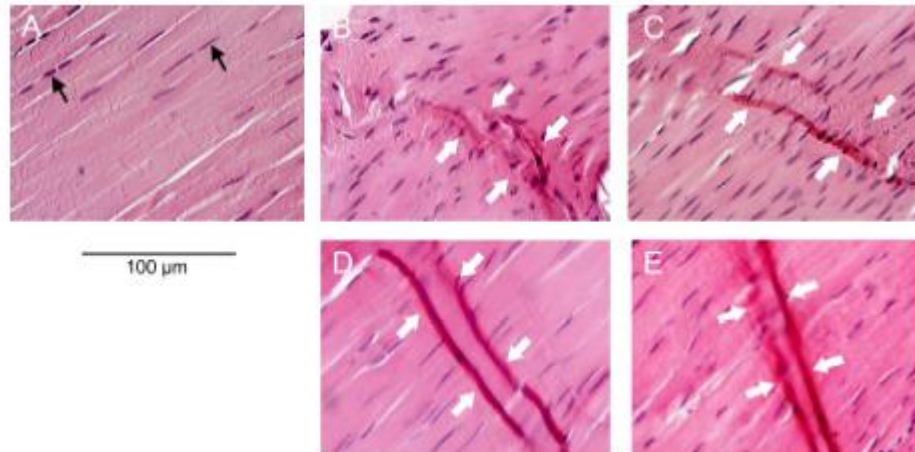
Figure 2.5. Representative histological sections from: A) non-injured MCL in a cage-control animal; B) scar region in a non-treated MCL at four weeks following injury; C) scar region in an IACFM-treated MCL at four weeks following injury; D) scar region in a non-treated MCL at 12 weeks following injury, and; E) scar region in an IACFM-treated MCL at 12 weeks following injury. Black arrows indicate fibroblasts aligned parallel to the collagen fibrils in a non-injured ligament. White arrows indicate scar region in injured ligaments.

Ligaments from injured, but not control, animals had granular tissue at low magnification (X25) on SEM and IACFM-treated ligaments appeared to have less surrounding granular tissue compared to non-treated ligaments, confirming the macroscopic observations (Figure 2.6). At higher SEM magnifications (X250-X6500), the scar region of IACFM-treated ligaments appeared to have improved collagen fiber bundle formation and orientation within the scar region than non-treated ligaments, supporting the light microscopy observations (Figure 2.7).
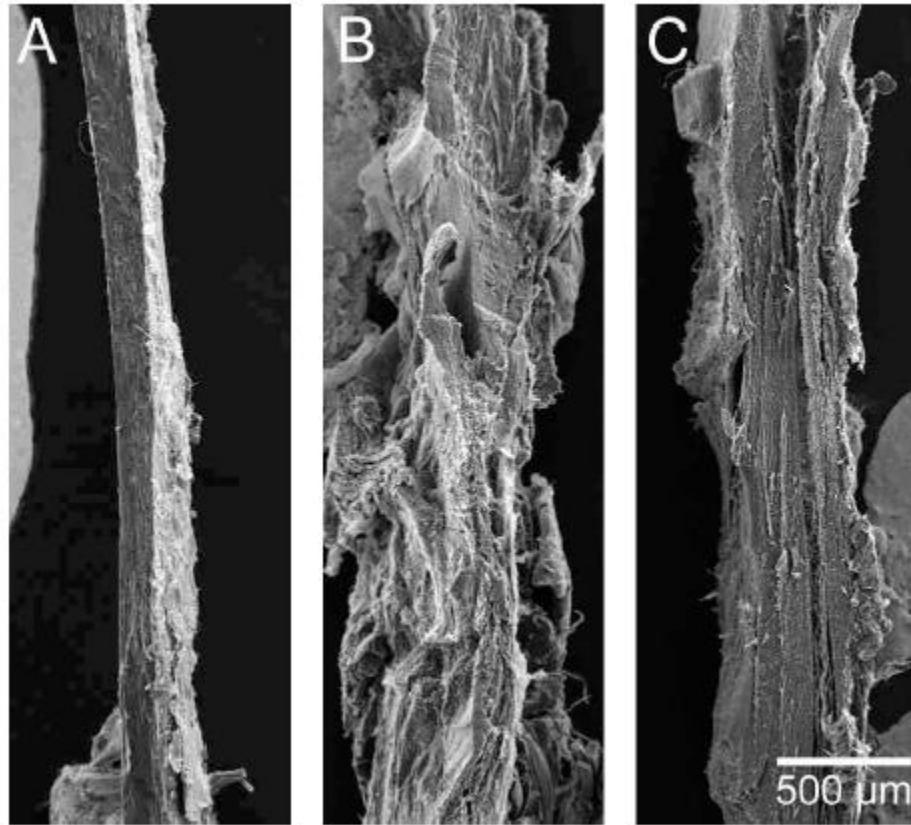
Figure 2.6. Representative scanning electron microscopy images at low magnification taken from a: A) intact ligament in a control animal; B) non-treated ligament at four weeks following injury, and; C) IACFM-treated ligament at four weeks following injury. Images were taken at low [X25] magnification. Note the close appearance of the IACFM-treated ligament (C) to the non-injured ligament from a control animal (A). Also, note the large amount of surrounding granulation tissue in the non-treated, but injured ligament (B) relative to the other two ligaments.
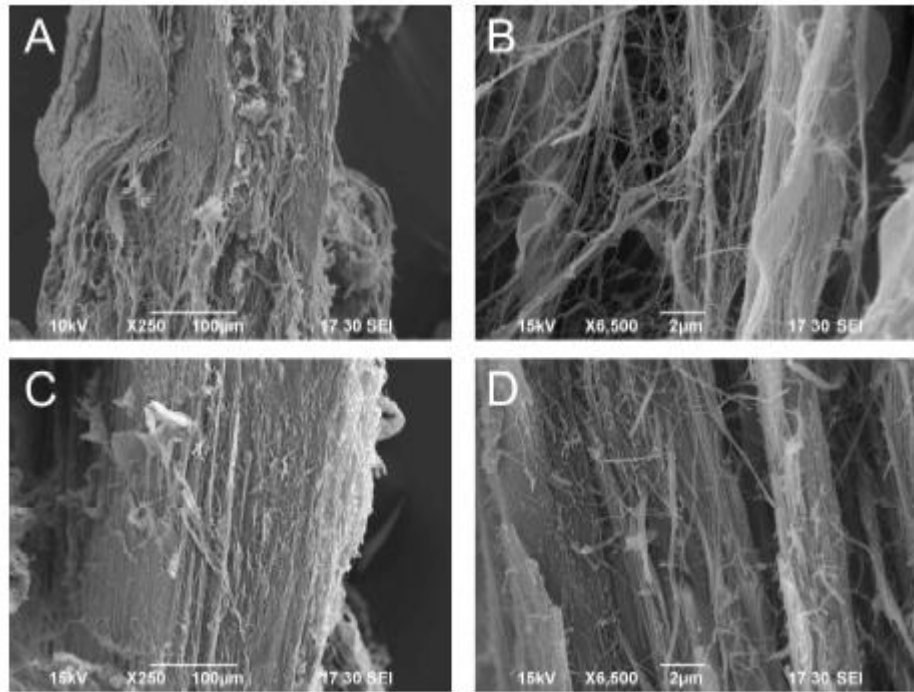
Figure 2.7. Representative scanning electron microscopy images taken from the scar region of a: A & B) non-treated, and; C & D) IACFM-treated ligament at four weeks following injury. Images were taken at: A & C) medium [X250], and; B & D) high [X6500] magnification. Note the improved collagen fiber bundle formation and orientation within the scar region of the IACFM-treated ligament (C, D) relative to that of the non-treated ligament (A, B).

2.4 Discussion

This set of studies investigated the potential utility of manual therapy in the form of IACFM on ligament healing. Results indicate that IACFM-treated ligaments were 43% stronger, 40% stiffer and able to absorb 57% more energy than contralateral, non-treated, injured ligaments at four weeks following injury. These mechanical differences may have resulted from favorable effects of IACFM on the organization of the underlying

collagen substructure, as suggested by preliminary light microscopy and SEM analyses. The latter needs to be confirmed by way of more in-depth quantitative analyses in future studies. In contrast, there was minimal effect of IACFM on ligament healing when assessed at 12 weeks following injury, with the only difference between IACFM- and non-treated ligaments being 15% greater stiffness in the former. Overall, the findings suggest that IACFM accelerates early tissue-level healing following ligament injury, but does little in terms of augmenting later healing.

The findings of the current study are interesting in that a relatively simple and practical manual therapy technique was found to enhance early recovery of ligament biomechanical properties following acute injury. How IACFM facilitates the restoration of ligament tensile mechanical properties following injury was not investigated in detail as the primary purpose was to provide proof-of-concept evidence for the utility of IACFM. However, preliminary light microscope and SEM assessments suggest that IACFM may enhance restoration of ligament biomechanical healing by optimizing the organization of the collagen substructure. The alignment and organization of newly formed collagen fibers in the direction of tensile loads during healing influences ligament mechanical properties.[49, 163] The qualitatively improved collagen fiber organization observed within the scar region of IACFM-treated ligaments is a possible explanation for why IACFM-treated ligaments had enhanced tensile mechanical properties.

Preliminary data indicate that IACFM accelerates the initial return of mechanical properties following acute ligament injury and improves ligament stiffness. These findings support a theoretically sound argument for the use of IACFM after acute capsular/extra-capsular ligament injury; however, careful interpretation of this controlled laboratory study is warranted until its findings are confirmed by clinical studies. Furthermore, these findings do not answer the important question of how healing is enhanced. Recent studies have found increased blood flow and angiogenesis positively

affects the return of mechanical properties in injured ligament.[164-165] Since a purported

benefit of massage techniques is increased tissue perfusion, potential mechanisms

underlying the beneficial effects of IACFM demonstrated thus far may involve

improvements in the vascular properties of injured knee ligaments.

CHAPTER THREE

The Effects of Instrument-Assisted Cross Fiber Massage on

Regional Blood Flow and Angiogenesis

3.1 Introduction

Initial findings in this research program demonstrated IACFM accelerated the
return of tissue-level mechanical properties in healing knee MCLs when assessed at four
weeks post-injury in an established animal model. While these findings support previous
studies suggesting the efficacy of IACFM, it did not address the important question of
how IACFM enhances ligament healing. Subsequent studies were designed to
investigate mechanisms that could contribute to the beneficial effects of IACFM on
ligament healing.

Based on the fact that each phase of ligament healing requires adequate blood
supply for the transport of cells and metabolites, it seemed logical that IACFM-induced
alterations in tissue perfusion could have influenced the ligament healing process.[41, 63, 73]
Therefore, the aim of this set of studies was to investigate the effect of IACFM on
regional vascular properties during early knee MCL healing in rodents. The specific
variables of interest were tissue perfusion and microvascular morphology. It was
hypothesized that IACFM-treated injured knees would demonstrate a) increased regional
tissue blood flow, either at some or all assessment points, and b) altered microvascular
morphology parameters suggesting angiogenesis during early ligament healing.

3.2 <u>Methodology</u>

3.2.1 Animals

Thirty adult, virgin, female, Sprague-Dawley rats (6 months; 280-300 g) were purchased from Harlan Spraque-Dawley, Inc. (Indianapolis, IN) and acclimated for a minimum of 7 days prior to experimentation. Animals were housed in standardized conditions with *ad libitum* access to standard rat chow and water at all times. All procedures were approved *a priori* by the Institutional Animal Care and Use Committee of Indiana University.

3.2.2 Ligament injury

All animals underwent surgery to create bilateral knee MCL injuries. The surgical procedure was the same as previously described in Chapter Two (Section 2.2.2).

3.2.3 IACFM intervention

IACFM was initiated one week following injury (post-acute) to allow completion of the initial inflammatory response/phase. The IACFM intervention was administered for one minute, three times per week for three weeks (total=9 treatment sessions) as previously described in Chapter Two (Section 2.2.3).

3.2.4 Assessment of superficial regional tissue perfusion

Regional superficial tissue perfusion was assessed *in vivo* in one set of animals (n=11) using noninvasive laser Doppler imaging (LDI). A desktop scanning laser-Doppler perfusion imager (MoorLDI2-IR™; Moor Instruments, Wilmington, DE) modified by the manufacturer for high resolution and maximum depth of penetration (2-3 mm) was used which possessed a laser diode source that produced near-infrared energy with a wavelength of 785 nm and nominal power of 2.25 mW. A low-frequency cut-off (250 Hz) was used to eliminate movement artifacts, while a high-frequency cut-off (15 kHz) was used to improve the signal-to-noise ratio.

LDI was performed in a standardized environment (22°C) with animals under inhalation anesthesia. The hindlimbs were shaved, and the animals were placed in dorsal recumbency on a warming pad (37°C) with both hindlimbs abducted to permit imaging of the medial knee joint region. Both hindlimbs were positioned within a single field of view to allow simultaneous scanning of the IACFM- and non-treated hindlimbs. The imager and animal were subsequently enclosed in an opaque screen to eliminate artifactual light during scanning. After animals were positioned they were left undisturbed for 10 minutes before scanning to reduce handing and environmental influences on tissue perfusion measures.[77]

Triplicate scans of each animal were performed without interim repositioning at the following time points: the day prior to surgically-induced MCL injury (pre-injury), immediately prior to each IACFM intervention (pre-IACFM), and at 0, 5, 10, 15 and 20 minutes, and 24 hours following IACFM intervention (post-IACFM). A final assessment was performed 1 week following the final IACFM intervention (4 week post-injury). As the IACFM- and non-treated hindlimbs were simultaneously scanned within each animal, the influences of subtle changes in ambient temperature and light and physiological status of

the animals were controlled. An identical region of interest (ROI) was selected over the medial knee region on the acquired 2D images, and mean flux values were obtained for both the IACFM- and non-treated hindlimbs. These flux values were averaged for the triplicate scans performed on each hindlimb at each assessment time point. The MCLs from these animals were harvested at 4 weeks post-surgery and used for assessment of morphological properties under SEM.

