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Biochemical and Physiological Dynamics in Ligament Injury & Healing

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Systems Engineering

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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ABSTRACT

Biochemical and Physiological Dynamics in Ligament Injury & Healing

Ligaments play a crucial role in the musculoskeletal system in maintaining joint stability. Their primary function is to guide and restrain skeletal motion playing a vital role by forming a flexible skeletal joint between bones. When ligament injury occurs, a wound healing cascade begins with platelet plug formation and ends with tissue remodeling. Rat Medial Collateral Ligament (MCL) surgical and sub-maximal injury models were developed to examine footprint gait patterns, biochemical response, and histological tissue samples. Animals (n=150) were randomized to three treatments, Platelet Rich Fibrin (PRF) treatment, Meloxicam, a non-steroid anti-inflammatory drug (NSAID), and untreated animals. We examined injury and treatment effects up to day twenty-eight post-injury. Results show that untreated animals' gait patterns recovered to normal function within seven days after injury, while anti-inflammatory drugs slowed functional recovery until day fourteen. The PRF-treated group showed the fastest functional recovery on day five. Biochemical evaluation with enzyme-linked immunoassay (ELISA) of the Vascular Endothelial Growth Factor (VEGF) showed a temporal effect with earlier peak VEGF concentration with PRF on day three compared to untreated animals peaking on day five and Meloxicam peaking on day seven. PRF reduces the time to functional recovery and correlates with early induction of VEGF. Even though NSAIDs manage the inflammatory response and pain, they retard functional recovery and VEGF induction. The histological assessment showed a temporal difference between day three and day twenty-eight regarding cellularity, collagen morphology and organization, and vascularity and set the stage to evaluate PRF and Meloxicam treatments on ligament healing. Establishing an animal model to study gross ligament injury and recovery sets the stage to study submaximal injury. Experiments have already designed an apparatus capable of producing submaximal injury. Initial studies have simulated mild ligamentous often experienced in different occupations requiring stooped posture work. Using a rodent model opens the opportunity to evaluate different approaches to improve ligament injury recovery and investigate various treatment methods.

To my Parents Abdulfattah & Sumaya

To my friend Brandon Miller, May Your Soul Rest in Peace

To my Children, Omar, Mohammad, and Tasneem

And to all Palestinian Farmers Under Occupation

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CHAPTER 1

INTRODUCTION

Musculoskeletal disorders (MSDs) are disorders of the soft tissues (muscles, tendons, and ligaments) and their surrounding structures (1). Musculoskeletal disorders (MSDs) account for 29–35% of all occupational injuries and illnesses in the US and keep workers from their jobs (2). The annual cost of Low Back Disorders (LBDs) exceeds $100 billion. In the US alone, 13 million people will develop LBDs annually due to their occupation, making it the most prevalent musculoskeletal problem in the workplace (3-5). For farmworkers, cumulative musculoskeletal injuries and LBDs pose high direct and indirect financial costs and direct human costs due to pain and discomfort (2, 6). About 600,000 workers in California perform stooped postures (bent forward and down at the waist and or mid-back while maintaining straight legs), increasing their risk of developing LBDs due to repeated stresses and exposures to the longitudinal spinal ligaments (3).

Skeletal ligaments are dense bands of collagenous fiber tissue that span a joint, restraining one bone to another forming a flexible skeletal joint (7, 8). Ligaments are pale from the limited blood supply (8). Ligaments play a crucial role in the musculoskeletal locomotive system in resisting tensile and torsional forces affecting the joint. Their primary function is to guide and restrain skeletal motion while keeping the joint structure intact (9).

Ligaments are passive tissues with viscoelastic properties susceptible to creep deformation under sustained static loading (10). Stretching viscoelastic tissue repeatedly for long periods exposes them to microdamage, “In the collagen fibers, irrespective of the load applied” (11). Depending on the loading level, injuries occur, and a repair process commences.

After an injury, ligaments undergo a series of wound healing events to restore original tissue structure and function; however, results vary. In most cases, repaired ligaments are inferior from a mechanical and functional standpoint (12-15). Four phases mark the repair process: restoration of hemostasis, inflammation, repair, and remodeling (16). During platelet activation and degranulation, growth factors are released from their granules, initiating other cascades necessary for wound healing (17). Biochemical, mechanical, and histological studies of healing ligament in a complete tear of the Medial Collateral Ligament (MCL) show healed ligaments with scar tissue that is mechanically inferior and biochemically abnormal in its composition and architecture, concluding that these properties do not return to normal (18).

With inferior mechanical properties, the ligament’s function is affected, leading to instability at the joint, and therefore susceptibility of other structures to injury is increased. The load distribution around the joint is disrupted, leading to altered contact mechanics with increased shear forces affecting cartilage and bones. This eventually leads to osteochondral degeneration and other joint chronic diseases (19, 20). In addition, muscles become less efficient in the locomotion system due to increased sliding between joint surfaces (19-21).

Even though the incidence and prevalence of ligament injuries in occupational settings are high, a fundamental understanding of the ligament’s response to injury and treatment is still lacking. Standard treatments produce inferior results that may lead to joint mechanical instability, thus increasing subsequent injuries and joint diseases. Because of these shortcomings, any current recommendation for ligament injury prevention in occupational settings such as farming tasks with

stooped postures will not succeed. Therefore, a basic understanding of the histological, biochemical, mechanical, and morphological response to ligament maximal and submaximal strain injury and repair is needed to guide occupational injury prevention and treatment recommendations.

This study examined the body's innate response to ligament injury at the early stages of healing. In addition, we developed a basis to study the efficacy of various treatment methods and injury mechanisms. Laboratory studies were designed and optimized to examine ligament injury and healing under different injury mechanisms. Finally, this study set the stage to examine ligament injuries in submaximal methods, simulating exposure to occupational injury. The hypothesis and specific aims follow.

Ligament Treatment Methods

The main standard treatments for ligament injuries are RICE (Rest, Ice, Elevate, Compression, and Elevation), NSAIDs (Non-Steroidal Anti-inflammatory Drugs), and a relatively new treatment with platelet-rich concentrates (e.g., Platelet Rich Fibrin or PRF). The goal of the RICE treatment is to reduce additional damage to injured tissue, decrease blood flow and enzymatic activity, which are expected to reduce edema and swelling and control pain (22, 23). NSAIDs reduce the inflammatory response by limiting the activity of Cyclooxygenase. Cyclooxygenase (COX) is a rate-limiting enzyme that converts arachidonic acid to prostaglandin H2, the precursor of prostaglandins, prostacyclin, and thromboxane (24). Thromboxane is involved in platelet aggregation, and prostaglandins mediate pain sensitization are inhibited with NSAIDs (25-28). Because of NSAIDs' action to reduce the inflammatory response, it serves to manage pain. However, in managing pain and the inflammatory response, they can also slow the healing response, which relies on platelet activation. The main goal of platelet treatment, such as PRF is to increase the availability of platelets containing biomolecules and growth factors responsible for repair at the site of the injury (29). By doing so, the platelets will mediate collagen expression and organization and shorten the time to mechanical and functional recovery (30-32).

OPEN RESEARCH QUESTION

Complete recovery of mechanical and functional properties like native ligament tissue is not currently achievable (33-35). Current literature focuses on restoring original tissue properties and time to recovery, and the level of healing varies depending on the treatment applied (12-15). There is insufficient evidence to support current treatment methods (NSAIDs and RICE) as effective treatments to restore ligament structure and function; however, they appear to be deployed to control pain and swelling, which are byproducts of repair and the inflammatory response (22, 36). Some authors argue that existing treatments only help relieve pain symptoms (19, 22, 25, 37). So, the improved return to normal mobility will mask any structural and functional restoration of the injured tissue (22, 37-39). Concerning PRF treatment, the lack of standardization in creating and activating platelets and experiments testing its efficacy and range of outcomes limits current support for using it as a standard treatment for ligament injuries (30-32).

Because of the uncertainty in the healing process after a ligament injury, the proper therapeutic intervention and any prevention recommendation in occupational settings such as farming tasks with stooped postures will also be uncertain. Therefore, a basic understanding of the histological, biochemical, and morphological response to maximal and sub-maximal strain injury will help guide the formulation of occupational injury prevention efforts and treatment strategies. In particular, the study will provide insight into the healing response to an injury similar

to that sustained in jobs requiring prolonged stooped posture, such as those commonly observed in farming, and will provide a basis to evaluate exposure-response schemes to guide prevention efforts and work policies.

Finally, conducting controlled laboratory experiments that induce injury is impossible with human subjects. Establishing an animal model will then permit experiments to simulate and examine different injury methods and treatments to examine their impact on healing.

MCL as a Model for Studying Ligaments

To investigate ligament injury-healing, an established animal model is needed. Many studies use the Medial Collateral Ligament (MCL) in experimental animal models due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (19, 40-43). Using the MCL to study ligament healing responses under different injury conditions will help elucidate our understanding of the underlying processes involved in the healing response within the context of ligament injury.

HYPOTHESIS

Hypothesis 1: PRF-treated ligaments will have a faster functional gait pattern recovery.

Hypothesis 2: Growth factors, VEGF, will be expressed fasted in the presence of PRF.

Hypothesis 3: NSAIDs will slow the healing response.

SPECIFIC AIMS

The project characterized the time-dependent functional, morphological, biochemical, and physiological changes in the ligament during recovery from injury. Even though many people suffer from a ligament injury, especially from medial collateral ligament (MCL) injury, the treatment approach remains somewhat ad hoc and does not arise from a specific understanding of the biochemistry of healing.

Our research aims to investigate ligament healing after a surgical ligament transection and submaximal ligament strain. The experiments characterized the time-dependent ligament repair by functional gait patterns, biochemistry, histomorphology, and tissue mechanics. The project had the following aims:

Aim 1: Develop a rat transection model, simulating severe ligament injury (Chapter 1 Appendix), and a submaximal injury model, simulating stooped posture injury described in chapter 5 (44).

Aim 2: Use biochemical and histological methods to characterize healing (45, 46).

Aim 3: Characterize mechanical tissue properties during healing (37).

Aim 4: Map with gait analysis the time course of functional recovery (47) .

Aim 5: Assess the impact of treatment intervention. We tested a commercially available Non-Steroid Anti-Inflammatory Drug (Meloxicam) and autologous Platelet Rich Fibrin (PRF) obtained from donor animals as described in Chapter 1 appendix (21).

BACKGROUND

Every joint in the human skeleton has several ligaments that are considered the primary restraints of the bones articulating around the joint (48). The role of ligaments to restraint a joint is complex when considering the various activities performed by individuals during normal functions at work and sports. Therefore, joint stability is a general role of ligaments without which the joint may subluxate, causing damage injury to the capsule, tendons, nerves, blood vessels, cartilage, discs of the spine, and the ligaments themselves. In addition, ligaments ensure stable motion of bones associated with a joint by providing travel in their prescribed anatomical tracks, ensuring contact pressure of the articular surfaces, and preventing separation of the bones from each other by increasing their tension with their viscoelastic properties (8).

When extreme trunk flexion is assumed (e.g., stooped posture), sudden onset of electrical silence called the flexion relaxation phenomenon occurs (49-52). The electrical activity of the erector spinae muscles abruptly stops after a specific amount of trunk flexion. A later continuation of electrical activity is observed when the trunk is extended from the fully flexed position at a location (sagittal angle), similar to that when the erector spinae stopped firing. For the musculoskeletal system to stay at equilibrium, the body passes the moment to passive tissues (ligaments, connective tissues, and discs) by inhibiting the erector spinae muscles. The response of muscle in this condition follows the length-tension curve. Passive tissue involvement in producing the total force becomes more dominant as the flexion bypasses that muscle group's normal joint range of motion. Therefore, during silence, a period of no muscle activation, vertebral ligaments produce most of the force needed to maintain posture or equilibrium.

Hindle et al. investigated the mechanical function of the human lumbar interspinous and supraspinous ligaments and found that they provide minimal support with minimal trunk flexion (5% of the counter moment). However, at full trunk flexion, maximum forward bend at the waist while the individual cannot bend any further with knees straight or slightly bent, and during the silence period, the interspinous ligament alone supported 75% of the load (53). Increased loading at this flexion range makes spinal ligaments more susceptible to microdamage, especially if the process is repeated without sufficient time to minimize creep deformation - increasing deformation under constant load (11).

Normal Ligament Structure, Physiology, and Function

Ligaments are made up of two-thirds water and one-third solid. The water contributes to cellular functions and viscoelastic behavior. The solid components of ligaments are majorly collagen. There are six types: type I, III, V, VI, XI, and XIV, accounting for approximately 75% of the dry weight. Proteoglycans, elastin, other proteins, and glycoproteins (actin, laminin, integrins) make up the remaining 25% (54-58). Type I collagen is the predominant type and roughly 85% of the total collagen content (59). During the formation of ligaments, triple-helical collagen molecules are aligned to form fibrils organized in a parallel manner and folded into fibers. The fibers are interconnected by cross-links which give collagen fibers high tensile strength. The cross-linked collagen created the extracellular matrix and the ligament structure (60-62). Ligaments also receive blood supply at their insertion sites. A uniform micro-vascular network travels along the epiligament and penetrates the ligament substances in a regular longitudinal pattern (63). This arrangement allows for a uniform and regular matrix synthesis and repair (64).

Knee Ligaments

The knee's ligaments provide stability, guide the motion of the femur and tibia, define contact mechanics between the femur and the tibia, and prevent excessive movement that can lead to dislocation (65). The bone structure of the knee joint is made up of the femur, the tibia, and the patella. The knee is a hinge joint held together by four ligaments; these ligaments connect the femur to the tibia. They are:

Anterior cruciate ligament (ACL): Located in the center of the knee, it controls the rotation and forward movement of the tibia.

Posterior cruciate ligament (PCL): Located in the center of the knee, it controls the backward movement of the tibia.

Medial collateral ligament (MCL): Gives stability to the inner part of the knee resisting valgus forces (66).

Lateral collateral ligament (LCL): Gives stability to the outer part of the knee resisting varus forces (66).

A layer of articular cartilage covers the weight-bearing surfaces of the knee (67). The lateral and the medial meniscus are located on either side of the joint between the cartilage surface of the femur and the tibia. The two menisci act as shock absorbers and work with the cartilage to reduce the stresses between the femur and the tibia (68).

Ligament Injuries

The four major ligaments in the knee are susceptible to injury. An injured ligament may be sprained, stretched, or ruptured. Ligament rupture may be partial or absolute. Symptoms associated with injured ligaments include painful swelling, tenderness around the knee, bruising, reduced knee movement, etc. The severity of the symptoms usually depends on the type of injury sustained (69). Despite the high incidence of ligament injuries in occupational and sports settings, a fundamental understanding of the tissue's response to injury remains unclear.

The MCL is a commonly injured ligament in the knee. In many cases, the body's natural healing process can repair the injury on its own with a full recovery to function. Although the MCL of the knee is known to heal spontaneously after injury, however, the associated disadvantage is that the morphology, biochemistry, and mechanical properties of the healed MCL have never returned to their original condition even after a long period (70). In addition, in most severe injuries, damage to the ligament can surpass the body's ability to fully repair and restore the ligament (14, 20, 44, 71-73).

Phases of Ligament Wound Healing

Ligament healing follows the four main phases of wound healing, starting with hemostasis and ending with remodeling (16). Three clinical ligament injury grade categories depend on the level of tear, with grade 1 associated with mild tears and grade 3 depicting a complete tear of the ligament (39, 74). A complex series of biochemical reactions and interactions among cells and mediators is an organized process consisting of four overlapping phases: hemostasis, inflammation, proliferation, and maturation and remodeling (75).

The endothelium is damaged when ligaments are disrupted due to injury and blood vessels are severed. It then exposes the basal lamina to blood plasma and peripheral blood cells (76). Hemostasis is achieved when damaged blood vessels are temporarily repaired by creating a platelet plug/clot. This step is dependent on the level of platelets recruited at the site of the injury (16, 76). In addition, the growth factors and biomolecules released from platelets play a significant role in

the deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (77). An influx of inflammatory cells follows activated platelets in the first 48 hours (76). This is followed by proliferation which is a set of four steps that begin at various periods in wound healing: (1) epithelialization, (2) angiogenesis, (3) granulation tissue formation, (4) and collagen deposition (16). Repair and remodeling of tissue occur following proliferation. Initially, the extracellular matrix, mainly made up of collagen, is deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet provides a basis for a new matrix (73). Next, the newly deposited collagen is reorganized into the structurally sound lattice, and the immature type III collagen is replaced with type I collagen that is structurally stronger (16, 76).

A detailed overview of each phase in wound healing is described herein:

Hemostasis Phase

The hemostasis (bleeding) stage is the first stage of wound healing; it is initiated immediately after an injury and usually lasts for hours. When an injury is sustained, the ligaments are typically disrupted, and blood vessels are severed; the blood vessels release blood plasma and peripheral blood cells into the wound site. Some signals are also released; these include the exposure of collagen on the blood vessel walls and the release of adenosine triphosphate (ATP) (16, 78).

The injured blood vessels then undergo vasoconstriction, and the endothelium and the platelets surrounding the injured area will activate the intrinsic coagulation cascade, which produces fibrin clots. This step is dependent on the level of platelets recruited at the site of injury (16). The Von Willebrand Factor (VWF) is a major mediator of platelet aggregation and clot formation (79). VWF is present in plasma, collagen extracellular matrix, platelets granules, and endothelial cells (80). Upon platelet contact with collagen and the VWF present in the extracellular matrix (ECM), platelets activate, change their shape, and begin degranulation, releasing their content of biomolecules and growth factors stored in their granules (76, 81, 82). This leads to further recruitment of platelets until a fibrin clot/scaffold is formed, leading to a stable plug and, therefore, restoration of hemostasis (76, 79, 83). Platelets adhere to collagen fibers which are exposed in the damaged endothelium using specific collagen receptor glycoproteins, forming a primary hemostatic plug. This process is called primary hemostasis. As the platelets are attached to the injury site, they rapidly bring about the upregulation of the high-affinity platelet integrin αIIbβ3, which mediates the platelet aggregation (16, 75, 82-84).

Once the platelets release their contents into the plasma, they stimulate local activation of plasma coagulation factors. These factors are responsible for triggering the generation of a fibrin clot dependent on the level of platelets recruited at the injury site (16). Exposure of blood plasma to tissue factors that are produced by sub-endothelial cells initiates an accelerated cascade of activated proteins which brings about fibrin formation (85-87). The fibrin clot acts as a temporary barrier that prevents excessive bleeding and limits the spread of infectious agents into the bloodstream. Fibrinogen usually undergoes modification by cleaving to produce fibrin monomers; these monomers then undergo polymerization and cross-linking reactions to form an intertwined, gelatin-like platelet plug that produces a stable clot (87, 88).

The fibrin clots play essential roles in the healing process; they serve as a scaffold for invading cells, and they help to concentrate the cytokines and the growth factor that are later involved in the wound healing (71, 76, 89, 90). The fibrin clots formed are made up of the following components: collagen, thrombin, platelets, and fibronectin. These factors mediate the release of signaling molecules such as cytokines and growth factors. Examples of growth factors

include Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β), and Vascular Endothelial Growth Factor (VEGF). These signaling molecules initiate the inflammatory phase (64, 77, 91).

Inflammatory Phase

Activated platelets are followed by an influx of inflammatory cells in the first 48 hours (76). The term "inflammation" comes from the Latin word *inflammare* (to set on fire). Roman Celsus in the 1st century AD is credited as first documenting the four cardinal signs of inflammation: *rubor et tumor cum calore* et dolore, which translates to; redness and swelling with heat and pain (92). The inflammation stage varies from hours to a maximum of seven days, and it begins immediately once the clots are formed (92-94). After releasing signaling molecules from the wound site during hemostasis, blood-borne inflammatory cells are recruited to the injury site. Chemoattractant molecules released by the platelets also increase vasodilation and vascular permeability, enhancing leukocytes' mobilization to the injury site (95). The incoming leukocytes recognize the plasma proteins (fibronectin, vitronectin, and thrombospondin), which are passively absorbed by the clots (96). As the inflammatory mediators are mobilized to the injury site, prostaglandins are secreted, and the surrounding blood vessels vasodilate to facilitate the increased cellular traffic of mediators which are drawn to the injury site. The inflammatory mediators drawn to the injury site include neutrophils, interleukin, cytokines, tumor necrosis factor, transforming growth factor, and bacterial products (27, 97-99).

Neutrophils are the first type of leukocytes that migrate from the bloodstream to the injury site and are tasked to kill pathogens and remove cellular debris (98). Effective neutrophil recruitment requires P-selectin, stored in alpha-granules of platelets and in Weibel–Palade bodies of endothelial cells, and is translocated to the cell surface of activated endothelial cells and platelets allowing them to interact with and capture/recruit neutrophils (99). Neutrophils play a major role in recruiting monocytes that infiltrate tissue and transform into tissue macrophages (77, 100, 101).

The neutrophils in the wound site are responsible for removing invading bacteria and cellular debris from the site. They do this by secreting degrading proteolytic enzymes, which digest bacteria and damaged tissues. The proteolytic enzymes are categorized into different types depending on the type of protein they degrade. The preexisting extracellular matrix in the wound area will be destroyed, while the matrix in the uninjured tissues will be protected by protease inhibitors (77). After functioning at the wound site, the neutrophils are destroyed by the apoptosis mechanism and are replaced by macrophages (16, 64, 102, 103). Monocytes in the surrounding tissues and blood are drawn into the injury area about 48 to 96 hours after injury. The monocytes transform into macrophages.

Macrophages further carry out functions within the healing region via phagocytosis of cellular debris, apoptosis, inflammatory cell and fibroblast recruitment, angiogenesis regulation, and scar tissue formation (76, 104). L-selectin is a cell adhesion molecule expressed on neutrophils and other leukocytes that regulates the capture and infiltration of monocytes at locations of inflamed or activated endothelium (99, 101, 105).

Macrophages are activated by type 1 cytokines such as interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) (43, 99). Macrophages exhibit potent microbial activity, releasing interleukin (IL)-12 and IL-23, which promote strong pro-inflammatory immune responses. Macrophages continue in pathogen killing by generating Nitric Oxide (NO). TNF and IL stimulate the macrophage iNOS to synthesize a vast quantity of NO, which react with peroxide ion oxygen radical to produce peroxynitrite and very toxic hydroxyl radicals (16, 106-108).

The damaged extracellular matrix is removed by matrix metalloproteinase (MMP); it clears away inflammatory debris and enables the migration of wounded cells through the extracellular matrix. MMP is expressed by macrophages, keratinocytes, fibroblasts, and monocytes in response to TNFα. In addition to the phagocytic action of macrophages, it also releases growth factors and cytokines that initiate the proliferative phase of the healing (109). These factors include PDGF, TGF-β, β-FGF, TNF-α, interleukin 1 (IL-1), and IL-6 (16). Even though lymphocytes are the last cells to infiltrate the wound site, they are essential in producing IL-2, which recruits fibroblasts.

The inflammatory stage of wound healing possesses some stop signals, which are referred to as checkpoint controllers of inflammation. Lipoxin is a stop signal for inflammation. Platelets and leukocytes are responsible for the biosynthesis of the lipoxin (110, 111). Lipoxin biosynthesis signifies the need to terminate the inflammation stage. When lipoxin appears at the wound site, neutrophils in peripheral blood will be exposed to prostaglandin E2; this results in a switch in eicosanoid biosynthesis from LTB4 to LXA4, which is the lipoxygenase product that terminates polymorphonuclear neutrophil infiltration (16).

The latter part of the inflammatory phase of MCL injury is characterized by substantial infiltration of blood vessels; the blood vessels which are initially localized in the epiligament move to the ligament proper (40, 63). This infiltration of blood vessels contributes to the local release of cytokines and growth factors to the injury site. As the blood vessels infiltrate the MCL, VEGF and endothelial cells increase and predominate in the forming granulation tissues (112). VEGF plays a multipurpose role in the healing process: it promotes collagen synthesis, inhibits apoptosis, and regulates vascular permeability (113-115). It also influences endothelial cells to regulate angiogenesis and ECM degradation, proliferation, migration, and tube formation.

Proliferative Phase

Proliferation is a set of four steps that begin at various time periods in wound healing: (1) Epithelialization, (2) Angiogenesis, (3) Granulation tissue formation, (4) and Collagen deposition (16). The migration of fibroblast initiates the prolifirative phase into the wound site. The migration is stimulated primarily by the PDGF that has been released by platelets and macrophages. PDGF stimulates fibroblastic proliferation, chemotaxis, and collagenase production (16). Several angiogenic factors mediate the formation of new blood vessels from pre-existing vessels. The most potent is the vascular endothelial growth factor (VEGF) (116). New vessel formation is critical for tissue regeneration due to increased metabolic rate and the need for oxygen supplied by blood vessels for oxidation and collagen formation from proline and lysine residues (76).

Fibroblasts and endothelial cells are the two major cells proliferating in this stage. In response to hypoxia, NO is produced by endothelial cells; NO stimulates more production of VEGF (14, 92). In turn, endothelial cells are influenced by VEGF to begin the synthesis of new capillary tubes. Angiogenesis is responsible for replacing damaged vasculature with granulation tissue. Fibroblasts, epidermal cells, vascular endothelial cells, and macrophages participate in angiogenesis by producing βFGF, TGF-β, and VEGF. The proliferative effects of VEGF are regulated by hypoxia which brings about VEGF-induced angiogenesis using adenosine. Furthermore, increased concentration of NO enhances the vasodilation of the endothelium and protects the regenerated tissue from the poisonous effects of ischemia and reperfusion injury (113).

Fibroblasts are mobilized into the MCL injury site from the surrounding tissues; they become activated and begin the synthesis of collagen. The main signals for recruiting fibroblasts are PDGF and Epidermal Growth Factor (EGF) and are derived from platelets and macrophages (16, 71). The wound fibroblasts synthesize collagen in response to TGF-β stimulation and

transform into myofibroblasts which mediate the contraction of the wound. TGF-β has other roles that it plays in this stage; it causes fibroblasts to synthesize type I collagen, decreases the production of MMP, enhances the production of tissue inhibitors of metalloproteinase, and increases the production of cell adhesion protein.

Apart from synthesizing structural protein, fibroblasts also produce the matrix metalloproteinases (MMPs), a set of proteolytic enzymes which enable fibroblast movement within the matrix. In the MCL, the synthesis of structural protein is regulated by two growth factors; TGF-β, which is secreted by both platelets and macrophages, and Connective Tissue Growth Factor (CTGF), which is secreted by fibroblasts (77, 117). Fibroblasts begin the synthesis of a provisional matrix made up of type III collagen, which is considered immature, glycosaminoglycans, and fibronectin in response to the PDGF stimulation (102). Epithelialization proceeds with the proliferation and migration of the epithelial cells, and the process is enhanced by EGF, keratinocyte growth factors (KGFs), and TGF-α.

The signal for turning off the activity for the proliferation stage comes from interferon-inducible protein, which inhibits EGF-induced fibroblast mobility, limiting fibroblast recruitment.

Maturation and Remodeling Phase

Repair and remodeling of tissue occur following proliferation. Initially, the extracellular matrix, mainly made up of collagen, is deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet provides a basis for a new matrix (73). This stage is characterized by the deposition of collagen in an organized manner. The newly deposited collagen is reorganized into the structurally sound lattice. The immature type III collagen is replaced with type I collagen that is structurally stronger, and wound strength is increased (16, 76). Glycosaminoglycans, proteoglycans, and other proteins are synthesized by fibroblasts and used for building up a temporary matrix framework which is later replaced by a stronger and more organized matrix made of collagen (19).

At the latter end of this stage, the inflammatory cells migrate out of the wound site, and cells that release growth factors become significantly reduced. The number of fibroblasts also begins to reduce gradually, but they continue to synthesize collagen. Cross-linking of collagen molecules is the terminal stage of this process. Healing of ligaments is slow and incomplete, creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (15, 76).

Heald Ligament Characteristics

Healing of ligaments is slow and incomplete, creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (15, 76). Saether et al. reported the differences that are observed in ligament structures after they have undergone the healing process (70); these differences include:

Histological changes: disorganized collagen, increased cell density, and metabolic rate.

Biochemical composition: reduced quantity of type 1 collagen, reduced amount of collagen fibrils, the excess quantity of glycosaminoglycans, larger proteoglycans, and fewer cross-links.

Biomechanical properties: Increased cross-sectional size, mild loss of structural properties, moderate loss of mechanical properties, and increased creep.

SUMMARY

Ligaments play a major functional role in the musculoskeletal system. When injured, the standard treatment methods are focused on pain management and return to function as appose to promoting time to recovery and healing of tissue to pre-injury properties. This study aimed to develop a rodent model to study ligament injury, provide a basis to evaluate treatment methods, and characterize tissue's response in maximal and submaximal injury models. Findings from this study will improve treatment methods, stooped work postures, injury exposure assessment approaches, and injury prevention policies.

CHAPTER 1 APPENDIX

EXPERIMENTAL PROTOCOLS

Surgical Procedure and MCL Transection Injury Protocol

1) Rats will be anesthetized with isoflurane inhalation (55, 118).
   a) Approx. 1-4% in O2 (2 L/min)
   b) Initially in a chamber and later moved to a nose mask

2) Their hind limbs are then shaven with clippers and washed with betadine solution on the medial side of the knee.

3) Under sterile conditions, a small incision (10 mm) is made in their skin on the knee joint of the right hind limb over the site of MCL.

4) The overlying connective tissue is dissected to expose the MCL.

5) **MCL Injury:** A 1-mm gap in the mid-substance is surgically created, and the gap is left without suturing and allowed to heal spontaneously.

6) The skin incision is closed using sutures.

7) After surgery, the rats are allowed free cage activity and monitored for infections and complications.

Sham Injury Protocol

1) Rats will be anesthetized with isoflurane inhalation
   a) Approx. 1-4% in O2 (2 L/min)
   b) Initially in the chamber and later moved to a nose mask
2) Their hind limbs are then shaven with clippers and washed with betadine solution on the medial side of the knee.
3) Under sterile conditions, a small incision (10 mm) is made in their skin on the knee joint of the right hind limb over the site of MCL.
   a) List tools used
4) The overlying connective tissue is dissected to expose the MCL.
5) Sham Injury: The MCL will be tugged gently using forceps without inducing injury.
6) The skin incision is closed using sutures.
7) After surgery, the rats are allowed free cage activity and monitored for infections and complications.

Platelet Rich Fibrin (PRF) Protocol

The PRF was prepared with a low speed and centrifuging technique as described in the literature and then optimized for experimental methods (29, 119, 120)

1. Rats will be anesthetized with Isoflurane inhalation.
2. Approx. 1-4% in O2 (2L/min)
3. Initially in a chamber and later moved to a nose mask
4. Blood will be collected via cardiac puncture into a 10 mm syringe
5. Blood will be emptied into individual 2 mm Eppendorf **tubes** with no anti-coagulant present
6. The collected blood is centrifuged at low speed (1300 rpm, 200 g) for 10 minutes (29).
7. A PRF clot is produced in the top layer of the centrifuge tubes (typically top 40% of the tube contains the PRF)
8. The PRF is validated by ensuring clotting has taken place and the plasma is not in liquid form.
9. The entire content of the Eppendorf is then stored at -80C until use.
10. When ready to use, the content of the tube is thawed on ice and the PRF is cut and used for the desired procedure.

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CHAPTER 2

GAITING

ABSTRACT

Ligaments play a crucial role in the musculoskeletal system in maintaining joint stability. When ligament injury occurs, a wound healing cascade begins with platelet plug formation and ends with tissue remodeling. A rat Medial Collateral Ligament (MCL) surgical injury model was developed to examine the role of modulating the initial recruitment of platelets and their impact on animal functional recovery. Animals (n=30) were randomized to an untreated group, a platelet-rich fibrin treatment group, and a Nonsteroidal Anti-inflammatory Drug (NSAID) treatment group. Footprint gait analysis was performed at select timepoints post-injury (Days 1, 3, 5, 7, 14, 28), and one parameter (Intermediate toe spread, ITS) was identified to show significant differences at early timepoints following injury. Results show that untreated animals' ITS recovered to normal function within seven days after injury while anti-inflammatory drugs slowed ITS functional recovery until after day fourteen. The PRF-treated group showed the fastest ITS functional recovery on day five. PRF makes available growth factors needed for wound healing. On the other hand, NSAIDs manage pain but probably inhibit the recruitment of platelets and thereby limit growth factors. Managing pain comes at the expense of early functional recovery. Using a rodent model opens the opportunity to evaluate different approaches to improve ligament injury recovery.

ABBREVIATIONS

ANOVA: Analysis of Variance
COP: Center of Plantar
COX: Cyclooxygenase
FBW: Front Paw Base Width
HBW: Hind Paw Base Width
IACUC: Institutional Animal Care and Use Committee
ITS: Intermediate Toe Spread
L-ITS: Left Intermediate Toe Spread
LOV: Left Overlap
LSL: Left Stride Length
MCL: Medial Collateral Ligament
MSL: Mean stride length
MX: Meloxicam
NSAIDs: Non-Steroidal Anti-inflammatory Drugs
PRF: Platelet Rich Fibrin
PRP: Platelet Rich Plasma
R-ITS: Right Intermediate Toe Spread
RICE: Rest, Ice, Compression, and Elevation
ROV: Right Overlap
RSL: Right Stride Length
TS: Toe spread

INTRODUCTION

Locomotion requires complex musculoskeletal and neurological coordination. Any deficit of the motor pathways, for example, due to injury, will lead to gait abnormalities (1, 2). Footprint analysis provides a simple and effective way of characterizing primary locomotor gait by detecting deficits associated with injury, age, or genetic manipulation. Differences in gait patterns are commonly used to characterize injury and healing response (3-6). Utilizing footprint analysis to examine functional recovery after ligament injury can serve as a valuable tool for analyzing different treatments to improve ligament injury recovery.

Investigating ligament injury-healing requires an animal model to with reproducible injury and to follow the functional recovery over time. Having a functional recovery test allows for a prospective temporal study to be performed. Many studies monitor the injury-recovery of the Medial Collateral Ligament (MCL) in e rodent models because the MCL is well characterized: ease of surgical access, reproducible injury model, and healing properties (7-11). This study has used the MCL model to investigate ligament injury-healing response under different treatment conditions. The result will help elucidate our understanding of the functional response to injury. The treatment methods under study are anti-inflammatory drugs and an emerging treatment using autologous platelet rich fibrin.

Wound Healing Phases

After an injury, ligaments undergo a series of wound healing events to restore tissue structure and function. The repair process is marked by four phases: restoration of hemostasis, inflammation, repair, and finally remodeling (12). When ligaments are disrupted, and blood vessels are severed, the endothelium is damaged. It then exposes the basal lamina to blood plasma and peripheral blood cells (13). Hemostasis is achieved when damaged blood vessels are temporarily repaired with the creation of a platelet plug/clot. This step is dependent on the level of platelets recruited at the site of injury (12). In addition, the growth factors and biomolecules released from platelets play a major role in the deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (14). Activated platelets are followed by an influx of inflammatory cells in the first 48 hours (13). This is followed by proliferation which is a set of four steps that begin at various time periods in wound healing: (1) epithelialization, (2) angiogenesis, (3) granulation tissue formation, (4) and collagen deposition (12). Repair and remodeling of tissue occur following proliferation. Initially, the extracellular matrix, mainly made up of collagen, is deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet providing a basis for a new matrix (15). The newly deposited collagen is reorganized into a structurally sound lattice, and the immature type III collagen is replaced with type I collagen that is structurally stronger and wound strength is increased (12, 13). Healing of ligaments is slow and incomplete, creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (13, 16).

Ligament Injury Treatment Methods

When ligaments are injured, there are three treatment methods to restore function. These treatments include RICE (Rest, Ice, Compression, and Elevation), NSAIDs (Non-Steroidal Anti-inflammatory Drugs), and a relatively new treatment with platelet rich concentrates (e.g., Platelet Rich Plasma/Fibrin or PRP/PRF) (17-22). The goal of the RICE treatment is to reduce additional damage to injured tissue, decrease blood flow and enzymatic activity, which are expected to reduce edema and swelling and control pain (22, 23). NSAIDs reduce the inflammatory response by

limiting the Cyclooxygenase activity. Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, which is the precursor of prostaglandins, prostacyclin, and thromboxane (24). Thromboxane is involved in platelet aggregation, and prostaglandins mediate pain sensitization. NSAIDs reduce the production of thromboxane and prostaglandins (19, 25-27). The main goal of platelet concentrates treatments such as PRF is to increase the availability of platelets, which contain biomolecules and growth factors responsible for repair at the site of injury (18). By adding platelets to the injury site, the platelets can increase the healing response and can mediate collagen expression and organization, which will then improve the time to mechanical and functional recovery (28-30).

Research Question

Complete recovery of functional and mechanical properties to that of native ligament tissue is not currently achievable (31-33). Current literature focuses on restoring original tissue functional properties and time to recovery with varying levels of healing based on the treatment applied (16, 34-36). The standard treatments with NSAIDs can manage pain, but their effectiveness in restoring ligament structure and function, however, appears moot (22, 37). Some authors argue that NSAIDs only help to relieve pain symptoms (11, 22, 25, 38). The improved return to normal mobility reflects success in pain management but masks any real structural and functional restoration of injured tissue (22, 38-40). With respect to PRF treatment, the lack of standardization in creating and activating platelets, in testing its efficacy, and in measuring quantitatively the range of outcomes limit the enthusiasm for deploying PRF as a standard treatment for ligament injuries (28-30).

Establishing a ligament injury animal model will open many opportunities to study ligament injury and treatments to restore function. The functional recovery characterization provides a critical basis to investigate the corresponding changes in signaling molecules, and histology, which will serve as a cross-reference to the gaiting observations. These findings together will paint a comprehensive picture of healing after a ligament injury.