3.2.5 Assessment of regional microvasculature morphology

Microvasculature morphology was assessed in a second set of animals (n=19) by micro-CT imaging of tissues perfused with a contrast agent, as previously described.[91-92] Animals were anesthetized at 4 weeks post-surgery (1 week following the final IACFM intervention) with an intraperitoneal injection of ketamine (60-80 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (7.5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). They were placed on a warming pad (37°C) in dorsal recumbency with their appendages splayed and fixed. The thoracic cavity was opened and the inferior vena cava located and severed to exsanguinate the animals. A 16-G cannula needle connected to a perfusion pump (Minipuls 2 Peristaltic Pump; Gilson Inc., Middleton, WI) was inserted through the left ventricle of the heart and into the ascending aortic. The vasculature was flushed with 0.9% normal saline containing heparin sodium (100U/ml) and sodium nitrite before being pressure fixed with 4% phosphate-buffered formalin. Formalin was flushed using heparinized saline and the vasculature perfused with a radiopaque silicone rubber containing lead chromate (Microfil MV-122, Flow Tech, Inc., Carver, MA). Following storage at 4°C overnight to allow contrast agent polymerization, the hindlimbs were removed and soaked in 10% neutral buffered formalin for 4 days to ensure complete tissue fixation.

Immediately prior to micro-CT imaging, the MCLs and their adjacent connective tissues were harvested and dissected to a standard sample size (length=1.5 cm, width=0.5 cm). Surrounding connective tissue was included in analyses as the MCL proper has limited vascularity and receives its blood supply from vessels in its epiligament and the adjacent peri-articular tissues (related discussion in Chapter One; Section 1.6.1). This approach maximized the number of vessels assessed, with the tissues containing these vessels also being injured during surgery and subsequently treated during IACFM intervention. Samples were custom mounted and positioned vertically on the computer-controlled rotation stage of a bench top micro-CT system (SkyScan 1172 high-resolution micro-CT; SkyScan, Kontich, Belgium) (Figure 3.1A) and scanned 359° around the vertical axis in rotation steps of 0.4° using an x-ray source operating at 50kV. The isotropic voxel size was 5.9 μm. Serial tomograms were reconstructed (Figure 3.1B) and images were binarized to segment the radiopaque microvasculature network from its surrounding connective tissue (threshold of 85 for blood vessels and 15 for connective tissue on a 0-255 scale).This was performed for the whole tissue and femoral, middle and tibial subregional thirds. Microvascular morphometric parameters for the volume of interest (VOI) included: vessel volume normalized to tissue volume (VV/TV; %), vessel thickness (V.Th; μm) (a.k.a. vessel diameter), vessel separation (V.Sp; μm), vessel number (V.N; /μm), and frequency distribution of V.Th.

3.2.6 Statistical analyses

IACFM effects on regional blood flow at each time point were principally determined using paired t-tests to compare in IACFM- and contralateral non-treated hindlimbs. Flux values in IACFM-treated hindlimbs were expressed relative to those

measured in non-treated hindlimbs (IACFM treated/non-treated) to provide a perfusion ratio for each individual animal. Intervention effects were further assessed at each timepoint by calculating mean perfusion ratios and their 95% CI. The 95% CIs not crossing 1 were considered statistically significant, as determined by single sample t-tests on the mean perfusion ratios with a population mean of 1. Intervention effects on regional microvasculature morphology were assessed using paired t-tests. All comparisons were with a level of significance set at 0.05.

3.3 Results

3.3.1 Regional tissue perfusion

A representative laser Doppler image of the left and right hindlimbs 1 week after the final IACFM treatment is depicted in Figure 3.2. There were no side-to-side differences in regional tissue blood flow in animals prior to surgically-induced MCL injury, or immediately prior to the initial IACFM intervention (all $p>0.05$). There was no immediate effect of IACFM on tissue perfusion, with IACFM- and non-treated hindlimbs having equivalent perfusion immediately, and 5, 10, 15 and 20 minutes following the initial IACFM treatment session (all $p>0.05$) (Figure 3.3A). However, when assessed 24 hours following the 4th and 9th (last) treatment sessions (15 and 26 days post-injury, respectively) IACFM-treated hindlimbs had significantly greater tissue perfusion than contralateral non-treated hindlimbs (all $p<0.05$) (Figures 3.3B). In addition to having increased perfusion at these times, IACFM-treated hindlimbs also had significantly greater perfusion when assessed 1 week following the final treatment session ($p<0.05$) (Figure 3.3B).

Figure 3.1. Methods for assessment of regional microvasculature morphology. A) Representative radiographic image of a tissue sample mounted and positioned in the micro-CT scanner. Ligament samples and their surrounding connective tissue were wrapped in clear plastic to prevent drying during the scanning procedure. Care was taken not to distort the tissue. The wrapped sample was then placed in a small clear plastic tube and secured with cotton packing and parafilm at the end of the tube out of view of the sample region, and mounted on the micro-CT stage. An angled cut was used to identify the femoral end of each sample (top of image).

B) Representative image of a reconstructed serial tomogram.

In both A) and B), single white asterisks indicate the plastic tube; white arrows point to the plastic wrap and black arrows denote blood vessels filled with radiopaque contrast agent. The double white asterisks in B) marks the empty space within the tube. The black asterisk in B) portrays the connective tissue. The ligament is not distinguishable from its surrounding connective tissue in either A) and B) due to similar radio-densities.
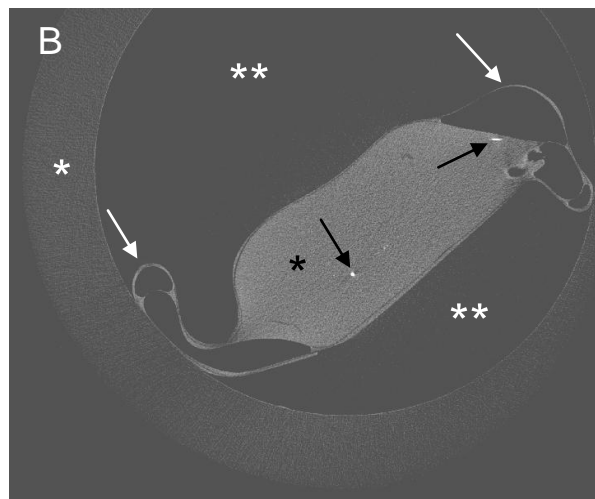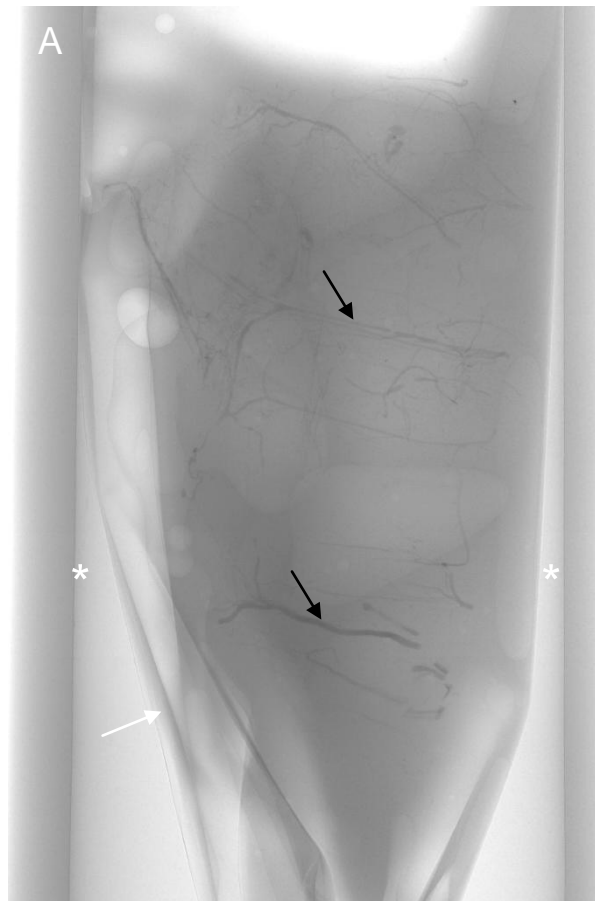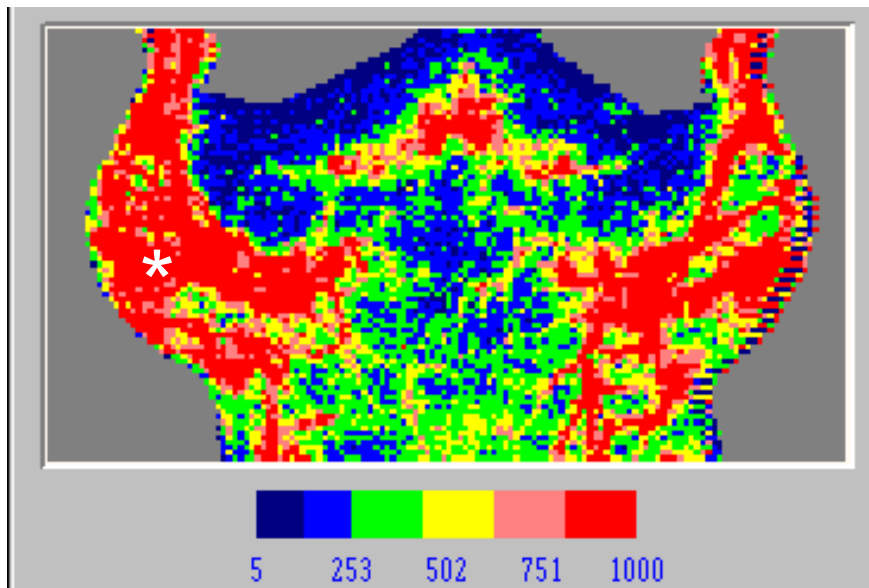
Figure 3.2. Representative laser Doppler image of the left and right injured hindlimbs 1 week after the final IACFM treatment. Tissue perfusion (flux) was increased in the IACFM-treated hindlimb (*) as indicated by the greater redness.
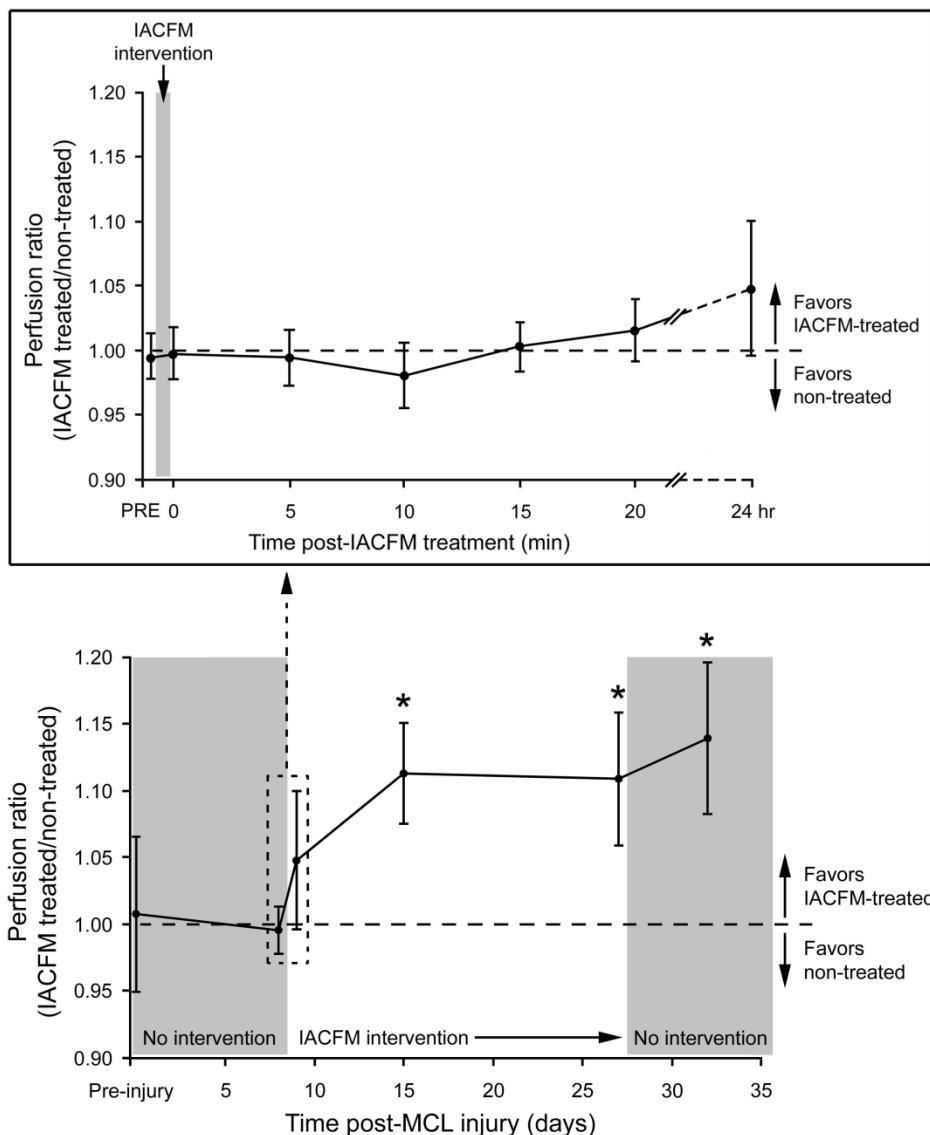
Figure 3.3. Effect of IACFM on regional tissue perfusion following knee MCL injury. A) IACFM had no immediate effect on tissue perfusion ratios, with ratios immediately prior (PRE) and after (0) the first IACFM treatment, at 5, 10, 15 and 20 minutes post-treatment, and at 24 hours post-treatment not differing significantly from 1 (i.e. perfusion in IACFM-treated = perfusion in non-treated). B) IACFM significantly increased tissue perfusion over time. Data during 'IACFM intervention' indicate perfusion ratios when assessed 24 hours following an IACFM treatment session. In addition to having increased perfusion when assessed at 24 hours post-treatment, IACFM treated hindlimbs had significantly greater perfusion when assessed one week following the final treatment session. Data represent the perfusion ratios (IACFM treated/non treated) between IACFM treated and non-treated hindlimbs, with error bars indicating the SEM. Ratios >1 indicate greater perfusion in IACFM-treated hindlimbs. * $p<0.05$, as determined by single sample t-tests with a population mean of 1.

3.3.2 Morphology of the microvasculature

A representative micro-CT image of the microvasculature morphology in the healing MCL and surrounding connective tissue is shown in Figure 3.4. There were no significant differences in VV/TV, V.Th, V.N or V.Sp between IACFM- and non-treated hindlimbs (all p=0.37 to 0.90) (Figure 3.5A, B, C). IACFM-treated hindlimbs had a higher proportion of blood vessel diameters, i.e. distribution of V.Th, within the 17.7 to <29.4 μm range compared to non-treated hindlimbs (p=0.02) in the tibial subregion (Figure 3.6A). There were no differences in the distribution of V.Th between IACFM- and non-treated hindlimbs in the femoral and middle subregions (all p>0.05) (Figure 3.6B, C).
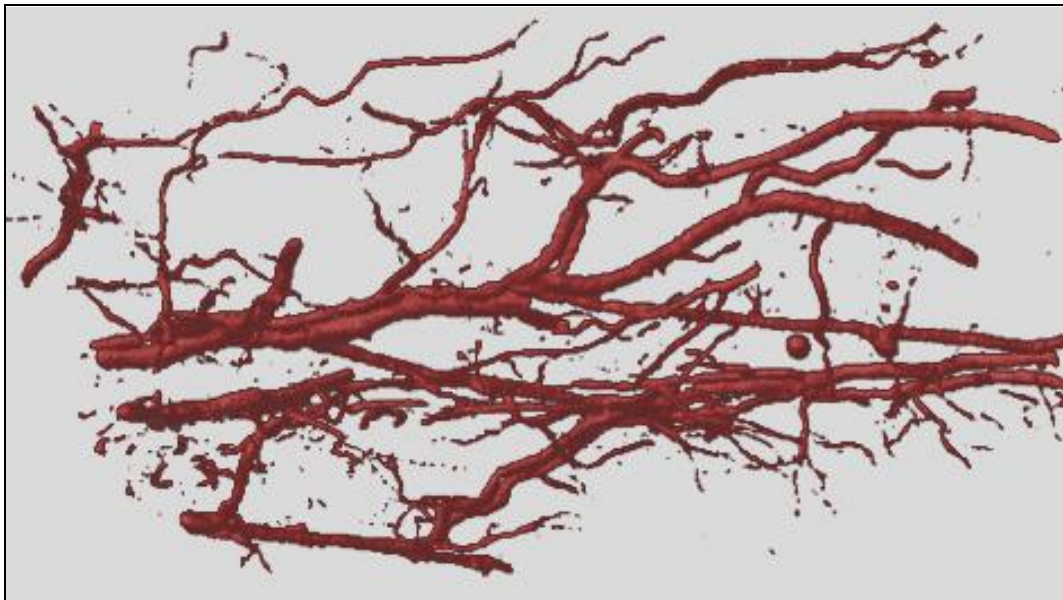
Figure 3.4. Representative micro-CT image of the microvasculature morphology in healing MCL and surrounding connective tissue. Blood vessels (shown in red) are distinguishable since they have been filled with radiopaque contrast agent. Connective tissue is radio-transparent at the blood vessel threshold and is not visible in this image.
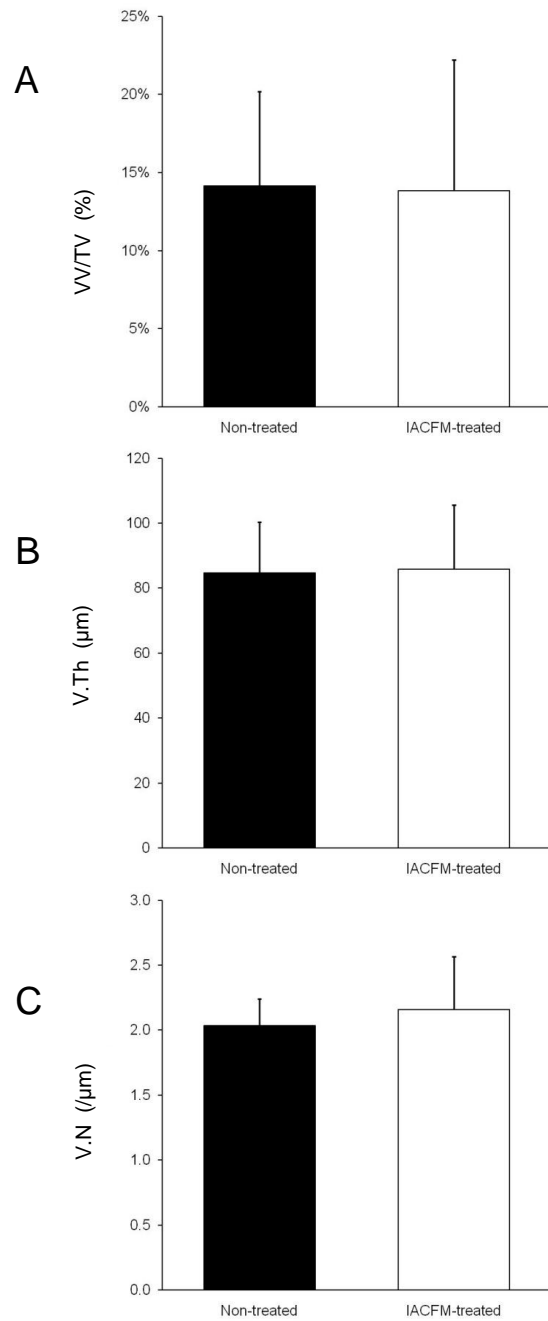
Figure 3.5. Microvasculature morphometric parameters in IACFM- and non-treated hindlimbs. There were no significant differences in A) vessel volume normalized to tissue volume (VV/TV), B) vessel thickness, a.k.a. vessel diameter (V.Th), and C) vessel number between IACFM- and non-treated hindlimbs (all *p*=0.37 to 0.90).

Figure 3.6. Frequency distribution of vessel thickness (diameter) in ligament subregions. A) IACFM-treated hindlimbs had a higher proportion of vessel diameters (vessel thickness [V.Th]) within the 17.7 to <29.4 µm range compared to non-treated hindlimbs (*p=0.02) in the tibial subregion. No differences were found in the distribution of vessel diameters between IACFM- and non-treated hindlimbs in the B) middle or C) femoral subregions (all p>0.05).

<u>3.4 Discussion</u>

Initial studies in this dissertation demonstrated IACFM accelerated the return of mechanical and histological properties following acute ligament injury. Since each phase of ligament healing requires adequate blood supply it was thought that IACFM-induced alterations in vascular properties were likely mechanisms underlying these preliminary findings.

Increased regional blood flow was found in IACFM-treated injured knee ligaments as compared to untreated contralateral injured ligaments; however, the increase was not observed immediately following intervention. This finding suggests IACFM does not lead to a direct increase in tissue perfusion due to vasodilation. Instead, IACFM-treated hindlimbs revealed increased tissue perfusion 24 hours following treatment sessions which persisted for 1 week following the last treatment. These findings suggested IACFM may stimulate angiogenesis when repeated applications are delivered over time.

Subsequent micro-CT analysis revealed a significant increase in smaller diameter blood vessels in the tibial portion of the IACFM-treated ligaments. Interestingly, this study found an increase in small blood vessels in the diameter range of arterioles. This is significant in that arterioles regulate blood flow through the capillary beds that they supply.[28, 166] Notably, the vascular expansion was only found in the tibial portion of the ligament. Given the definitive increase in regional blood blow, the investigators expected all portions of the ligament would have demonstrated increased angiogenesis. It is possible IACFM-treated vessels were relatively more dilated while in living tissue compared to those in untreated tissues indicating a greater functional response versus a structural change. Or, possibly, the regional variation could be related to normal vascular anatomy of the region or transitional zone characteristics at the MCL tibial insertion

(Chapter One). Another likely factor could be varying treatment pressures in different subregions of the ligament. For example, greater pressure may have been generated in the tibial portion due to direct compression of the ligament between the rigid treatment device and the underlying bone. This would correspond to findings in a prior study that demonstrated higher fibroblastic activity in IASTM-treated tendons in response to higher pressures.[158]

LDI was used to measure regional blood flow. Other authors have exposed the MCL to obtain measures more specific to the MCL;[77] however, exposing the MCL would not have allowed for multiple *in vivo* measures over time in response to repeat IACFM interventions as commonly performed in the clinic. Furthermore, IACFM intervention is clinically delivered through the skin, affecting many tissues.

Micro-CT imaging was used *ex vivo* to assess blood vessel morphological parameters in response to IACFM intervention. The ligament proper and surrounding connective tissue of a standardized sample size was analyzed for morphological vascular parameters of a selected VOI. This approach was chosen since ligaments are relatively avascular and rely on surrounding connective tissue for blood supply as described in Chapter One, section 1.6.1. This research study was able to resolve vessels down to 5.9 µm diameter, which is consistent with other investigations.[92,83]

In conclusion, IACFM significantly increased tissue perfusion over time. Furthermore, a significant increase in smaller diameter blood vessels in the distal portion of IACFM-treated ligaments was found. These findings suggest improved vascular properties may be a factor underlying the accelerated IACFM-treated knee ligament healing previously demonstrated. Mechanotransduction of the IACFM treatment forces is a possible mechanism for the enhanced biomechanical, histological and vascular properties found in IACFM-treated healing knee ligaments throughout this research program.

CHAPTER FOUR

Mechanotransduction as a Mechanism for the Therapeutic Effects of

Instrument-Assisted Cross Fiber Massage

4.1 Introduction

Preliminary studies in this dissertation suggest CFM, specifically IACFM, has

beneficial effects on biomechanical, histological and vascular properties in healing knee

ligaments in rats (Chapters Two and Three). Positive effects of CFM and IACFM have

been previously reported in animal model and clinical studies (Chapter One). Mechanical

forces influence the growth and shape of virtually every tissue and organ. It is known

that cells are mechanosensitive to their surrounding environment and that physical

forces play an important role in the organization, growth, maturation and function of

tissues.[167] It follows that an essential mechanism underlying the therapeutic effects of

CFM, and arguably all forms of STM, may be mechanotransduction.

Mechanotransduction is defined as processes whereby a mechanical stimulus is

converted into biochemical responses that affect cellular functions.[168-169] It is involved in

homeostasis, and tissue healing and repair, and implicated in some disease processes

such as vascular atherogenesis.[170] Load signaling pathways have been considered with

multiple tissues, e.g. bone,[168, 171-172] tendon,[173] cartilage,[174] ligament,[175] and intervertebral

discs.[176] Mechanotransduction has been implicated as a mechanism for the treatment

effects of modalities such as low intensity pulsed ultrasound in bone[177] and ligament

healing;[22] acupuncture;[178-179] therapeutic exercise[180] in tendon, muscle,[181] and bone;[182]

and functional activities.[183] Nonetheless, there is still much to learn about the

mechanisms by which individual cells sense mechanical signals and transduce them into

intracellular biochemical responses and gene expression.

Manual therapy interventions offer conservative treatment alternatives to invasive procedures and pharmaceuticals. A common denominator for all STM approaches is that they exert a physical force on the soft tissue which may be detected and converted into a signal with downstream effects on the ECM, vascular function and structure, neurosensory system, and the tissue healing and repair process. This purpose of this chapter is to introduce the concept of mechanotransduction as a mechanism for the therapeutic effects of CFM, whether administered by hand or instrument-assisted.

4.2 Background on tissue adaptation models

The concept of load-signaling pathways is not new. Wolff's law (Julius Wolff, 1892) predicted that remodeling of bone occurs in response to physical stresses. This model led to a later concept that bone is deposited in sites subjected to stress and reabsorbed from sites with minimal stress; essentially, it asserts mechanical stress is responsible for determining the architecture of bone.[184] The piezoelectric effect, i.e. the ability of some materials to generate an electric potential in response to applied mechanical stress, was used to explain this relationship between the structure and function in bone. Davis' law is the connective tissue corollary of Wolff's law that describes how soft tissue models along imposed demands.[184]

The mechanostat theory, introduced by Frost in the 1980s, depicts the modulation of bone modeling or remodeling by mechanical strain. It is therapeutically useful in that it suggests a window of optimal load for signaling a physiological response.[172] The concept of tensegrity (Fuller, 1965) offers a model of cell architecture that helps explain how the mechanical behavior of the cell emerges from physical interactions among different molecular filament systems forming the cytoskeleton and the nucleus. The model contends cell tensile elements (actin filaments) impose a pre-

stress on cells that is countered by compression-resistive elements (microtubules).[185-187] More recent literature describes the concept of mechanotransduction.

4.3 Mechanotransduction

Mechanotransduction is defined as the process of mechanical forces influencing intracellular signaling and subsequent cell behaviors. It involves a four-stage cell-mediated theory including mechanocoupling, biochemical coupling, signal transmission and effector response, summarized in Table 4.1.[169, 182]

Table 4.1 Summary of stages in mechanotransduction process

Mechanocoupling	Physical transduction of loads (mechanical force) applied to tissue into local mechanical signals that can be detected by tissue cells. Involves physical derangement or perturbation of the cell, either directly or indirectly.
Biochemical coupling	Process whereby the local mechanical signal is converted into a biochemical signal. Cells sense a load using mechanosensitive structures.
Signal transmission	Process in which a sensor cell signals an effector cell.
Effector response	Cellular outcomes that may lead to protein synthesis, remodeling and reabsorption of its matrix. Leads to a tissue level response.