Study Aim

This experiment aims to establish an MCL injury in a rat model and characterize injury recovery with gaiting parameters under different treatment conditions. The experiments utilized observed and analyzed footprint analysis to detect significant gait differences during recovery. A multitude of studies have utilized the methodology (4, 6, 41-46). Observational gait or footprint analysis is a critical tool used to test rodent models due to its utility in producing quantifiable physical and behavioral data for a given disease, injury, or drug effects on locomotion (47, 48). Detecting ligament injury at early stages allows for temporal tracking and examining animal locomotion at different stages of wound healing and under different treatment conditions. A gait footprint test follows a rodent's locomotion during a walk along a straight path towards a target enclosure. When animals are impaired, the footprint placements become more variable, allowing for tracking progression towards healing under different conditions. Gaiting performance during recovery will test the efficacy of anti-inflammatory drugs (Meloxicam) and platelet-rich concentrates (PRF).

MATERIALS AND METHODS

Animals

The study was approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC). Thirty skeletally mature male Wistar rats (The Jackson Laboratory) were used as an animal model for ligament healing. The animals were randomly assigned to 3 treatment groups: Untreated, Platelet Rich Fibrin, and Meloxicam (n=10/group). Animals were fed rat chow and housed in pairs per cage under the care of a university veterinarian. Detailed surgical procedure is described in detail in chapter 1. Briefly, a surgically transected, rather than torn, Medial Collateral Ligament (MCL) of the knee was used as an experimental model to create a uniform defect for healing. The surgical transection was done on the right side and sham injury on the left side as described in the literature (49).

To obtain footprints for further analysis, animals' paws are coated with non-toxic paints using fine paintbrushes with two different colors for the front and hind paws, and the animal is allowed to walk along a narrow, paper-covered corridor, leaving a track of footprints. Once the footprints have dried, measurements are taken in centimeters manually using a pencil and ruler. In this study, eight parameters were measured: right and left stride lengths, front and hind paws base widths, paws overlap, mean stride length, toe spread, and inner toe spread (6, 50).

Testing Apparatus Setup

The testing apparatus is set up with a corridor leading to an enclosed goal box at one end, as depicted in Fig. 1. The experimental-room environment is kept constant (with respect to temperature, humidity, and light intensity) for all test sessions (42, 48, 50). Each animal is given an identification number used to label the corner of each sheet of white paper so that each rat has its own sheet for each run. At the beginning of each run, the sheet of paper is placed lengthwise on the floor of the runway, and the sides of the paper are wider than the width of the corridor to ensure all footprints are captured.

Animal Training Procedure

Animals require acclimatization and training on the testing apparatus to ensure a successful test run (50). Animals are transferred in their home cages from the holding room to the experimental room and allowed to habituate to the experimental room for at least 60 min. After acclimatization, a subject is removed from their cage and held by the scruff of the neck. A simulation of painting the paws is performed by passing a dry paintbrush on the paws. Animals are then immediately placed at the end of the sheet of paper opposite the goal box and allowed to run over the paper to the goal box. The researcher would then initially tap on the table without touching the animal to encourage movement and then animals are allowed to walk freely towards the goal chamber. Performance should be stable before testing and data collection.

Treatment Groups

There are three treatment groups in this study:

- **Untreated:** Surgical injury to animals with no additional treatment and animals are allowed to heal under normal conditions.
- **Meloxicam Treatment:** Subcutaneous Meloxicam injection (Ostilox, 5 mg/mL, MWI/VetOne, Boise, ID, USA) at a dose of 0.2 mg/kg at the site of MCL transection immediately after surgery.

- **Platelet Rich Fibrin Treatment:** Previously prepared PRF from donor animals and stored at -80 degrees Celsius is thawed out on the ice and sutured with resorbable sutures internally at the site of MCL transection followed by suturing the skin.

Experimental Run Procedure

At the time of performing an experiment, a small quantity of each nontoxic paint is placed into separate Petri plates. While holding an animal by the scruff of the neck, forepaws are painted one color (blue) and the hind paws the other color (red). Two different paintbrushes are used for each color, and just enough paint is used to cover the pads of the paws to minimize smudging or blurred footprints during testing and data collection. Once paws are painted, animals are immediately placed at the end of the sheet of paper opposite the goal box. This process is repeated to produce three runs for each animal using a fresh sheet of the paper and reapplying paint for each trial. The sheet of paper is removed from the runway and allowed to dry in a well-aerated room before storing. In cases where the resulting footprint pattern is poor (for example, the paint smudges or the rat stops walking midway through the trial), the animal is given a second trial. Animals are returned to their home cages after the final trial, and at the end of the day, all cages are returned to the holding room. All testing apparatus and surfaces are cleaned apparatus thoroughly with 70% ethanol.

Gait Parameters and Data Collection

Eight parameters are collected from the footprints collected from the experimental runs. The method to obtain these parameters is described by Carter and is listed below and shown in figure 3 (50).

- **Center of Plantar (COP)**: The center of the paw print is marked by two lines. One line is horizontal across the print, starting just below the most lateral toe. The other is a vertical line from the toe to the heel of the paw print.
- **Hind Paw Base Width (HBW)**: The distance between the left and ride center of the hind paws.
- **Right Stride Length (RSL)**: The distance between the forepaw center and back paw center for the right side. Stride length and width require clear sequential prints where the forefoot region is well defined in paint.
- **Left Stride Length (LSL)**: The distance between the forepaw center and back paw center for the left side.
- **Toe spread (TS)**: The distance between the most lateral and distal toes. Toe spread does not require sequential prints for scoring, only clear prints of the first and last toes on a single foot.
- **Inner toe spread (ITS):** The distance between the innermost toes on the hind paw. ITS is measured on both the right and left sides resulting in **R-ITS and L-ITS.**
- **Right and Left Paws overlap (ROV, LOV):** The distance between the center of the planter for the front and hind limbs on the right and left side, respectively.
- **Front Paw Base Width (FBW)**: The distance between the left and ride center of the front paws.
- **Mean stride length (MSL)**: The distance between the center of the front paws and the center of the hind paws.

Data Processing and Analysis

Data was compiled in excel sheets for further analysis. Statistical analysis used Minitab (Minitab, LLC. (2021). *Minitab*. Retrieved from https://www.minitab.com) and Microsoft Excel. Significance was determined by General Linear Model analysis of variance (ANOVA) using an alpha (α) value of 0.05 and post hoc analysis was done using Tukey and Dunnett methods where applicable (4, 50).

RESULTS

Testing Apparatus

The testing apparatus shown in figure Fig. 2-1 includes a plexiglass corridor with white butcher paper cut to 6" widths for the entire length of the track that measured 40". The goal chamber is a dark enclosure with animal bedding placed inside to motivate the animals to walk towards the goal chamber. Fig. 2-2 shows a sampling footprint with manual data measurements. The measurements are marked on the paper and then later transferred to an excel sheet for data keeping and analysis. A visual sample of the different parameters from one run is shown in Fig. 2-3.

Pilot Study Results

A pilot study was initially performed using one animal for each treatment. Three runs were done for each animal, and 6 to 9 data points for each parameter were obtained. Initial results are shown in tables 2-1 to 2-15 and Figures 2-4 to 2-15. Fig. 2-4 to 2-9 show box plots for parameters that did not indicate any significant difference from control levels as determined by ANOVA in Tables 2-1 to 2-7. These parameters were LSL, RSL, HBW, MBW, L-ITS, LOV, and ROV. Based on this finding, these parameters were not considered for any future experiments. Fig. 2-10 to Fig. 2-12 show parameters (FBW, LTS, RTS) that had a significant difference from control levels as determined with single-factor ANOVA in tables 2-8, 2-10, and 2-12. However, upon further analysis and post hoc analysis of the different time points using the Tukey method (Tables 2-9, 2-11, and 2-13), these parameters did not show significant differences at early stages of healing and therefore were not considered for future studies. Fig. 2-13 shows boxplots for the untreated group's pilot study gait analysis results for the (A) Right Inner Toe Spread (R-ITS) and (B) the Left Inner Toe Spread (L-ITS). The R-ITS shows an initial significant decrease and a recovery after day sixteen as proven by ANOVA in Table 2-14 and Tukey Post Hoc-Analysis in Table 2-15. For Meloxicam treatment, Fig. 2-14 Shows the (A) Right Inner Toe Spread (R-ITS) and (B) for the Left Inner Toe Spread (L-ITS) with an initial decrease and a sooner recovery than untreated animals as shown in ANOVA and Tukey Post-Hoc Analysis Tables 2-16 and 2-17. Tables 2-18 and 2-19 show the Meloxicam L-ITS ANOVA and post hoc analysis results which indicates that the control group is not significantly different from all other groups while days 14 and 22 are different from each other. Fig. 2-15 shows the Platelet Rich Fibrin (PRF) group's pilot results for the (A) Right Inner Toe Spread (R-ITS) and (B) for the Left Inner Toe Spread (L-ITS) with no apparent initial dip as seen in the untreated and MX groups. Table 2-20 shows the R-ITS to not have a significant difference between the control and the different time points. On the other hand, ANOVA results and post-hoc analysis for L-ITS (Tables 2-21 and 2-22) show a significant difference, with the control group not being different from days 3, 6, and 22. The average ITS on days 2, 10, and 14 is higher than control levels on the sham side.

Full Study Results

Each experimental group randomly received ten animals. After two days of acclimatization in the housing facility, animals underwent initial gait testing, and thee runs of footprints were collected for each animal. The surgical procedure was undertaken to induce ligament and sham injury on the same day. The surgical procedure is described in chapter 1 appendix. Following injury, gait testing was performed on Days 1, 3, 5, 7, 14, and 28. Fig. 2-16 shows ITS results for the untreated group. Initially, the R-ITS decreased from control levels preinjury and gradually over the study period approached control levels within seven days. ANOVA and Dunnett post hoc analysis for the untreated group with the sham pre-injury ITS acting as the control is displayed in tables 2-23 and 2-24, and Fig. 2-20. Significant differences were realized between the control and the injured limb on days 1 to 7. All other time points for the injured and sham limbs were the same as the controls. Fig. 2-17 shows ITS results for the MX group. With Meloxicam treatments R-ITS showed a similar initial significant decrease (Table 2-25) and recovery to control levels after 14 days, as shown by Dunnett post hoc analysis (Table 2-26 and Fig. 2-21). For the PRF group, Fig. 2-18 shows ITS results with PRF-treated animals showing a significant change in R-ITS gait patterns (Table 2-27) and grouping of days 1, 3, and 5 on the injured limb marking an early recovery by day seven as shown in the Dunnett post hoc analysis in Table 2-28 and Fig. 2-22.

A normalized ITS Table was calculated for each timepoint based on the average ITS value on day zero as the baseline for each treatment. The resulting data is displayed in Fig. 2-19. The L-ITS for all treatments were unchanged from baseline. However, on day one, the R-ITS dropped 17% for the untreated group, 12% for the MX group, and 8% for the PRF group, with a gradual return to baseline on subsequent days. The chart also shows a steeper recovery of R-ITS with PRF treatment compared to MX and untreated animals.

DISCUSSION

Among the most challenging problems faced by musculoskeletal injury investigators are the selection of appropriate evaluation methods for recovery of function during repair (5). Having a reliable method to examine changes in gait patterns allows for evaluating different treatment methods in a systematic and standardized fashion. Footprint analysis methods use variations from the normal uninjured state of gaiting and compare them to gait patterns of animals under different treatment conditions. Initial and subsequent gait results in this study show that functional evaluation methods can be used to examine MCL healing response following a surgical transection injury as well as provide a utility to evaluate the impact of treatment methods.

To date, there is not one single solution to help restore injured ligaments to their original native state (31-33). However, varying levels of functional healing or return to function based on the treatment are achievable, as seen in this study. Nonetheless, there is insufficient evidence to support standard treatments for all types of ligament injury; however, some clinical treatments appear to be deployed to control pain and swelling which are byproducts of the wound healing and inflammatory response (16, 22, 34, 36, 37, 51). Furthermore, some authors argue that standard treatments (e.g., NSAIDs) only help relieve pain symptoms with no other utility for restoring structure or function (11, 22, 25, 38).

Animal Model

To investigate ligament injury and healing response, an animal model was developed. The Medial Collateral Ligament (MCL) was chosen as the experimental rodent model due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (7-11). Animals and procedures were conducted as described previously

Footprint Analysis

After MCL injury, initial results showed that the Inner Toe Spread (ITS) could serve as a reliable marker for tracking the recovery of function. Other studies have used toe spread and intermediate toe spread as a marker for recovery (52, 53). The mechanism of ITS in the context of ligament injury is unclear; however, we speculate it is due to weight shifting and favoring the sham injury limb leading to reduced spread between the inner toes. Similar behavior of changes in ITS was observed in studies investigating sciatic nerve injury and recovery (54-56).

Statistical analysis of ITS for the untreated group shows that the ITS for the MCL injured limb decreased significantly between day one and day seven post-injury and recovered to control levels after day seven. On the other hand, ITS for the sham injury in untreated animals was the same as control levels for all time points. For the Meloxicam-treated animals, the ITS for the injured limb decreased significantly between day one and day fourteen post-injury and recovered to control levels after day fourteen. Similarly, ITS for the sham injured limb with Meloxicam treatment was the same as control levels for all time points. With PRF treatment, the ITS for the injured MCLs decreased significantly between day one and day five post-injury. ITS then recovered to control levels after day five while sham MCLs remained at control level for all time points. This observation for sham MCLs is similar in the untreated and Meloxicam groups.

Impact of MCL Injury and Healing Phases on Gait Patterns

Untreated Group

The untreated animals' gait results for ITS is a control reference for the timeframe needed for recovery to function with no intervention. In this study, untreated animals recovered to function after seven days of injury, and this appears to be consistent with the start and end of the inflammatory phase which typically lasts for five days depending on the extent of injury (13, 57). Following trauma to ligament tissue, an influx of immune cells to purge injured tissue of debris and stimulate cytokines that are important to further healing processes and events. Inflammatory cells are released, and a retraction of the disrupted ligament is noted. In addition, a blood clot is formed, which is later resorbed and replaced with a denser cellular infiltrate (58, 59). This, together with fluid from damaged cells, produces swelling within the joint, putting pressure on nerve endings causing pain. This sensation of pain and discomfort appears to impact the amount of pressure animals are willing to exert on their injured paws and thereby reducing the inner toe spread. Subsequent recovery to control levels suggests that animals tolerate more pressure on the injured limb and perhaps less pain sensation and can therefore mobilize to preinjury ITS levels.

Meloxicam Group

With Meloxicam treatment, the delay in recovery of ITS until 14 days post-injury can potentially be reasoned with the impact of anti-inflammatory drugs on the production of Cyclooxygenase and subsequently on platelet recruitment and wound healing response. Activated platelets degranulate releasing the content of their granules which are rich in growth factors necessary for further steps in wound healing. When Meloxicam, a non-steroidal anti-inflammatory drug (NSAID), is administered, the pathway to producing Cyclooxygenase (COX) is impacted by means of reducing the biosynthesis of prostaglandins and thromboxane through direct inhibition of cyclo-oxygenase, and consequently, reduction in pain and inflammation (60). COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, which is the precursor of several molecules, including prostaglandins, prostacyclin, and thromboxane (24). Meloxicam is a COX-2 preferential inhibitor and bears the same effect on the production of

thromboxane and prostaglandins as other NSAIDs with studies showing 77% inhibition of thromboxane (20, 60).

Thromboxane is involved in platelet aggregation and prostaglandins mediate pain sensitization are both inhibited with NSAIDs (19, 25-27). Thromboxane stimulates activation of new platelets as well as increases platelet aggregation (61, 62). Limiting its production with NSAIDs has a direct impact on the developing thrombosis, or fibrin plug, which allows for restoration of hemostasis as well as providing a fibrin mesh that is rich in growth factors that are released slowly during the process of wound healing (18, 63).

As platelets aggregate, they become activated and change their shape and degranulate releasing their content of biomolecules and growth factors stored in their granules (13, 64, 65). This leads to further recruitment of platelets until a fibrin clot or scaffold is formed leading to a stable plug and, therefore, restoration of hemostasis (13, 66, 67). In addition, the growth factors and biomolecules released from platelets play a major role in subsequent wound healing events such as deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (14).

Based on our gaiting observation after MX treatment of delayed return to control levels (after day fourteen) for the ITS, it appears that interfering with initial stages of wound healing as in hindering platelet recruitment and aggregation with COX inhibition and consequently making available less growth factors and biomolecules leads to delays in functional recovery. With MX treatment, the content of platelets that could be released at the injury site is also reduced. Subsequently, this may lead to less intensity of inflammation, proliferation, and repair phases (68-70). And therefore, ITS functional recovery may be delayed in recovery by extended sensation of pain due to possibly an extension in the inflammatory phase and extended swelling and expression of prostaglandins.

PRF Group

Platelet concentrates and initially Platelet Rich Plasma (PRP) was first introduced in 1998, emphasizing the rich growth factor content following platelet degranulation (71, 72). The main goal of platelet concentrates treatments is to increase the availability of platelets that contain biomolecules and growth factors responsible for repair at the site of injury (18, 30, 63, 73, 74). An increased level of response is sought so that collagen expression and organization are ultimately improved, thereby reducing the time to mechanical and functional recovery (28-30).

Our results show that R-ITS was significantly different from the control group for days one through five, thus returning to normal function at an earlier timepoint compared to untreated controls at day seven and Meloxicam at day fourteen. This faster recovery to function with PRF could be interpreted with the availability and proximity of biomolecules and growth factors released from activated platelets slowly and steadily out of the fibrin mesh (18). Similar effects were observed with rat osteoblasts where PRF released autologous growth factors gradually and expressed a stronger and more durable impact on proliferation and differentiation (75).

In contrast with Meloxicam treatment, PRF has no interference with the inflammatory phase of wound healing. On the contrary, we speculate that wound healing reaches the proliferative phase and remodeling phases sooner and with higher intensity than untreated groups due to the "shortcut" of making available activated platelets at the site of injury by means of PRF.

Recent studies suggest that platelet rich concentrates possess analgesic properties, thus providing pain relief at the site of injection (72, 76). With pain suppression, animals are more likely to mobilize sooner, and early mobility has been argued to better heal various soft tissue injuries (11, 22, 77). Hauser et al. argue that early controlled resumption of activity after ligaments and tendons injury has beneficial effects, including enhanced cellular synthetic and proliferative

effects, increased strength, size, matrix organization, and collagen content of ligaments and tendon (11). Although our study did not investigate pain suppression with PRF, it is an area worth further investigation.

CONCLUSION

Ligaments play a major functional role in the musculoskeletal system. When injured, the standard treatment methods are focused on pain management and return to function as appose to promoting time to recovery and healing of tissue to preinjury properties. This study aimed to provide a basis to discriminate between the functional gaiting performance of injured and healing MCL under different treatment conditions. With untreated animals, recovery to function took seven days, while with Meloxicam treatment, animals recovered to function after fourteen days. The fastest recovery to function was realized after five days with PRF treatment which appears to have an effect on speeding up the wound healing response by making available platelets and growth factors released in a steady and "natural" manner as opposed to NSAIDs which interfere with platelet aggregation and have the opposite effect on the timeliness of wound healing.

Learning from this study provides a basis for examining ligament injury and healing in a controlled laboratory setting and provides insight into the use of current and emerging treatment methods. For example, ligament treatment methods should focus on treating the injury and improving the wound healing process as in PRF treatment while at the same time allowing for managing pain without interfering with platelet aggregation and activation processes necessary for making available growth factors that are integral for healing. For instance, alternative pain suppression treatments other than NSAIDs would be worth investigating further to allow for early mobility without hindering natural healing.

CHAPTER 2 FIGURES
Figure 2-1

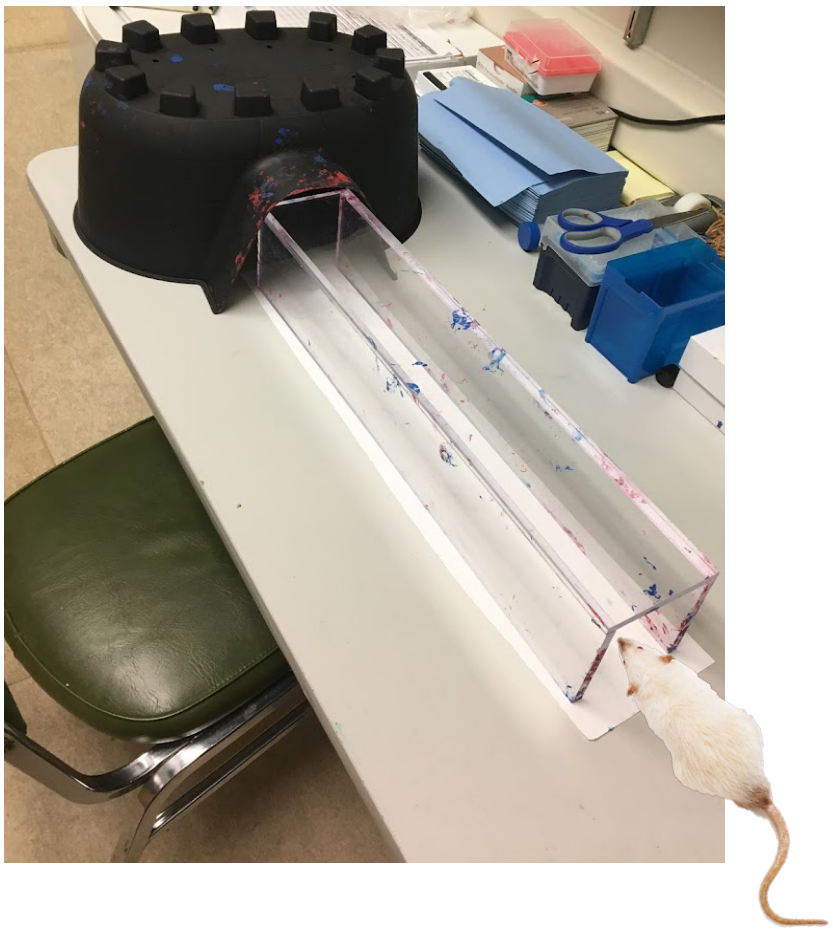


Figure 2-1
Gait testing apparatus. The apparatus includes a plexiglass corridor (a) with white butcher paper (b) cut to 6" widths for the entire length of the track that measured 40". The goal chamber (c) is a dark enclosure with animal bedding placed inside to motivate the animals to walk towards the goal chamber. Animals are initially placed at the beginning of the corridor. The researcher would then initially tap on the table without touching the animal to encourage movement and then animals are allowed to walk freely towards the goal chamber. This process is repeated 3 times per animal. The strips of paper are inspected for each run visually to ensure at least two to four clear consecutive footprints for the forepaws, and hind paws are observed without any smudges. When completed, animals are placed back in their cages. The testing area and tunnel are then wiped down with ethanol or an equivalent cleaning solution between each animal.

Figure 2-2

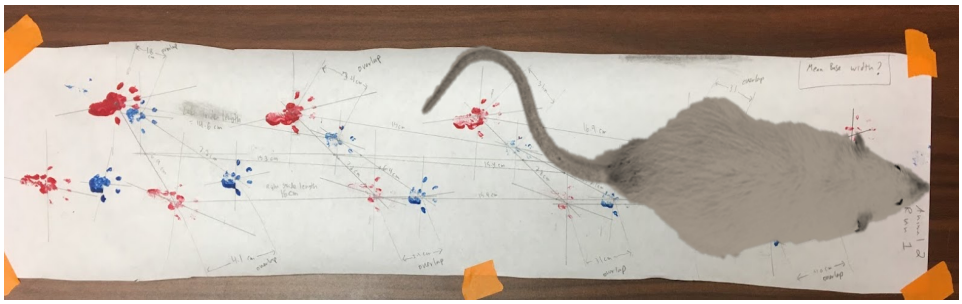


Figure 2-2
A sample of footprints and measurements. To obtain footprints, the animals' paws and the center of the foot are fully coated using a small paintbrush with non-toxic paints. Two different colors are used for the front and hind paws (red for hind paw and blue for front paw in our case) and the animal is allowed to walk along a narrow, paper-covered corridor, leaving a track of footprints. Once the footprints have dried, measurements can be taken from the prints manually. Measurements are not taken from the beginning and ending regions on the sheet of paper as the animals are changing the walking speed at the start and end of the tunnel. Before scoring, the paper with footprints is allowed to dry fully overnight. Eight parameters were measured: **right and left stride lengths**, **front and hind paws base widths**, **paws overlap**, **mean stride length, toe spread, and inner toe spread** (50).

Figure 2-3

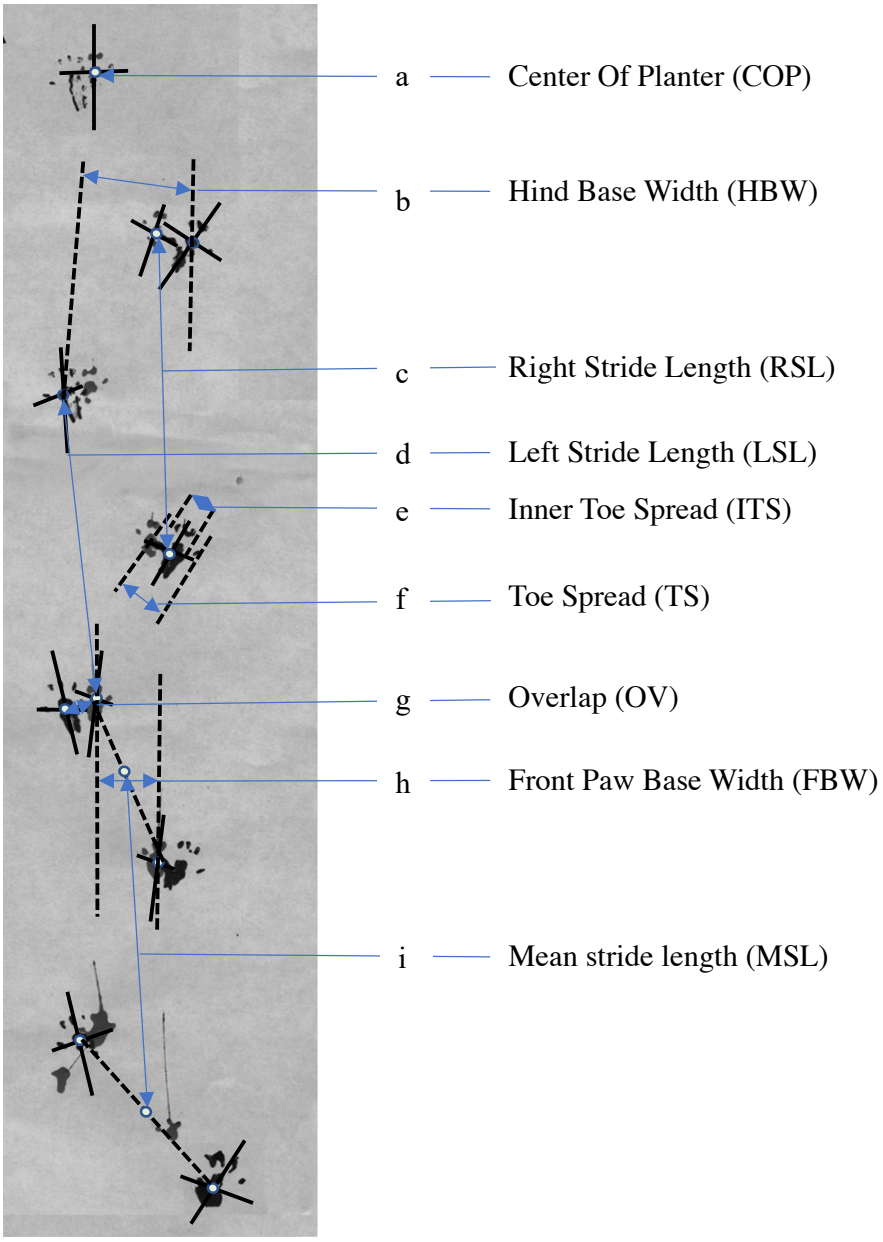


Figure 2-3
Gait Parameters. The diagram shows a sample run of an animal's gait and markings for data collection. Data collection of the parameters is done manually using a ruler and pencil. The center of the paw is determined as described, and then measurements are obtained. Two to three data points were recorded of each parameter within each run. Each animal completed their run 3 times and therefore, 6 to 9 data points were obtained for each parameter.

Figure 2-4

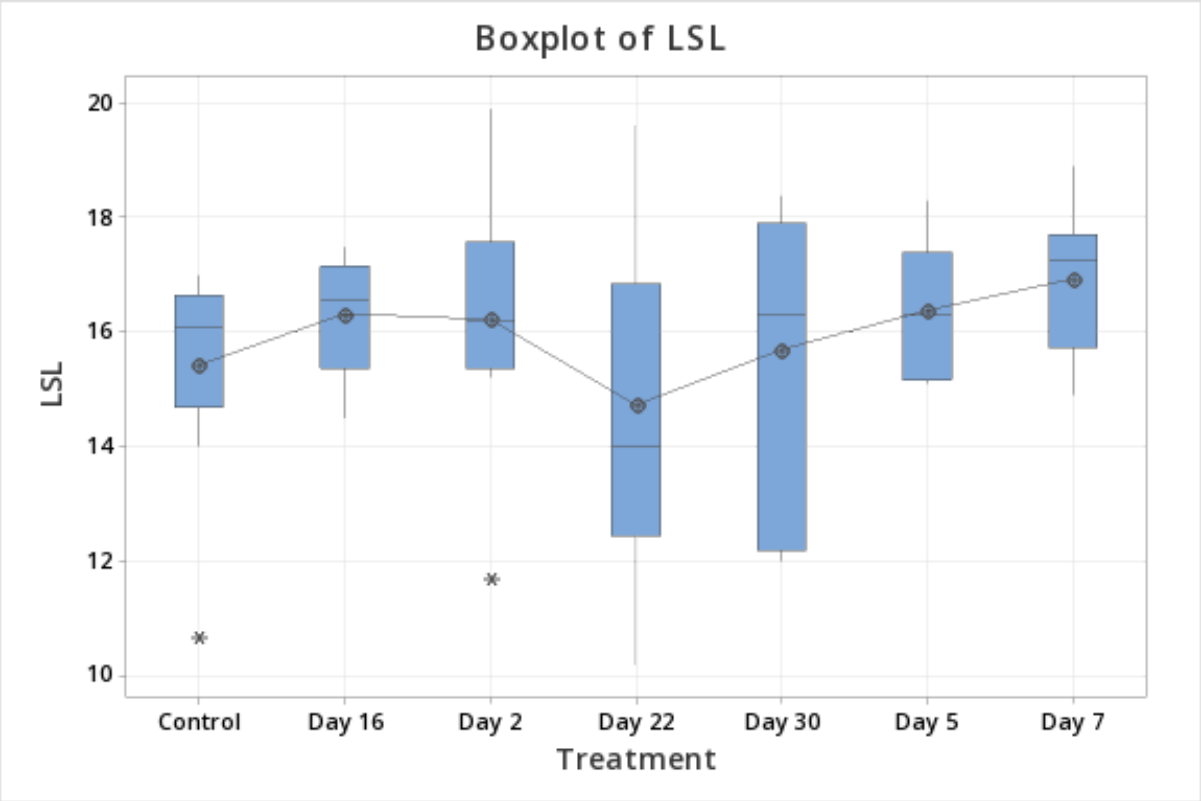


Figure 2-4
Pilot Results for Left Stride Length (LSL). Boxplot showing left stride length (LSL) results at each timepoint.

Figure 2-5

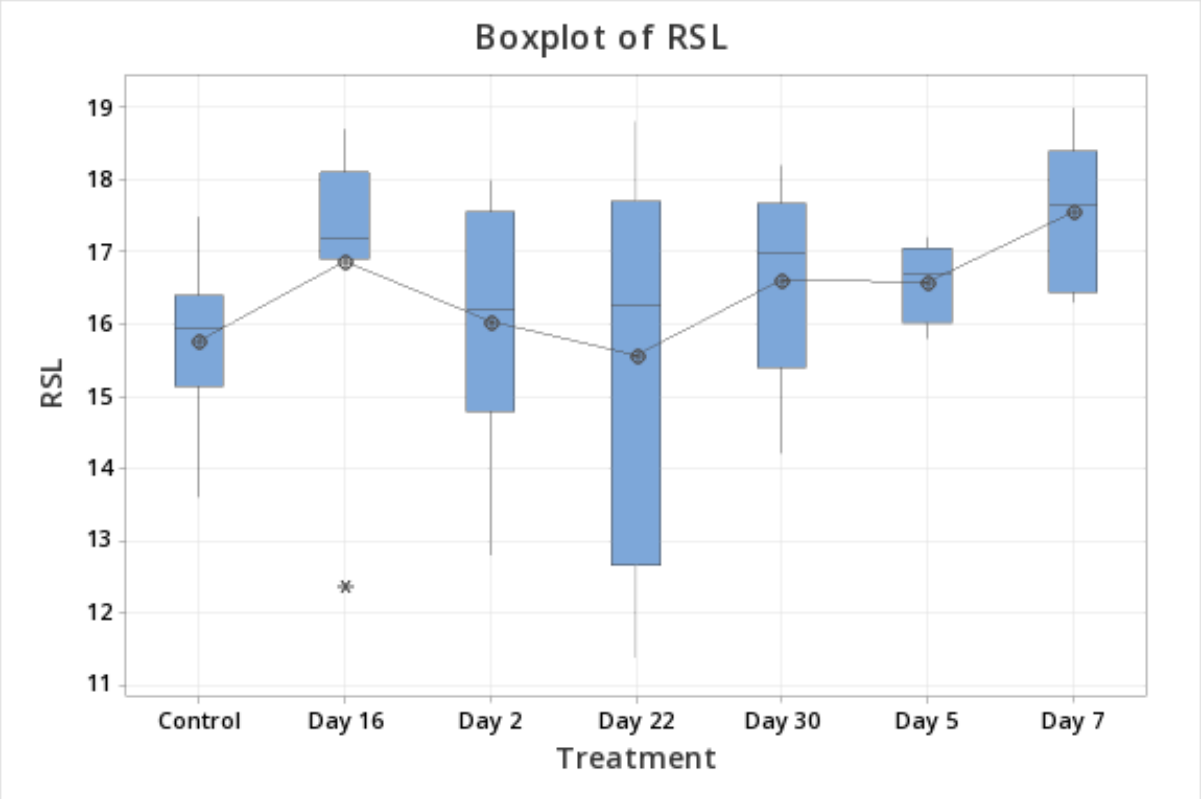


Figure 2-5
Pilot Results for Right Stride Length (RSL). Boxplot showing right stride length (RSL) results at each timepoint.

Figure 2-6

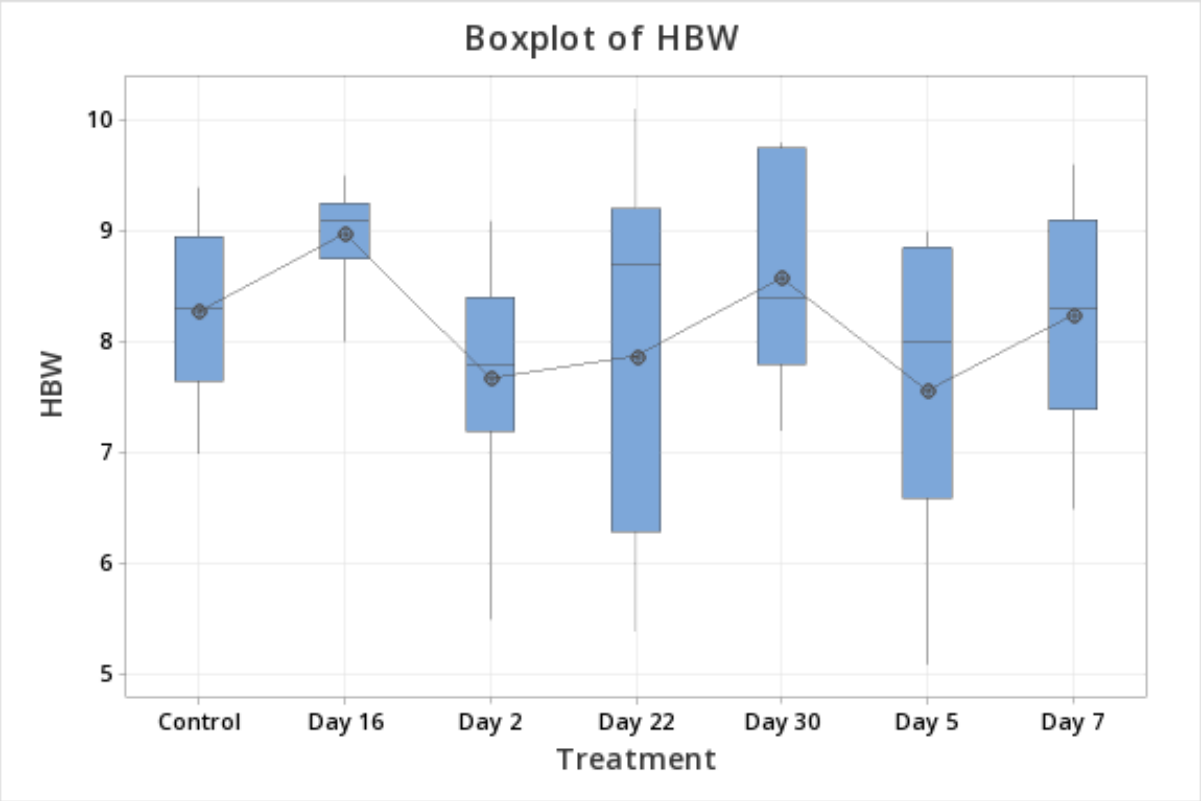


Figure 2-6
Pilot Results for Hind Base Width (HBW). Boxplot showing hind base width results at each timepoint.

Figure 2-7

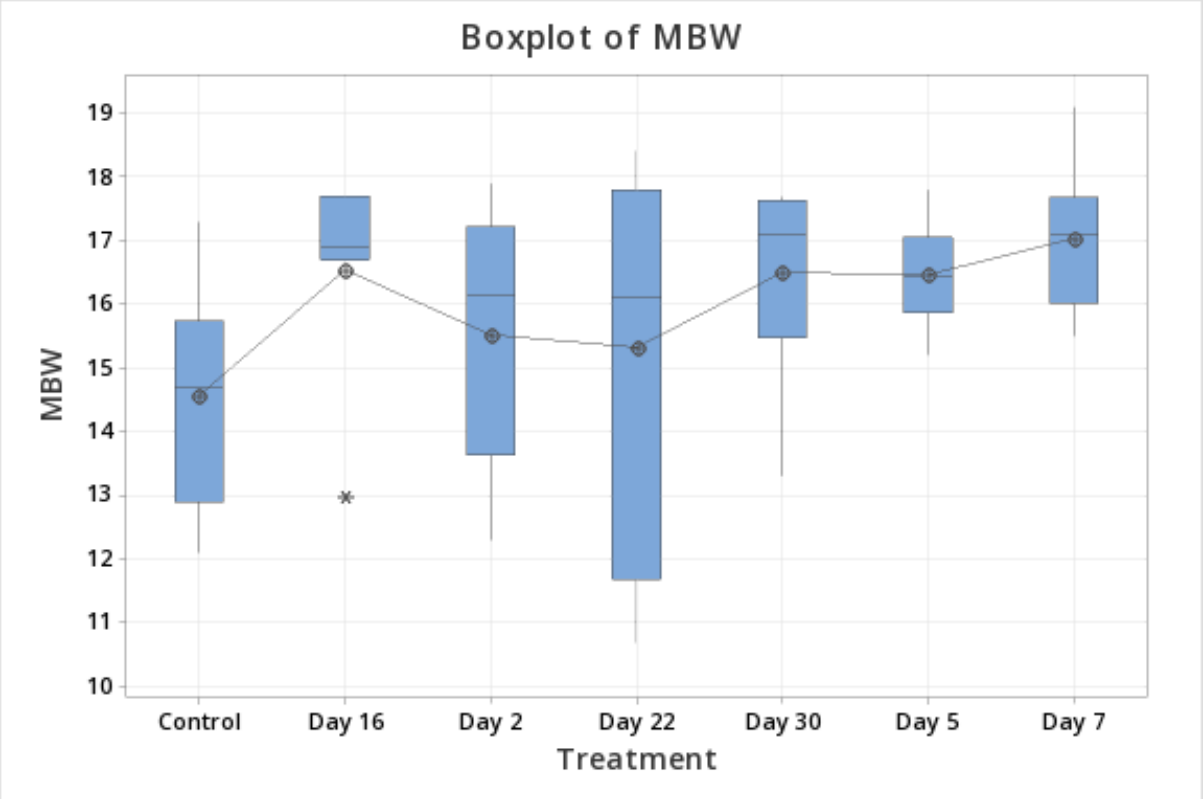


Figure 2-7
Pilot results for Mean Base Width (MBW). Boxplot showing mean base width (MBW) results at each timepoint

Figure 2-8

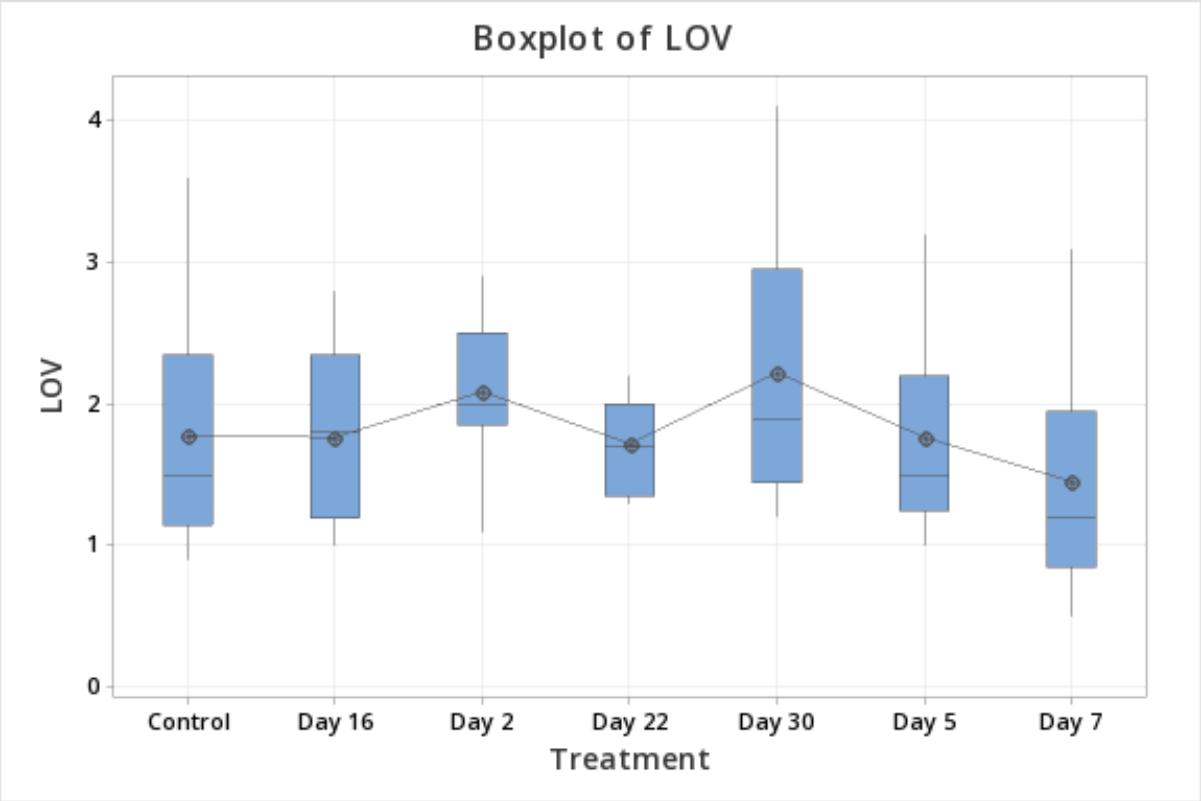


Figure 2-8
Pilot Study Results for Left Overlap (LOV). Boxplot for Left Overlap (LOV) showing results for each time point.

Figure 2-9

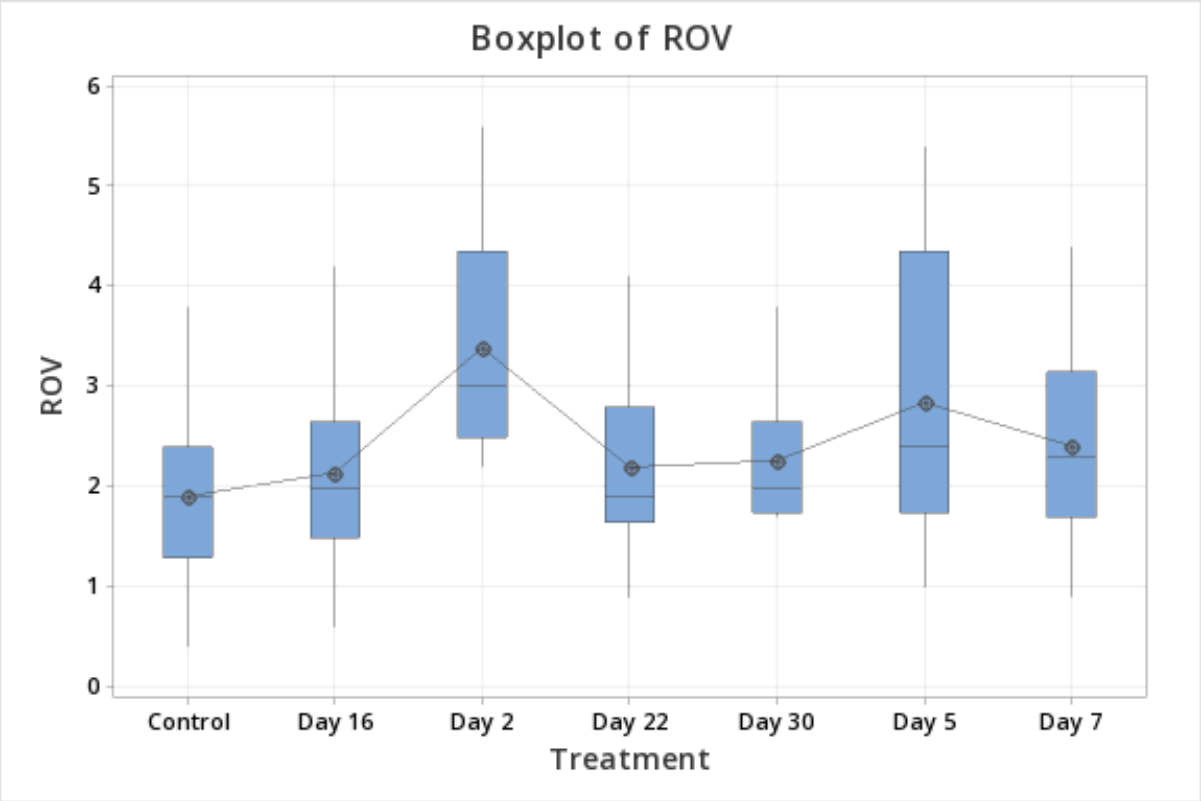


Figure 2-9
Pilot Study Results for Right Overlap (ROV). Boxplot for Right Overlap (ROV) showing results for each time point.

Figure 2-10

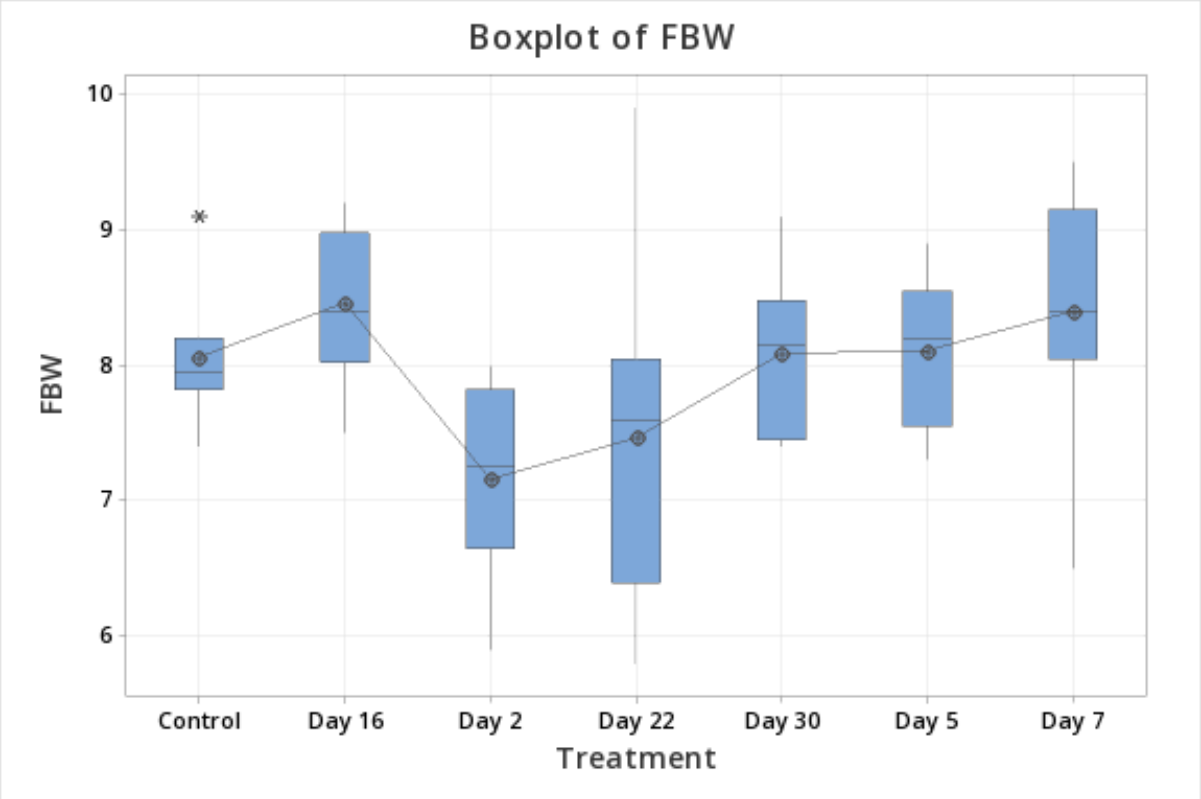


Figure 2-10
Pilot Study Results for Fore Base Width (FBW). Boxplot showing fore base width results at each timepoint.

Figure 2- 11

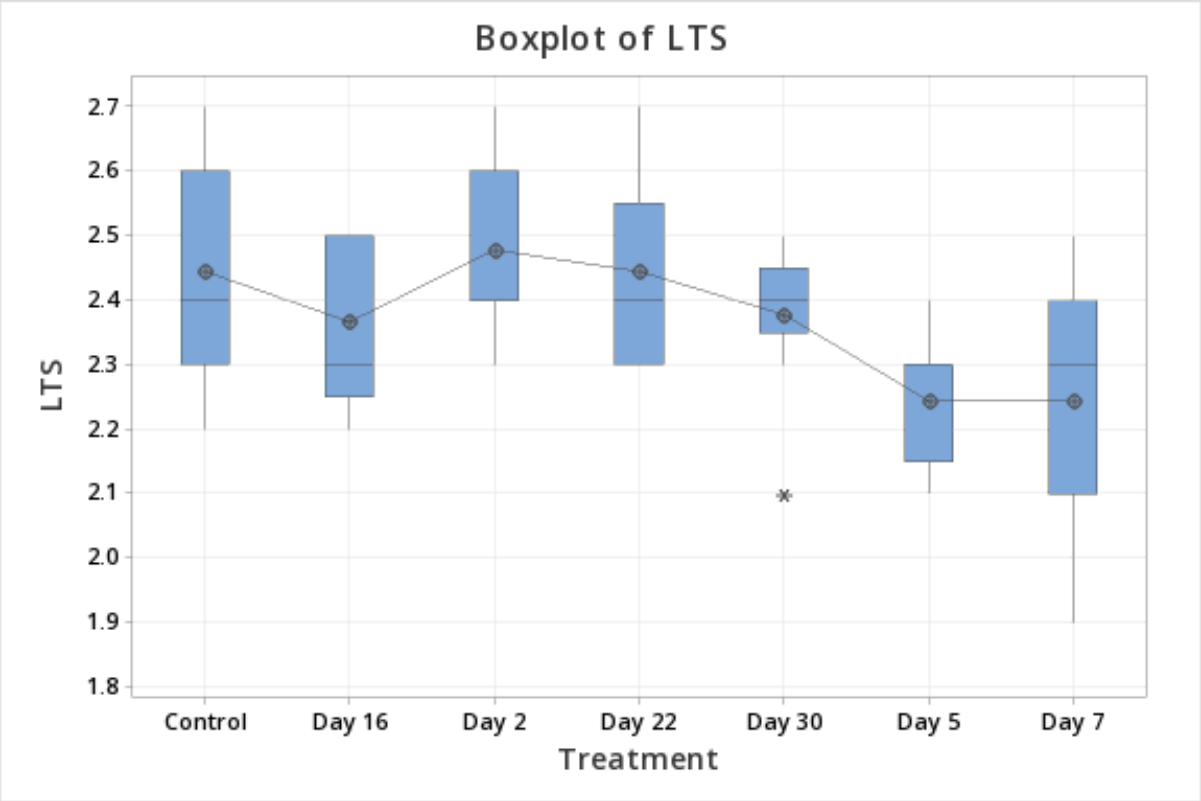


Figure 2-11
Pilot results for Left Toe Spread (LTS). Boxplot showing Left Toe Spread (LTS) results at each timepoint.

Figure 2-12

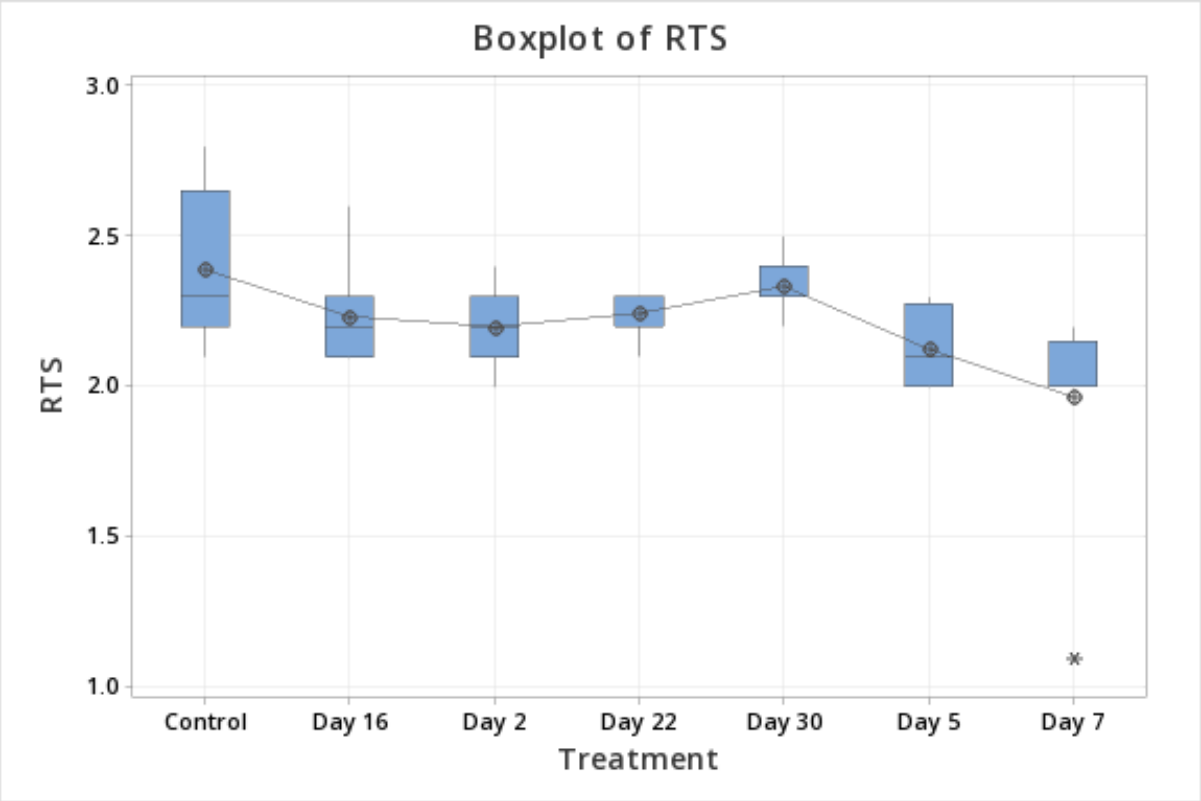


Figure 2-12
Pilot Study Results for Right Toe Spread (RTS). Boxplot showing Right Toe Spread (RTS) results at each timepoint.

Figure 2-13

Figure 2-13 (A): Untreated Group Pilot Study Results for Right Inner Toe Spread (R-ITS)

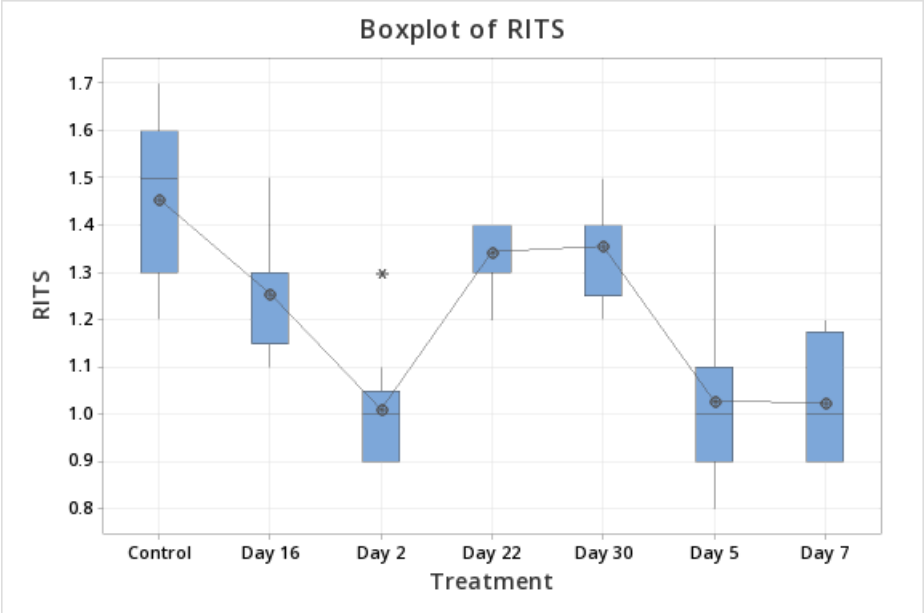


Figure 2-13 (B): Untreated Group Pilot Study Results for Left Inner Toe Spread (L-ITS)

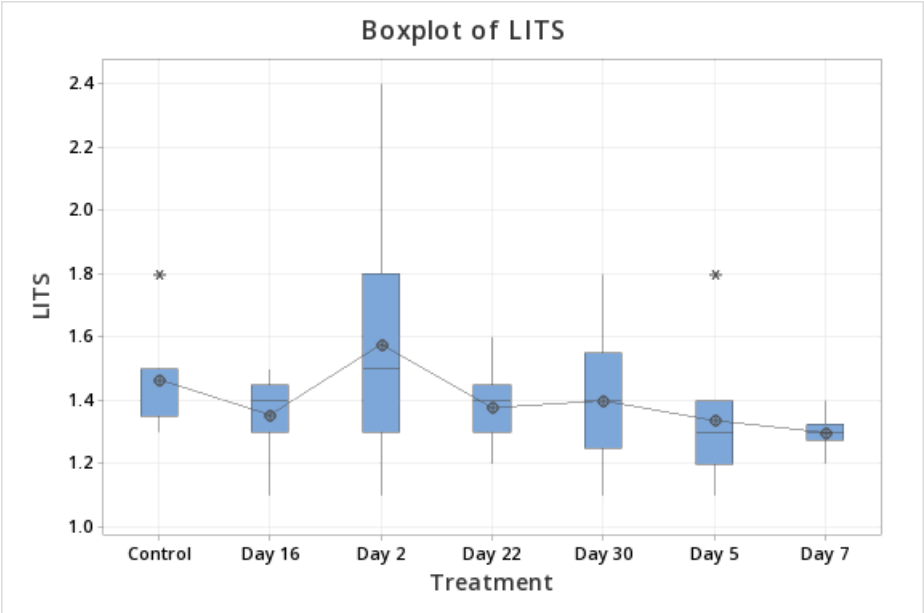


Figure 2-13
Pilot Study Results for Inner Toe Spread - Untreated. A) Boxplot for Right Inner Toe Spread (R-ITS) showing results for each time point. Control animals did not receive any additional treatment aside from the transection and sham injury. B) Boxplot for Left Inner Toe Spread (L-ITS) showing results for each time point.

Figure 2-14

Figure 2-14 (A): Meloxicam (MX) Treatment Pilot Study Results for Right Inner Toe Spread (R-ITS)

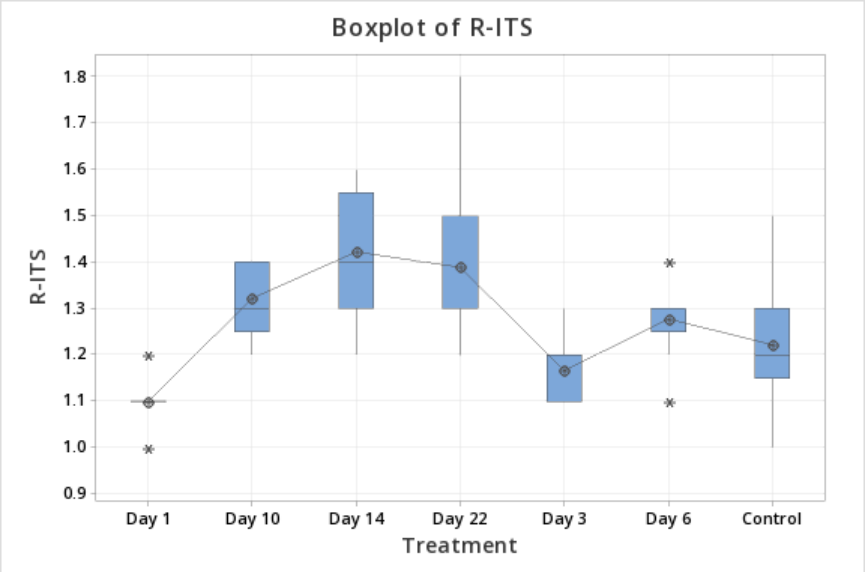


Figure 2-14 (B): Meloxicam (MX) Treatment Pilot Study Results for Left Inner Toe Spread (L-ITS)

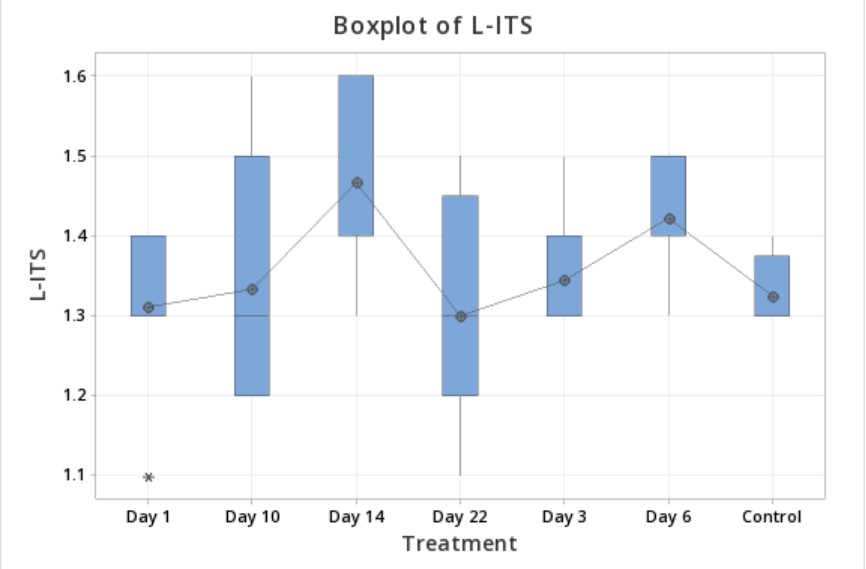


Figure 2-14
Pilot Results for Inner Toe Spread - Meloxicam (MX) Treatment. A) Boxplot for Right Inner Toe Spread (R-ITS) showing results for each time point. Meloxicam-treated animals received an MCL transection injury on the right side and sham injury on the left side. Injection of MX at the site of ligament injury was done immediately after surgery. B) Boxplot for Left Inner Toe Spread (L-ITS) showing results for each time point. Meloxicam-treated animals received an MCL transection injury on the right side and sham injury on the left side. Injection of MX at the site of ligament injury was done immediately after surgery.

Figure 2-15

Figure 2-15 (A): Platelet Rich Fibrin (PRF) Treatment Pilot Study Results for Right Inner Toe Spread (R-ITS)

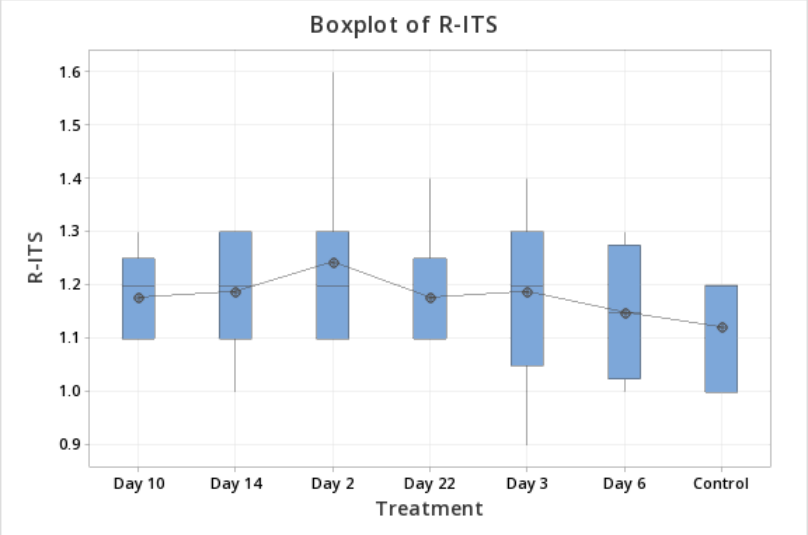


Figure 2-15 (B): Platelet Rich Fibrin (PRF) Treatment Pilot Study Results for Left Inner Toe Spread (L-ITS)

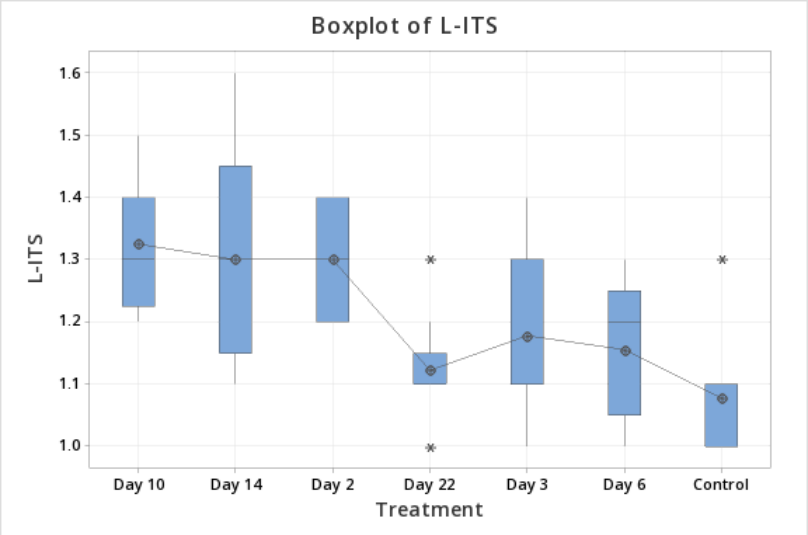


Figure 2-15

Pilot Results for Inner Toe Spread - Platelet Rich Fibrin (PRF) Treatment. A) Boxplot for Right Inner Toe Spread (R-ITS) showing results for each time point. PRF-treated animals received an MCL transection injury on the right side and sham injury on the left side. Placement of PRF at the site of ligament injury was done immediately after injury and the PRF was secured with absorbable sutures at the site of MCL injury during surgery. PRF was previously prepared from donor animals. B) Boxplot for Left Inner Toe Spread (L-ITS) showing results for each time point. PRF-treated animals received an MCL transection injury on the right side and sham injury on the left side. Placement of PRF at the site of ligament injury was done immediately after injury and the PRF was secured with absorbable sutures at the site of MCL injury during surgery. PRF was previously prepared from donor animals.

Figure 2-16

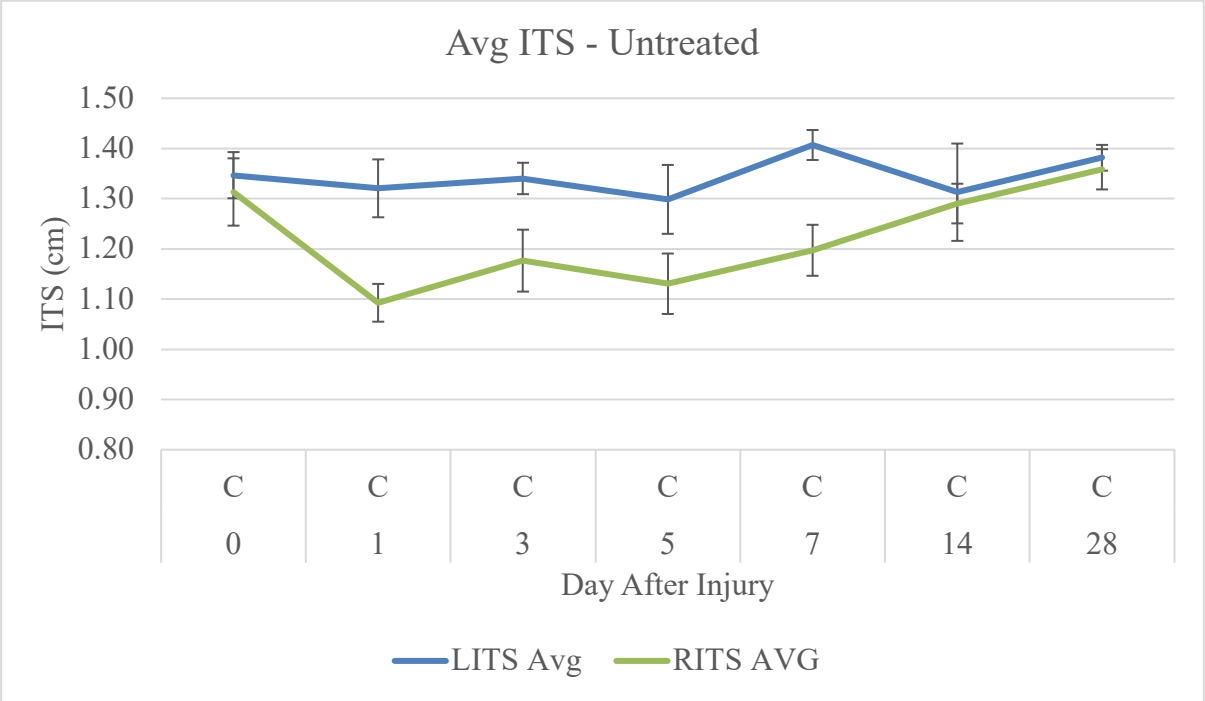


Figure 2-16
Inner Toe Spread for Untreated Animals. Mean ITS and standard deviation for each time point for both left and right ITS of untreated animals.

Figure 2-17

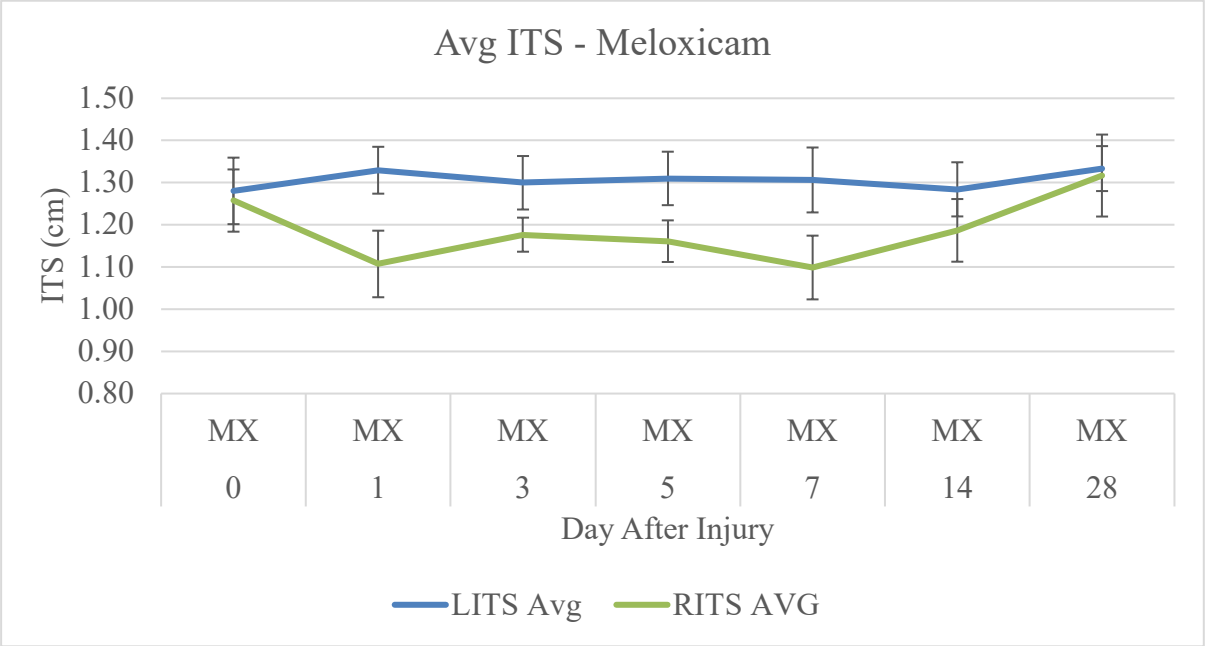


Figure 2-17
Inner Toe Spread for Meloxicam-treated Animals. Mean ITS and standard deviation for each time point for both left and right ITS of Meloxicam-treated animals.