4.3.1 Mechanocoupling

Mechanocoupling is the physical transduction of mechanical force to a form that can be detected by cells. For example, in connective tissue, mechanical loads (stress) imposed by movement cause local deformations (strain) in the ECM detected by a sensor cell. Fibroblasts are sensor cells in connective tissue. The transduction of mechanical forces to a signal the cell can detect is effected by the rate, magnitude and

frequency of the applied load.[169, 188] For example, one study using IASTM on injured rat

Achilles tendons found fibroblast proliferation depended on the magnitude of applied

pressure.[158]

Fluid shear, compression and tension (stretch) stresses from the surrounding

environment are stimuli for mechanical signaling in cells. Experiments involving

mechanical stress on cells have been done mostly on cultured fibroblast, endothelial and

smooth muscle cells using either cyclic stretch or prolonged shear.[97, 121, 189] Results of

studies have shown tensile stress imposed on fibroblasts in static culture causes actin

filaments to reorganize from an irregular orientation to organized bundles ("stress fibers")

associating with focal adhesions (FAs) without significant change in tubulin or

intermediate fibers.[190] Other studies have found that fluid flow creates shear stress on

cell membranes which induces rapid release of prostaglandins, e.g. PGE_2, PGI_2. These

particular prostaglandins can alter fluid viscosity to a more serous-like consistency which

facilitates cell migration.[191] Once the mechanical stimulus is detected by the cell, it is

translated by mechanosensors it into a cellular response.

4.3.2 Biochemical coupling

Biochemical coupling is defined as the transduction of a local mechanical signal

into an intracellular biochemical response ultimately leading to gene expression.

Mechanical signaling is transduced into a biochemical response through at least four

independent but interacting mechanosensors: a) integrins, b) stretch activated calcium

(Ca^{++}) channel opening, c) G-protein activation in the lipid bilayer, or d) cytoskeleton

deformation; and as of yet, unidentified mechanoreceptors. Although the processes are

complex and not fully understood, it is known that a broad range of mechanical stimuli

can act through divergent or common signaling pathways. The desired gene expression is achieved by the integration of direct and indirect processes.[97]

a) Direct processes in mechantransduction

Deformations of the ECM can have a direct effect on the translation of a mechanical stimulus into a cellular response through the ECM-integrin-cytoskeleton axis. Load-signaling pathways in connective tissues typically involve different types of cell matrix adhesion contacts, e.g. FAs. Integrins are cell surface receptors that associate with FAs to create a transmembrane axis that physically couples the ECM with cytoplasmic constituents to transmit external stimuli directly to the internal environment of the cell.[97] Integrins are transmembrane glycoproteins with α and β subunits that bind specifically to ECM proteins (e.g. fibronectin, collagen); and, intracellularly with the cytoskeleton and other cytoplasmic components via adapter structural proteins (e.g. vinculin, talin, zyxin, paxillin).[190] It is thought that integrins within FAs function primarily as stretch sensors ('strain gauges') that trigger downstream signaling pathways in response to changes in mechanical stress, i.e. reorganization of cytoskeletal components, redistribution of intracellular forces.[97]

The cytoskeleton filament network in turn has physical connections to the nucleoplasmic surface. The nuclear membrane is connected to nuclear scaffolds that may connect to discrete DNA regions which can directly alter gene transcription.[192] The exact nature of this process remains unknown; however, there is evidence for mechanical coupling between the cell surface and nucleus enabling the entire cell to function as a single, mechanically-coupled system. For example, mechanical forces promote structural rearrangements within the cytoplasm and nucleus that allow for faster conduction of events than chemical diffusion or translocation based signal propagation, in the order of milliseconds compared to seconds respectively.[190]

b) Indirect processes in mechantransduction

The translation of mechanical stimuli by sensor cells may be indirect, involving various intracellular signaling cascades. One prominent signaling cascade is stretch activated ion channel opening. For instance, stretch activated Ca^{++} channel opening allows an influx of Ca^{++} which activates Ca^{++}-dependent signaling paths. Alternatively, a cellular mechanotransduction pathway could activate an available transcription factor, or induce its transcription, which then binds to a regulator element in an ECM gene promoter.[33, 97]

4.3.3 Signal transmission

The biochemical signal in the sensor cell can be propagated to an effector cell via two pathways. First, a fibroblast can sense the mechanical deformation and the same cell acts as the effector cell leading to increased synthesis of ECM components. This may occur by direct upregulation of synthesis of matrix products (i.e. tenascin-C, an anti-adhesion molecule) or autocrine mechanisms such as synthesis of growth factors that act on the sensor cell itself. Secondly, intercellular signaling may be propagated from a sensor cell to other effector cells via cell-cell communication (i.e. via gap junctions) or by paracrine mechanisms, including growth factors (e.g. TGF-β) or signaling molecules (e.g. nitric oxide [NO], prostaglandins), that act on surrounding cells.[33]

4.3.4 Effector response

The effector cell response is the cellular outcome that leads to the final tissue-level response. Downstream effects of a mechanical stimulus may include synthesis and

local release of growth factor, cytokines, vasoactive substances, degradative enzymes and structural matrix elements.[182]

A combination of complex processes mediates the integration of chemical and physical signals responsible for the control of cell behavior and tissue properties. The exact nature of these processes remains a matter of ongoing investigations. It's plausible that IACFM exerts a physical force that has downstream effects on the connective tissue matrix, vascular function and structure, and neuromodulation which deserve consideration.

4.4 Mechanotransduction and the connective tissue matrix

The improved biomechanical and histological properties demonstrated during early healing in IACFM-treated knee ligaments in this dissertation possibly reflect the mechanotransduction of the IACFM stimulus into cellular responses leading to alterations of the connective tissue matrix. A probable mechanotransduction pathway for the effect of IACFM on the connective tissue matrix is the ECM-integrin-cytoskeletal axis previously described in section 4.3.3.a. Figure 4.1 illustrates a simplified sequence for a clinical perspective of a possible direct signaling cascade in response to this form of IASTM. In this scenario, the cascade is initiated when a rigid instrument is used to apply a mechanical load (stress) to the body surface which generates deformations (strain) in the ECM of underlying soft tissue. The physical force is then transduced by integrins in the plasmalemma into an intracellular response. The signal is subsequently transmitted through the cytoskeleton filaments to the nucleolemma and nuclear scaffold which may upregulate gene expression. This could result in increased mRNA levels, translation and protein synthesis culminating in altered cell function and tissue-level response.[190]
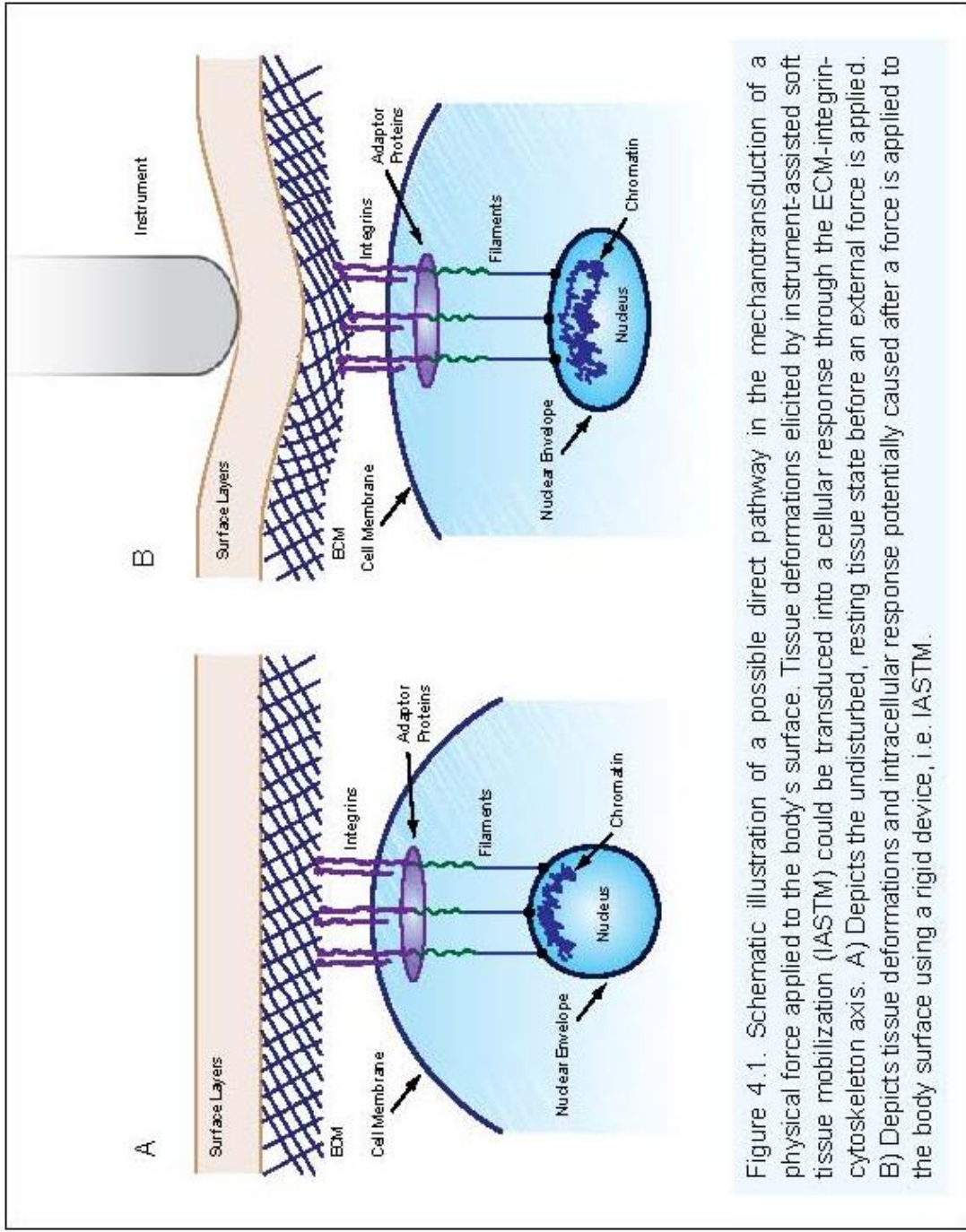
Figure 4.1. Schematic illustration of a possible direct pathway in the mechanotransduction of a physical force applied to the body's surface. Tissue deformations elicited by instrument-assisted soft tissue mobilization (IASTM) could be transduced into a cellular response through the ECM-integrin-cytoskeleton axis. A) Depicts the undisturbed, resting tissue state before an external force is applied. B) Depicts tissue deformations and intracellular response potentially caused after a force is applied to the body surface using a rigid device, i.e. IASTM.

- original illustration

Each step of the described mechanotransduction process as it relates to a CFM force requires further substantiation, and provides a level of inquiry for future investigations on the effects of manual therapies in normal and injured tissue. For example, at the level of mechanocoupling, studies could explore the effects of stress (i.e. magnitude, rate, duration, direction, type) from CFM. The type of stress interpreted at the cell surface may be tensile, compressive or shear in nature. Biochemical processes triggered by CFM stimulation including signaling pathways, gene expression (i.e. mRNA levels) and protein levels (i.e. of transcription factors) and signal transmission processes involving levels of growth factors and signaling molecules involved in could be determined. The effector response could be analyzed by monitoring changes in cell activity and ECM composition, e.g. proliferation, tenascin-C or collagen levels, in response to IACFM treatment.

IACFM may affect FA formation. The mechanical link between FAs and the surrounding ECM and cytoskeleton allows bidirectional communication between the inside and outside of cells.[192-193] It is the transmission of load through FAs that controls their maturation (i.e. size) or disassembly. In fact, the size of FAs has been found nearly proportional to the local mechanical stress to which they are subjected.[97, 194] In order to provide additional insight into mechanotransduction processes and the connective tissue response to non-invasive physical forces, future studies could aim at establishing the effects of various manual therapy applications such as IACFM on FA size (maturation), specific connections of FAs with the ECM or cytoskeletal components, alterations in proteins associated with FAs, and consequent cytoskeletal organization.

As a final consideration, it is known that the 3D composition of the ECM is adapted specifically to changes in load (i.e. fiber alignment, fiber size, interfiber distance, cross-linking). Interestingly, studies have found when the 3D ECM is compressed (as into a sheet) or has increased cross-linking; a rigid versus porous ECM is produced that

alters distribution of adhesion proteins.[192, 194] The 3D ECM composition in response to IACFM should be explored since connective tissue manipulation may help to restore its architecture to one more conducive of functional mobility and strength.

4.5 Mechanotransduction and vascular function and morphology

Studies in this dissertation also demonstrated positive vascular effects of IACFM in healing knee ligaments. It is conceivable that the intermittent force from CFM intervention may load vascular tissue directly or through tension in the ECM, or may alter fluid flow (i.e. rate, impulse) in a manner that facilitates vascular function and growth.

Oxygen levels, circumferential stretch from blood pressure impulses, shear stress from blood flow, and a variety of growth factors are among stimuli that regulate vessel growth and remodeling. The primary trigger for adult angiogenesis is tissue ischemia; however, flow-mediated mechanotransduction from shear stress has a profound effect on the functional expression of endothelial cell (EC) and vascular smooth muscle cell (VSMC) proteins, including transcription factors, enzymes, growth factors, signaling molecules, integrins and adhesion molecules.[68] Blood pressure impulses cause circumferential stretching to ECs and VSMCs, while shear stress from flowing blood acts on ECs lining the vessels. ECs do respond to stretch, but shear seems to be a main determinant of EC function.

As in fibroblasts, a number of mechanosensors in ECs and VSMCs have been proposed to mediate load signaling pathways in vascular tissue. Forces are typically transmitted in ECs from the apical cell domain (where shear is applied) through the cytoskeleton to basolateral domains (where integrins exist that interact with the ECM). Subsequent changes in classic signaling pathways and multiple transcription factors mediate the response of ECs and VSMC to changes in flow and stretch. For instance,

flow-mediated mechanotransduction may lead to NO release resulting in vessel dilation.[68] This provides a plausible explanation for the enhanced tissue perfusion despite the modest degree of angiogenesis found in IACFM-treated knees in this dissertation; although, future study is required.