Figure 2-18

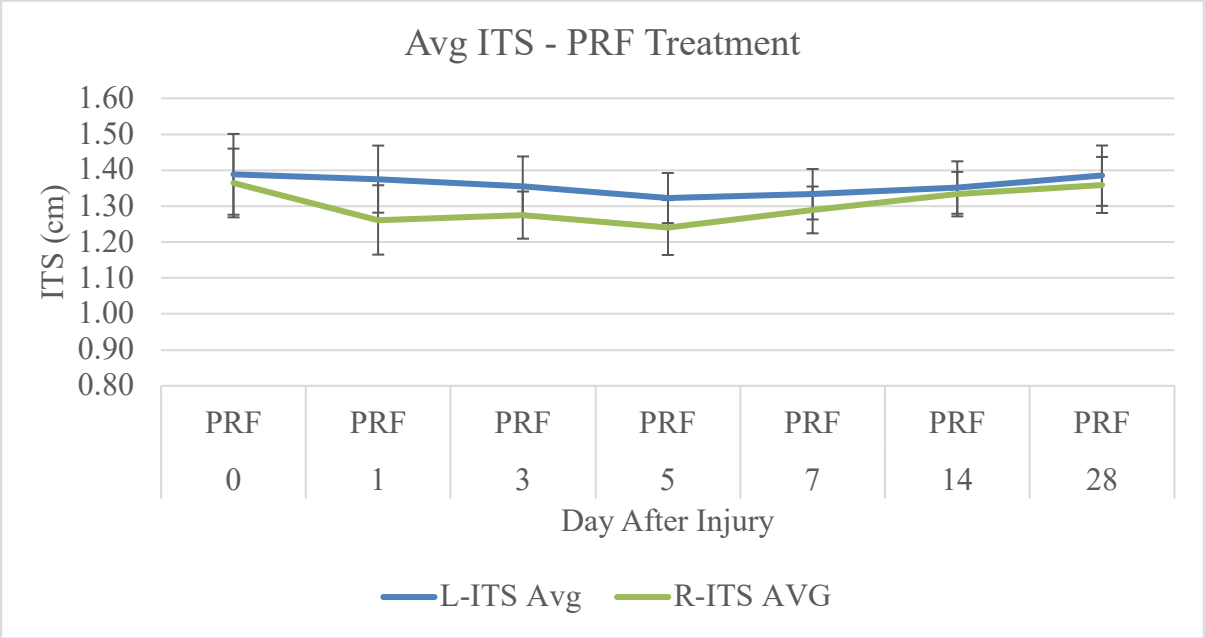
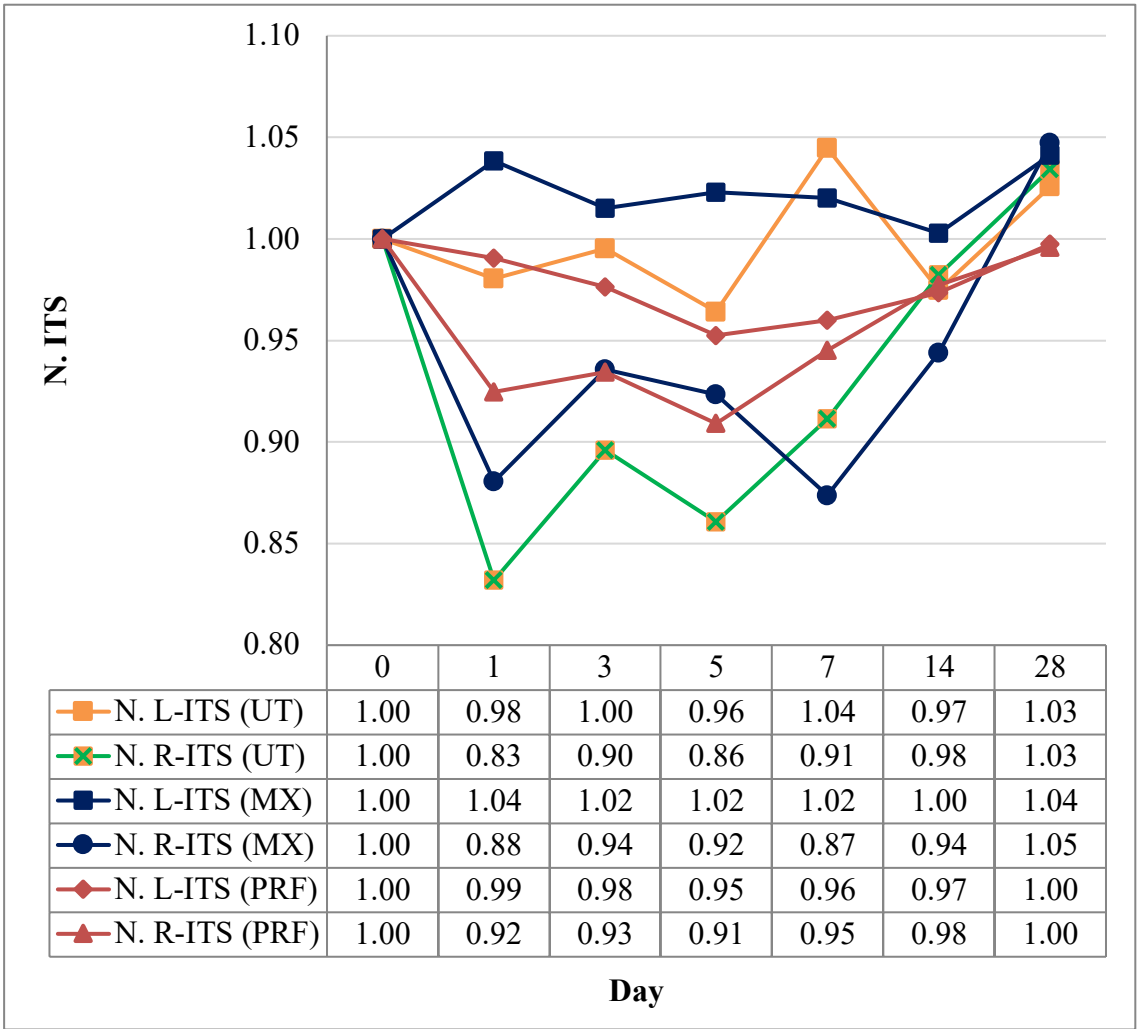


Figure 2-18
Inner Toe Spread for PRF-treated Animals. Mean ITS and standard deviation for each time point for both left and right ITS of PRF-treated animals.

Figure 2-19



	0	1	3	5	7	14	28
N. L-ITS (UT)	1.00	0.98	1.00	0.96	1.04	0.97	1.03
N. R-ITS (UT)	1.00	0.83	0.90	0.86	0.91	0.98	1.03
N. L-ITS (MX)	1.00	1.04	1.02	1.02	1.02	1.00	1.04
N. R-ITS (MX)	1.00	0.88	0.94	0.92	0.87	0.94	1.05
N. L-ITS (PRF)	1.00	0.99	0.98	0.95	0.96	0.97	1.00
N. R-ITS (PRF)	1.00	0.92	0.93	0.91	0.95	0.98	1.00

Figure 2-19
Normalized ITS for All Treatments. ITS results for all treatments were normalized based on the initial average ITS value on day 0 pre-injury.

Figure 2-20

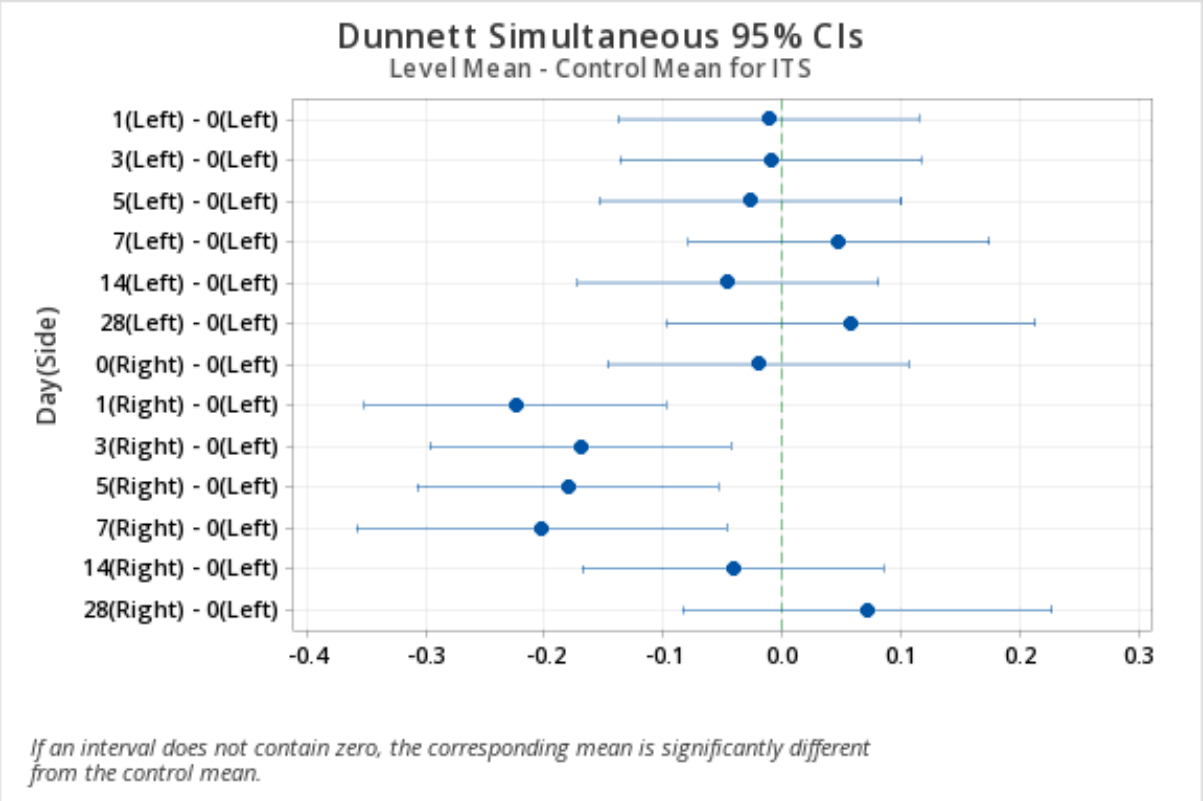


Figure 2-20
Untreated Group's Dunnett Simultaneous 95% Confidence Intervals. The diagram shows confidence intervals for the untreated group and compares the sham side pre-surgery as the control to the mean ITS on each day for both sham and injured limbs. The diagrams show days 1 to 7 of the injured side to be significantly different from the control whereas all other means are the same as the control.

Figure 2-21

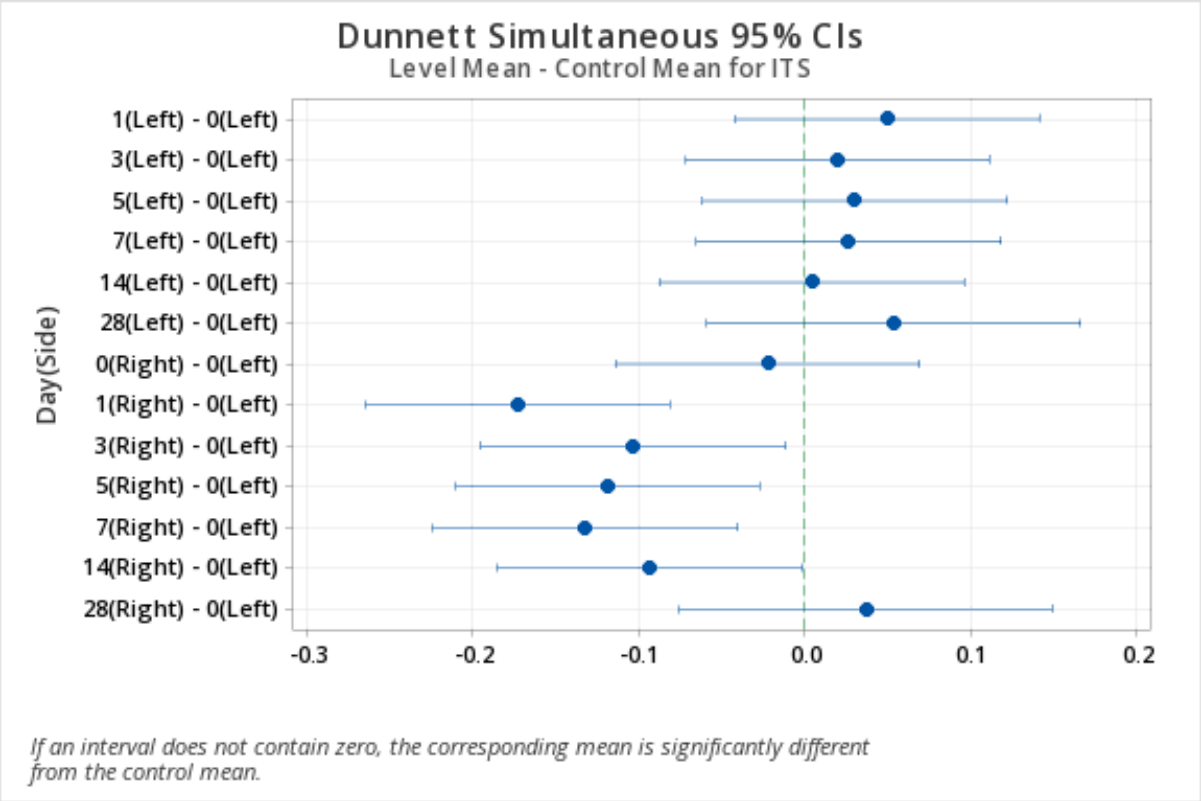


Figure 2-21
MX Group's Dunnett Simultaneous 95% Confidence Intervals. The diagram shows confidence intervals for the MX group and compares the sham side pre-surgery as the control to the mean ITS on each day for both sham and injured limbs. The diagrams indicate that days 1 to 14 of the injured side to be significantly different from the control whereas all other means are the same as the control.

Figure 2-22

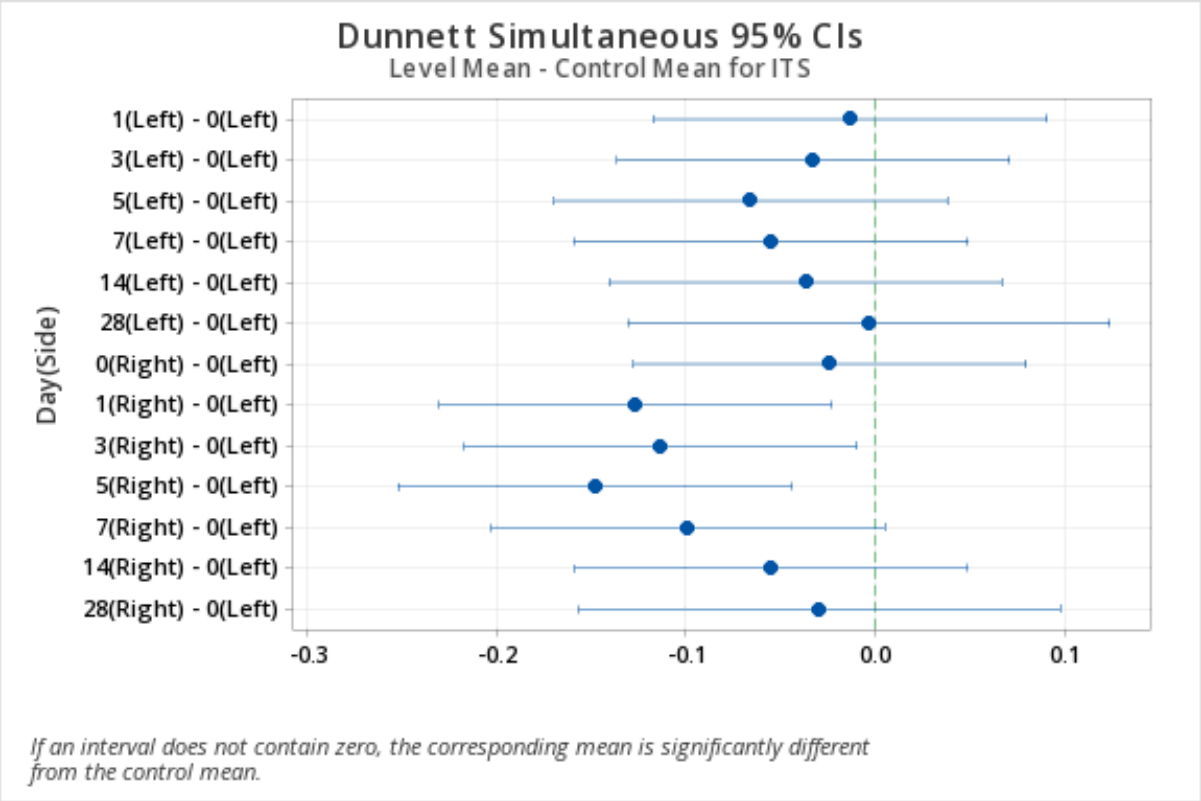


Figure 2-22
PRF Group's Dunnett Simultaneous 95% Confidence Intervals. The diagram shows confidence intervals for the PRF group and compares the sham side pre-surgery as the control to the mean ITS on each day for both sham and injured limbs. The diagrams indicate that days 1 to 5 of the injured side to be significantly different from the control whereas all other means are the same as the control.

CHAPTER 2 TABLES

Table 2-1: Analysis of Variance Table for Left Stride Length (LSL) of Untreated Group's Pilot Study. ANOVA results show LSL to be unaffected due to injury over the study period. DF = Degree of Freedom, Adj SS = Adjusted Sum of Squares, Adj MS= Adjusted Mean Square. Error in the table refers to residual error. The F-Value addresses the amount of variation between the treatments (the four temperatures) with respect to the variation within the treatments (denoting the error). A large ratio of the mean squares (the F-statistic) implies that the amount of variation explained by Treatment is large in comparison with the residual error.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	24.57	4.09	0.91	0.50
Error	46	207.79	4.52		
Total	52	232.36			

Table 2-2: ANOVA table for Right Stride Length (RSL) of Untreated Group's Pilot Study. ANOVA results show RSL to be unaffected due to injury over the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	19.59	3.27	1.10	0.38
Error	43	127.22	2.96		
Total	49	146.80			

Table 2-3. ANOVA table for Hind Base Width (HBW) of Untreated Group's Pilot Study. ANALYSIS OF VARIANCE results showing HBW to be unaffected due to injury over the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	13.75	2.3	1.91	0.10
Error	56	67.34	1.20		
Total	62	81.09			

Table 2-4. ANALYSIS OF VARIANCE table for Mean Base Width (MBW) of Untreated Group's Pilot Study. ANALYSIS OF VARIANCE results showing FBW to be unaffected due to injury over the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	32.13	5.36	1.51	0.2
Error	41	145.35	3.55		
Total	47	177.48			

Table 2-5. ANOVA table for L-ITS of the Untreated Group's Pilot Study. ANOVA results show no significance for the L-ITS difference between all timepoint.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.43	0.072	1.55	0.18
Error	52	2.43	0.047		
Total	58	2.87			

Table 2-6. ANOVA table for LOV of the Untreated Group's Pilot Study. ANOVA results show no significant difference for LOV between all timepoint.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	3.45	0.58	1.14	0.35
Error	56	28.32	0.51		
Total	62	31.77			

Table 2-7. ANOVA table for the ROV of the Untreated Group's Pilot Study. ANOVA results show no significant difference for ROV between all timepoint.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	13.67	2.28	1.93	0.092
Error	56	66.11	1.18		
Total	62	79.78			

Table 2-8. ANOVA table for the Fore Base Width (FBW) of the Untreated Group's Pilot Study. ANOVA results show FBW to show some significant differences between groups due to injury over the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	11.46	1.91	3.17	0.010
Error	52	31.37	0.60		
Total	58	42.83			

Table 2-9. Grouping Information for FBW of Untreated Group's Pilot Study Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey analysis showed no significant difference between control and the first 30 days after injury. This indicates that the FBW is not a good indicator for tracking injury and healing response in the initial period following injury.

Treatment	N	Mean	Grouping	
Day 16	8	8.46	A	
Day 7	9	8.40	A	
Day 5	9	8.11	A	B
Day 30	8	8.088	A	B
Control	8	8.063	A	B
Day 22	9	7.47	A	B
Day 2	8	7.16		B

Means that do not share a letter are significantly different.

Table 2-10. ANOVA Table for LTS of Untreated Group's Pilot Study. ANOVA results for LTS show some significant differences between groups due to injury over the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.49	0.081	3.86	0.0030
Error	56	1.18	0.021		
Total	62	1.67			

Table 2-11. Grouping Information for LTS of Untreated Group's Pilot Study Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey analysis shows that all days are not significantly different from the control and therefore the LTS is not a good measure for tracking healing response after injury.

Treatment	N	Mean	Grouping	
Day 2	9	2.48	A	
Day 22	9	2.44	A	B
Control	9	2.44	A	B
Day 30	9	2.38	A	B
Day 16	9	2.37	A	B
Day 7	9	2.24		B
Day 5	9	2.24		B

Means that do not share a letter are significantly different.

Table 2-12. ANOVA Table for RTS of Untreated Group's Pilot Study. ANOVA results show a significant difference for RTS between the timepoints.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	1.031	0.17	4.86	0.00
Error	55	1.95	0.035		
Total	61	2.98			

Table 2-13. Grouping Information for RTS of the Untreated Group's Pilot Study Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey analysis shows that all days are not significantly different from the control except for day seven and therefore the RTS is not a good measure for tracking healing response after injury, especially for immediate days post-injury.

Treatment	N	Mean	Grouping	
Control	9	2.39	A	
Day 30	9	2.33	A	
Day 22	9	2.24	A	
Day 16	9	2.23	A	B
Day 2	9	2.20	A	B
Day 5	8	2.13	A	B
Day 7	9	1.97		B

Means that do not share a letter are significantly different.

Table 2-14. ANOVA Table for the R-ITS of the Untreated Group's Pilot Study. ANOVA results showing Right Inner Toe Spread (RITS) to be significantly different between the different timepoints in the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	1.78	0.29	16.85	0.00
Error	53	0.93	0.017		
Total	59	2.70			

Table 2-15. **Grouping Information for Untreated Group's Pilot Study R-ITS Using the Tukey Post-hoc Statistics Method and 95% Confidence.** Tukey analysis shows the grouping of the R-ITS at different timepoints. The untreated group is significantly different from time points at days 2, 5, and 7. In addition, the untreated group is not significantly different from days 30 and 22. These results make the R-ITS a great candidate for further study and analysis to act as a marker for ligament healing response post-injury.

Treatment	N	Mean	Grouping		
Control	9	1.46	A		
Day 30	9	1.36	A	B	
Day 22	9	1.34	A	B	
Day 16	9	1.26		B	
Day 5	7	1.029			C
Day 7	8	1.025			C
Day 2	9	1.01			C

Means that do not share a letter are significantly different.

Table 2-16. ANOVA Table for R-ITS of Meloxicam Treatment's Pilot Study. ANOVA results showing right inner toe spread (R-ITS) of MX treatment to be significantly different between the different timepoints in the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.74	0.12	9.16	0.00
Error	56	0.75	0.013		
Total	62	1.49			

Table 2-17. Grouping Information for R-ITS of Meloxicam Treatment Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey analysis shows the grouping of the R-ITS at different timepoints. The control group (Day 0, Pre-Injury) is significantly different from time points at days 1, and 3. In addition, the control group is not significantly different from day twenty two. These results make the R-ITS a great candidate for further study and analysis to act as a marker for early detection of ligament injury and healing response with MX treatment.

Treatment	N	Mean	Grouping			
Day 14	9	1.42	A			
Day 22	9	1.39	A	B		
Day 10	9	1.32	A	B	C	
Day 6	9	1.28	A	B	C	
Control	9	1.22		B	C	D
Day 3	9	1.17			C	D
Day 1	9	1.10				D

Means that do not share a letter are significantly different

Table 2-18. ANOVA Table for L-ITS of Meloxicam Treatment – Pilot Study. ANOVA results show the Left Inner Toe Spread (L-ITS) of MX treatment to be significantly different between the different timepoints in the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.21	0.035	3.09	0.011
Error	55	0.62	0.011		
Total	61	0.83			

Table 2-19. Grouping Information for L-ITS of Meloxicam Treatment Using the Tukey Post-hoc Statistics Method and 95% Confidence for the Pilot Study. Tukey analysis shows the grouping of the L-ITS at different timepoints. The results show that the control group is not significantly different from any other timepoint.

Treatment	N	Mean	Grouping	
Day 14	9	1.47	A	
Day 6	9	1.42	A	B
Day 3	9	1.34	A	B
Day 10	9	1.33	A	B
Control	8	1.33	A	B
Day 1	9	1.31		B
Day 22	9	1.30		B

Means that do not share a letter are significantly different

Table 2-20. Analysis of Variance for R-ITS of Platelet Rich Fibrin (PRF) Treatment – Pilot Study. ANOVA results show no significant difference for R-ITS for PRF treatment between all timepoint.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.076	0.0127	0.84	0.54
Error	55	0.83	0.015		
Total	61	0.90			

Table 2-21. Analysis of Variance for L-ITS of PRF Treatment – Pilot Study. ANOVA results showing Left Inner Toe Spread (L-ITS) of PRF treatment to be significantly different between the different timepoints in the study period.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Treatment	6	0.51	0.086	6.16	0.00
Error	55	0.76	0.014		
Total	61	1.28			

Table 2-22. Grouping Information for L-ITS of PRF Treatment Using the Tukey Post-hoc Statistics Method and 95% Confidence – Pilot Study. Tukey analysis shows the grouping of the L-ITS at different timepoints. The control group is significantly different from time points at days 2, 10, and 14. In addition, the control group is not significantly different from days 3, 6, and 22. These results are unique in that the sham injury side showed a significant difference between the different time points. The average for days 2, 10, and 14 seem to indicate a larger ITS on the sham injury side and could possibly be an indication the animal is placing more weight on that limb and perhaps more time to allow an increase in the separation between the inner toes.

Treatment	N	Mean	Grouping	
Day 10	8	1.33	A	
Day 2	9	1.30	A	
Day 14	9	1.30	A	
Day 3	9	1.18	A	B
Day 6	9	1.16	A	B
Day 22	9	1.12		B
Control	9	1.08		B

Means that do not share a letter are significantly different.

Table 2-23. Analysis of Variance Untreated Group – Full Study. ANOVA results showing Inner Toe Spread (ITS) to be significantly different between the different timepoints and between injured and sham sides in the study period for the untreated group.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Side	1	0.36	0.36	36.10	0.00
Day(Side)	12	0.62	0.052	5.22	0.00
Error	111	1.10	0.010		
Total	124	2.13			

Table 2-24. Grouping Information for Untreated Group Using the Dunnett Post-hoc Statistics Method and 95% Confidence – Full Study. Dunnett analysis for the untreated group shows that Days 1 to 7 post-injury for the injured limb are significantly indifferent; however, they differ from the sham limb on all days. ITS recovery to control levels for the untreated group occurs after day seven post-injury.

Day(Side)	N	Mean	Grouping
0(Sham) (Control)	10	1.33	A
28(Injured)	5	1.40	A
28(Sham)	5	1.39	A
7(Sham)	10	1.38	A
3(Sham)	10	1.32	A
1(Sham)	10	1.32	A
0(Injured)	10	1.31	A
5(Sham)	10	1.30	A
14(Injured)	10	1.29	A
14(Sham)	10	1.29	A
3(Injured)	10	1.16	
5(Injured)	10	1.15	
7(Injured)	5	1.129	
1(Injured)	10	1.11	

Means not labeled with the letter A are significantly different from the control level mean.

Table 2-25. Analysis of Variance for MX – Full Study. ANOVA results showed Inner Toe Spread (ITS) to be significantly different between the different timepoints and between injured and sham sides in the study period for the MX group.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Side	1	0.39	0.39	75.05	0.00
Day(Side)	12	0.24	0.020	3.87	0.00
Error	116	0.60	0.0052		
Total	129	1.31			

Table 2-26. Grouping Information for MX Group Using the Dunnett Post-hoc Statistics Method and 95% Confidence – Full Study. Dunnett analysis for the MX group shows that Days one to fourteen post-injury for the injured limb are significantly indifferent however they are different from the sham limb on all days. ITS recovery to control levels for the untreated group occurs after day fourteen post-injury.

Day(Side)	N	Mean	Grouping
0(Left) (Control)	10	1.28	A
28(Left)	5	1.33	A
1(Left)	10	1.33	A
28(Right)	5	1.32	A
5(Left)	10	1.31	A
7(Left)	10	1.31	A
3(Left)	10	1.30	A
14(Left)	10	1.28	A
0(Right)	10	1.26	A
14(Right)	10	1.19	
3(Right)	10	1.18	
5(Right)	10	1.16	
7(Right)	10	1.15	
1(Right)	10	1.11	

Means not labeled with the letter A are significantly different from the control level mean.

Table 2-27. **Analysis of Variance for PRF – Full Study**. ANOVA results showing Inner Toe Spread (ITS) to be significantly different between the different timepoints and between injured and sham sides in the study period for the PRF group.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Side	1	0.094	0.094	14.15	0.00
Day(Side)	12	0.16	0.014	2.03	0.027
Error	116	0.77	0.0067		
Total	129	1.04			

Table 2-28. Grouping Information for PRF Group Using the Dunnett Post-hoc Statistics Method and 95% Confidence – Full Study. Dunnett analysis for the PRF group shows that Days 1 to 5 post-injury for the injured limb are significantly indifferent however they are different from the sham limb on all days. ITS recovery to control levels for the untreated group occurs after day five post-injury.

Day(Side)	N	Mean	Grouping
0(Left) (Control)	10	1.39	A
28(Left)	5	1.38	A
1(Left)	10	1.38	A
0(Right)	10	1.36	A
28(Right)	5	1.36	A
3(Left)	10	1.36	A
14(Left)	10	1.35	A
14(Right)	10	1.33	A
7(Left)	10	1.33	A
5(Left)	10	1.32	A
7(Right)	10	1.29	A
3(Right)	10	1.28	
1(Right)	10	1.26	
5(Right)	10	1.24	

Means not labeled with the letter A are significantly different from the control level mean.

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CHAPTER 3

ELISA

ABSTRACT

Ligaments play a crucial role in the musculoskeletal system in maintaining joint stability. When ligament injury occurs, a wound healing cascade is initiated starting with platelet plug formation and ending with tissue remodeling. A rat Medial Collateral Ligament (MCL) surgical injury model was developed to examine the role of modulating the initial recruitment of platelets and their impact on animal functional recovery. Animals (n=60) were randomized to a no-treatment group, a Platelet Rich Fibrin (PRF) treatment group and a Nonsteroidal Anti-inflammatory Drug (NSAID) treatment group with 20 animals assigned to each group and five animals assigned to each timepoint under study. Enzyme-Linked Immunosorbent Assay (ELISA) was performed on select timepoints post-injury (Days 1, 3, 5, 7) and concentrations of Vascular Endothelial Growth Factor (VEGF) in homogenized MCL tissue was measured. Results show that untreated animals' peak VEGF concentration is achieved on day five after injury while anti-inflammatory drugs (NSAIDs) treatment showed VEGF peak concentration on day seven. The PRF-treated group showed the fastest time to peak VEGF concentration at day three post-injury.

PRF reduces the time needed to aggregate and recruit platelets and makes available signaling biomolecules and growth factors needed for wound healing. Other hand, NSAIDs manage pain but probably inhibits recruitment of platelets and thereby limit growth factors, making their application controversial with regards to wound healing and tissue repair. With Meloxicam treatment, MCLs still managed to reach a high concentration of growth factors but it was delayed. Using a rodent model opens the opportunity to evaluate different approaches to improve ligament injury recovery and investigation of various treatment methods and injury mechanisms.

ABBREVIATIONS

ANOVA: Analysis of Variance
COX: Cyclooxygenase
ELISA: Enzyme-Linked Immunoassay
IACUC: Institutional Animal Care and Use Committee
MCL: Medial Collateral Ligament
MX: Meloxicam
NSAIDs: Non-Steroidal Anti-inflammatory Drugs
PRF: Platelet Rich Fibrin
PRP: Platelet Rich Plasma
RICE: Rest, Ice, Compression, and Elevation
VEGF: Vascular Endothelial Growth Factor

INTRODUCTION

Skeletal ligaments are dense bands of collagenous fiber tissue that span a joint restraining one bone to another forming a flexible skeletal joint (1, 2). Ligaments are described as pale from the limited blood supply (2). As a "first line of defense" to mechanical loading on joints, skeletal ligaments play a crucial role in the musculoskeletal locomotive system in resisting tensile and torsional forces affecting the joint. Their main function is to guide and restrain skeletal motion while keeping the joint structure intact (3).

Ligaments are passive tissues with viscoelastic properties susceptible to creep deformation under sustained static loading (4). Stretching viscoelastic tissues repeatedly for long periods exposes them to microdamage, "In the collagen fibers, irrespective of the load applied" (5). Depending on the level of loading, injuries occur, and a repair process commences.

After injury, ligaments undergo a series of wound healing events to restore original tissue structure and function however results vary and, in most cases, repaired ligaments are inferior from a mechanical and functional standpoint (6-9). The repair process is marked by four phases: restoration of hemostasis, inflammation, repair, and finally remodeling (10). Biochemical, mechanical, and histological studies of healing ligament in a complete tear of the medial collateral ligament (MCL) show healed ligament with scar tissue that is mechanically inferior and biochemically abnormal in its composition and architecture concluding that these properties do not return to normal (11).

Treatment methods of injured ligaments vary with no clear efficacy in restoring structure or function. The different treatment methods impact the biochemical response of the various wound healing cascades. Specifically, the impact on platelets aggregation and recruitment seems to be influenced by common treatments such as Non-Steroid Anti-inflammatory Drugs (NSAIDs) and emerging treatments such as platelet rich concentrates. Ultimately during platelet activation and degranulation, growth factors are released from their granules initiating other cascades necessary for wound healing and restoration of structure and function (12). One of these factors is Vascular Endothelial Growth Factor (VEGF) which has known effects in promoting vascular function and angiogenesis and recent evidence suggests that platelets are the main contributors of VEGF (13-15).

Examining the biochemical response at the early stages of healing will provide an insight into healing and the impact of current and emerging treatment methods.

Wound Healing Phases

Three clinical ligament injury grade categories depend on the level of the tear, with grade 1 associated with mild tears and grade 3 depicting a complete tear of the ligament (16, 17). Ligament healing follows the four main phases of wound healing starting with hemostasis and ending with remodeling (10).

The endothelium is damaged when ligaments are disrupted and blood vessels are severed. It then exposes the basal lamina to blood plasma and peripheral blood cells (18). Hemostasis is achieved when damaged blood vessels are temporarily repaired by creating a platelet plug/clot. This step is dependent on the level of platelets recruited at the site of injury (10, 18). In addition, the growth factors and biomolecules released from platelets play a major role in the deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (19). Activated platelets are followed by an influx of inflammatory cells in the first 48 hours (18). This is followed by proliferation which is a set of four steps that begin at various time periods in wound healing: (1) epithelialization (2) angiogenesis, (3) granulation tissue formation, (4) and collagen deposition (10). Repair and remodeling of tissue occur following proliferation and initially the extracellular

matrix mainly made up of collagen, is deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet providing a basis for a new matrix (20). The newly deposited collagen is reorganized into a structurally sound lattice and the immature type III collagen is replaced with type I collagen that is structurally stronger and wound strength is increased (10, 18). Healing of ligaments is slow and incomplete creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (9, 18).

Studies show that the mechanical properties of the healing or remodeled ligaments are inferior to the native tissue (20-25). In some studies, ligaments show a regain of stiffness and strength that is 40% to 80% of normal and inferior creep properties with elongation that is twice as much as normal tissue with suggestions that deformations may be permanent or long-lasting (23). With inferior mechanical properties, ligament's function is affected leading to instability at the joint and therefore susceptibility of other structures to injury is increased (e.g., cartilage and meniscus tears) in addition to the inefficacy of muscles in the locomotion system due to increased sliding between joint surfaces (23, 26, 27). With altered mechanical properties, the load distribution around the joint is disrupted leading to altered contact mechanics with increased shear forces affecting cartilage and bones and eventually leading to osteochondral degeneration or osteoarthritis and other joint chronic diseases (23, 26).

Ligament Injury Treatment Methods

Investigating ligament injury-healing requires an animal model to with reproducible injury and to follow recovery over time. Many studies monitor the injury-recovery of the Medial Collateral Ligament (MCL) in rodent models because the MCL is well characterized: ease of surgical access, reproducible injury model, and healing properties (23, 28-31). This study has used the MCL model to investigate ligament injury-healing response under different treatment conditions. The result will help elucidate our understanding of the biochemical response to injury. The treatment methods under study are anti-inflammatory drug, Meloxicam, and an emerging treatment using autologous Platelet Rich Fibrin (PRF).

When ligaments are injured, there are three treatment methods to help restore function, yet they are impactful on a biochemical level and in some cases may have a direct impact on platelet recruitment. Some studies suggest that interfering with platelet recruitment plays a major role in the outcome of this cascade (32). These treatments include RICE (Rest, Ice, Compression, and Elevation), NSAIDs (Non-Steroidal Anti-inflammatory Drugs), and a relatively new treatment with platelet rich concentrates (e.g., Platelet Rich Plasma/Fibrin or PRP/PRF) (27, 33-37). The goal of the RICE treatment is to reduce additional damage to injured tissue, decrease blood flow and enzymatic activity which are expected to reduce edema and swelling and control pain (37, 38).

NSAIDs reduce the inflammatory response by limiting the Cyclooxygenase activity. Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, which is the precursor of prostaglandins, prostacyclin, and thromboxane (39). Thromboxane is involved in platelet aggregation, and prostaglandins mediate pain sensitization. NSAIDs reduce the production of thromboxane and prostaglandins (35, 40-42).

The main goal of platelet concentrates treatments such as PRF is to increase the availability of platelets, which contain biomolecules and growth factors responsible for repair at the site of injury (34). Amongst the different types of platelet concentrates, PRF is unique because it is formed as a gel-like substance with activated platelets in a fibrin network without the need for anti-coagulants and modifying centrifugation protocols to allow for gel-like formation structure (43, 44). The added benefit of PRF is that is not in a liquid form, making it possible localize further

and concentrate platelets where desired (33, 45). By adding platelets to the injury site, the platelets can increase the healing response and mediate collagen expression and organization, which then may improve the time to mechanical and functional recovery (46-48).

Biochemical Assessment of Growth Factors Using Enzyme-Linked Immunoassay (ELISA)

Quantitative laboratory methods serve to identify the presence and concentration of molecules in biological fluids. Assays for antibody production depend upon the measurement of the interaction of antibody with the antigen of interest (49). Enzyme-Linked Immunosorbent Assay (ELISA) shows visible light color changes with antigen-antibody reactions with enzyme-linked conjugate and enzyme-substrate chemistry. ELISA is a powerful method for detecting and quantifying a specific protein in a complex biological mixture. Engvall and Perlmann originally described the method in 1971 which enables analysis of protein samples immobilized in microplate wells using specific antibodies (49, 50). The unique advantage of ELISA is that it allows detection with high specificity using antibodies or antigens developed for very low-concentration molecules such as peptides/proteins, hormones, vitamins, and drugs (50-52).

ELISAs are versatile in that there are six main variations that are deployed depending on the desired outcome and the type of sample being assayed (52). Amongst the different variations, Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than other methods and do not require a purified antigen (49). To detect an antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen (Fig. 3-1). The unbound antigen is washed out and a different antigen-specific antibody conjugated to an enzyme (i.e., developing reagent) is added, followed by another incubation. Unbound conjugate is washed out and the substrate is added. After another incubation, the degree of substrate hydrolysis is observed with varying color changes, and the optical density is measured with laboratory instruments, a plate reader. The amount of antigen in the test solution is proportional to the amount of substrate that it reacts to and is hydrolyzed (49, 50). A standard antigen with known concentrations is used to develop a standard curve used to calculate unknown antigen concentrations.

This experiment utilized ELISA to detect the impact on growth factors concentration at the site of injury during recovery. The utility of ELISA in producing a quantitative assessment of biochemical processes is a critical tool used to test rodent models for a given disease, injury, or drug effects on wound healing phases.

Research Void

When ligaments are injured, the innate immune response and wound healing take effect with the goal of restoring tissue structure and function. Tracking the impact of treatment methods on the concentration of biomolecules involved in signaling will test the efficacy of such treatments.

Ligament biochemical signaling and characterization provide a critical basis to investigate the associated and corresponding changes in histology and functional recovery. Together, these findings will paint a comprehensive picture of healing after a ligament injury.

The standard treatments with NSAIDs can manage pain, but their effectiveness in restoring ligament structure and function, however, remains controversial (37, 53). Some authors argue that NSAIDs only help to relieve pain symptoms (23, 37, 40, 54). With respect to PRF treatment, the lack of standardization in creating and activating platelets, in testing its efficacy, and in measuring quantitatively the range of outcomes limit the enthusiasm for deploying PRF as a standard treatment for ligament injuries (46-48).

Study Aim

Time to peak growth factor concentration is of critical importance as the subsequent cascades are temporal in nature and are dependent on the initial release of such factors. VEGF promotes angiogenesis and an early increase in concertation may play an important role in the healing response. In this study, we examined if treatment methods play a role in the temporal concentration profiles of VEGF and the time to peak concertation.

We established an MCL injury in a rat model and characterized the initial biochemical response under different treatment conditions. Anti-inflammatory drugs (Meloxicam) and platelet rich concentrates (PRF) are common and emerging treatments and both in have an impact on platelet recruitment and aggregation at the site of injury. The biochemical impact of such treatments on VEGF concentrations is under study using quantitative ELISA methods.

MATERIALS AND METHODS

Animals

The study was approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC). Thirty skeletally mature male Wistar rats (The Jackson Laboratory) were used as an animal model for ligament healing. The animals were randomly assigned to 3 treatment groups: Untreated, Platelet Rich Fibrin, and Meloxicam (n=20/group) with five animals randomly assigned to each day after injury for MCL tissue harvest. Animals were fed rat chow and housed in pairs per cage under the care of a university veterinarian. Detailed surgical procedure is described in detail in chapter 1. Briefly, a surgically transected, rather than torn, Medial Collateral Ligament (MCL) of the knee was used as an experimental model to create a uniform defect for healing. After two days of acclimatization in the housing facility, the surgical procedure was undertaken to induce ligament transection and sham injuries for each animal. Following injury, animals are returned to their cages and allowed free-roaming, access to water, and ambulation. Surgical transection was done on the right side and sham injury on the left side as described in the literature (55).

Treatment Methods

The three treatment groups in this study are:

- **Untreated:** Surgical injury to animals with no additional treatment and animals are allowed to heal under normal conditions.
- **Meloxicam Treatment:** Subcutaneous Meloxicam injection (Ostilox, 5 mg/mL, MWI/VetOne, Boise, ID, USA) at a dose of 0.2 mg/kg at the site of MCL transection immediately after surgery.
- **Platelet Rich Fibrin Treatment:** Previously prepared PRF from donor animals and stored at -80 degrees Celsius is thawed out on ice and sutured with resorbable sutures internally at the site of MCL transection followed by suturing the skin.

Tissue Harvest and Storage

MCL tissue was harvested on days 1,3, 5, and 7 post-injury. The animals were sacrificed in a carbon dioxide (CO2) chamber to effect. Hindlimbs were skinned, and the superficial fascia that is not adherent to the ligament or its surrounding connective tissue was carefully cut and reflected. At the time of sacrifice, the MCLs were carefully dissected without disturbing the scar region. The MCL is removed using a scalpel with the blade placed under the MCL on the fibula side, and moved distally from the knee joint to free the MCL at the insertion site of the fibula.

Careful attention to avoid removing bone tissue will be observed and, in such cases, where the bone is removed, the insertion site was approximated, and bone tissue was cut transversally leaving behind ligament tissue. The scalpel is then moved under the MCL towards the femoral side freeing the superficial MCL from the meniscal attachment (deep MCL) and moving distally from the knee towards the femoral attachment site of the MCL. Similarly, careful attention to avoid removing bone tissue. MCLs are immediately placed in pre-labeled aluminum foil for flash freezing in liquid nitrogen (Fig. 3-2). The tissue is later stored at -80 degrees Celsius freezer.

Homogenization procedure

BioRad Bio-PlexTM Cell Lysis Kit (Bio-Rad, Hercules, CA), 2 mL Eppendorf tubes and stainless-steel beads mixture containing a 0.9–2.0 mm stainless steel bead blend, 2.5 mm stainless steel balls (Next Advance, Averill Park, NY) were used (Fig. 3-3). The lysis buffer was prepared on the day of tissue homogenization per manufacturer instructions by mixing: 9.9 mL Lysis Buffer, 40 µL Factor 1, 20 µL Factor 2, 40 µL PMSF/DMSO using a benchtop vortex. The final cell lysis buffer was put on ice until added to each Eppendorf tube with MCL tissue samples. Each tube is labeled on the cap and on the tube itself. The weight of each sample was captured before hominization. Each tube received 500 µL of wash buffer that was kept on ice, and the tissue was rinsed by pipetting up and down. The wash buffer was then removed from each tube, and 500 µL previously prepared Lysis Solution (Bio-Rad, Hercules, CA) was added with stainless steel beads mixture.

The MCLs are then lysed using a TissueLyser (TissueLyser II, Qiagen, Valencia, CA, USA) shown in Fig. 3-4 for a total of 4 min at 30 Hz with a 1 min pause to put the tubes on the ice at the middle of lysing procedure. The 1 min pause is to protect protein integrity by controlling temperature as the lysing procedure can produce heat. Samples are checked at the end of the 4 minutes to ensure there are no chunks of tissue left. In cases chunks of tissue are detected, lysing was resumed for another 1–2 min. Homogenized tissue is then placed on ice and then into a -80°C freezer and the next day, they are thawed on ice (Fig. 3-5), vortexed and then centrifuged @ 4,500 g for 4 min. The supernatant is then aliquoted into two 250 µL samples and used for ELISA assay or placed in a -80°C freezer for future use. Due to low protein density in ligament tissue samples, no sample dilutions were performed.

ELISA Procedure

To follow the biochemical response to injury in a quantitative method, sandwich Enzyme-Linked Immunosorbent Assay (ELISA) methods were used (56). We assayed for Vascular Endothelial Growth Factor (VEGF) (57, 58). There are variations of ELISA methods and the assay employed in this study is the quantitative sandwich enzyme immunoassay technique. Antibody specific for rat VEGF are pre-coated and fixed onto a microplate base. When standards and samples are pipetted into the wells, any VEGF present is bound by the immobilized antibodies. After removing any unbound substances through washing steps, a biotin-conjugated antibody specific for VEGF is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development is stopped, and the intensity of the color is measured with lab instruments.

On the day of ELISA procedure, aliquoted samples are thawed on ice for 30 to 45 min. The VEGF ELISA kit #MBS703527 (MyBioSource, San Diego, CA) is pre-coated with target protein antibodies and has a detection range of 3.9 pg/ml-250 pg/ml. The minimum detectable

dose of rat VEGF is typically less than 0.97 pg/ml. The sensitivity of this assay or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. Per the manufacturer's literature, the assay has high sensitivity and specificity for the detection of rat VEGF. No significant cross-reactivity or interference between rat VEGF and analogues was observed.

Standard Preparation

The standard is reconstituted with 1.0 ml of sample diluent provided in the ELISA kit. This reconstitution produces a stock solution of 250 pg/ml. The standard is then mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making serial dilutions. 250 µl of sample diluent is then pipetted into each tube. The stock solution is then used to produce a 2-fold serial dilution while mixing each tube thoroughly before the next transfer. The undiluted Standard serves as the high standard (250 pg/ml). Sample Diluent serves as the zero standard (0 pg/ml).

Assay Procedure

All reagents and samples are brought to room temperature before use. Samples are centrifuged after thawing on ice before the assay. All reagents, working standards, and samples are prepared as directed by the manufacturer. The assay layout sheet is determined for samples under study and to identify the number of wells to be used. The standard and samples are assayed in duplicates. 100µl of standard and sample per well is added and the plate is covered with adhesive strip and incubated for 2 hours at 37°C. The liquid is removed from each well and 100µl of Biotin-antibody is added to each well and the plate is covered with a new adhesive strip then incubated for 1 hour at 37°C. Each well is aspirated and washed using wash buffer (200µl) with a multi-channel pipette allowing each wash cycle 2 minutes to complete. 100µl of HRP-avidin (1x) to each well is added then incubate for 1 hour at 37°C. 90µl of TMB Substrate is then added to each well and the plate is incubated for 15-30 minutes at 37°C. In darkroom conditions, 50µl of Stop Solution is added to each well using a multichannel pipette (Fig. 3-6). The plate is then read within 5 minutes of adding the stop solution using a plate reader spectrophotometer FilterMax (Molecular Devices, Sunnyvale, California). To construct a standard curve, the average of duplicate readings for each standard and sample is calculated, and we then subtract the average zero standard optical density. We then plotted the mean absorbance for each standard on the x-axis against the concentration on the y-axis and drew the best fit curve through the points on the graph. The fit equation was then used to calculate the concentrations of the unknown samples based on the absorbances read using the plate reader. Data is then exported into excel files and stored for future statistical analysis.

Pilot Study

Optimization of laboratory procedures and methods using the VEGF ELISA kit and tissue samples obtained was performed.

Data Processing and Analysis

Statistical analysis used both Minitab (Minitab, LLC. (2021). *Minitab*. Retrieved from https://www.minitab.com) and Microsoft Excel. Significance was determined by General Linear Model nested design analysis of variance (ANOVA) using an alpha (α) value of 0.05 and post hoc analysis was done using Tukey pairwise comparison method (59, 60).

RESULTS

Pilot Study Results

VEGF was measured on day five after the transection of the right MCL ligament. The left ligament served as the sham control. VEGF ELISA pilot results (Fig. 3-7) show that on Day 5 after injury, animals that received no treatment had a 3-fold increase in VEGF expression in comparison to sham controls (27.1 pg/ml and 8.8 pg/ml). In the PRF-treated animals VEGF expression increases 2-folds (20.1 pg/ml and 9.7 pg/ml) and Meloxicam (MX) treated animals showed roughly no difference on day five after injury between the sham and injured MCLs (10.9 pg/ml and 13.8 pg/ml).

Full Study Results

ELISA measures the concentration of the target protein, VEGF, in prepared samples. Table 3-1 shows summary statistics of means and standard deviations for the different time points and injury conditions in each of the treatment groups.

Each experimental group randomly received twenty animals with five animals randomly assigned to each day after injury for MCL tissue harvest. After two days of acclimatization in the housing facility, the surgical procedure was undertaken to induce ligament transection and sham injuries for each animal. The surgical procedure is described in chapter 1 appendix. Following injury, animals are returned to their cages and allowed free-roaming, access to water, and ambulation.

Untread Group Results

VEGF concentration of injured and sham MCLs for the Untreated group is displayed in Fig. 3-8 showing the largest difference between the sham and injured MCLs is on day five followed by day seven. Table 3-2 shows the ANOVA results with significant differences detected. Tukey Post-hoc analysis (Table 3-3) for the untreated group shows the injured side on day five to be significantly higher VEGF concentration (25.41 pg/ml) than all other days and injury conditions. Day seven came in second (15.50 pg/ml) and is also significantly higher than other days and injury conditions. Day one and three of the injured MCLs were not different from the sham VEGF concentrations. In addition, sham MCLs are the same in one group on all days following the injury. Fig. 3-9 provides a summary of the Tukey results for the untreated animals.

Meloxicam Group Results

VEGF concentration of injured and sham MCLs for the Meloxicam group is displayed in Fig. 3-10 showing the largest difference between the sham and injured MCLs is on day seven followed by day five. Table 3-4 shows the ANOVA results with significant differences detected. Tukey Post-hoc analysis (Table 3-5) for the Meloxicam group shows the injured MCLs on day seven to be significantly higher VEGF concentration (72.96 pg/ml) than all other days and injury conditions. Injured MCLs' VEGF concentration on day five came in second (40.10 pg/ml) and day three came in third (31.82 pg/ml) with both belonging to the same group and being significantly higher than all other days and injury conditions except for day seven. The injured MCL on day one was not different from the sham VEGF concentrations. In addition, sham MCLs are the same under one group on all days following the injury. Fig. 3-11 provides a summary of the Tukey results for the Meloxicam-treated animals. Meloxicam treatment indicates a delay in peak VEGF concentration compared to untreated animals.

Platelet Rich Fibrin Group Results

VEGF concentration of injured and sham MCLs for the PRF group is displayed in Fig. 3-12 showing the largest difference between the sham and injured MCLs is on day three. Table 3-6 shows the ANOVA results with significant differences detected. Tukey Post-hoc analysis (Table 3-7) for the PRF group shows the injured MCLs on day three to have significantly higher VEGF concentration (61.77 pg/ml) than all other days and injury conditions. Injured MCLs VEGF concentration on day five came in second (19.66 pg/ml) and is significantly higher than all other days and injury conditions except for injured MCLs on day three. The injured MCL on Day 1 and Day 7 were not different from the sham VEGF concentrations. In addition, sham MCLs are the same under one group on all days following the injury. Fig. 3-13 provides a summary of the Tukey results for the PRF-treated animals. PRF treatment indicates an earlier timepoint for peak VEGF concentration compared to the untreated and the Meloxicam groups.

Summary of All Treatment Results

Fig. 3-14 to 3-16 show VEGF concentration with the day after injury for all treatments and injury conditions. There is no difference between sham and injured MCL on day one for all injury conditions. Injured MCLs' VEGF concentration on day three shows a peak for PRF treatment and an increased concentration for MX treatment compared to sham. Day 5 continues to show MX with elevated concentration for injured MCLs and the highest level of VEGF for the UT group compared to sham occurred on this day. Finally, Day 7 shows peak VEGF concentration for MX and returns to control levels for all other treatment groups and injury conditions.

In summary, the time to peak VEGF concertation for the injured MCLs with the UT group is on day five, for MX is on day seven, and for PRF is on day three. The sham injury had no effect on the MCL VEGF concertation on all days after injury.

DISCUSSION

Initial and subsequent ELISA results in this study show that biochemical evaluation methods can be used to examine MCL healing response following a surgical transection injury as well as provide a utility to evaluate the impact of treatment methods.

To date, there is not one single solution to help restore injured ligaments to their original native state (61-63). There is insufficient evidence to support standard treatments such as RICE and NSAIDs for all types of ligament injury however some clinical treatments appear to be deployed to control pain and swelling which are byproducts of the wound healing and inflammatory response (6, 8, 9, 37, 53, 64). Furthermore, some authors argue that standard treatments (e.g., NSAIDs) only help in relieving pain symptoms with no other utility for restoration of structure or function (23, 37, 40, 54).

Animal Model

To investigate ligament injury and healing response, an animal model was developed. The Medial Collateral Ligament (MCL) was chosen as the experimental rodent model due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (23, 28-31). Furthermore, animals have bilateral MCLs allowing for sham injury on one side to serve as an internal control. Animal injury procedures were conducted as described previously.

After MCL injury, results show that that the timing of peak VEGF concentration can be modulated with the treatment method applied. Interventions that modulate the level of platelet recruitment appear to subsequently impact the amount of growth factors released from the process of degranulation and release of biomolecules from the platelet plug developed during the initial

hemostatic phase of the wound healing (14). The ramifications of reduced growth factors necessary for subsequent biochemical signaling can be impactful on various cascades of events necessary for restoring structure and function.

VEGF, the growth factor understudy, is a potent angiogenic factor that promotes the proliferation of vascular endothelial cells and new vasculature at the site of injury. A healing wound has increased metabolic needs and is highly responsive to changes in the oxygen supply (65). Collagen production requires adequate oxygen supply necessary for the hydroxylation of proline and lysine residues required for chemical bonds to form appropriately creating a mature collagen (18, 65).

Investigating the concentration of VEGF with NSAIDs and PRF treatments in relation to untreated animals provided us with insight into the impact of treatments applied on the temporal nature of growth factors concentrations within the MCL tissue.

Pilot Study

In normal tissue response, literature shows that VEGF peaks between days 3 and 7 as part of the normal wound healing response (8). For our pilot study, we performed an ELISA on day five to examine the efficacy of our methods. Our results shown in Fig. 3-12 indicate that on day five after injury, the PRF-treated animals had a lower injured/sham VEGF concentration ratio (~ 2-fold increase) when compared to the non-treated animals (~3-fold increase). This observation is noteworthy as the increase with PRF-treated animals was expected to be higher than non-treated animals. One possible explanation is that the growth factor expression is shifted to an earlier time point. Another possibility is that VEGF expression with PRF or MX treatment may decrease relative to the untreated ligament (8).

In either case, the pilot study results allowed us to optimize the testing procedure and increase our focus on the first seven days-post-injury to investigate the observation of a lower concentration of VEGF for PRF-treated animals than initially anticipated. In other words, a closer examination of VEGF concentration during the first seven days post-injury was needed to better understand the biochemical response based on the different treatments applied. Understanding how PRF and Meloxicam may have a temporal impact on growth factor concentration within injured ligaments was of interest and set the stage for the larger study.

Full Study

Statistical analysis of VEGF concentration for the untreated group shows a significant difference between the sham and injured MCLs timepoints (Table 3-2). The injured MCL on day five had the highest concentration post-injury and recovered to control levels after day seven (Table 3-3). On the other hand, the sham injury in untreated animals was the same as control levels for all time points. For the Meloxicam-treated animals, VEGF for the injured MCLs increased significantly (Table 3-4) from sham MCLs on days 3, 5, and 7 post-injury with a peak on Day seven which was significantly higher than all other timepoints and injury conditions (Table 3-5). Similarly, VEGF for the sham injured limb with Meloxicam treatment was the same as control levels for all time points. With PRF treatment, VEGF concentration also showed a significant difference between timepoints and injury conditions (Table 3-6). The injured MCLs peaked on day three post-injury and recovered to control levels on day seven, while the sham injury in those animals was the same as control levels for all time points similar to what was observed in the untreated and Meloxicam group (Table 3-7).

Untreated Group

Untreated animals reached peak VEGF concentration on day five post-injury which appears to be consistent with the inflammatory phase, which typically lasts for 5-7 days (18, 66).

Following trauma to ligament tissue, a blood clot is formed to achieve hemostasis (1, 10, 67). The hemostasis (bleeding and clotting) stage is the first stage of wound healing; it is initiated immediately after an injury occurs and it usually lasts a few hours. Platelets adhere to collagen fibers which are exposed following damage to the endothelium, forming a primary hemostatic plug. The injured blood vessels then undergo vasoconstriction and the endothelium and the platelets surrounding the injured area will activate the intrinsic coagulation cascade which produces fibrin clots. The fibrin clots formed are made up of the following components: collagen, thrombin, platelets, and fibronectin. The fibrin clot acts as a temporary barrier that prevents excessive bleeding and limits the spread of infectious agents into the bloodstream (68). As platelets continue to aggregate, they activate, degranulate, and release their granule's contents stimulating the local release of growth factors. The fibrin clot plays an important role in the healing process and serves as a scaffold for invading cells, and they help to concentrate the cytokines and release of growth factors in a temporal nature (10, 19, 69, 70). Examples of growth factors include platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF).

These signaling molecules initiate the inflammatory response which starts immediately as the clot is forming and lasts for a few days after injury (71). In the untreated group, the release of VEGF peaked on day five and returned to initial conditions on day seven. This is consistent with the wound healing response described and acts as a basis for comparison with other treatment conditions.

Meloxicam Group

With Meloxicam treatment time to peak VEGF concentration is delayed until day seven post-injury. This may be due to the impact of anti-inflammatory drugs on the production of Cyclooxygenase and subsequently on platelet recruitment and wound healing response.

Activated platelets degranulate releasing the content of their granules which are rich in growth factors necessary for further steps in wound healing. When Meloxicam, a non-steroidal anti-inflammatory drug (NSAID), is administered, the pathway to producing Cyclooxygenase (COX) is impacted by means of reducing the biosynthesis of prostaglandins and thromboxane through direct inhibition of cyclo-oxygenase, and consequently, reduction in pain and inflammation (72). COX is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, which is the precursor of several molecules, including prostaglandins, prostacyclin, and thromboxane (39). Meloxicam is a COX-2 preferential inhibitor and bears the same effect on the production of thromboxane and prostaglandins as other NSAIDs with studies showing 77% inhibition of thromboxane (36, 72).

Thromboxane is involved in platelet aggregation and prostaglandins mediate pain sensitization are both inhibited with NSAIDs (35, 40-42). Thromboxane stimulates activation of new platelets as well as increases platelet aggregation (73, 74). Limiting its production with NSAIDs has a direct impact on the developing thrombosis, or fibrin plug, which allows for restoration of hemostasis as well as providing a fibrin mesh that is rich in growth factors that are released slowly during the process of wound healing (34, 75). NSAIDs have also been found to contribute to excessive scar formation which is essentially related to interference with the inflammatory phase and the biomolecules and growth factors released that are necessary for an adequate platelet plug formation (76).

Based on our observations of VEGF concentration after MX treatment, there appears to be a temporal effect delaying the time to peak concentration until Day 7. This could be due to the impact NSAIDs may have on the development of a platelet plug and possibly increased chance of forming a weaker plug susceptible to further bleeding and subsequently slow but steady aggregation of platelets (32, 77).

PRF Group

Our results show that VEGF concentration for the injured MCLs was significantly higher than the sham MCLs on day three and returned to normal levels on day five. The early peak VEGF concentration on day three with PRF compared to the untreated group on day five appears to be due to the availability and proximity of biomolecules and growth factors released from activated platelets of the fibrin mesh (34). Similar effects were observed with rat osteoblasts where PRF released autologous growth factors gradually and expressed a stronger and more durable impact on proliferation and differentiation (78). The introduction of PRF at the site of injury immediately after injury appears to have shortened the timeframe for platelet aggregation and activation and therefore allowed for earlier release of growth factors from the fibrin plug. In contrast with Meloxicam treatment, PRF did not hinder the inflammatory phase of wound healing.

Platelet concentrates and initially Platelet Rich Plasma (PRP) was first introduced in 1998, emphasizing the rich growth factor content following platelet degranulation (79, 80). The main goal of platelet concentrates treatments is to increase the availability of platelets that contain biomolecules and growth factors responsible for repair at the site of injury (34, 48, 75, 81, 82). By doing so, an increased level of response is sought so that ultimately collagen expression and organization are improved and thereby reducing the time to mechanical and functional recovery (46-48).

Recent studies suggest that platelet rich concentrates possess analgesic properties thus providing pain relief at the site of injection (80, 83). With pain suppression, animals are more likely to mobilize sooner, and early mobility has been argued to better healing in various soft tissue injuries due to its ability to promote blood circulation (23, 37, 84). Hauser et al argue that early controlled resumption of activity after ligaments and tendons injury has beneficial effects including enhanced cellular synthetic and proliferative effects, increased strength, size, matrix organization and collagen content of ligaments and tendon (23). Although our study did not investigate pain suppression with PRF, it is an area worth further investigation.

CONCLUSION

Ligaments play a major functional role in the musculoskeletal system. When injured, the standard treatment methods focus on pain management and return to function as apposed to promoting recovery and healing of tissue to preinjury properties. The goal of this study was to provide a basis to discriminate between healing MCLs under different treatment conditions. With untreated animals, the wound healing phases were not impacted, and peak VEGF concentration was achieved at day five while with Meloxicam treatment, VEGF concentration steadily increased over the study period and peaked on day seven.

The fastest time to peak VEGF concertation was realized on day three with PRF treatment which appears to influence speeding up the wound healing response by making available platelets and growth factors as opposed to NSAIDs which interfere with platelet aggregation and have the opposite effect on platelet aggregation and fibrin plug formation during hemostasis. Meloxicam treatment was delayed to day seven but did not hinder achieving high VEGF concentration like that achieved with PRF (Fig. 3-14).

Learning from this study provides a basis for examination of ligament injury and healing in a controlled laboratory setting and provides insights on the use of current and emerging treatment methods. For example, ligament treatment methods should focus on treating the injury and improving the timeliness of the wound healing process as in PRF treatment while at the same time allowing for managing pain without interfering with platelet aggregation and activation processes necessary for making available growth factors that are integral for healing. For instance, alternative pain suppression treatments other than NSAIDs would be worth investigating further to allow for early mobility without hindering natural healing. Other future studies may also investigate PRF treatment with pain suppression treatment that does not interfere with platelet function.

In this study, biochemical investigation using ELISA showed that it is possible to discriminate between normal healing and different treatment methods and provided a basis to draw conclusions about different treatment methods.

CHAPTER 3 FIGURES

Figure 3-1

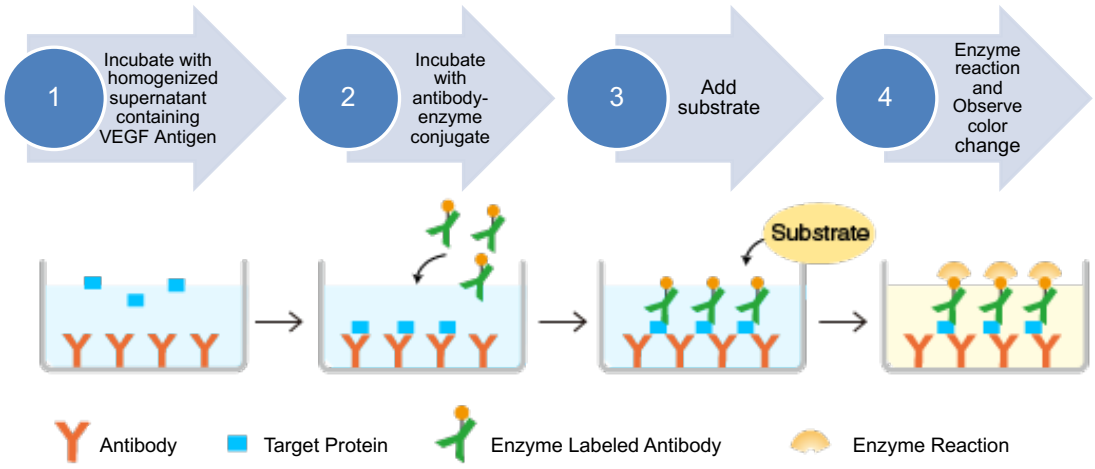

Figure 3-1
Enzyme-Linked Immunoassay (ELISA). Diagram shows a pictogram of the steps involved in sandwich ELISA analytical method.

Figure 3-2

Figure 3-2
Injured Medial Collateral Ligament (MCL). Image shows an untreated MCL removed at day seven after injury. The healing ligament shows the two ends of the transfected ligament (white color) and a **translucent tissue/ matrix formation.**

Figure 3-3

Figure 3-3
Eppendorf tube with steel beads mixture. Image shows the MCL (white tissue) with stainless steel bead mixture and Bio-Rad lysis buffer solution prior to tissue homogenization steps.

Figure 3-4

Figure 3-4
TissueLyser. Lab Equipment shown used for hominization of tissue. The frequency and duration are set for oscillating the tissue placed in Eppendorf tubes along with stainless steel beads and tissue lysis buffer solution. TissueLyser was set for 4 minutes at 30 Hz with a pause for 1 min halfway to place the tubes in ice to reduce the chance of protein loss.

Figure 3-5

Figure 3-5
Homogenized tissue thawing on ice after being stored at -80 degrees. Tissue is allowed to thaw on ice to protect protein integrity prior to starting the ELISA protocol.

Figure 3-6

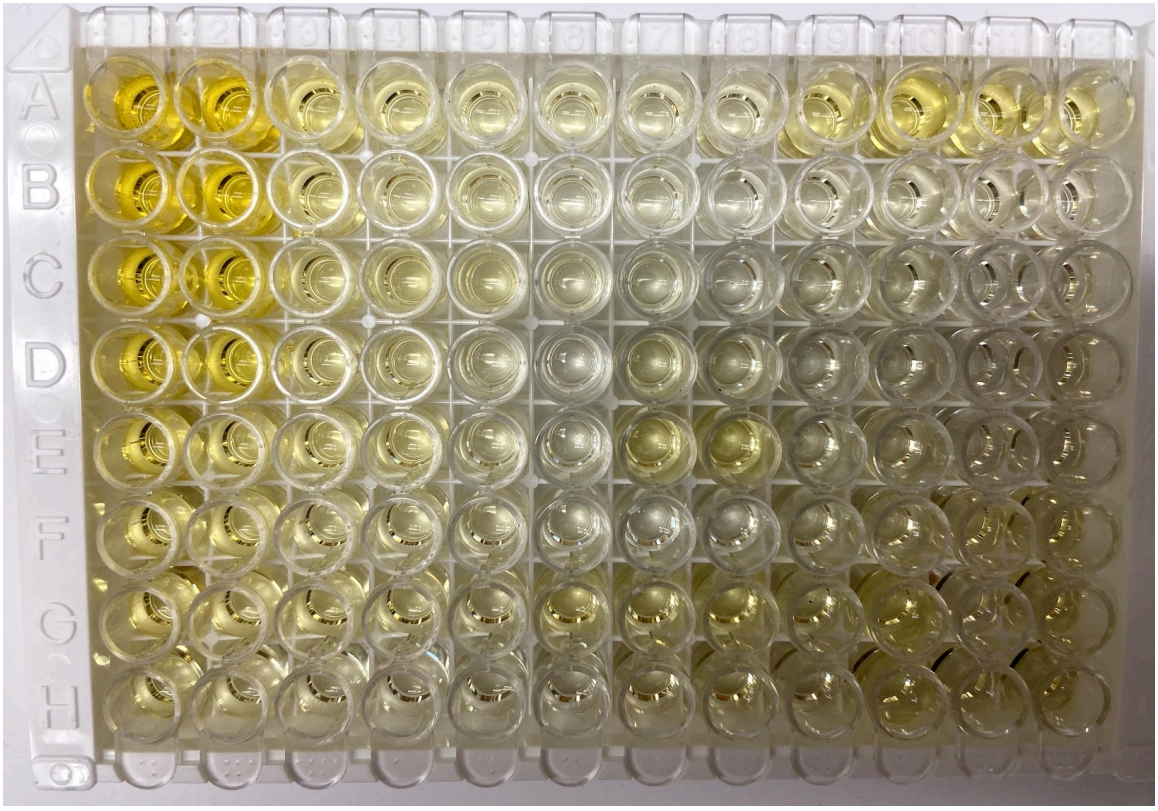

Figure 3-6
ELISA plate after adding the stop solution. ELISA plate showing different coloration indicative of the concentration of the target protein (VEGF) at the conclusion of the protocol. This plate is read using a plate reader measuring the optical density. Standard curve is calculated based on known concentrations which is later used to calculate the concentration in each well.

Figure 3-7

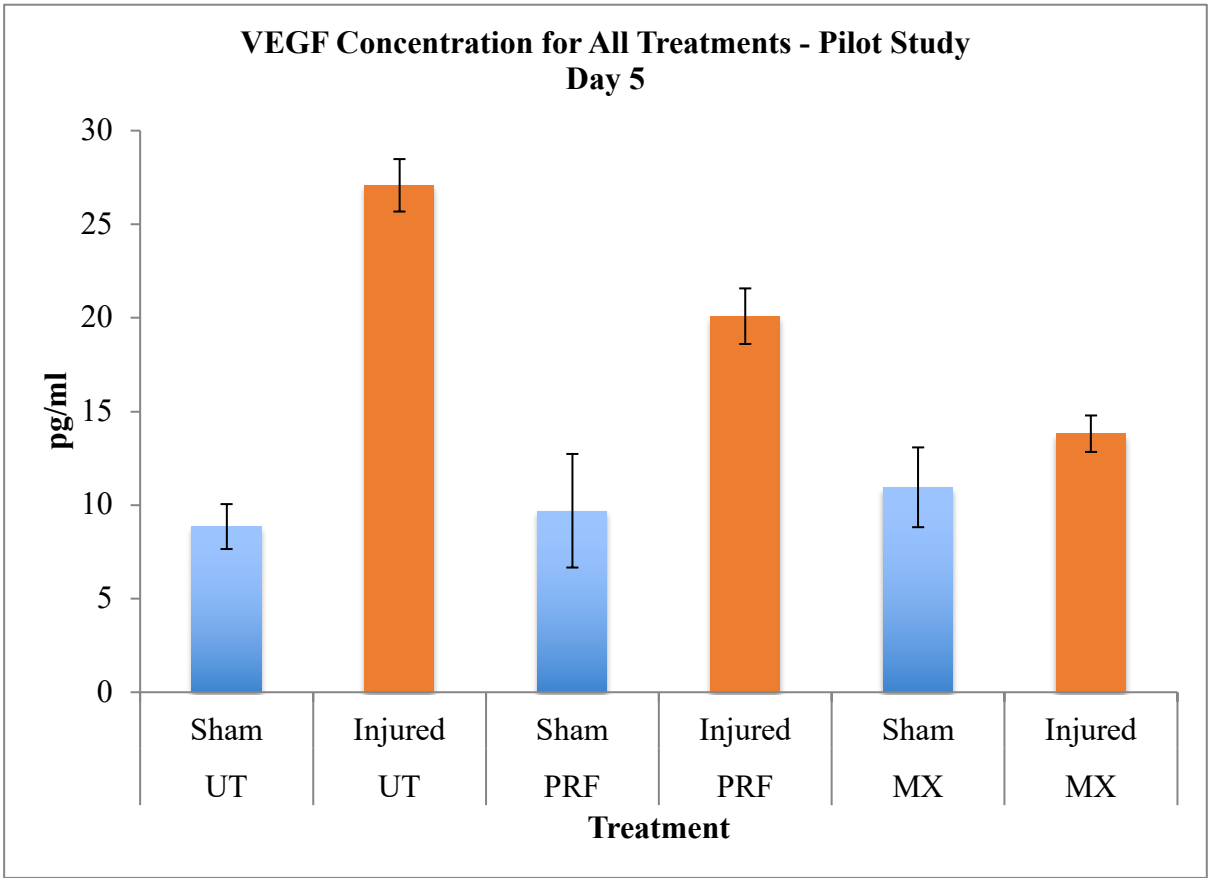

Figure 3-7
VEGF Concentration for Pilot Study. Figure shows pilot study VEGF concentrations for all treatments on Day 5 after injury.

Figure 3-8

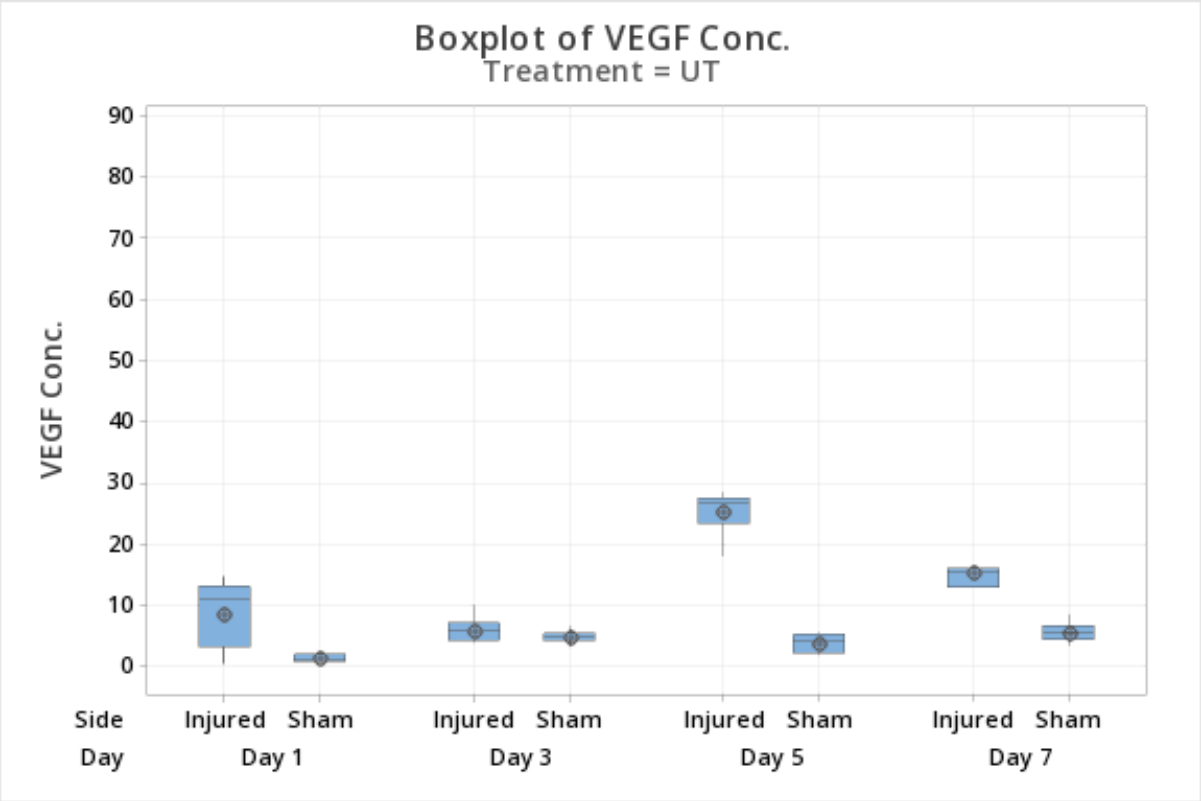

Figure 3-8
ELISA VEGF concentration of injured and sham MCLs for the Untreated group. Box plot shows injured and sham MCLs' VEGF concentrations measured by ELISA for the untreated animals. The largest difference between the sham and injured MCLs is on day five followed by day seven.