Shear is a key determinant of EC function; however, there is evidence suggesting a reciprocal relation between the ECM and EC activity. For instance, the ECM can modulate how flow-sensitive ECs respond to this force during matrix remodeling. The ECM provides a scaffold through which new vessels migrate. The ECM helps regulate angiogenesis, in part by modulating normal integrin receptor expression and ECM-endothelial cell interactions, although the details are not fully understood.[170] Mechanical stimulus may also be a factor in mast cell degranulation, macrophage recruitment and endothelial cell migration; all of which are essential for directing tissue vascularization.[67-68] Future inquiries on EC and/or VSMC cellular activity in response IACFM stimulation may provide insight into the mechanisms underlying the beneficial effects of this modality.

4.6 Mechanotransduction and neuromodulation

The neurological effects of IACFM were not addressed in this dissertation; however, it is logical that this manual therapy could impact neurosensory and neuromuscular processes. Ligament innervation has been implicated for normal ligament healing and function. For instance, one study showed denervation of rabbit MCLs impaired healing.[195] Comparatively, another study revealed injured rat MCLs treated directly with exogenous nerve growth factor had increased nerve density and improved biomechanical properties.[164]

Neurosensory stimulation provides information to an organism about its external and internal environment, and facilitates reflexive modulation of organs, systems and immune effector functions. Mechanosenory cells respond to physical forces (e.g. sound, touch, joint motion, and muscle, tendon, and capsule stretch receptors) and transmit a facilitory or inhibitory response. Each sensor cell has distinct cytoskeletal elements and extracellular components. Sensory mechanoreceptors have different structures but all have transduction channel proteins which are not yet well characterized.[196] Neurosensory transduction is thought to result primarily from the direct gating of transduction channels. In excitable cells, (mechanosenory nerve and muscle cells), stretch sensitive cation channels (i.e. mechanically gated of K^+ channels) are more likely to act as strain gauges than integrins as in connective tissue.[197]

It is questionable whether the neuromodulatory effects of CFM techniques are facilitory or inhibitory. A recent study found CFM reduced the excitability of the flexor carpi radialis motor neuron pool.[151] Other studies have shown massage of the forearm and hand to improve motor performance including increased power grip strength following exercise fatigue.[147]

Analgesia is a reported benefit of CFM interventions. Most often CFM is not painless for a patient to receive. Apparently, CFM provides a form of counter-irritation (noxious stimulation) for pain modulation that leads to an analgesic response. Blocked nocioception due to inhibition of pain signals via the gate-control mechanism is a possible mechanism for the analgesic effects of IACFM.[198-199] Reduced pain can lead to muscle relaxation which allows for more normal neuromuscular movement patterns and functional progression.[200]

IACFM may influence the ANS, perhaps indirectly, i.e. via pain reduction. Autonomic nervous system (ANS) effects have been implicated as an underlying mechanism for some manual therapies, e.g. eliciting a shift from a sympathetic state to a

more parasympathetic response. Pressure applied by massage approaches may stimulate vagal activity, reduce stress hormones and decrease arousal leading to increased parasympathetic activity (e.g. decreased heart rate, blood pressure, respiratory rate).[123, 201]

The neuromusculoskeletal system requires adequate neuromodulation for full, pain-free function to occur normally and during tissue healing and repair. More research on the effects of mechanical soft tissue manipulation on neurosensory and neuromuscular modulation including pain, muscle performance, and ANS response is indicated.

In summary, mechanotransduction is a mechanism for CFM approaches to affect the neuromusculoskeletal and vascular systems with consequent clinical implications. Several perspectives must be considered in the decision to utilize manual therapy interventions clinically.

4.7 Clinical perspectives on mechanotransduction and IACFM

Manual therapies involve the application of specifically selected forces directed to the body tissues to induce desired outcomes. The concept of mechanotherapy was recently re-introduced with respect to therapeutic exercise as "the employment of mechanotransduction for the stimulation of tissue and repair."[180] IACFM is a form of mechanotherapy; however, the rationale to use it, or any soft tissue manipulation technique, depends on several factors, e.g. the stage of tissue healing and repair (acute vs. chronic). For example, IACFM should not be used acutely in a manner that would interrupt the healing process; but on the other hand, it should be introduced early enough to avoid the formation of covalent cross-links in newly deposited, disorganized collagen fibers. Overall patient condition (i.e. co-morbidities), associated pathology,

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tissue structure and type and the desired treatment response (i.e. pro-inflammatory vs. non-inflammatory) must also be considered.[144-145]

In practice, manual therapy treatment parameters (e.g. load frequency, rate, direction and duration) are adjusted to deliver a mechanical stimulus to the tissue in order to elicit a desired response (e.g. decreased pain; increased strength, mobility, function). For instance, IACFM is often used more aggressively (i.e. higher load with chronic, degenerative conditions such as tendinosis,) reportedly to introduce a controlled amount of micro-trauma to the tissue in order to re-initiate the healing cascade. On the other hand, it is suggested IACFM be used less aggressively (i.e. lower load) in the case of an acute tendinitis for the purposes of desensitization and preventing adhesions formation.[144-145]

Guidelines for manual therapy applications are founded mostly in clinical observations and physiological rationale which require validation. The theory behind many approaches rests on how the pressure is applied. Although one animal study examined the effects of varying massage pressures,[158] human subjects studies have not considered this variable.[201] Complex models of soft tissue mechanics have been developed; however, a conceptual framework for STM approaches based on the mechanotransduction of the mechanical stimulus that typifies a given technique would be advantageous for establishing practice guidelines.

A framework for STM approaches can be imagined that would classify techniques based on the nature of their mechanical stimulus in context of the desired response. The mechanical stimulus could be initially defined by its load (i.e. high vs. low load) and frequency (i.e. high vs. low frequency). Formulating such a framework would require future effort, including dose response studies. One of the first challenges would be to quantifiably define high versus low load and frequency.

A conceptual framework of STM approaches based on the mechanical stimulus, possible indications and potentially desired treatment response is outlined in Table 4.2.[30, 115, 145, 202] The framework in Table 4.2 is not meant to be exhaustive, but it is presented for illustrative purposes. Other treatment parameters should eventually be integrated and a greater variety of manual approaches could be included to develop a more comprehensive framework. Such a structure would help to establish a common perspective for clinical utility in selecting and evaluating different manual therapies for various conditions. It would also facilitate the design of research projects aimed at establishing optimal dose responses for different techniques, comparing manual therapy approaches to each other or other modalities, and evaluating the summative effects of physical interventions, e.g. the effects of therapeutic exercise and massage on tissue healing.

In conclusion, mechanotransduction is a proposed mechanism for the beneficial effects of IACFM, a form of manual therapy, demonstrated in this dissertation. A greater understanding of how a manually delivered mechanical stimulus is transduced into a therapeutic response will foster improved therapeutic outcomes. Better characterization of STM approaches based on the mechanical stimulus delivered to the tissue will support research and clinical decision making processes.

Table 4.2 Conceptual framework for soft tissue mobilization approaches based on the mechanical stimulus.

	High Load High Frequency	High Load Low Frequency	Low Load High Frequency	Low Load Low Frequency
STM Approach	CFM IACFM Petrissage	IC* deep pressure Slow, deep sweeping techniques IASTM	Cutaneous brushing or stroking techniques Effleurage IASTM	IC light pressure IASTM
Possible Indications	Tendinosis Chronic muscle holding Chronic scar mobilization	Deep, latent trigger points Chronic fascial tightness	Tendinitis Scar desensitization Acute edema Active trigger point	Active trigger points Acute, painful scar
Desired Treatment Responses	Pro-inflammatory Increased tissue extensibility	Non-inflammatory Increased tissue extensibility	Non-inflammatory Fluid movement Desensitization	Non-inflammatory Desensitization

IC* stands for ischemic compression

CHAPTER FIVE

Summary and Future Directions

5.1 Dissertation summary

Studies in this dissertation investigated the effects of IACFM on knee MCL healing using an established rodent model. Initial studies found that IACFM-treated injured ligaments, have a) enhanced mechanical properties at 4 weeks post-injury, suggesting accelerated healing, and b) improved stiffness at 12 weeks post-injury, possibly indicating augmented healing compared to contralateral untreated, injured ligaments. Light microscopy revealed qualitative improvements in fibroblast proliferation and fiber alignment. Subsequent SEM observations confirmed the latter. Histological findings may be related to the improved healing found in IACFM-treated ligaments.

Succeeding studies considered vascular properties in IACFM-treated versus untreated healing knee ligaments in rats. LDI assessment revealed enhanced regional blood flow over time with repeated IACFM application. Subsequent micro-CT analysis found regional alterations in microvasculature structure possibly suggesting angiogenesis. These vascular findings may be related to the accelerated healing found in IACFM-treated ligaments.

Mechanotransduction was proposed in concept as a mechanism for the therapeutic effects of IACFM found in this research project. Factors involving mechanotransduction and the connective tissue matrix, vasculature and neuromodulation were considered in greater detail, and clinical implications discussed.

5.2 Strengths and limitations of the present research project

There are noteworthy strengths of studies in this dissertation. First, findings from this research program help to fill a void in the literature regarding the effects of manual therapy interventions. Irrespective of the longevity and popularity of its use for a variety of conditions, relatively little quantitative research on the benefits of massage techniques has been performed. Studies in this dissertation demonstrated quantitative improvements on a tissue-level in the biomechanical and vascular properties of healing knee ligaments using a type of massage, IACFM. This is important, because manual therapies, including IACFM, offer readily available and cost-effective alternatives to invasive (i.e. surgical) and possibly toxic interventions (i.e. pharmaceuticals) in the management of musculoskeletal disorders. Secondly, the research that has been done on manual therapies is limited not only in quantity but quality.[123, 201] Thus, another primary strength is the experimental design of studies in this research project. The use of a within-subject study design enabled more accurate investigation of IACFM tissue-level effects. By comparing IACFM-treated versus contralateral untreated injured MCLs in the same subject, it helped to control for potential confounding effects of other variables, e.g. activity levels, treatment pressure, genetic and environmental factors.

In light of its strengths, there are acknowledged limitations with studies in this dissertation. First, studies were performed in an animal model wherein the knee MCL was injured via surgical transection. Although this is a highly reproducible and established model for the preclinical testing of ligament injuries,[7, 14, 49, 203] the ability of the model to predict the clinical scenario wherein ligaments are injured via excessive tensile load has not been established. Second, the size of rodent tissues in relation to those of humans raises scaling issues in terms of intervention introduction and response. This issue was addressed by introducing IACFM to rodent ligaments with the same tool and

force as used clinically to treat similarly sized ligaments. Using a pressure sensor could have standardized the pressure given; however, this was controlled for by a single individual administering the treatment. Third, between-animal differences in activity levels may have influenced ligament healing rates as activity has previously been shown to mediate healing of isolated connective injuries in rodents.[158, 204] Again, these variables were controlled for by establishing IACFM effects within-animal design. Fourth, the current studies did not consider IACFM effects on clinically-measurable outcomes such as recovery from symptoms (e.g. pain, fatigue) which can influence function. The restoration of mechanical and vascular properties are important preclinical outcomes; however, it is feasible that IACFM can affect tissue-level properties without influencing symptom recovery. Despite these limitations, IACFM-treated knee ligaments demonstrated improvements in mechanical, histological and vascular properties. Adequate ligament strength is needed for ligament function, and adequate perfusion is essential to all stages of ligament healing; however, many questions remain.

5.3 Future directions

This research program found positive effects of IACFM in healing knee ligaments on a tissue level in rats; however, future is study is needed to further substantiate its mechanisms and benefits. Research projects could aim to establish the effects of IACFM treatment on connective tissue matrix characteristics and associated cellular and molecular-level events, vascular properties and clinical outcomes.

Future research projects that establish the effects of IACFM treatment on characteristics of the connective tissue matrix that modulate tissue tensile strength and stiffness are indicated. In order to have such effects, IACFM must influence the fibroblastic cells responsible for producing collagen. A candidate pathway for

investigation at each level of the mechanotransduction process is the ECM-integrin-cytoskeleton axis as discussed in Chapter Four. In brief, studies considering the tissue-level effects of IACFM may include analysis of collagen content (e.g. total collagen, relative proportions of collagen type I and type III), non-collagenous content (e.g. proteoglycans), and fibril diameter and alignment. The degree of mature, non-reducible collagen cross-link content (e.g. hydroxlysyl pyridinoline) could also be analyzed.[38] Investigations on the cellular and molecular-level events in response to IACFM stimulation may consider DNA content, mRNA expression of candidate genes responsible for the observed tissue-level effects (e.g. gene expression for collagens, proteoglycans, growth factors, collagenases), protein levels and localization including focal adhesion localization and maturation.

Additional research is needed to further resolve the effects of IACFM on ligament vascular properties. For instance, other methods, which may be more precise, e.g. laser speckle imaging, could be used to determine alterations in blood flow in response to soft tissue manipulation techniques.[205-206] Also, future studies may use other contrast mediums and imaging methods to determine vascular volume, and could consider measuring vascular morphological parameters of the ligament proper only.[66, 85-87] Studies investigating cellular and molecular mechanisms underlying vascular function in response to IACFM treatment are also needed. Atypical ligament cell populations may be assessed, especially those that produce angiogenic factors associated with ligament injury, e.g. mast cells[207] or macrophages;[208-209] or peri-endothelial cells. These cell populations are mediated by a variety of factors and express an array of vasoactive peptides, growth factors and cytokines. Gene expression of angiogenic factors and their receptors (i.e. VEGF, VEGFR, Ang-1, FGF, PDGF-β, TGF-β) at the transcript level[38] along with resultant protein levels and localization of angiogenic factors should be examined. The role of the ECM in angiogenesis in response to IACFM treatment is

another avenue for future investigation, since it provides a scaffold through which new vessels migrate. The ECM also regulates angiogenesis in part by modulating integrin expression and ECM-endothelial cell interactions.[68]

Research from a more clinical perspective is warranted. The load and frequency used in this research program were based on clinical recommendations for IACFM application to a structure of similar size; however, dose response studies are needed. Future research should aim to establish the effects of IACFM force magnitude, frequency and duration on connective tissue. For example, one study in this dissertation found IACFM ligaments to have enhanced stiffness at 12 weeks post-injury indicating IACFM may augment ligament healing; however this time period appeared too short to establish whether the final product of the ligament healing process is enhanced with this intervention. To demonstrate more conclusively whether IACFM augments ligament healing, longer-term effects (i.e. 6 months post-injury) should be investigated. A discrete treatment modality (i.e. IACFM) and a dense, regular connective tissue type (i.e. ligament) were focused on for experimental control; however, studies could consider other forms of STM and tissue types. Moreover, the combined effects of other modalities, e.g. exercise, and IACFM should also be explored. Finally, clinical trials are needed to establish the therapeutic effects of IACFM on pain, impairment and function in human subjects.

In conclusion, this dissertation provides insight into the beneficial effects of a manual therapy intervention, IACFM, on ligament healing using an animal model. These findings are compelling given the current healthcare climate which demands evidence-based practice and reduced costs in an aging population. As essential mechanisms underlying the effects of soft tissue manipulation are better understood, a greater potential for manual therapies in the conservative management of connective tissue dysfunctions can be derived.

[RESEARCH REPORT]

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Instrument-Assisted Cross-Fiber Massage Accelerates Knee Ligament Healing

L igament injuries account for up to 50% of sporting injuries,[6] with the majority being to capsular and extracapsular ligaments (such as the knee and ankle collateral ligaments).[11] Injuries to these ligaments have traditionally been thought to heal in a straightforward manner; however, preclinical studies have shown that ligament healing occurs by the formation of a reparative scar, rather than via regeneration, which leaves a deficiency in mechanical properties at the completion of healing.[7,29] This persistent tissue weakness, combined with any residual neuromuscular deficiency, may explain why a history of ligament injury is a strong risk factor for subsequent injury.[14,23] Some patients (up to a third) also continue to experience significant symptoms even up to 3 years following capsular or extracapsular ligament injury,[25] and injuries to these ligaments can contribute to the development of osteoarthritis.[24]

To address the short- and long-term consequences of capsular and extracapsular ligament injuries, there is a need for simple interventions that facilitate early recovery (accelerate healing) and/or result in a better final outcome (augment healing). By accelerating tissue-level healing, the injured tissue may be less susceptible to reinjury during early rehabilitation and the individual may be able to return to function quicker. By augmenting tissue-level healing, the final product of the healing process may be enhanced such that the healed tissue more closely approximates that of the native tissue.

Cross-fiber massage (CFM) may be a method for accelerating and/or augmenting capsular and extracapsular ligament healing. CFM refers to the application of specifically directed forces transverse to the direction of the underlying collagen substructure in order to induce physiological and/or structural tissue changes.

- **STUDY DESIGN:** Controlled laboratory study.

- **OBJECTIVES:** To investigate the effects of instrument-assisted cross-fiber massage (IACFM) on tissue-level healing of knee medial collateral ligament (MCL) injuries.

- **BACKGROUND:** Ligament injuries are common and significant clinical problems for which there are few established interventions. IACFM represents an intervention that may mediate tissue-level healing following ligament injury.

- **METHODS:** Bilateral knee MCL injuries were created in 51 rodents, while 7 rodents were maintained as ligament-intact, control animals. IACFM was commenced 1 week following injury and introduced 3 sessions per week for 1 minute per session. IACFM was introduced unilaterally (IACFM-treated), with the contralateral, injured MCL serving as an internal control (nontreated). Thirty-one injured animals received 9 IACFM treatments, while the remaining 20 injured animals received 30 treatments. Ligament biomechanical properties and morphology were assessed at either 4 or 12 weeks postinjury.

- **RESULTS:** IACFM-treated ligaments were 43.1% stronger ($P<.05$), 39.7% stiffer ($P<.01$), and could absorb 57.1% more energy before failure ($P<.05$) than contralateral, injured, nontreated ligaments at 4 weeks postinjury. On histological and scanning electron microscopy assessment, IACFM-treated ligaments appeared to have improved collagen fiber bundle formation and orientation within the scar region than nontreated ligaments. There were minimal differences between IACFM-treated and contralateral, nontreated ligaments at 12 weeks postinjury, although IACFM-treated ligaments were 15.4% stiffer ($P<.05$).

- **CONCLUSION:** IACFM-accelerated ligament healing, possibly via favorable effects on collagen formation and organization, but had minimal effect on the final outcome of healing. These findings are clinically interesting, as there are few established interventions for ligament injuries, and IACFM is a simple and practical therapy technique. *J Orthop Sports Phys Ther 2009;39(7):506-514. doi:10.2519/jospt.2009.2997*

- **KEY WORDS:** biomechanics, complementary therapies, medial collateral ligament, physical therapy, sports medicine

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It differs from other massage techniques in that there is little motion between the therapist's contact and the patient's skin. Instead, CFM involves moving the skin and subcutaneous tissues over deeper connective tissues to exert controlled mechanical forces on the latter. As the reparative cells (fibroblasts) responsible for producing collagen and forming a scar following ligament injury are mechanosensitive,[4,26] it is theorized that CFM facilitates matrix production and the restoration of tissue-level mechanical properties.

An addition to the practice of CFM has been the use of rigid instruments, with the resultant technique referred to as instrument-assisted CFM (IACFM). IACFM appears to be effective in promoting tissue remodeling, with Davidson et al[5] and Gehlsen et al[9] having found increased fibroblast recruitment and activation in an animal model of Achilles tendon injury. Results of clinical pilot studies also suggest that IACFM reduces symptoms in individuals with carpal tunnel syndrome, patellar tendinopathy, and chronic ankle pain.[1,17,30]

Based on the hypothesized mechanical mode of action of IACFM and preliminary evidence demonstrating its potential efficacy, the aim of this study was to examine the short- and long-term effects of IACFM on tissue-level healing of knee medial collateral ligament (MCL) injuries in an established animal model. The primary variable of interest was ligament mechanical properties, as the ultimate outcome of any healing process in a load-bearing tissue (such as a ligament) is the restoration of mechanical properties. The secondary variable of interest was ligament morphology, as this may explain differences in tissue mechanical properties.

METHODS

Animals

FIFTY-EIGHT 6-MONTH-OLD, VIRGIN, female Sprague-Dawley rats (body mass, 280-300 g) were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, IN) and acclimated for a minimum of 7 days prior to experimentation. Animals had *ad libitum* access to standard rat chow and water at all times, and were housed 2 per standard size cage (length, 40 cm; width, 20 cm; height, 20 cm). All procedures were approved a priori by The Institutional Animal Care and Use Committee of Indiana University.

Ligament Injury

Fifty-one animals underwent surgery on entry to the study to create bilateral knee MCL injuries of their hindlimbs (injured animals). The remaining 7 animals served as age-matched, ligament-intact cage controls and were not operated on (control animals). Following a preoperative subcutaneous dose (0.05 mg/kg) of buprenorphine hydrochloride analgesia (Buprenex; Reckitt & Colman Pharmaceuticals Ltd, Richmond, VA), surgical anesthesia was achieved using a mixture of ketamine (60-80 mg/kg) (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (7.5 mg/kg) (Sedazine; Fort Dodge Animal Health), introduced intraperitoneally. Using a sterile technique, a 5-mm longitudinal incision was made over 1 knee's medial joint line, and the MCL sharply transected at the joint line using a size-11 scalpel blade. This resulted in complete disruption of the MCL at its midsubstance and transverse to the underlying collagen fiber alignment. No ligament material was removed, and the ligament ends were juxtaposed but not sutured prior to closing of the skin incision with a single subcuticular absorbable suture. The procedure was repeated on the contralateral knee to create bilateral injuries. All animals demonstrated normal, symmetrical hindlimb use upon recovery from surgery and were allowed normal cage activity (without access to exercise wheels) for the duration of the study.

Intervention

IACFM was performed using a rigid tool fabricated from stainless steel (GT6; Graston Technique, TherapyCare Resources, Indianapolis, IN). The GT6 instrument was used because it is designed to apply
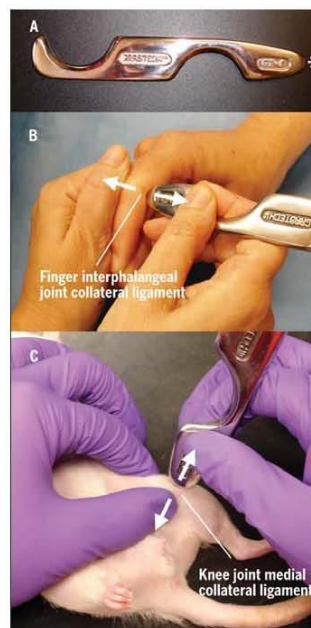
FIGURE 1. Instrument-assisted cross-fiber massage (IACFM) intervention. (A) The rigid Graston Technique GT6 tool fabricated from stainless steel has a tapered tip,* which permits treatment of small structures. IACFM of a (B) human finger, and (C) similar-size rodent knee joint medial collateral ligament using the GT6 tool. Arrows indicate the direction of movement/force application perpendicular to the collagen substructure of the ligament.

force through its tip to small structures, such as finger collateral ligaments in humans (in the present study, rat knee-size ligaments) (FIGURE 1). IACFM was initiated 1 week postoperatively (postinjury) to allow the initial inflammatory response/phase of ligament healing to subside. This initial delay in the introduction of IACFM is consistent with its suggested clinical use following an acute injury. IACFM was administered with the animals under isoflurane anesthesia (3% at 1.5 L/min for initial knockdown in a plastic container, and 1.5% at 1.5 L/min via a face mask for maintenance of anesthesia). Approximately 250 to 300 g of instrument downward force was applied during treatment. This

force is equivalent to that previously used to demonstrate benefits of IACFM on injured rat Achilles tendons,[8] and was determined by using the massage instrument on a force plate, with kinesthetically similar pressure to that which would be used clinically to treat a ligament of comparable size at an equivalent tissue depth (eg, collateral ligament of a human interphalangeal joint). Thirty-one injured animals were treated 3 times per week for 3 weeks (total treatments, 9), while the other 20 injured animals were treated 3 times per week for 10 weeks (total treatments, 30). The number of treatments in the latter animals is more than would typically be introduced in a clinical setting; however, these were implemented to maximize the potential of finding any long-term benefit of IACFM. IACFM was applied to the left MCL in injured animals for 1 minute per session (IACFM-treated). This treatment duration was based on the recommended clinical use of IACFM for the treatment of isolated tissue lesions[3] and evidence from previous preclinical studies demonstrating the efficacy of short-duration IACFM interventions.[5,8] The contralateral injured MCL in these animals served as an internal control and did not receive IACFM (nontreated). The 7 control animals were not treated with IACFM.

Assessment Time Points and Specimen Preparation

Animals were euthanized postinjury at either 4 weeks (all animals treated for 9 sessions [n = 31] and 2 control animals) or 12 weeks (all animals treated for 30 sessions [n = 20] and 5 control animals). Animals euthanized at 4 weeks had both hindlimbs harvested and prepared for mechanical testing (injured animals, n = 18), scanning electron microscopy (injured, n = 11; control animals, n = 2), or histological assessment (injured animals, n = 2). Animals euthanized at 12 weeks had both hindlimbs removed and prepared for mechanical testing (injured, n = 17; control animals, n = 4) or histological assessment (injured, n = 3; control animals, n = 1).

Mechanical Testing

Ligament mechanical properties were assessed as previously described.[27,28] Hindlimbs destined for mechanical testing were initially stored at −80°C, with the knee tissues intact. Postmortem storage of ligaments by freezing does not influence their mechanical properties.[33] On the day of mechanical testing, the hindlimbs were allowed to thaw to room temperature in phosphate-buffered saline (PBS). Femoral-MCL-tibia (FMT) complexes were prepared by dissecting clear extraneous tissue (including the joint capsule and adherent medial meniscus), while keeping the MCL and its insertion sites hydrated with PBS. The femoral and tibial insertions of the MCL were left intact, and the proximal tibia growth plate was removed to permit more space within the knee joint during testing. MCL thickness and width were measured optically at the knee joint line, and MCL area estimated using an elliptical geometry.[20] Each FMT complex was placed in a customized testing jig, with the knee joint positioned in 70° flexion, for MCL testing. This position appears to load all ligament fibers simultaneously.[20] The femoral and tibial portions were embedded in Wood's low-melting-point metal (bismuth alloy LMA-117; Small Parts, Inc, Miami Lakes, FL) for fixation. The jig was coupled to an electromagnetic material testing device (TestBench 200 N ELF LM-1; EnduraTEC Systems Group, Bose Corp, Minnetonka, MN), equipped with a 50-N load cell (**FIGURE 2A**). This system possesses a force and displacement resolution of 0.01 N and 0.001 mm, respectively. A preload of 0.05 N was applied and the ligaments were preconditioned by cyclically loading at 1 Hz for 10 cycles to 1% strain to reduce the effect of deep freezing on low-load mechanical properties.[33] The ligaments were unloaded and allowed to recover for 1.5 minutes, while being kept moist with PBS. Following tissue recovery, ligaments were again preloaded (0.05 N) and pulled to tensile failure in displacement control at a rate of 0.8 mm/s (~10%/s). Force and
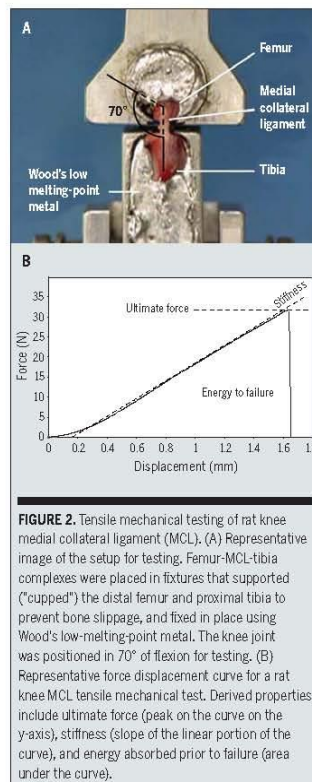
FIGURE 2. Tensile mechanical testing of rat knee medial collateral ligament (MCL). (A) Representative image of the setup for testing. Femur-MCL-tibia complexes were placed in fixtures that supported ("cupped") the distal femur and proximal tibia to prevent bone slippage, and fixed in place using Wood's low-melting-point metal. The knee joint was positioned in 70° of flexion for testing. (B) Representative force displacement curve for a rat knee MCL tensile mechanical test. Derived properties include ultimate force (peak on the curve on the y-axis), stiffness (slope of the linear portion of the curve), and energy absorbed prior to failure (area under the curve).

displacement data were collected at 100 Hz, and the mechanical properties of ultimate force (N), stiffness (N/mm), and energy to failure (mJ) obtained from the force-displacement curves (**FIGURE 2B**).