Figure 3-9

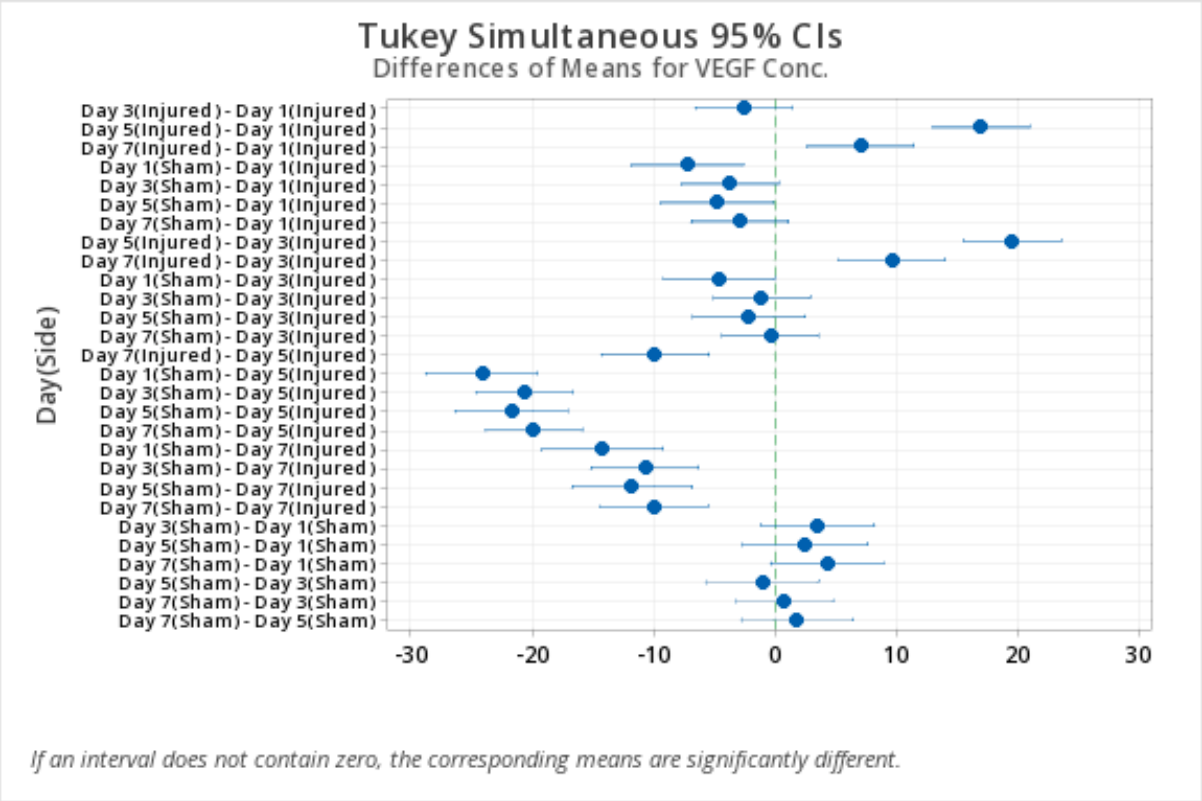

Figure 3-9
Tukey Post-hoc Statistics analysis method for untreated animals. VEGF ELISA post-hoc analysis shows that injured limbs on days 5 and day seven are significantly higher than the rest of the timepoints and injury conditions. In addition, days 5 and 7 are significantly different from each other with day five having a higher concentration of VEGF.

Figure 3-10

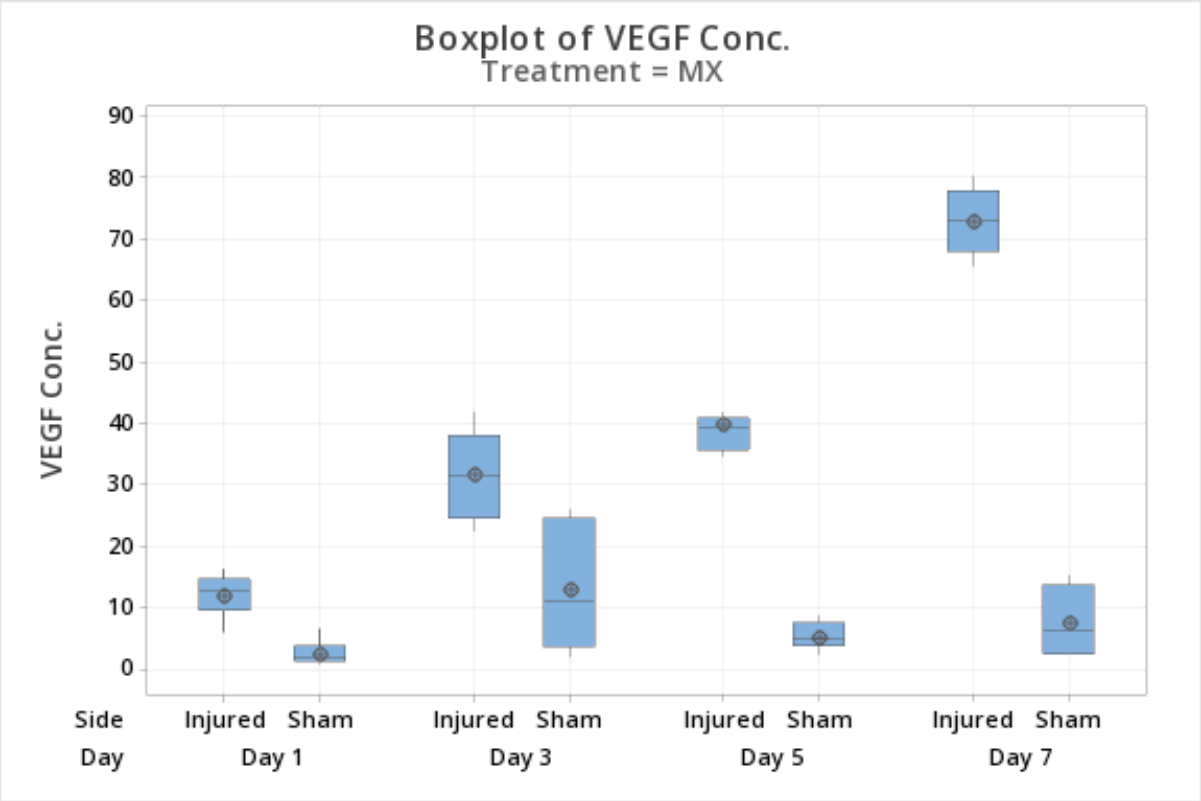

Figure 3-10
ELISA VEGF concentration of injured and sham MCLs for the Meloxicam Treatment. Box plot shows injured and sham MCLs' VEGF concentration measured by ELISA. The largest difference between the sham and injured MCLs is on day seven followed by day five.

Figure 3-11

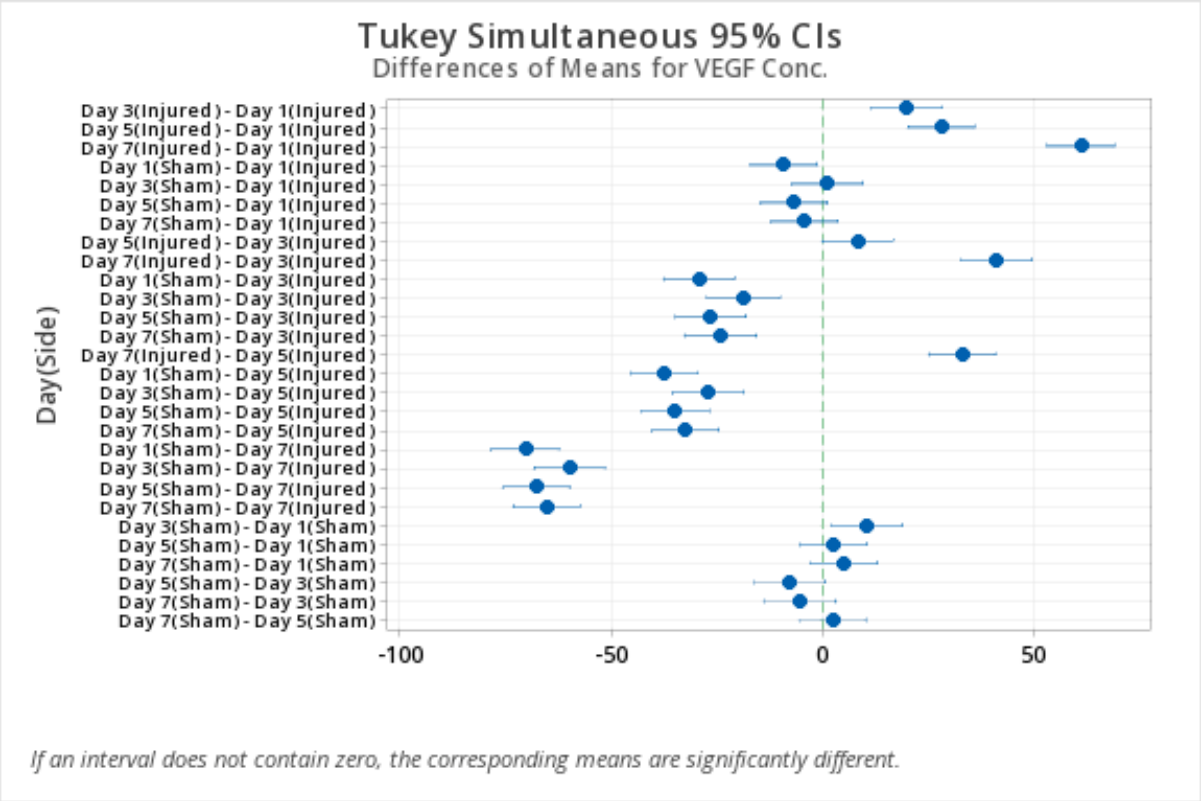

Figure 3-11
Tukey Post-hoc Statistics analysis method for Meloxicam Treatment. VEGF ELISA post-hoc analysis shows that injured MCL on day seven has significantly higher concentration than the rest of the timepoints and injury conditions. In addition, days 3 and 5 are significantly higher than the rest of the timepoints except for Day 7. Meloxicam treatment indicates a delay in VEGF expression compared to untreated animals.

Figure 3-12

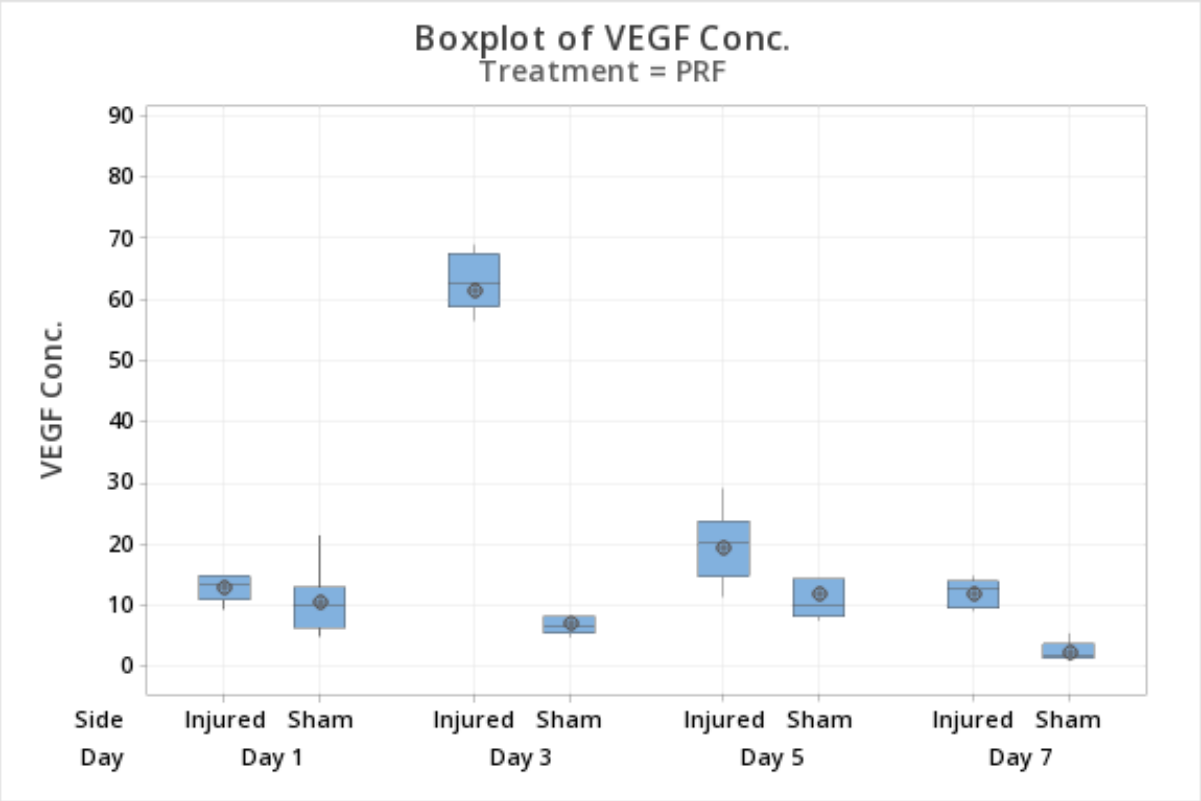

Figure 3-12
ELISA VEGF concentration of injured and sham MCLs for the PRF Treatment. Box plot shows injured and sham MCLs' VEGF concentration measured by ELISA. The largest difference between the sham and injured MCLs is on day three.

Figure 3-13

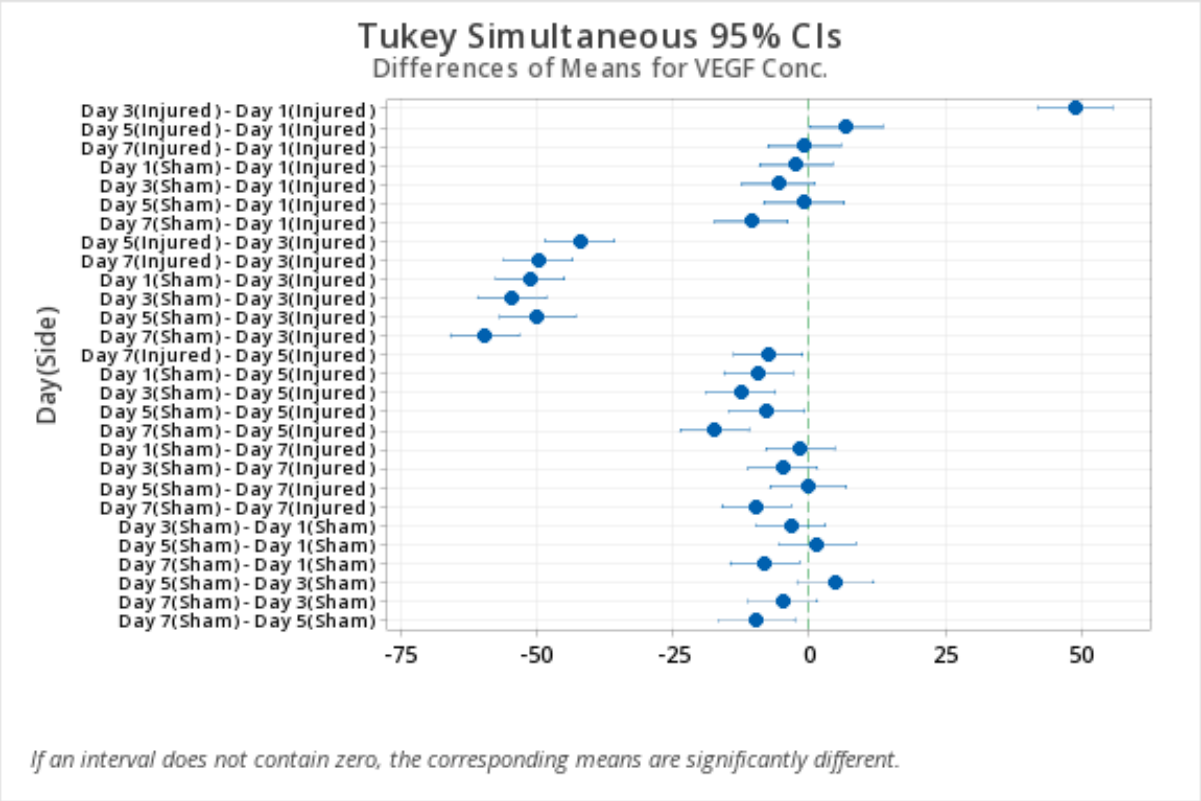

Figure 3-13
Tukey Post-hoc Statistics analysis method for PRF Treatment. VEGF ELISA post-hoc analysis shows that injured MCL on day three has significantly higher concentration than the rest of the timepoints and injury conditions. In addition, day five is significantly higher than the rest of the timepoints except for Day 7. MCLs treated with PRF show a shorter timeframe to an increased VEGF concentration compared to sham MCLs within the same animal. VEGF concentrations for sham MCLs are the same on all days following injury.

Figure 3-14

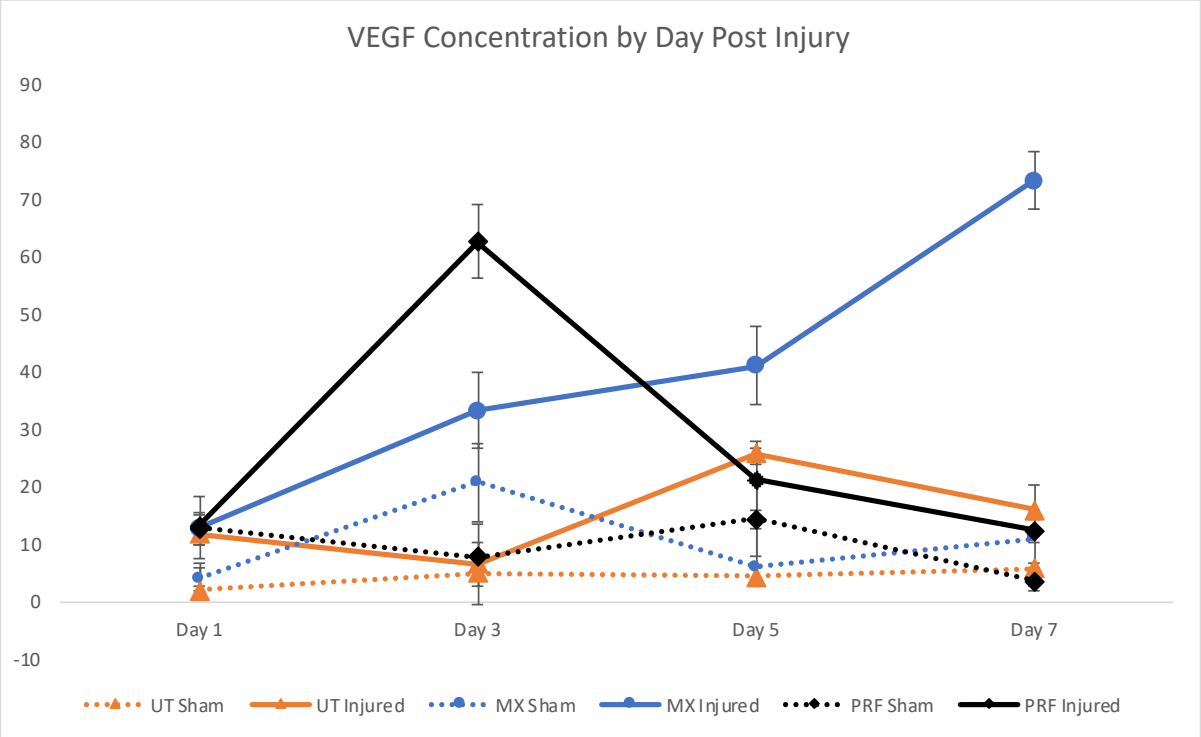

Figure 3-14
VEGF Concentration by Day after Injury - All Treatments. The figure shows the concentration of VEGF for all treatments by day. The time to peak VEGF for the UT is on day five, for MX is on day seven, and for PRF is on day three. Time to peak VEGF concentration varies with treatment.

Figure 3-15

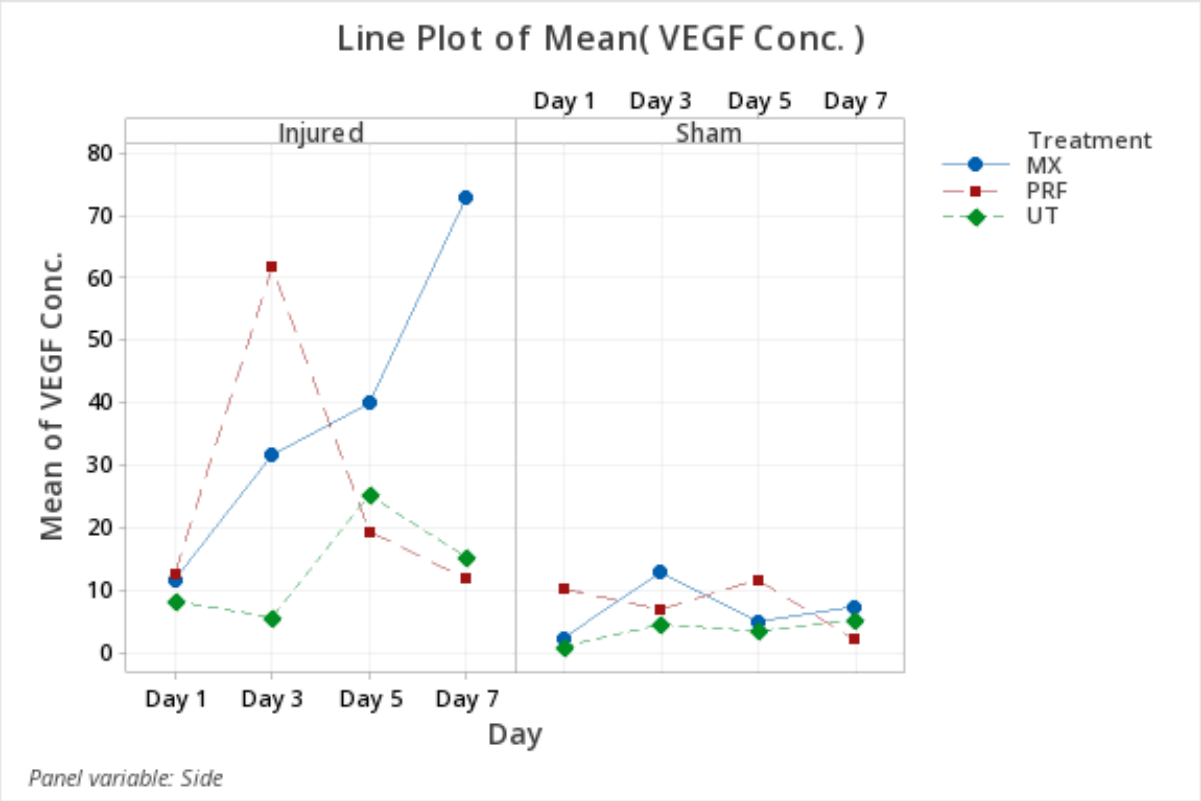

Figure 3-15
VEGF Concentration by Day after Injury - All Treatments. The figure shows a line plot for VEGF concentration for all treatments by day and injury conditions. The time to peak VEGF for the UT is on day five, for MX is on day seven, and for PRF is on day three. The sham injury had no effect on the MCL VEGF concertation.

Figure 3-16

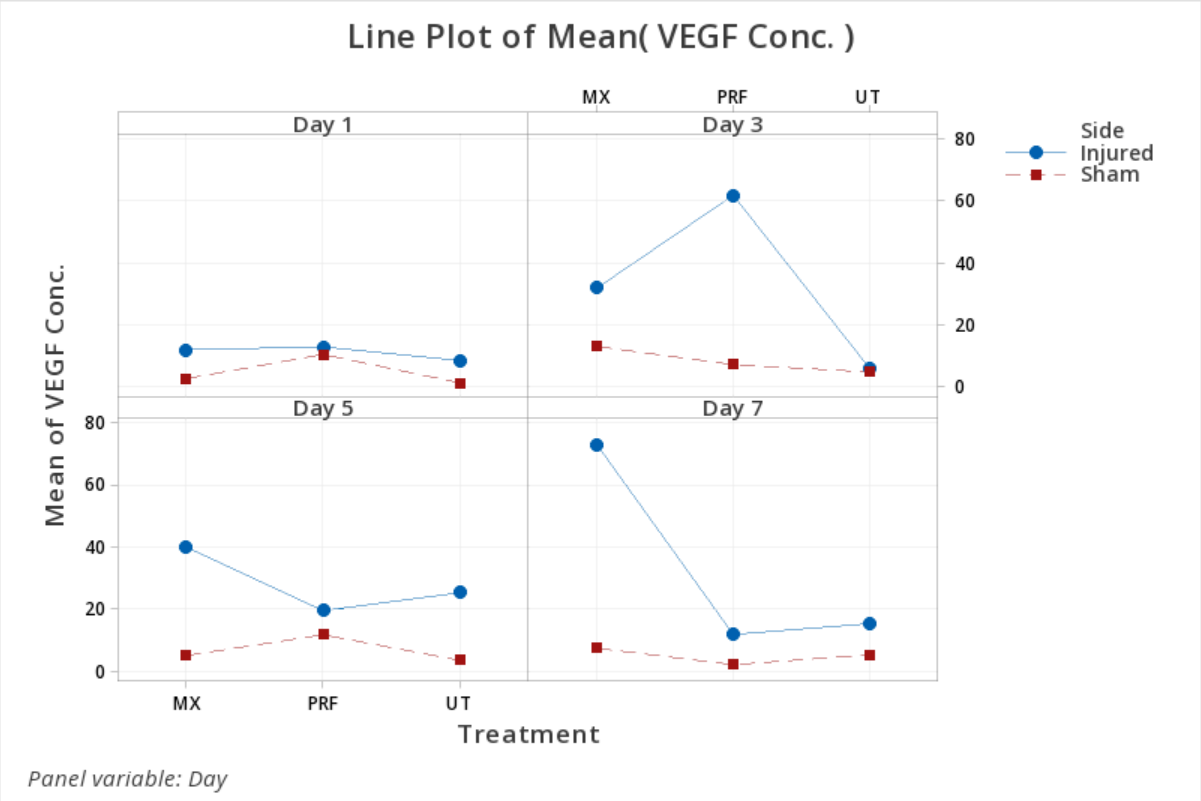

Figure 3-16
VEGF Concentration by Day after Injury - All Treatments. Figure shows a line plot for VEGF concentration with the day after injury acting as the panel variable for all treatments and injury conditions. There is no difference between sham and injured MCL on day one for all injury conditions. Day 3 shows a peak for PRF and an increase for MX compared sham. Day 5 shows MX with elevated concentration and the highest level for the UT compared sham. Day 7 shows peak VEGF for MX. Time to peak VEGF for the UT is on day five, for MX is on day seven, and for PRF is on day three. Sham injury had no effect on the MCL VEGF concertation on all days after injury.

CHAPTER 3 TABLES

Table 3-1. Summary Statistics. Injured and sham MCL VEGF average concentrations and standard deviation for each timepoint under study.

		MX		PRF		UT	
		Injured	**Sham**	**Injured**	**Sham**	**Injured**	**Sham**
Day 1	**Average**	12.87	3.97	13.18	12.88	11.83	1.98
	STDEV	2.83	1.93	1.87	5.40	3.11	0.64
Day 3	**Average**	33.29	20.87	62.57	7.89	6.56	5.00
	STDEV	6.68	7.03	6.36	2.61	2.01	1.02
Day 5	**Average**	40.97	6.13	21.27	14.48	25.79	4.38
	STDEV	6.75	2.02	5.34	6.65	2.72	1.29
Day 7	**Average**	73.29	11.11	12.39	3.74	16.00	5.91
	STDEV	4.84	4.40	1.94	1.79	2.90	1.52

Table 3-2. Analysis of Variance Table for VEGF ELISA - Untreated Group. ANOVA results showing significant differences observed with over the study period between sham and injured MCLs as well as between the side nested within day after injury.

Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	**P-Value**
Side	1	1640.40	1640.38	201.86	0.00
Day(Side)	6	2347.00	391.16	48.14	0.00
Error	61	495.70	8.13		
Total	68	4403.40			

DF = Degree of Freedom, Adj SS = Adjusted Sum of Squares, Adj MS= Adjusted Mean Square, Error = Residual Error.

Table 3-3. Grouping Information for the Untreated Group Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey Post-hoc analysis for the untreated group shows the injured side on day five and day seven to be significantly different from each other and to also be higher than all other days and injury conditions. VEGF concentrations for sham MCLs are the same on all days following injury.

Day(Side)	N	Mean	Grouping				
Day 5(Injured)	10	25.41	A				
Day 7(Injured)	7	15.50		B			
Day 1(Injured)	10	8.48			C		
Day 3(Injured)	10	5.91			C	D	
Day 7(Sham)	10	5.52			C	D	E
Day 3(Sham)	10	4.77			C	D	E
Day 5(Sham)	6	3.72				D	E
Day 1(Sham)	6	1.27					E

Means that do not share a letter are significantly different.

Table 3-4. Analysis of Variance Table for VEGF ELISA – Meloxicam Treatment. ANOVA results show significant differences observed over the study period between sham and injured sides as well as between the side nested within a day after injury.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Side	1	19356.00	19355.90	601.80	0.00
Day(Side)	6	19774.00	3295.60	102.47	0.00
Error	68	2187.00	32.20		
Total	75	42361.00			

Table 3-5. Grouping Information for the Meloxicam Treatment Using the Tukey Post-hoc Statistics Method and 95% Confidence. Tukey Post-hoc analysis for the Meloxicam group shows the injured MCL on day seven to be significantly higher than all other days and injury conditions. Injured MCL VEGF concentrations on days 3 and 5 are the same and are significantly less than day seven while being significantly higher than all other days and injury conditions. VEGF concentrations for sham MCLs are the same on all days following injury.

Day(Side)	N	Mean	Grouping			
Day 7(Injured)	10	72.96	A			
Day 5(Injured)	10	40.10		B		
Day 3(Injured)	8	31.82		B		
Day 3(Sham)	8	13.05			C	
Day 1(Injured)	10	11.96			C	
Day 7(Sham)	10	7.61			C	D
Day 5(Sham)	10	5.24			C	D
Day 1(Sham)	10	2.63				D

Means that do not share a letter are significantly different.

Table 3-6. Analysis of Variance Table for VEGF ELISA – PRF Treatment. ANOVA results show significant differences observed over the study period between sham and injured sides as well as between the side nested within a day after injury.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Side	1	6394.00	6393.71	311.61	0.000
Day(Side)	6	16952.00	2825.28	137.70	0.000
Error	67	1375.00	20.52		
Total	74	25575.00			

Table 3-7. **Grouping Information for the PRF Treatment Using the Tukey Post-hoc Statistics Method and 95% Confidence.** Tukey Post-hoc analysis for the PRF group shows the injured MCL on day three to be significantly higher than all other days and injury conditions. Injured MCL VEGF concentrations on day five is significantly less than day three while being significantly higher than all other days and injury conditions. VEGF concentrations for sham MCLs are the same on all days following injury.

Day(Side)	N	Mean	Grouping			
Day 3(Injured)	10	61.77	A			
Day 5(Injured)	10	19.66		B		
Day 1(Injured)	8	12.89			C	
Day 7(Injured)	10	12.03			C	
Day 5(Sham)	7	11.92			C	
Day 1(Sham)	10	10.44			C	
Day 3(Sham)	10	7.17			C	D
Day 7(Sham)	10	2.32				D

Means that do not share a letter are significantly different.

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CHAPTER 4

HISTOLOGY

ABSTRACT

Ligaments play a crucial role in the musculoskeletal system in maintaining joint stability. When ligament injury occurs, a wound healing cascade is initiated starting with platelet plug formation and ending with tissue remodeling. A rat Medial Collateral Ligament (MCL) surgical injury model was developed to examine histological tissue sections. Animals (n=20) were randomized to 4 equal groups of 5 animals for histological examination on days 3, 7, 14, and 28 post-injury. Paraffin-embedded sections were examined under light microscopy with Hematoxylin and Eosin (H&E) staining using the Bonar assessment criteria. Results show marked differences between injured and sham sections as well as a temporal difference between day three and day twenty-eight with regards to cellularity, collagen morphology and organization as well as vascularity.

Using a rodent model opens the opportunity to evaluate ligament histology and morphology. Future work will examine histological MCL sections for different treatment methods such as platelet-rich concentrate and anti-inflammatory drugs as well as different submaximal injury mechanisms representative of occupational injuries.

ABBREVIATIONS

ANOVA: Analysis of Variance
COX: Cyclooxygenase
ELISA: Enzyme-Linked Immunoassay
IACUC: Institutional Animal Care and Use Committee
MCL: Medial Collateral Ligament
MX: Meloxicam
NSAIDs: Non-Steroidal Anti-inflammatory Drugs
PRF: Platelet Rich Fibrin
PRP: Platelet Rich Plasma
RICE: Rest, Ice, Compression, and Elevation

INTRODUCTION

Skeletal ligaments are dense bands of collagenous fiber tissue that span a joint restraining one bone to another forming a flexible skeletal joint (1, 2). Ligaments are described as pale from the limited blood supply (2). As a "first line of defense" to mechanical loading on joints, skeletal ligaments play a crucial role in the musculoskeletal locomotive system in resisting tensile and torsional forces affecting the joint. Their main function is to guide and restrain skeletal motion while keeping the joint structure intact (3).

Ligaments are passive tissues with viscoelastic properties susceptible to creep deformation under sustained static loading (4). Stretching viscoelastic tissues repeatedly for long periods exposes them to microdamage, "In the collagen fibers, irrespective of the load applied" (5). Depending on the level of loading, injuries occur, and a repair process commences.

After injury, ligaments undergo a series of wound healing events to restore original tissue structure and function however results vary, and in most cases, repaired ligaments are inferior from a mechanical and functional standpoint (6-9). The repair process is marked by four phases: restoration of hemostasis, inflammation, repair, and finally remodeling (10). During platelet activation and degranulation, growth factors are released from their granules initiating other cascades necessary for wound healing and restoration of structure and function (11). Biochemical, mechanical, and histological studies of healing ligament in a complete tear of the medial collateral ligament (MCL) show healed ligament with scar tissue that is mechanically inferior and biochemically abnormal in its composition and architecture concluding that these properties do not return to normal (12).

Examining the histological and morphological tissue response at early stages of healing will provide an insight on natural healing and a basis to examine various treatment methods and injury mechanisms.

Wound Healing Phases

There are three clinical ligament injury grade categories depending on the level of tear with grade 1 associated with mild tears and grade 3 depicting a complete tear of the ligament (13, 14). Ligament healing follows the four main phases of wound healing starting with hemostasis and ending with remodeling (10).

When ligaments are disrupted and blood vessels are severed, the endothelium is damaged. It then exposes the basal lamina to blood plasma and peripheral blood cells (15). Hemostasis is achieved when damaged blood vessels are temporarily repaired with the creation of a platelet plug/clot. This step is dependent on the level of platelets recruited at the site of injury (10, 15). In addition, the growth factors and biomolecules released from platelets play a major role in the deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (16). Activated platelets are followed by an influx of inflammatory cells in the first 48 hours (15). This is followed by proliferation which is a set of four steps that begin at various time periods in wound healing: (1) epithelialization (2) angiogenesis, (3) granulation tissue formation, (4) and collagen deposition (10). Repair and remodeling of tissue occurs following proliferation and initially the extracellular matrix mainly made up of collagen, is deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet providing a basis for a new matrix (17). The newly deposited collagen is reorganized into structurally sound lattice and the immature type III collagen is replaced with type I collagen that is structurally stronger and wound strength is increased (10, 15). Healing of ligaments is slow and incomplete creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (9, 15).

Studies show that the mechanical properties of the healing or remodeled ligaments are inferior to the native tissue (17-22). In some studies, ligaments show a regain of stiffness and strength that is 40% to 80% of normal and inferior creep properties with an elongation that is twice as much as normal tissue with suggestions that deformations may be permanent or long-lasting (20). With inferior mechanical properties, ligament's function is affected leading to instability at the joint and therefore susceptibility of other structures to injury is increased (e.g., cartilage and meniscus tears) in addition to the inefficacy of muscles in the locomotion system due to increased sliding between joint surfaces (20, 23, 24). With altered mechanical properties, the load distribution around the joint is disrupted leading to altered contact mechanics with increased shear forces affecting cartilage and bones, and eventually leading to osteochondral degeneration or osteoarthritis and other joint chronic diseases (20, 23).

Research Void

When ligaments are injured, the innate immune response and wound healing takes effect with the goal of restoring tissue structure and function. Tracking natural wound healing with histological methods will provide an additional layer of examination to assess healing and tissue repair as well as allow for testing the efficacy of different treatments and injury conditions. These findings together with biochemical and functional recovery will paint a comprehensive picture of healing after a ligament injury.

Study Aim

In this study, we examined histological sections and soft tissue assessment methods. We established an MCL injury in a rat model and optimized histological techniques.

MATERIALS AND METHODS

Animals

The study was approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC). Twenty skeletally mature male Wistar rats (The Jackson Laboratory) were used as an animal model for ligament healing. The animals were untreated and 5 subjects were randomly assigned to select timepoints after injury for MCL tissue harvest on days 3, 7, 14, and 28. Animals were fed rat chow and housed in pairs per cage under the care of a university veterinarian. Detailed surgical procedure is described in detail in chapter 1. Briefly, a surgically transected, rather than torn, Medial Collateral Ligament (MCL) of the knee was used as an experimental model to create a uniform defect for healing. After two days of acclimatization in the housing facility, the transection surgical procedure was undertaken to induce ligament transection and sham injuries for each animal. Surgical transection was done on the right side and sham injury on the left side as described in the literature (25). Following injury, animals are returned to their cages and allowed free-roaming, access to water, and ambulation.

Tissue Harvest and Fixation

MCL tissue was harvested on days 3,7, 14, and 28 post-injury. The animals were sacrificed in a carbon dioxide (CO2) chamber to effect. Hindlimbs were skinned and the superficial fascia that is not adherent to the ligament or its surrounding connective tissue was carefully cut and reflected. The overlying connective tissue is dissected to expose the MCL. The MCLs were carefully dissected without disturbing the scar region. The bulk of the proximal musculature was removed. The femur and tibia/fibula were then cut transversely at approximately the midpoint

leaving the MCL and the knee joint intact (Fig. 4-1). The harvested femur-MCL-tibia/fibula complex is then fixed as a whole in 4% formalin for 48 hours and then placed in 70% ethanol for up to 14 days (Fig. 4-2).