Scanning Electron Microscopy

Immediately after harvest, specimens for scanning electron microscopy were placed in a custom limb frame that held the knee positioned in 70° flexion. The MCL was exposed and drip fixed for 1 hour with 2.5% glutaraldehyde in 0.1 mol sodium cacodylate buffer (pH 7.4) (Electron Microscopy Services, Hatfield, PA). After drip fixation, the MCLs were removed using a microsurgical scalpel, with the femoral insertion marked by an angled

cut. Any adherent tissue was removed under a dissecting microscope. Ligaments were then rinsed twice in buffered solution and dehydrated by immersing for 15 minutes each in fresh solutions of 70%, 95%, and 100% ethyl alcohol. They were subsequently immersed in liquid nitrogen, placed on precooled microscope slides, and fractured under a dissecting microscope in the sagittal plane from the femoral to tibial end using one half of a precooled, double-edged stainless steel razor blade (Electron Microscopy Sciences, Hatfield, PA). The ligament samples were then critical point dried (Samdri model 780A; Tousimis Research Corp, Rockville, MA), mounted on 10-mm scanning electron microscopy specimen mount blocks using nonconductive adhesive tabs (Ted Pella, Inc, Redding, CA), and surrounded by colloidal silver paste (Electron Microscopy Sciences, Hatfield, PA). After drying overnight in a vacuum dessicator with dissecant, the samples were sputter coated with gold-palladium (Polaron, Energy Beam Sciences, East Gramby, CT) for 1.75 minutes, and stored in a vacuum dessicator with desiccant until imaged. The samples were imaged on a scanning electron microscope (JSM-6390LV; JEOL Ltd, Peabody, MA), using a 5-kV accelerating voltage and working distance of 11 mm. The ligaments were aligned at low magnification (approximately ×25) by orienting the femoral end of the ligament to the top of the screen, and the residual and scar regions were identified. The morphology of collagen fibrils and fibers for each ligament in the residual and scar tissue regions were examined at magnifications of ×250 to ×11000, and digitally imaged.

Histology

Ligaments for histology were fixed under tension in 4% paraformaldehyde at 4°C for 48 hours. They were subsequently dehydrated in graded alcohols, washed with 2 changes of xylene, and infiltrated and embedded in paraffin, using a Shandon automatic tissue processor (Thermo Electron Corp, Waltham, MA). Sagittal
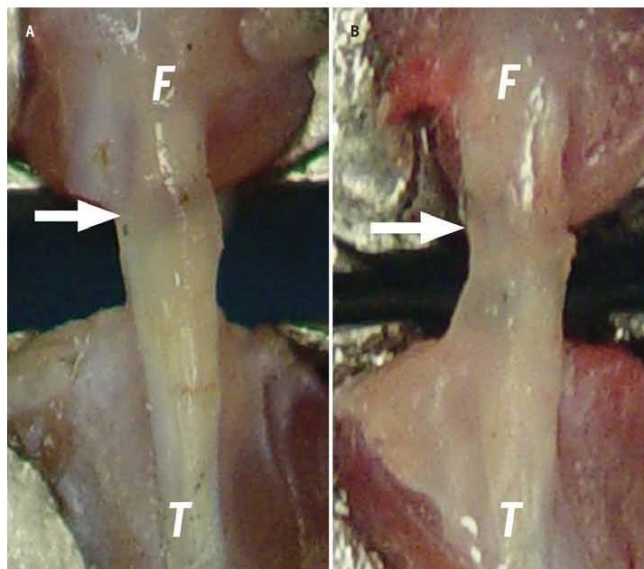
FIGURE 3. Representative images of surgically transected, untreated rat knee medial collateral ligaments (MCL) at (A) 4 and (B) 12 weeks following injury. Arrow indicates location of scar (initial injury). Abbreviations: F, femoral origin of the MCL; T, tibial insertion of the MCL.

plane thin (4 μm) sections were cut using a rotary microtome (Reichert-Jung Model 2050; Reichert-Jung, Heidelberg, Germany), mounted onto microscope slides, and stained with Harris hematoxylin and eosin on a linear stainer (Shandon Linistain GLX; Thermo Electron Corp, Waltham, MA). Three sections per specimen were qualitatively assessed under light microscopy using a Nikon Optiophot 2 microscope (Nikon, Inc, Garden City, NY).

Statistical Analyses

Statistical analyses were performed using SPSS, Version 16.0 (SPSS Inc, Chicago, IL). All comparisons were 2-tailed, with a level of significance set at 0.05. Unpaired t tests were performed to assess time (4 versus 12 weeks postinjury) and group (injured versus control animals) effects on body mass. IACFM effects were principally determined using paired t tests to compare IACFM-treated and

contralateral nontreated MCLs. Paired t test results were subsequently confirmed by calculating mean percent differences between IACFM-treated and nontreated MCLs [(IACFM-treated – IACFM-nontreated) ÷ nontreated × 100%], which were analyzed using single sample t tests with a population mean of 0%.

RESULTS

Animal Characteristics

THERE WERE NO OPERATIVE OR POSToperative complications. Animals assessed at 12 weeks postinjury were significantly heavier than those assessed at 4 weeks (mean ± SD, 291.4 ± 13.2 g versus 313.5 ± 22.6 g; $P<.05$). There were no differences in weight between injured and control animals ($P = .76$).

Ligament Macroscopic Morphology

All surgically induced ligament defects were bridged with scar tissue at the time

FIGURE 4. Effect of 9 sessions (1 minute per session) of instrument-assisted cross-fiber massage (IACFM) on knee medial collateral ligament (MCL) mechanical properties assessed at 4 weeks following injury. IACFM-treated ligaments had greater (A) ultimate force, (B) stiffness, and (C) energy to ultimate force than contralateral, nontreated ligaments. *Indicates P<.05. †Indicates P<.01, as determined with paired t test. The dashed line indicates mean mechanical properties within uninjured MCLs from age-matched, cage control animals. Error bars indicate SD.

FIGURE 5. Effect of 30 sessions (1 minute per session) of instrument-assisted cross-fiber massage (IACFM) on knee medial collateral ligament (MCL) mechanical properties assessed at 12 weeks following injury. IACFM-treated ligaments had (A) equivalent ultimate force, (B) enhanced stiffness, and (C) equivalent energy to failure than contralateral, nontreated ligaments. *Indicates P<.05, as determined with paired t test. The dashed line indicates mean mechanical properties within uninjured MCLs from age-matched, cage-control animals. Error bars indicate SD.

of harvest. At 4 weeks postinjury, the injured region was clearly distinguishable from the uninjured ligament tissue by the presence of a thickened, somewhat translucent, pinkish scar (**FIGURE 3A**). In comparison, ligaments at 12 weeks postinjury had difficult-to-see whitish scars that were relatively indistinguishable from the uninjured tissue, and the thickness of the scar region was continuous with that of

the uninjured portions of the ligament (**FIGURE 3B**). There were no grossly observable differences between IACFM-treated and nontreated ligaments at either 4 or 12 weeks postinjury; however, nontreated ligaments often had more adhesions and granular tissue, and were more difficult to harvest than IACFM-treated ligaments. Mean ± SD cross-sectional area of IACFM-treated and nontreated

ligaments did not differ significantly at either 4 (5.46 ± 1.01 mm² versus 5.16 ± 1.55 mm²; P = .45) or 12 (3.80 ± 1.02 mm² versus 4.09 ± 0.79 mm²; P = .29) weeks postinjury.

Ligament Mechanical Properties
At 4 weeks postinjury, IACFM-treated ligaments could resist 6.4 N (95% confidence interval [CI], 1.6 to 11.2 N; P = .01) greater force than contralateral nontreated ligaments (**FIGURE 4A**). This was reflected by IACFM-treated ligaments having 43.1% (95% CI, 8.2% to 78.0%; P = .02) greater mean difference in tensile strength than nontreated ligaments. Similarly, IACFM-treated ligaments had 4.9 N/mm (95% CI, 2.4 to 7.4 N/mm; P = .001) (**FIGURE 4B**) and 5.8 mJ (95% CI, 0.7 to 10.9 mJ; P<.05) (**FIGURE 4C**) greater stiffness and energy to failure at 4 weeks postinjury than nontreated ligaments, respectively. This was reflected by IACFM-treated ligaments being 39.7% (95% CI, 15.9% to 63.5%; P<.01) stiffer and being able to absorb 57.1% (95% CI, 3.4% to 110.9%; P = .04) greater energy before failure than nontreated ligaments.

At 12 weeks postinjury, IACFM-treated ligaments had 2.6 N/mm (95% CI, 0.2 to 5.0 N/mm; P<.05) greater stiffness than nontreated ligaments, resulting in the former being 15.4% (95% CI, 0.1%-30.7%; P<.05) stiffer (**FIGURE 5B**). However, there were no differences at 12 weeks postinjury between IACFM-treated and nontreated ligaments in ultimate force (1.1 N; 95% CI, −2.6 to 4.7 N; P = .54) (**FIGURE 5A**) or energy to failure (−0.6 mJ; 95% CI, −6.7 to 5.5 mJ; P = .84) (**FIGURE 5C**). Mechanical properties of ligaments in injured animals at both 4 and 12 weeks postinjury remained inferior to intact, noninjured ligaments from control animals (P<.05).

Ligament Microscopic Morphology
Light microscopy of noninjured ligaments from control animals revealed a uniform appearance of tightly packed, well-aligned collagen fibrils with interspersed fibroblasts aligned parallel to
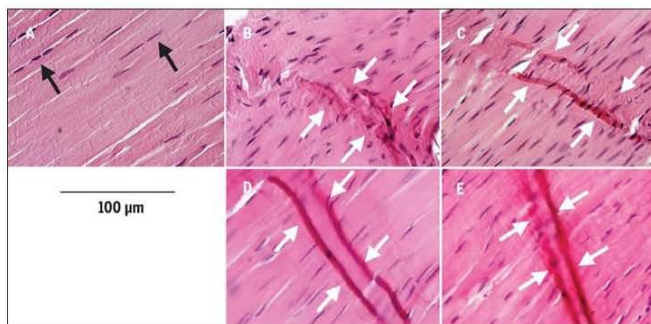
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FIGURE 6. Representative histological sections from (A) noninjured knee medial collateral ligament (MCL) in a cage-control animal, (B) scar region in a nontreated MCL at 4 weeks following injury, (C) scar region in an instrument-assisted cross-fiber massage (IACFM)-treated MCL at 4 weeks following injury, (D) scar region in a nontreated MCL at 12 weeks following injury, and (E) scar region in an IACFM-treated MCL at 12 weeks following injury. Black arrows indicate fibroblasts aligned parallel to the collagen fibrils in a noninjured ligament. White arrows indicate scar region in injured ligaments.
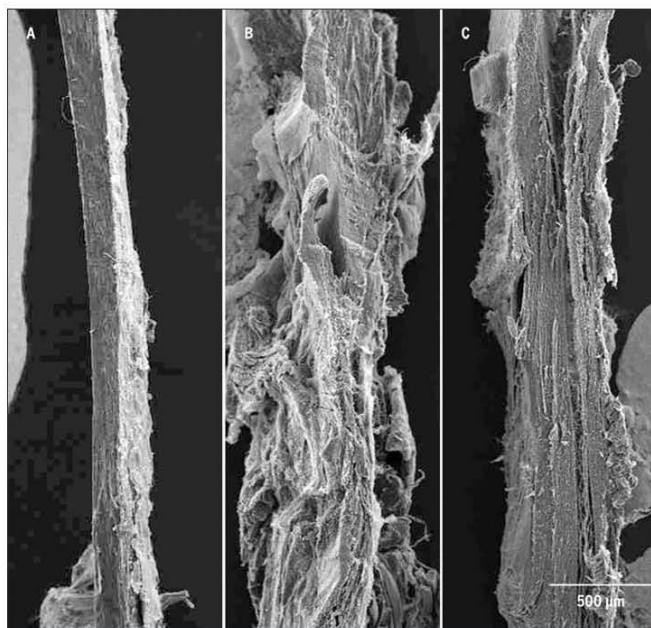

FIGURE 7. Representative scanning electron microscopy images taken from an (A) intact knee medial collateral ligament (MCL) in a control animal, (B) nontreated MCL at 4 weeks following injury, and (C) instrument-assisted cross-fiber massage (IACFM)-treated MCL at 4 weeks following injury. Images were taken at low (×25) magnification. Note the close appearance of the IACFM-treated ligament (C) to the noninjured ligament from a control animal (A). Also, note the large amount of surrounding granulation tissue in the nontreated, but injured ligament (C) relative to the other 2 ligaments.

the fibrils (**FIGURE 6A**). In contrast, ligaments from injured animals appeared to have scar morphology with extracellular matrix disorganization and hypercellularity, particularly at 4 weeks postinjury (**FIGURE 6B-E**). The scar region of IACFM-treated ligaments at 4 weeks postinjury also appeared to have greater cellularity, with collagen fiber bundles appearing to be orientated more along the longitudinal axis of the ligament than observed in contralateral nontreated ligaments (**FIGURE 6B-C**). At 12 weeks postinjury, there were limited histological differences between IACFM-treated and nontreated ligaments (**FIGURE 6D-E**).