Histological Processing Procedure

On the day of embedding, the MCL is removed using a scalpel with the blade placed under the MCL on the fibula side and moved distally from the knee joint to free the MCL at the insertion site of the fibula. Careful attention to avoid removing bone tissue was observed and, in such cases, where the bone is removed, the insertion site was approximated, and bone tissue was cut transversally leaving behind ligament tissue. The scalpel is then moved under the MCL towards the femoral side freeing the superficial MCL from the meniscal attachment (deep MCL) and moving distally from the knee towards the femoral attachment site of the MCL. Similarly, careful attention to avoid removing bone tissue.

MCLs are immediately placed in screen histological white cassettes with the MCL's skin side up and femur side pointing towards the top of the cassette secured with polyester foam biopsy pads (Histo Pals^{TM,} Bio Pals, Inc. San Rafael CA) as shown in Fig. 4-3. The pads will ensure samples maintain orientation after removal and placement in the cassettes. The cassettes are labeled with the sample number and are then processed overnight using autotechnicon laboratory equipment (Fig. 4-4). The equipment processes tissue samples through a series of steps starting with dehydrating the samples in increasing concentrations of ethanol 70%, 80%, 95%, 100%. This is followed by a rinse of the samples with 50% Toluene/50% 100% Alcohol then a rise in Toluene followed by a rinse in Xylene. The samples are then held in paraffin wax until embedded into paraffin wax blocks.

Paraffin Embedding Procedure

Embedding in paraffin wax will ensure the preservation of tissue as well as allow for sectioning and creation of microscope slides. A histoembedder is used for this process. Cassettes with proper sample numbers are prepared before starting the embedding process. Ligaments are placed in the smallest possible mold when embedding. We embedded samples with the skin side and femoral side registered in a specific orientation to maintain the registration of tissue as they are embedded (Fig. 4-5). This will allow us to section the MCL and obtain sections in a side view cross-section which will provide tissue images with the skin and joint sides on one slide.

A small amount of the wax is placed on the base of the mold and the ligament is carefully placed in the desired position and the wax is allowed to cool to hold the ligament in place. The labeled cassette is then placed on top of the mold and additional wax is dispensed to create the mold (Fig. 4-6). In some cases, the ligament segment is not straight and best effort to embed in the desired orientation was sought.

Sectioning Procedure

We ensure blocks are frozen at -20 degrees for at least 24 hours prior to sectioning

And we also ensured that blocks are not left out of the -20-degree freezer prior to sectioning for more than 2-3 minutes. When "facing" the block, careful attention should be observed to the initial speed of sectioning at 15 micros to avoid damage to the block. After facing the block, it is placed back in a -20 degrees freezer for 5 minutes with the sectioning face placed down on the freezer ice before sectioning to ensure the proper temperature for proper sectioning results. We then placed the block back in the microtome and section at 5 microns. We obtain six sections from

each sample and then mount sections on slides and place on dehydrating rack overnight and cover plated the next day.

Staining Procedure of Paraffin Sections

Hematoxylin and Eosin (H&E) were used to stain MCL histological sections. This staining technique displays various cytoplasmic, nuclear, and extracellular matrix characteristics. Hematoxylin stains nucleic acids in dark deep blue-purple color while eosin is pink and stains proteins nonspecifically. A typical finished tissue is stained blue at the nuclei whereas the cytoplasm and extracellular matrix have varying degrees of pink staining (26).

The following steps were followed to stain MCL sections with H&E.

1. Deparaffinize in Toluene: **Three times at five minutes each.**
2. 50/50 100% EtOH and Toluene: **One time at 3 min.**
3. Hydrate in EtOH (100%, 100%, 95% 80%): **Two minutes in each EtOH concentration.**
4. Rehydrate in Deionized water (in hood): **One minute.**
5. Rehydrate in Deionized water (at sink): **One minute.**
6. (Hematoxylin) Gill's III Stain-Undiluted (Filter before and after use): **Three minutes.**
7. Rinse in Deionized water (at sink): **One minute.**
8. Scott's Tap Substitute: **One minute.**
9. Rinse in Deionized water (at sink): **Two minutes.**
10. Dehydrate in 70% EtOH: **One minute.**
11. Polyscience Eosin Y: **10 seconds.**
12. Dehydrate in EtOH (80%): **5 dips**
13. Dehydrate in EtOH (95%): **5 dips**
14. Dehydrate in EtOH (95%): **5 dips**
15. Dehydrate in EtOH (100%): **15 dips**
16. Dehydrate in EtOH (100%): **Two minutes.**
17. 50/50 100% EtOH and Toluene: **Two minutes.**
18. Clear in Toluene then Xylene: **Three minutes each.**
19. Mount and Coverslip.

Pilot Study

Optimization of tissue fixing, embedding, and sectioning methods was performed to achieve desirable results.

Image Analysis

A 12 Megapixel video camera, Leica DFC 500, mounted on a light microscope, Olympus BH 2, was used to view and capture MCL section images at a magnification of 4x. ImageJ, a freeware application available from the National Institutes of Health (NIH) was used to view and process images for further assessment.

RESULTS

Laboratory Optimization Techniques

Tissue Fixing Optimization

Initially, MCL tissue was removed from animals and fixed directly in a 4% formalin solution. The resulting MCLs upon sectioning did not maintain orientation and were twisting producing poor sections and images. This issue was resolved by fixing tissue as a complex femur-MCL-tibia/fibula. The resulting MCL after fixing in 4% formalin and then in 70% ethanol were desirable with no twisting during embedding steps or sectioning of MCL tissue.

Tissue Embedding Optimization

Tissue embedding in paraffin wax required maintenance of orientation from the moment the MCL tissue was excised from the animal (Fig. 4-3, 4-4, and 4-5). Prior to sectioning, maintaining registration was achieved by scoring one corner of the paraffin block at the direction of skin side of the MCL and femoral end. When 5-micron sections were obtained using a microtome, the scored sides were picked up from the water bath in the same direction keeping the registration intact on the slides. This is critical as paraffin film will be dissolved during processing.

Sectioning Optimization

The angle of sectioning was set at 5 degrees. Paraffin blocks were best sectioned when kept frozen at -20 degrees for at least 24 hours prior to sectioning and when sectioning is done within 2-3 minutes of removal from the freezer.

Staining optimization

Increasing the length of time to deparaffinize tissue sections in toluene at the start and end of the staining steps improved the quality of the slides.

Histological Assessment Results

We used criteria described in the Bonar scoring system for the ligament assessment (27). Bonar assessment examined sections for changes in fibroblast morphology, collagen bundle characteristics and variations in vascularity. A more detailed criteria for histological examination is described below:

- ***Collagen Fibers***
    - ***Fiber Arrangement.***
        - In the normal ligament tissue, the fibers are parallel to each other and well organized.
        - Abnormal tissue shows various degrees of loss of this ordered arrangement.
    - ***Fiber Structure.***
        - Normal ligaments show closely packed collagen fibers lying parallel to each other, with slight waviness.
        - Increased waviness and separation of the fibers accompany slight and moderate changes.
        - Markedly abnormal specimens show loss of the finer fiber structure.

- ***Fibroblasts:***
  - ***Cellularity***.
    - The whole slide is assessed for areas of increased cellularity.
  - ***Fibroblast Nuclei.***
    - Fibroblasts of normal ligaments have flattened, or spindle-shaped, nuclei arranged in rows between the collagen fibers.
    - With increasing degrees of abnormality, the number of nuclei markedly increases.
    - The nuclei have a more rounded appearance in abnormal fibroblast.
- ***Vascularity***.
  - Vascular bundles usually run parallel alongside the collagen fibers. The number of these vascular bundles increases with degeneration of tissue.

Histological Assessment of Sham Ligaments

Fig. 4-7 shows the untreated sham MCL on day three Post Injury. The MCL shows organized collagen fibers with normal crimping and minimal cellularity. Elongated nuclei with minimal cytoplasm indicate mature cells in the body of the ligament. Minimal vasculature is observed in the body of the ligament with no clusters of capillaries observed. The same observations were made on all sham MCLs on all other days post-injury (Fig. 4-9, 4-11 and 4-13).

Day 3 Histological Assessment

Fig. 4-8 shows the untreated injured MCL on day three post-injury. The MCL section shows highly disorganized collagen fibers and increased cellularity across the entire body of the MCL. Rounded nuclei indicate immature cells in the body of the ligament and increased vasculature and clusters of capillaries is observed in the body of the ligament. Separation of fibers and loss of architecture is observed.

Day 7 Histological Assessment

Fig. 4-10 shows the untreated Injured MCL on day three Post Injury. MCL continues to show highly disorganized collagen fibers with loss of crimping and increased cellularity across the entire body of the MCL. Rounded nuclei with cytoplasm indicate immature cells in the body of the ligament. Increased vasculature and clusters of capillaries are observed in the body of the ligament. Separated fibers and loss of architecture are observed. The ligament is more organized than on day three.

Day 14 Histological Assessment

Fig. 4-12 shows the untreated Injured MCL on day fourteen Post Injury. MCL shows disorganized collagen fibers with minimal regain of crimping and continued increased cellularity across the entire body of the MCL. Nuclei are less rounded indicate maturing cells in the body of the ligament. The vasculature is less visible in the body of the ligament. The ligament appears more organized than day seven.

Day 28 Histological Assessment

Fig. 4-14 shows the untreated injured MCL on day twenty-eight Post Injury. MCL shows more organized collagen fibers with regain of collagen crimping. The continued increase in cellularity across the entire body of the MCL is observed. Nuclei are more rounded in certain parts of the ligament indicating maturing cells in the body of the ligament. Vasculature and clusters of

capillaries are not visible in the body of the ligament. The ligament is more organized than day fourteen.

DISCUSSION

The Bonar assessment method used for examining injured MCLs over the study period examined cellularity, vascularity, and collagen organization (27). Histological and morphological examination of MCL tissue shows noticeable differences between healing and sham injury response following a surgical transection. After MCL injury, results show noticeable temporal histological changes between day three and day twenty-eight post-injury (Fig. 15). The histological sections on day twenty-eight show a remarkable cellular activity indicating that healing and repair remain underway with no visible restoration of the tissue to its original state.

To investigate ligament injury and healing response, an animal model was developed. The Medial Collateral Ligament (MCL) was chosen as the experimental rodent model due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (20, 28-31). Furthermore, animals have bilateral MCLs allowing for sham injury on one side to serve as an internal control. Animal injury procedures were conducted as described previously.

After ligament injury and as platelets continue to aggregate, they activate, degranulate, and release their granule's contents stimulating the local release of growth factors. These signaling molecules initiate the inflammatory response which starts immediately as the clot is forming and lasts for a few days after injury (32). The outcome of these is the recruitment of various cellular components during inflammation and proliferation which is observed through histological sections.

Future Work

The assessment method used in this study laid the stage for further studies examining treatment methods. A cross-examination of the effects of Platelet Rich Fibrin (PRF) and Non-Steroid Anti-Inflammatory Drugs (NSAIDs) is underway. Experiments were performed using PRF and Meloxicam as treatment methods and MCL tissue was embedded in paraffin blocks for histological assessment and analysis. Due to delay in research during the pandemic, these experiments were not carried fully, and post-doctoral research is planned to draw comparisons between histological findings in untreated, Meloxicam, and PRF. Subsequently, an overall comparison between histological, biochemical, and functional recovery will be performed to paint a more comprehensive picture of ligament injury and healing under different treatment methods.

CONCLUSION

Ligaments play a major functional role in the musculoskeletal system. When injured, the standard treatment methods are focused on pain management and return to function as appose to promoting time to recovery and healing of tissue to preinjury properties. The goal of this study was to provide a histological examination of untreated healing MCL tissue and set the stage for further assessment under different treatments with Meloxicam and PRF. In addition, sub-maximal (i.e., mild non-surgical injury) examination using histological methods will be performed under different treatment methods.

Investigating the histological and morphological outcomes of MCL injury provided us with insight on the impact of injury and the progression of healing during the first 28 days after injury. In this study, the histological investigation showed that it is possible to discriminate between healing and sham MCLs and provided a basis for further analysis of different treatment methods. Injured ligaments showed temporal changes between day three and day twenty-eight however,

these changes were not in alignment with our observation during functional recovery. It appears that animals recover to function much sooner than structure is restored.

Future studies will utilize learnings from this histological examination of healing ligaments and study the effect of other treatment methods.

CHAPTER 4 FIGURES

Figure 4-1

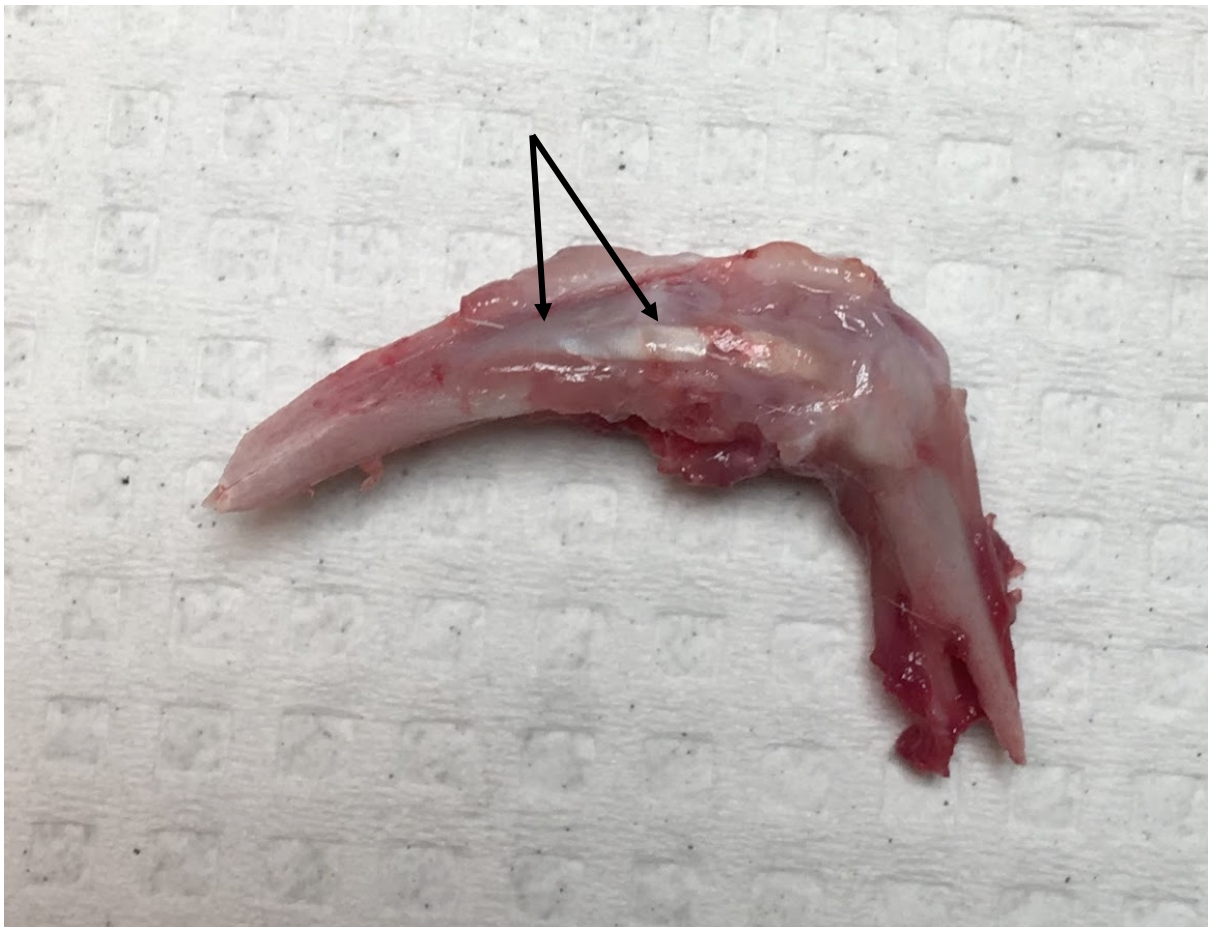

Figure 4-1
Harvested Femur-MCL-Tibia/Fibula complex. The image shows the harvested tissue prior to embedding. The MCL (arrows) is one continuous structure and arrows point to the two ends of the transaction with the middle surgically transected region showing active wound healing.

Figure 4-2

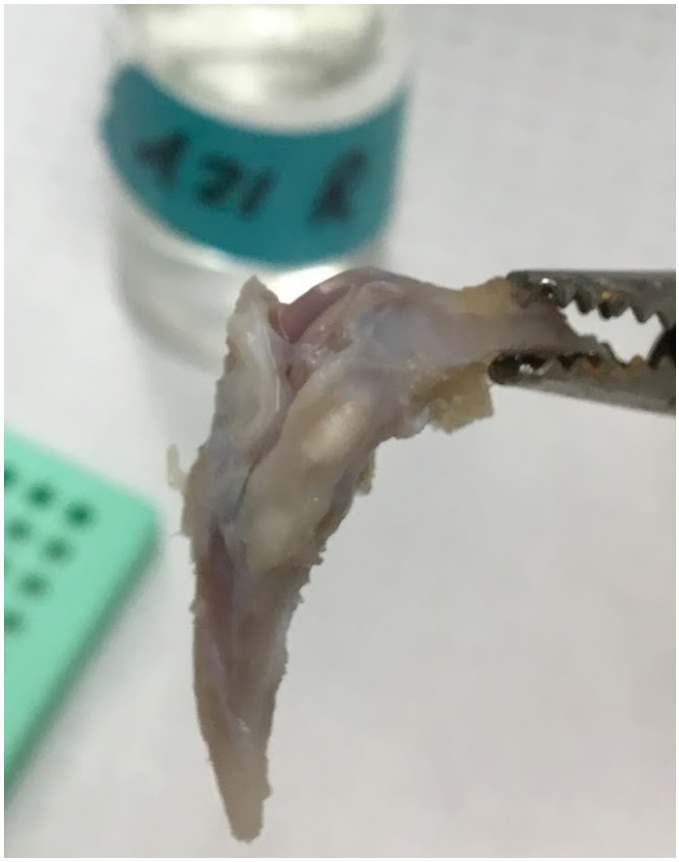

Figure 4-2
The image shows fixed Femur-MCL-Tibia/Fibula complex after removal from 70% EtOH.

Figure 4-3

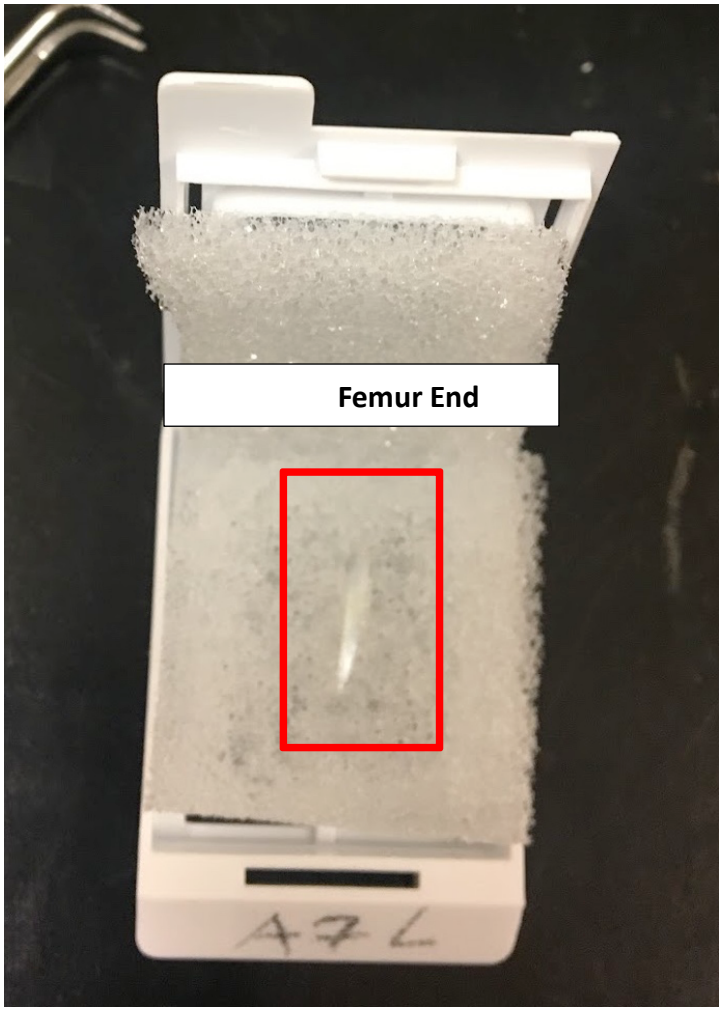

Figure 4-3
The figure shows dissected MCL and placed in the processing cassette with the biopsy pads. The pads will maintain the desired orientation of the MCL once removed from its anatomical position. The femur end is depicted on the picture and the skin side is kept pointing up at this step.

Figure 4-4

Figure 4-4
Autotechnicon. Laboratory equipment for processing histological tissue samples through a series of steps starting with dehydrating the samples in increasing concentrations of ethanol 70%, 80%, 95%, 100% followed 50% Toluene/50% 100% alcohol then toluene followed by a rinse in Xylene. The samples are then held in paraffin wax.

Figure 4-5

Figure 4-5
The figure shows the MCL orientation in the histological embedding mold. The base of the mold is coated with melted wax and the MCL is then placed in the desired orientation with the femur end and skin side oriented as shown.

Figure 4-6

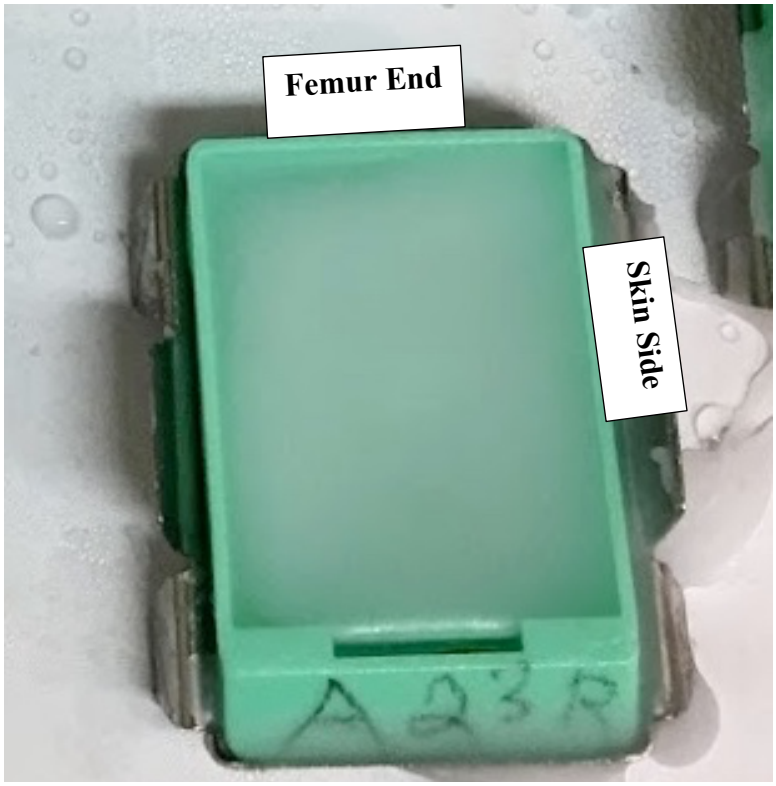

Figure 4-6
Figure shows the embedded MCL tissue sample with the cassette placed and additional wax added. Sample numbers are captured on the cassette in pencil. Orientation is depicted on the image.

Figure 4-7

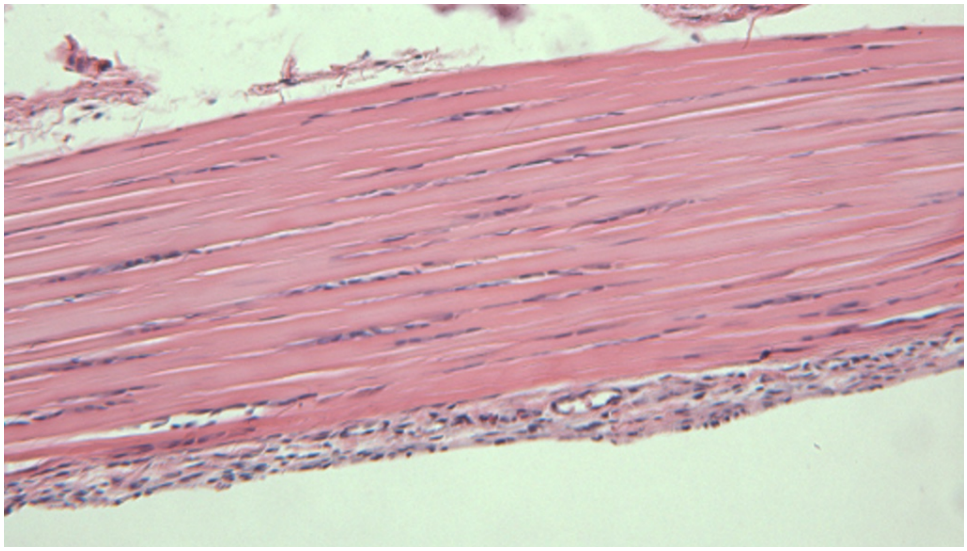

Figure 4-7
Untreated Sham MCL on Day 3. MCL shows organized collagen fibers with normal crimping and minimal cellularity. Elongated nuclei with minimal cytoplasm indicate mature cells in the body of the ligament. Minimal vasculature is observed in the body of the ligament with no clusters of capillaries observed.

Figure 4-8

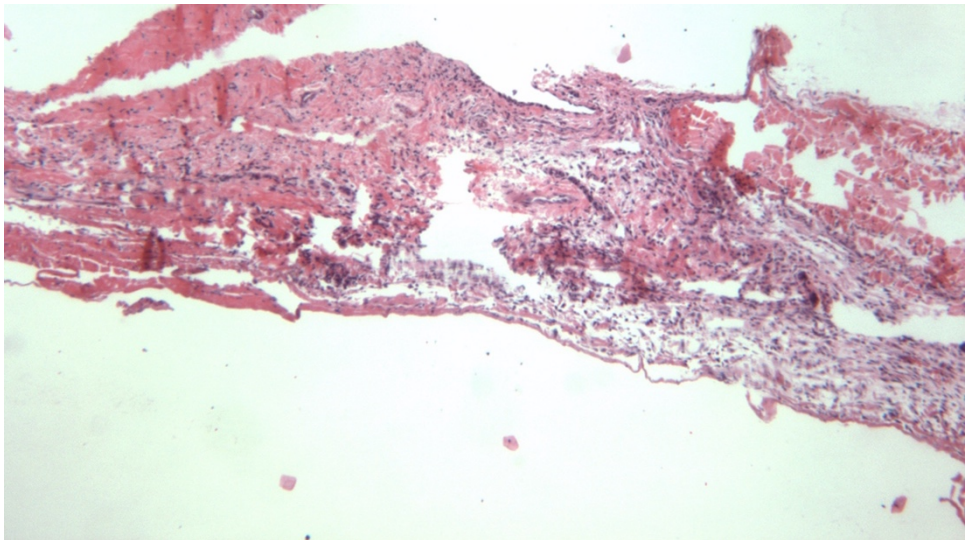

Figure 4-8
Untreated Injured MCL on Day 3 Post Injury. MCL shows highly disorganized collagen fibers and increased cellularity across the entire body of the MCL. Rounded nuclei indicate immature cells in the body of the ligament. Increased vasculature and clusters of capillaries is observed in the body of the ligament. Separation of individual as well as markedly separated fibers and loss of architecture is observed.

Figure 4-9

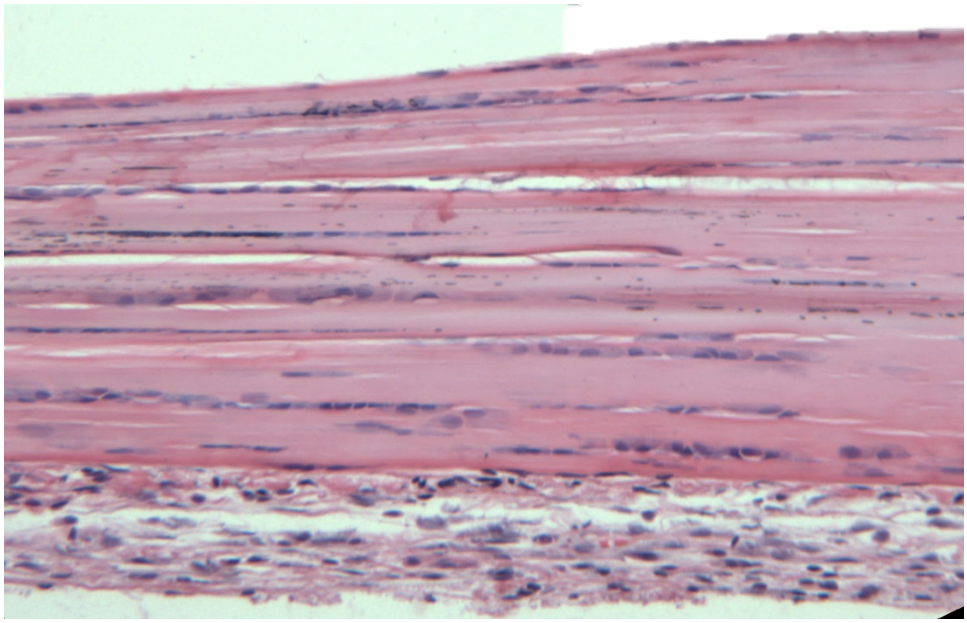

Figure 4-9
Untreated Sham MCL on Day 7. MCL shows organized collagen fibers with normal crimping and minimal cellularity. Elongated nuclei with minimal cytoplasm indicate mature cells in the body of the ligament. Minimal vasculature is observed in the body of the ligament with no clusters of capillaries observed.

Figure 4-10

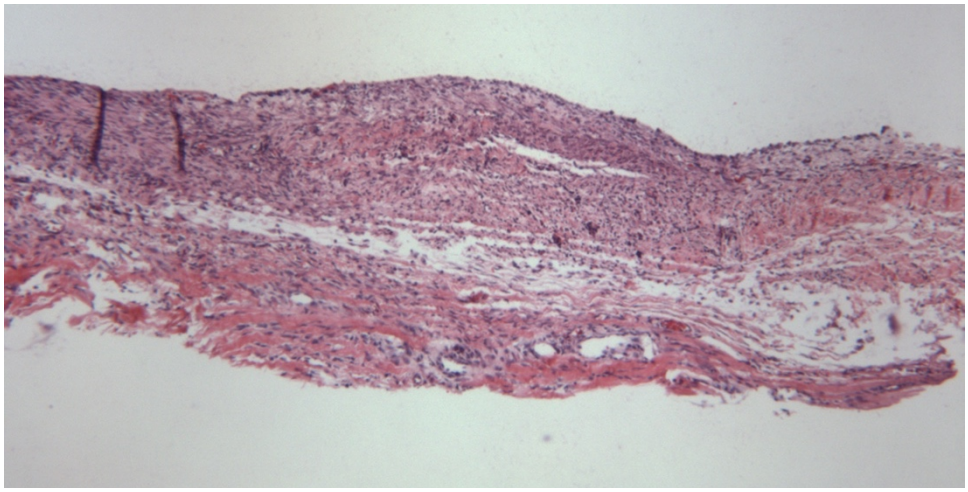

Figure 4-10
Untreated Injured MCL on day seven Post Injury. MCL shows highly disorganized collagen fibers with loss of crimping and increased cellularity across the entire body of the MCL. Rounded nuclei with cytoplasm indicate immature cells in the body of the ligament. Increased vasculature and clusters of capillaries is observed in the body of the ligament. Separation of individual as well as markedly separated fibers and loss of architecture is observed. The ligament is more organized than day three.

Figure 4-11

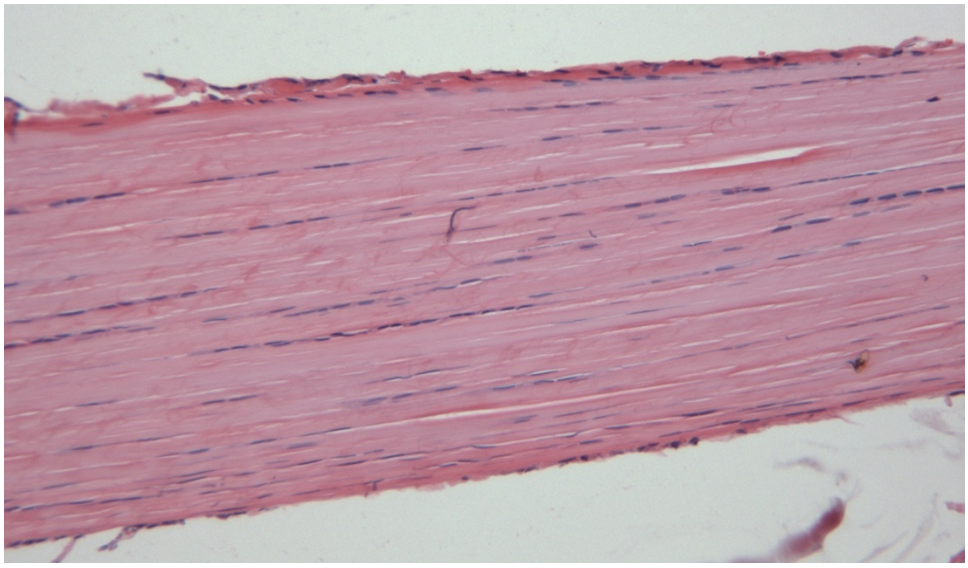

Figure 4-11
Untreated Sham MCL on Day 14. MCL shows organized collagen fibers with normal crimping and minimal cellularity. Elongated nuclei with minimal cytoplasm indicate mature cells in the body of the ligament. Minimal vasculature is observed in the body of the ligament with no clusters of capillaries observed.

Figure 4-12

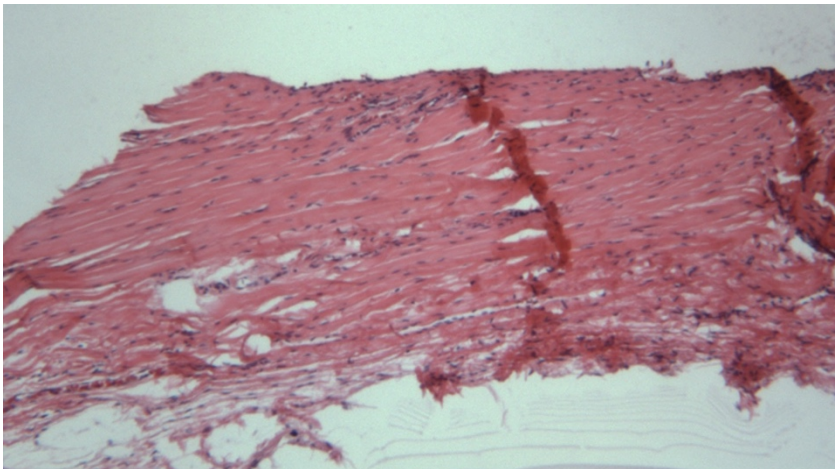

Figure 4-12
Untreated Injured MCL on Day 14 Post Injury. MCL shows disorganized collagen fibers with minimal regain of crimping and continued increased cellularity across the entire body of the MCL. Nuclei are less rounded indicate maturing cells in the body of the ligament. Vasculature and clusters of are less visible in the body of the ligament. The ligament is more organized than day seven.

Figure 4-13

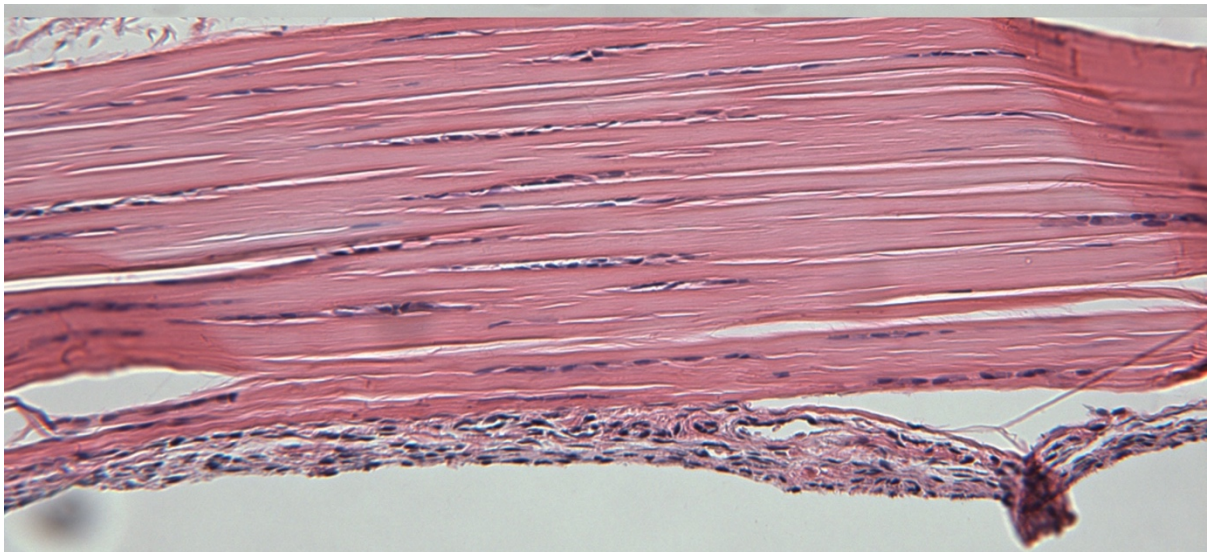

Figure 4-13
Untreated Sham MCL on Day 28. MCL shows organized collagen fibers with normal crimping and minimal cellularity. Elongated nuclei with minimal cytoplasm indicate mature cells in the body of the ligament. Minimal vasculature is observed in the body of the ligament with no clusters of capillaries observed.