Ligaments from injured, but not control, animals had granular tissue at low magnification (×25) on scanning electron microscopy and IACFM-treated ligaments appeared to have less surrounding granular tissue compared to nontreated ligaments, supporting the macroscopic observations (**FIGURE 7**). At higher scanning electron microscopy magnifications (×250-×6500), the scar region of IACFM-treated ligaments appeared to have improved collagen fiber bundle formation and orientation within the scar region compared to nontreated ligaments, supporting the light microscopy observations (**FIGURE 8**).

DISCUSSION

THIS STUDY INVESTIGATED THE POtential utility of manual therapy in the form of IACFM on ligament healing. Results indicate that IACFM-treated ligaments were 43% stronger, 40% stiffer, and able to absorb 57% more energy than contralateral, nontreated, injured ligaments at 4 weeks following injury. These mechanical differences may have resulted from favorable effects of IACFM on the organization of the underlying collagen substructure, as suggested by preliminary light microscopy and scanning electron microscopy analyses. The latter needs to be confirmed by way of more in-depth quantitative analyses in future studies. In contrast, there was
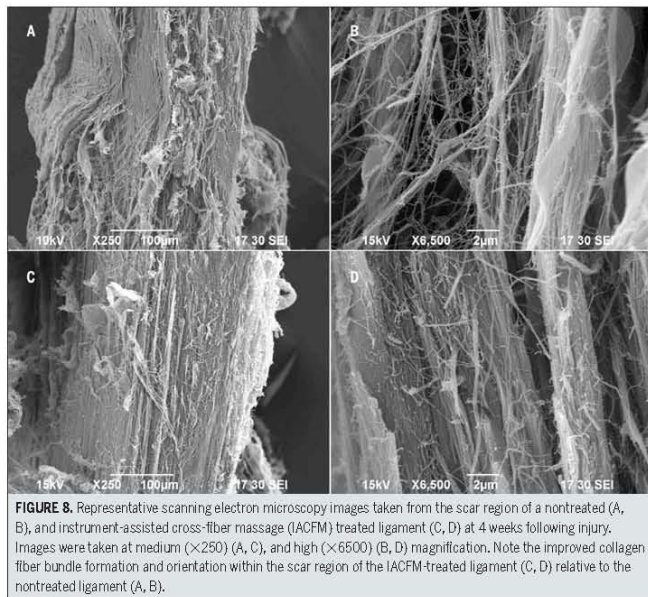
FIGURE 8. Representative scanning electron microscopy images taken from the scar region of a nontreated (A, B), and instrument-assisted cross-fiber massage (IACFM) treated ligament (C, D) at 4 weeks following injury. Images were taken at medium (×250) (A, C), and high (×6500) (B, D) magnification. Note the improved collagen fiber bundle formation and orientation within the scar region of the IACFM-treated ligament (C, D) relative to the nontreated ligament (A, B).

minimal to no effect of IACFM on ligament healing when assessed at 12 weeks following injury, with the only difference between IACFM-treated and nontreated ligaments being 15% greater stiffness in the former. Overall, the findings of this study suggest that IACFM accelerates early tissue-level healing following ligament injury, but does little in terms of augmenting healing.

The findings of the current study are interesting in that a relatively simple and practical manual therapy technique was found to enhance early recovery of ligament biomechanical properties following acute injury. This may be clinically relevant, as there are currently limited established treatment options for mediating tissue-level ligament healing. It is clear from preclinical and clinical studies that surgery with or without immobilization is not indicated for most capsular and extracapsular ligament injuries. This holds true for both partial- and full-thickness ligament tears, with comparative studies showing con-

servative treatment and surgical repair producing similar outcomes irrespective of the extent of the initial ligament damage.[9,13,22] Consequently, there is a need to establish interventions other than surgery for influencing ligament healing. Numerous preclinical studies have investigated the utility of novel interventions targeting ligament healing, including the use of gene therapies, growth factors, biological scaffolds, stem cell therapies, and biophysical modalities.[10,15,18,32] While each of these directions has shown promise in influencing ligament healing, the techniques are far from being translated into the clinical realm and their eventual costs may prohibit wide use in mainstream clinical practice. In contrast, IACFM may have clinical utility, as it is currently readily available and practical from the sense that gains in ligament biomechanical properties were produced in the current study using a relatively limited number of short treatment sessions (9 total sessions of 1-minute duration each).

How IACFM facilitates the restoration of ligament tensile mechanical properties following injury was not investigated in detail in the current study, as the primary purpose was to provide proof-of-concept evidence for the utility of IACFM. However, preliminary light microscope and scanning electron microscopy assessments suggest that IACFM may enhance restoration of ligament biomechanical healing by optimizing the organization of the collagen substructure. Collagen (in particular, type 1 collagen) is the primary load-bearing molecule in ligament that endows tensile strength, and is ordered hierarchically into fibrils and fibers.[21] The alignment and organization of newly formed collagen fibers in the direction of tensile loads during healing influences ligament mechanical properties.[19,20] The qualitatively improved collagen fiber organization observed within the scar region of IACFM-treated ligaments in the current study is a possible explanation for why IACFM-treated ligaments had enhanced tensile mechanical properties. This will be the focus of future quantitative studies into IACFM effects on ligament morphology.

In addition to more detailed studies into IACFM effects on ligament morphology, studies are planned to explore potential molecular mechanisms by which IACFM generates its biomechanical effects. Our working hypothesis is that IACFM has an underlying effect on collagen, which may include effects on its synthesis, maturation, and/or cross-linking. To have such effects, IACFM must influence the fibroblastic cells responsible for producing collagen. This potential effect is supported by previous work that found that IACFM increases fibroblast recruitment and activation in a rodent Achilles tendon injury model.[5,8] It is plausible that IACFM presents a direct mechanical stimulus to the extracellular matrix, which is subsequently transduced into a cellular response. A candidate mechanotransduction pathway for this response deserving of future investigation is the extracellular matrix-integrin-cytoskeleton

axis. Integrins are a family of glycoprotein molecules that connect extracellularly with the extracellular matrix and intracellularly with the cytoskeleton and other cytoplasmic constituents.[12] This creates a transmembrane axis that mechanically links the extracellular matrix with the cytoplasmic constituents of the cell to transmit external stimuli (such as those associated with IACFM) directly to the internal environment of the cell to alter gene expression and protein synthesis.

The current study provides supportive evidence for the potential clinical use of IACFM in the treatment of capsular and extracapsular ligament injuries; however, the findings need to be carefully interpreted in light of acknowledged limitations. First, the study was performed in an animal model, wherein the knee MCL was injured via surgical transection. This is a highly reproducible and established model for the preclinical testing of interventions for ligament injuries[16,20,29,31]; however, the ability of the model to predict the clinical scenario wherein ligaments are injured via excessive tensile load has not been established. Second, the size of rodent tissues in relation to those of humans raises scaling issues in terms of intervention application and response. We addressed this issue by performing IACFM to rodent ligaments with the same tool and force as those used clinically to treat similar-size ligaments (finger collateral ligaments). Third, between-animal differences in activity levels may have influenced ligament-healing rates, as activity has previously been shown to mediate healing of isolated MCL injuries in rodents.[2] We controlled for this possibility in the current study by establishing within-animal IACFM effects, wherein IACFM-treated ligaments were compared to contralateral nontreated ligaments that were presumably exposed to equivalent activity levels. Fourth, the current study did not consider IACFM effects on clinically measurable outcomes, such as recovery from symptoms such as pain. The restoration of mechanical properties is the ultimate tissue-level outcome

of the ligament-healing process and is an important preclinical outcome; however, it is plausible that IACFM accelerates ligament biomechanical healing without influencing symptom recovery. Also, the establishment of IACFM benefits via the ex vivo testing of MCL tensile properties with the knee at 70° flexion may not represent the most clinically translatable outcome. While testing at 70° knee flexion is reported to load all fibers of the rat MCL simultaneously,[20] it is plausible that alternative joint positions provide better tests of functionally important portions of the ligament. Similarly, as the MCL was isolated for mechanical testing, the contribution of other structures that contribute to the in vivo resistance of knee valgus forces were not assessed. It is possible that other passive and active restraints are able to compensate for injury to the MCL in the clinical setting, reducing the potential clinical effect size of IACFM during ligament healing.

CONCLUSION

IN SUMMARY, THIS STUDY SUGGESTS that IACFM may accelerate early tissue-level healing following acute capsular/extracapsular ligament injury but it has minimal to no effect in terms of augmenting the overall outcome of the ligament-healing process. This finding supports a theoretically sound argument for the use of IACFM after acute ligament injury; however, careful interpretation of this controlled laboratory study is warranted until its findings are confirmed by clinical studies. ◉

■ KEY POINTS
FINDINGS: IACFM accelerated early tissue-level healing following acute capsular/extracapsular ligament injury but had minimal to no effect in terms of augmenting the overall outcome of the ligament-healing process.
IMPLICATION: IACFM is a relatively simple and practical therapy technique that may facilitate earlier return of ligament tissue-level biomechanical properties,

enabling quicker return to function with less susceptibility to reinjury.
CAUTION: Careful interpretation of this controlled animal study is warranted until its findings are confirmed by clinical studies.

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CURRICULUM VITAE

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Education

2010: Doctor of Philosophy, Department of Anatomy and Cell Biology

Indiana University, Indianapolis

1993: Master of Science, Physical Therapy, University of Indianapolis, Indianapolis

1983: Bachelor of Science, Physical Therapy, Indiana University, Indianapolis

Certifications

1993: National Certification in Therapeutic Massage and Body Work

1993: Certified Manual Therapist

Honors and Awards

2007: Indiana University Trustees Teaching Award, IUPUI

2007: National APTA Distinguished Teaching Award, Nominee

2006: St. Vincent Research Symposium Allied Health Research Award

2003: Indiana University Trustees Teaching Award, IUPUI

1993: Academic Excellence Award – University of Indianapolis

1983: Francis Ekstam Outstanding Student Award – Indiana University

Grants

American Massage Therapy Association (2007) co-investigator, $19,253

TherapyCare Resources, Inc. (2004) $5,000; (2006), $3,000

Professional Memberships

1983-present: American Physical Therapy Association – Orthopedic, Education Sections

2003-present: American Association of Anatomists

Professional Experience

1998-present: Associate Clinical Professor, School of Health and Rehabilitation Sciences, Department of Physical Therapy Doctor of Physical Therapy Program

1983-present: Licensed Physical Therapist. Practiced in a variety of settings; specializing in Spine and Orthopedic Rehabilitation and Manual Therapy

1994-2002: GrasTech, Inc., Director of Physical Therapy and Education

<u>Conferences Attended</u> (Platform and Poster Presentations)

Gundeck E, Klene F, Bayliss A, LOGHMANI MT. Instrument-assisted soft tissue mobilization for treatment of a finger PIP joint strain in a guitarist: a case study. (Poster)

LOGHMANI MT, Warden SJ. Instrument-assisted cross fiber massage improves regional blood flow in healing knee ligaments. National APTA Combined Sections Meeting 2010. (Platform)

Bayliss AJ, Klene FJ, Gundeck EL, LOGHMANI MT. Treatment of a patient with post-natal calf pain utilizing instrument-assisted soft tissue mobilization. National APTA Combined Sections Meeting 2010. (Platform)

Klene F, Gundeck E, Hassett S, LOGHMANI MT, Bayliss A. An instrument-assisted soft tissue mobilization approach for the treatment of trochanteric bursitis: a case study. IUPUI Research Day 2009. (Poster)

LOGHMANI MT, Warden SJ, Burr D. Biomechanical and Histological Effects of Instrument-Assisted Cross Fiber Massage on Acute and Long-Term Ligament Healing. ACSM 2008. (Poster)

LOGHMANI MT, Warden SJ. Long-Term Effects of Instrument-Assisted Cross Fiber Massage on Healing Medial Collateral Ligament – National APTA Combined Sections Meeting 2007. (Platform)

Hayes D, LOGHMANI MT, Lubitz R, Moore E. A Comparison of Two Instrument Assisted Soft Tissue Mobilization Techniques: Effects on Therapist discomfort/fatigue and Treatment Time. National APTA Combined Sections Meeting 2007. (Platform)

LOGHMANI MT, Warden SJ. Instrument-Assisted Cross Fiber Massage Accelerates Knee Ligament Healing in Rats – National APTA Combined Sections Meeting 2006. (Platform)

CAREY-LOGHMANI MT, Ploski M, Sweney L. The Graston Technique of Soft Tissue Mobilization. APTA Combined Sections Meeting, Seattle 1999. (Poster)

<u>Publications</u>

LOGHMANI MT, Bayliss A, Gundeck E, Klene F. Instrument-assisted soft tissue mobilization for treatment of a finger PIP joint strain in a guitarist: a case study. (In progress)

Klene F, Gundeck E, Hassett S, LOGHMANI MT, Bayliss A. An instrument-assisted soft tissue mobilization approach for treatment of hip pain. The Edge. December 2009.

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Burke JR, Buchberger DJ, CAREY-LOGHMANI MT, Dougherty PE, Greco DS, Dishman JD. A pilot study comparing two manual therapy interventions for carpal tunnel syndrome. Journal of Manipulative and Physiological Therapeutics. Jan 2007.

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LOGHMANI MT, Hammer WI. "Graston Technique of instrument-assisted soft tissue mobilization". Functional Soft Tissue Examination and Treatment by Manual Methods 3rd Ed. Hammer WI editor. Jones & Bartlett Publisher, 2006. (Book Chapter)

CAREY-LOGHMANI et al. Graston Technique Training Manual 2ND ed. Published 2001.

CAREY-LOGHMANI et al. Graston Technique Training Manual 1ST ed. Published 1998.