Figure 4-14

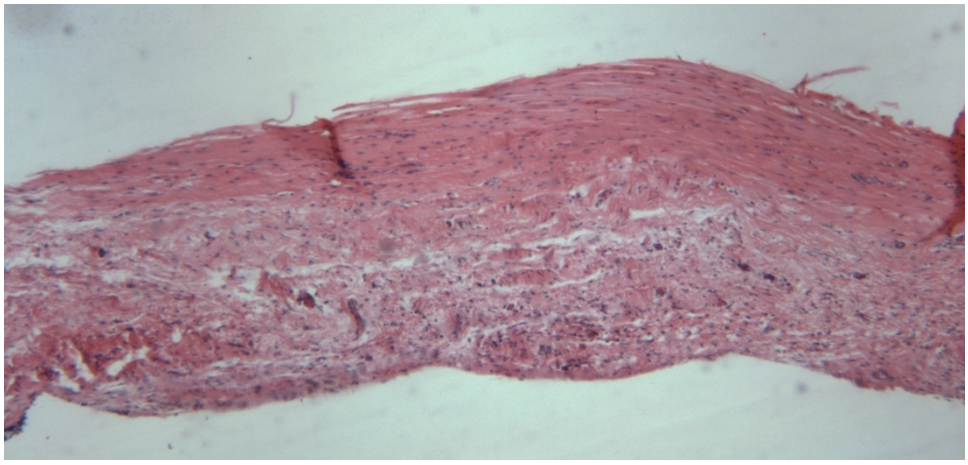

Figure 4-14
Untreated Injured MCL on Day 28 Post Injury. MCL shows more organized collagen fibers with regain of collagen crimping. Continued increase in cellularity across the entire body of the MCL is observed. Nuclei are more in certain parts of the ligament indicating maturing cells in the body of the ligament. Vasculature and clusters of capillaries are not visible in the body of the ligament. The ligament is more organized than day fourteen.

Figure 15

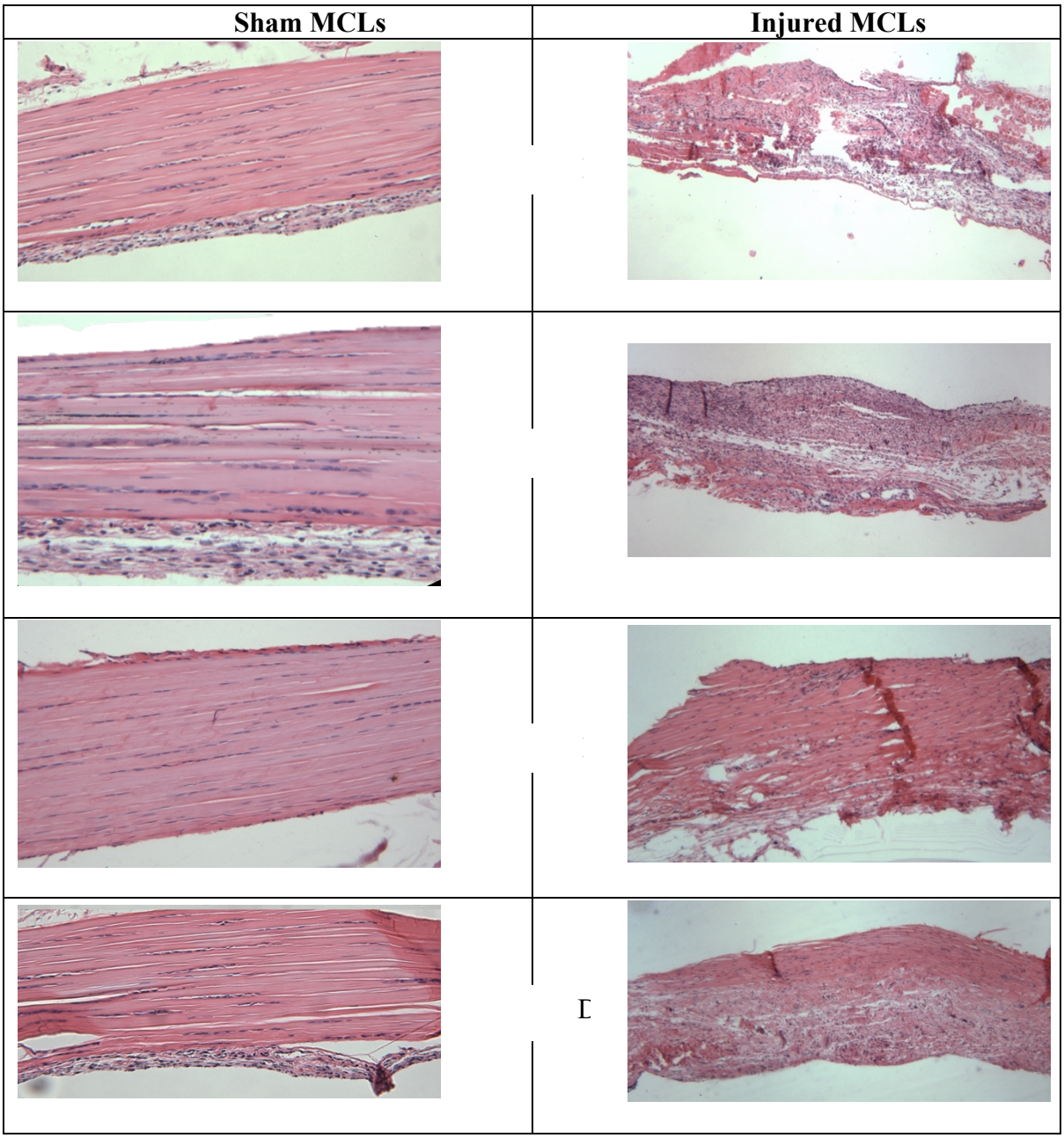

Figure 15
Untreated Injured and Sham MCL sections from Day 3 to Day 28. The figure shows the MCL sections for sham ligaments and injured ligaments. The change in histology between the injured MCLs is clearly shown with initial disruption of fibers and increased cellularity with improvements in histology by day twenty-eight. The Sham ligaments look the same across all days.

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CHAPTER 5

SUBMAXIMAL MCL INJURY

ABSTRACT

Skeletal ligaments play a crucial role in maintaining joint stability while protecting other anatomical structures within and around the joint. When a sub-maximal ligamentous injury occurs, a cascading wound healing response is initiated, starting with platelet plug formation, inflammation, and ending with remodeling. A novel injury-inducing apparatus was developed herein to induce a sub-maximal injury to the Medial Collateral Ligament (MCL) while exposing the knee joint to a valgus position. This will allow for examining the wound healing cascade following injury under occupationally representative conditions such as those observed in a stooped posture. In addition, ligament healing will be examined under treatments with platelet rich concentrate as well as non-steroid anti-inflammatory drugs. To study ligament healing under occupationally relevant exposure conditions, we will use animal models subjected to submaximal injury using our novel apparatus and conduct already optimized experiments described in earlier chapters with gait pattern analysis for functional recovery, biochemical evaluation of growth factors using enzyme-linked immunoassay, measure the stiffness and the ultimate force of ligament tissue using material testing, as well as histological evaluation.

ABBREVIATIONS

COX: Cyclooxygenase
ELISA: Enzyme-Linked Immunoassay
IACUC: Institutional Animal Care and Use Committee
MCL: Medial Collateral Ligament
MX: Meloxicam
NSAIDs: Non-Steroidal Anti-inflammatory Drugs
PRF: Platelet Rich Fibrin
PRP: Platelet Rich Plasma
RICE: Rest, Ice, Compression, and Elevation

INTRODUCTION

In the US, musculoskeletal disorders (MSDs) account for 29–35% of all occupational injuries and illnesses and keep workers from their jobs (1). The annual cost of Low Back Disorders (LBDs) exceeds $100 billion. In the US alone, 13 million people will develop LBDs annually due to their occupation making it the most prevalent musculoskeletal problem in the workplace (2-4). LBDs result from direct stimulation to the facet joints, pressure on the annulus of the disc, or pressure on the longitudinal ligaments of the spine (4). About 600,000 workers in California perform work in stooped postures (bent forward and down at the waist and or mid-back while maintaining straight legs) increasing their risk of developing LBDs due to repeated stresses and exposures to the longitudinal spinal ligaments (2).

After injury, ligaments undergo a series of wound healing events to restore original tissue structure and function however results vary and, in most cases, repaired ligaments are inferior from a mechanical and functional standpoint (5-8). The repair process is marked by four phases: restoration of hemostasis, inflammation, repair, and finally remodeling (9). During platelet activation and degranulation, growth factors are released from their granules initiating other cascades necessary for wound healing and restoration of structure and function (10). Biochemical, mechanical, and histological studies of healing ligament in a complete tear or submaximal injury conditions of the medial collateral ligament (MCL) conclude that ligament do not return to normal or pre-injury conditions (11). Results show healed ligament with scar tissue that is mechanically inferior and biochemically abnormal in its composition and architecture (12-17).

Developing laboratory models and experimental procedures to examine tissue response to submaximal injury during the early stages of healing will provide an insight into natural healing and a basis to examine various treatment methods.

Ligaments Structure and Function and Occupational Relevance to Stooped Posture

Skeletal ligaments are dense bands of collagenous fiber tissue that spans a joint restraining one bone to another forming a flexible skeletal joint (18, 19). They are pale from the limited blood supply (19). As a "second line of defense" to joint injuries after muscles and tendons, skeletal ligaments play a crucial role in the musculoskeletal locomotive system in resisting tensile and torsional forces affecting the joint and stimulating antagonist musculature to preserve joint structure from injury (20-22). Their main function is to guide and restrain skeletal motion while keeping the joint structure intact (23).

Ligaments are passive tissues with viscoelastic properties susceptible to creep deformation under sustained static loading (24). Stretching viscoelastic tissues repeatedly for long periods exposes them to microdamage, "In the collagen fibers, irrespective of the load applied" (25). When extreme trunk flexion is assumed (e.g., stooped posture), sudden onset of electrical silence called the *flexion relaxation phenomenon* occurs (26-28). The electrical activity of the *erector spinae* muscles abruptly stops after a specific amount of trunk flexion. A later continuation of electrical activity is observed when the trunk is extended from the fully flexed position at a location (sagittal angle), similar to that when the *erector spinae* stopped firing. For the musculoskeletal system to stay at equilibrium, the body passes the moment to passive tissues (ligaments, connective tissues, and discs) by inhibiting the erector spinae muscles. The response of muscle in this condition follows the length-tension curve. Passive tissue involvement in producing the total force becomes more dominant as the flexion bypasses the normal joint range of motion for that muscle group. Therefore, during silence, a period of no muscle activation, vertebral ligaments produce most if not all the force that is needed to maintain posture or equilibrium. Hindle et al. investigated the mechanical function of the human lumbar interspinous and supraspinous ligaments and found that

they provide minimal support with minimal trunk flexion (5% of the counter moment). However, at full trunk flexion, maximum forward bend at the waist while the individual cannot bend any further with knees straight or slightly bent, and during the silence period, the interspinous ligament alone supported 75% of the load (29). With increased loading at this range of flexion, spinal ligaments are more susceptible to microdamage especially if the process is repeated without sufficient time to minimize creep deformation - increasing deformation under constant load (25).

Complete recovery of mechanical and functional properties similar to that of native ligament tissue is not currently achievable (30-32). Studies show that the mechanical properties of the healing or remodeled ligaments are inferior to native tissue (8, 16, 33, 34). In some studies, ligaments show a regain of stiffness and strength that is 40% to 80% of normal (35, 36). Remodeled ligaments have inferior creep properties with elongation that is twice as much as normal tissue with suggestions that deformations may be permanent or long-lasting (15). With inferior mechanical properties, ligament function is affected leading to joint instability and changed kinematics and therefore susceptibility of other structures to injury and disease is increased (e.g., cartilage and meniscus tears) (37, 38). With altered mechanical properties of ligaments under sustained or static loading, the load distribution around the joint is disrupted leading to increased shear forces affecting cartilage and bones, and eventually leading to osteochondral degeneration or osteoarthritis and other joint chronic diseases (15, 39). In addition, muscles efficiency is affected in the locomotive system due to increased sliding between joint surfaces (35).

MCL as a Model for Studying Ligaments

To investigate ligament injury-healing, an established animal model is needed. Many studies use the Medial Collateral Ligament (MCL) in experimental animal models due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (15, 40-43). Using the MCL to study ligament healing responses under different injury conditions will help elucidate our understanding of the underlying processes involved in the healing response within the context of ligament injury.

Ligament Injury and Healing Response

There are three ligament injury grade categories depending on the level of tear with grade 1 associated with mild tears and grade 3 depicting a complete tear of the ligament (44, 45). Injury to ligaments initiates a repair process marked by four phases starting with restoration of hemostasis and start of inflammation, repair, and finally remodeling (9).

When ligaments are disrupted and blood vessels are severed, the endothelium is damaged exposing the basal lamina to blood plasma and peripheral blood cells (46). Hemostasis is achieved when damaged blood vessels are temporarily repaired with the creation of a platelet plug/clot. This step is dependent on the level of platelets recruited at the site of injury (9). The Von Willebrand Factor (VWF) is a major mediator of platelet aggregation and clot formation (47). VWF is present in plasma, on collagen extracellular matrix, in platelets granules, and in endothelial cells (48). Upon platelet contact with collagen and the VWF present in the extracellular matrix (ECM), platelets activate, changing their shape and begin degranulation releasing their content of biomolecules and growth factors stored in their granules (46, 49, 50). This leads to further recruitment of platelets until a fibrin clot/scaffold is formed leading to a stable plug and therefore restoration of hemostasis (46, 47, 51). In addition, the growth factors and biomolecules released from platelets play a major role in the deposition of extracellular matrix, chemotaxis, epithelialization, and angiogenesis (52).

Activated platelets are followed by an influx of inflammatory cells in the first 48 hours (46). Neutrophils are the first type of leukocytes that migrate from the bloodstream to the site of injury and are tasked to kill pathogens and remove cellular debris (53). Effective neutrophil recruitment requires P-selectin which is stored in alpha-granules of platelets and in Weibel–Palade bodies of endothelial cells, and is translocated to the cell surface of activated endothelial cells and platelets allowing them to interact with and capture/recruit neutrophils (54). Neutrophils play a major role in recruiting monocytes that infiltrate tissue and transform into tissue macrophages (52, 55, 56). Macrophages further carry out functions within the healing region via phagocytosis of cellular debris, apoptosis, inflammatory cell and fibroblast recruitment, angiogenesis regulation, and scar tissue formation (46, 57). L-selectin is a cell adhesion molecule expressed on neutrophils and other leukocytes that regulates the capture and infiltration of monocytes at locations of inflamed or activated endothelium (54, 56, 58).

Proliferation is a set of four steps that begin at various time periods in wound healing: (1) Epithelialization (2) Angiogenesis, (3) Granulation tissue formation, (4) and Collagen deposition (9). The formation of new blood vessels from pre-existing vessels is mediated by several angiogenic factors. The most potent is the vascular endothelial growth factor (VEGF) (59). New vessel formation is critical for regeneration of tissue, due to increased metabolic rate and need for oxygen supplied by blood vessels for oxidation and formation of collagen from proline and lysine residues (46).

Repair and remodeling of tissue occur following proliferation and initially the extracellular matrix which is mainly made up of collagen gets deposited in a haphazard fashion creating a preliminary network that is mechanically weaker yet providing a basis for a new matrix (13). The newly deposited collagen is reorganized into a structurally sound lattice and the immature type III collagen is replaced with type I collagen that is structurally stronger and wound strength is increased (9, 46). Healing of ligaments is slow and incomplete creating an inferior repaired ligament, never reaching original tissue strength, due to the disorganized nature of the initial matrix and the slow progression during remodeling (8, 46).

Ligament Treatment Methods

The main standard treatments for ligament injuries are RICE (Rest, Ice, Elevate, Compression, and Elevation), NSAIDs (Non-Steroidal Anti-inflammatory Drugs), and a relatively new treatment with platelet rich concentrates (e.g., Platelet Rich Fibrin or PRF). The goal of the RICE treatment is to reduce additional damage to injured tissue, decrease blood flow and enzymatic activity which are expected to reduce edema and swelling and control pain (60, 61). NSAIDs interfere with the inflammatory response by limiting the production of Cyclooxygenase. Cyclooxygenase (COX) is a rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H2, which is the precursor of several molecules, including prostaglandins, prostacyclin, and thromboxane (62). Thromboxane is involved in platelet aggregation and prostaglandins mediate pain sensitization are both inhibited with NSAIDs (63-66). The main goal of platelet concentrates treatments such as PRF is to increase the availability of platelets that contain biomolecules and growth factors responsible for repair at the site of injury (67). By doing so, an increased level of response is sought and mediated so that collagen expression and organization are improved and thereby improving the time to mechanical and functional recovery (68-70).

Current literature focuses on restoring original tissue properties and time to recovery and the level of healing varies depending on the treatment applied (5-8). There is insufficient evidence to support RICE and NSAIDs as effective treatments to restore ligament structure and function

however they appear to be deployed to control pain and swelling which are byproducts of repair and the inflammatory response (60, 71). Some authors argue that RICE and NSAIDs only help to relieve pain symptoms (15, 60, 63, 72). So, the improved return to normal mobility will mask any real structural and functional restoration of injured tissue (44, 60, 72, 73). With respect to PRF treatment, the lack of standardization in creating and activating platelets and experiments testing its efficacy and range of outcomes limits current support for using it as a standard treatment for ligament injuries (68-70).

RESEARCH VOID

Evaluating an efficacious approach to treat ligamentous tissue injury requires an understanding of the biochemistry and physiology of healing under simulated occupational injury conditions in controlled laboratory settings. Such a study will provide an objective basis to discriminate between exposure and treatment methods. In particular, the study will provide insight into the healing response to an injury similar to that sustained in jobs requiring prolonged stooped posture, such as those commonly observed in farming and will provide a basis to evaluate exposure-response schemes to guide prevention efforts and work policies.

AIMS

The aims of this study are to investigate ligament healing after a sub-maximal injury using a novel MCL stretching apparatus in vivo. We will characterize the time-dependent histological, biochemical, functional gait assessment, and tissue mechanics to elucidate our understanding of the efficacy of common and emerging treatments (NSAIDs and PRF) under different injury exposure conditions.

MATERIALS AND METHODS

Animals

The study was approved by the University of California at Davis Institutional Animal Care and Use Committee (IACUC). Two skeletally mature male Wistar rats (The Jackson Laboratory) were used as animal models. The animals did not receive any injury and were used for optimizing the design of the MCL submaximal stretching apparatus. Animals were fed rat chow and housed in pairs per cage and allowed free-roaming, access to water, and ambulation, and kept under the care of a university veterinarian.

Design and 3D printing Software

All 3D models were designed in Autodesk Fusion 360 (Autodesk, INC. (2021). Fusion 360. Retrieved from (https://www.autodesk.com/products/fusion-360) and the G-Code for 3D printers were created in Autodesk Print Studio.

3D Printer

Creality Ender 3D printer (https://www.creality.com/goods-detail/creality-ender-3-s1-3d-printer) was used to print all parts. The printer has a maximum print size of 487 x 453 x 622 mm. Polylactide resin (PLA) plastic was used on all components of the MCL stretch apparatus.

RESULTS

MCL Stretching Brace

Fig. 5-1 shows a prototype of a brace designed to induce an MCL stretch on animal models. The results did not achieve desired stretching of MCL as securing and keeping the brace on the animal at the proper anatomical location was not possible.

MCL Stretching Device

Fig. 5-2 to 4 show a working prototype of the MCL stretching device. The design allows for controlling the stretching angle as well as providing a platform to secure the animal in place. Figure 5 shows the device in use with an animal under anesthesia. Significant strapping was required to keep the limb under study in place. A redesign of the limb holding mechanism was done with a vice mechanism and is shown in Fig. 5-6. Cushioning with gauze was still required to hold the limb in place along with taping the limb to ensure an initial secure hold. Wire nuts were tightened to effect while ensuring minimal stress is placed on the tibia/fibula. Fig. 5-7 to 12 provide engineering drawings of the prototype.

DISCUSSION

To investigate ligament injury and healing response in a submaximal injury condition representative of occupational exposures, an in vivo injury-inducing apparatus was designed and tested on an animal model. The device was validated to create a valgus knee position which in turn places strain forces on the MCL leading to submaximal injury. Stretching the Medial Collateral Ligament (MCL) was possible while keeping the animal secured.

Medial Collateral Ligament (MCL) was chosen as the experimental rodent model due to its well-characterized healing properties, ease of access, and ability to reproduce injury conditions (15, 40-43). Furthermore, animals have bilateral MCLs allowing for sham injury on one side to serve as an internal control. The highest MCL strains occur at full extension in the posterior region of the MCL proximal to the joint line during valgus loading (74). This confirms our understanding that this region may be most vulnerable to injury under these loading conditions and therefore our experiment was designed to induce a valgus loading around the knee joint.

In this experiment and prototype development, stretching the MCL was possible while keeping the animal secured. Repeatable injury conditions were achieved with the protype however additional optimization may still be necessary to ensure consistency with degree at which MCL stretching is achieved. In other words, the extent of MCL strain needs to be measured to ensure injury conditions are consistent.

Future Work

The submaximal injury conditions achieved in this experiment will allow us to induce simulated occupational injuries and exposures to animal models. Future experiments will build on techniques developed with histological, biochemical and gait testing performed in the complete transection studies. In addition, cross-examination of the effects of Platelet Rich Fibrin (PRF) and Non-Steroid Anti-Inflammatory Drugs (NSAIDs) is will be performed for submaximal injury conditions. Furthermore, mechanical testing will be performed to examine the extent of mechanical restoration of ligament tissue compared to uninjured ligaments. Figure 13 shows a diagram of the mechanical testing apparatus as well as the expected force-displacement figure. Details of the apparatus and parameters used are also depicted with measurements of the load to failure, displacement, stiffness and energy to failure. We speculate that return to pre-injury

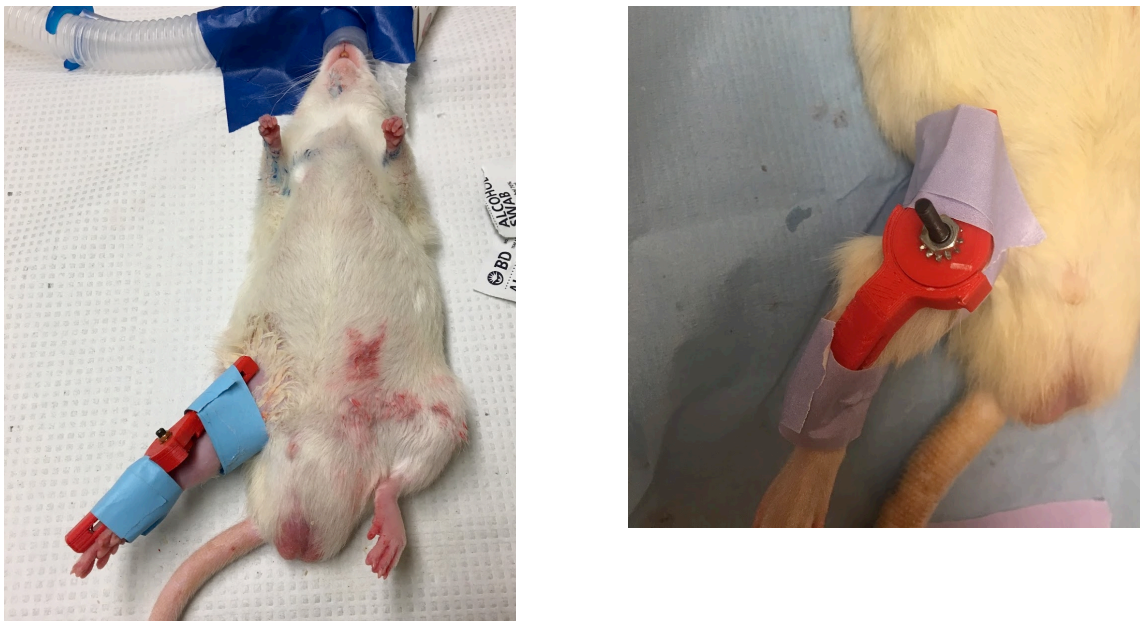
mechanical performance may not be possible however we hypothesized that PRF treatment will allow a faster return to optimal ligament structure. We also suspect that NSAIDs will have the opposite effect of delaying return of mechanical performance to approach pre-injury conditions. These hypotheses will be validated with stiffness measurements and ultimate force to failure which is expected to both be higher for PRF than NSAIDs with both approaching pre-injury conditions but never achieving it.

Due to delays in research during the pandemic, these experiments were not carried fully, and post-doctoral research is planned to conduct sub-maximal injury conditions simulating occupation exposure. This will allow us to draw comparisons between histological findings in untreated, Meloxicam and PRF-treated animals.

CONCLUSION

Ligaments play a major functional role in the musculoskeletal system. When injured, the standard treatment methods are focused on pain management and return to function as appose to promoting time to recovery and healing of tissue to preinjury properties. The goal of this study was to develop a submaximal injury condition within animal models under controlled laboratory conditions. Future studies will allow examination of the injury outcomes with histology, mechanical testing and biochemical evaluation of growth factors involved in wound healing. In addition, further assessment under different treatments with Meloxicam and PRF will be carried out.

CHAPTER 5 FIGURES
Figure 5-1



A B

Figure 5-1
MCL Stretch Brace Prototype. The images in A and B show a 3D printed design of an MCL stretch brace. The prototype brace was intended to create a submaximal MCL injury however it failed during a pilot experiment at producing desirable results. The brace did not keep its position during testing.

Figure 5-2

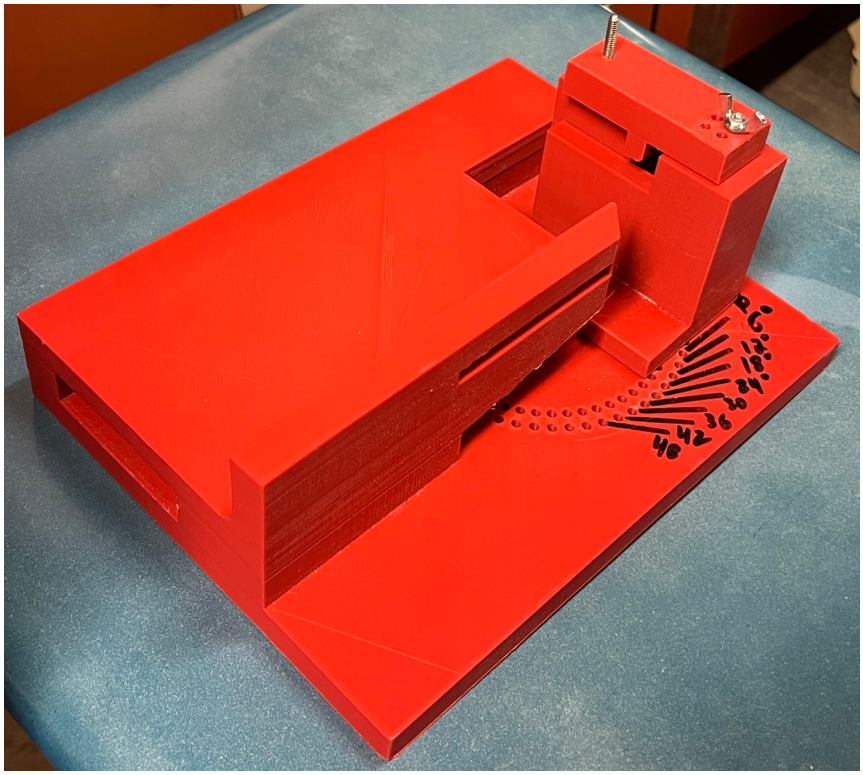


Figure 5-2
MCL Stretch Device Prototype. The image shows a 3D printed design of the MCL stretch device. The device allows for controlled degrees of MCL stretching in vivo.

Figure 5-3

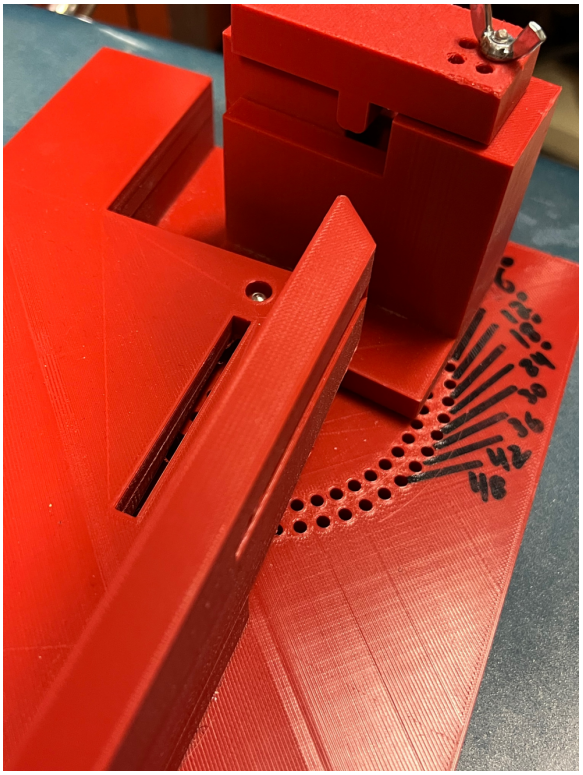


Figure 5-3
MCL Stretch Device Prototype. A close-up image showing the MCL stretch device. Degrees of stretching are in increments of 6 degrees starting with zero and up to 48 degrees.

Figure 5-4

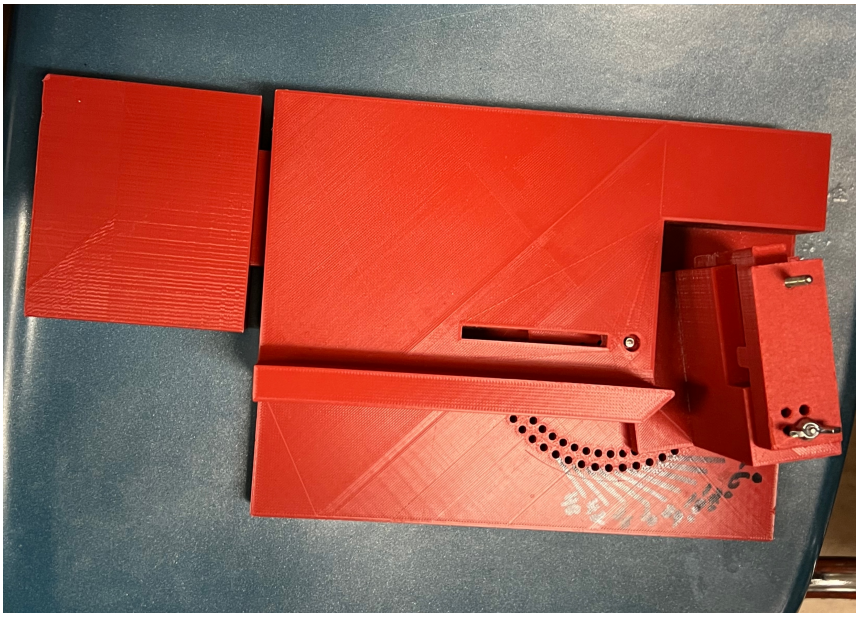
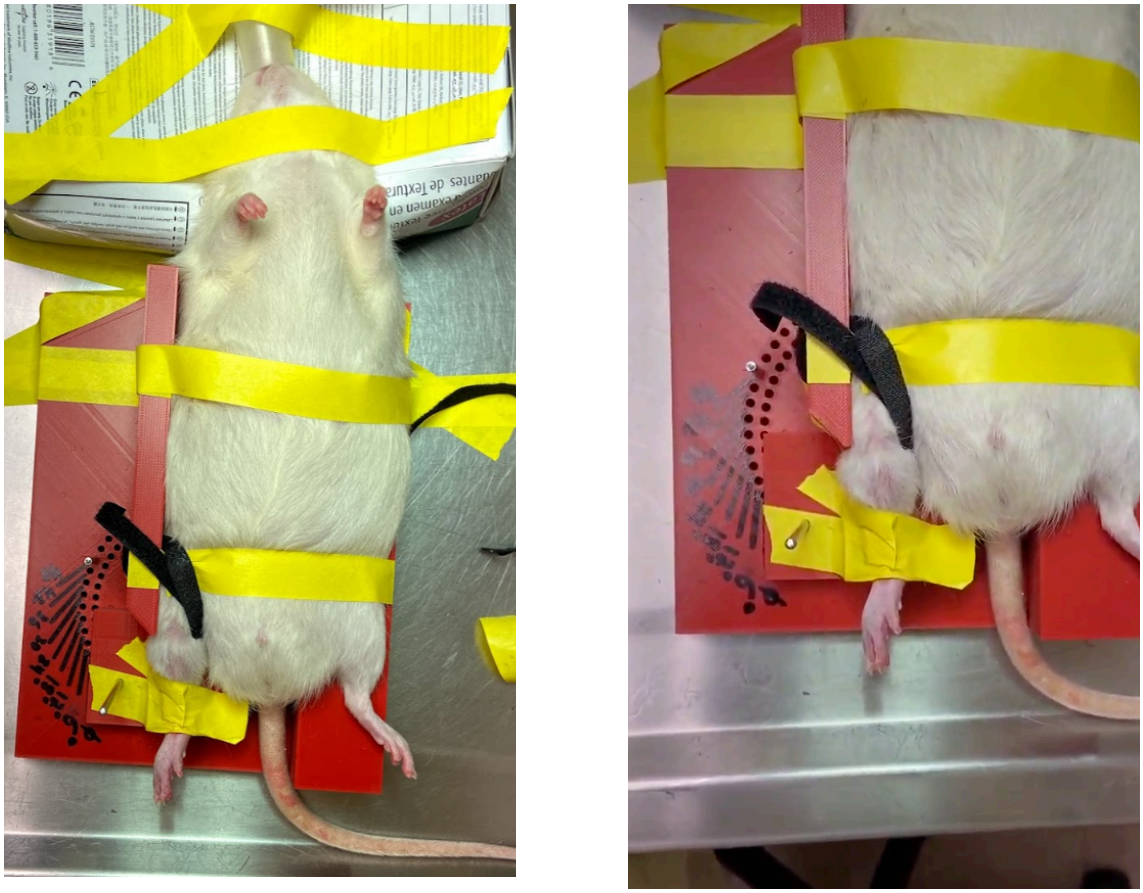


Figure 5-4
MCL Stretch Device Prototype – Top View. Top view of the 3D printed MCL stretch device. The Added extension allows for the head of the animal to rest and for anesthesia equipment to be secured to the base.

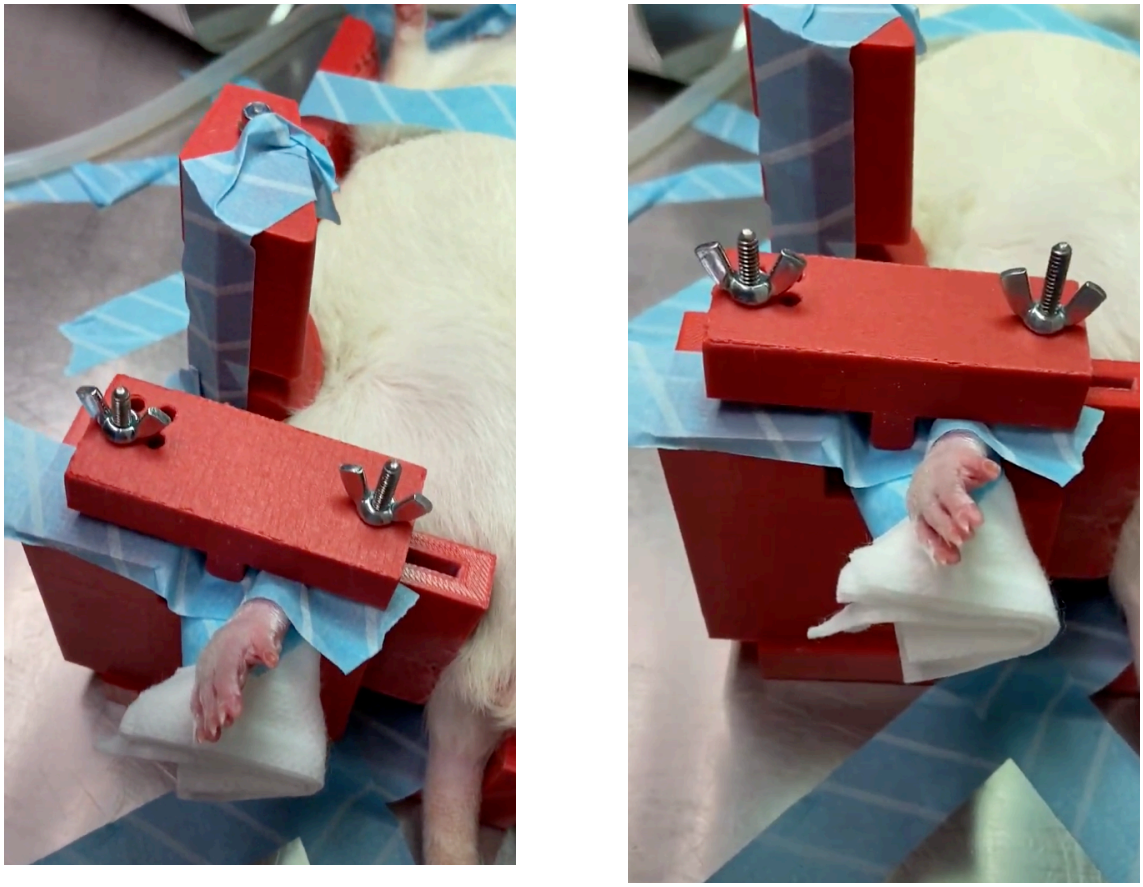
Figure 5-5



A **B**

Figure 5-5
Pilot run with the MCL Stretch Device Prototype. Animal testing of the stretch device. A) Full view showing anesthetized animal. B) shows a close-up view of the knee stretching mechanism. A significant amount of strapping and holding the animal in pace was necessary with this prototype.

Figure 5-6



A **B**

Figure 5-6
Modified lower hindlimb holding vice design. Animal testing of the modified stretch device. A and B show different view angles of the vice design used to hold the lower limb in place. This design allowed for a more secure and stable holding of the limb with minimal sliding. Cushioning with gauze was still required to hold the limb in place along with taping the limb to ensure an initial secure hold. The wire nuts were tightened to effect while ensuring minimal stress is placed on the tibia/fibula.

Figure 5-7

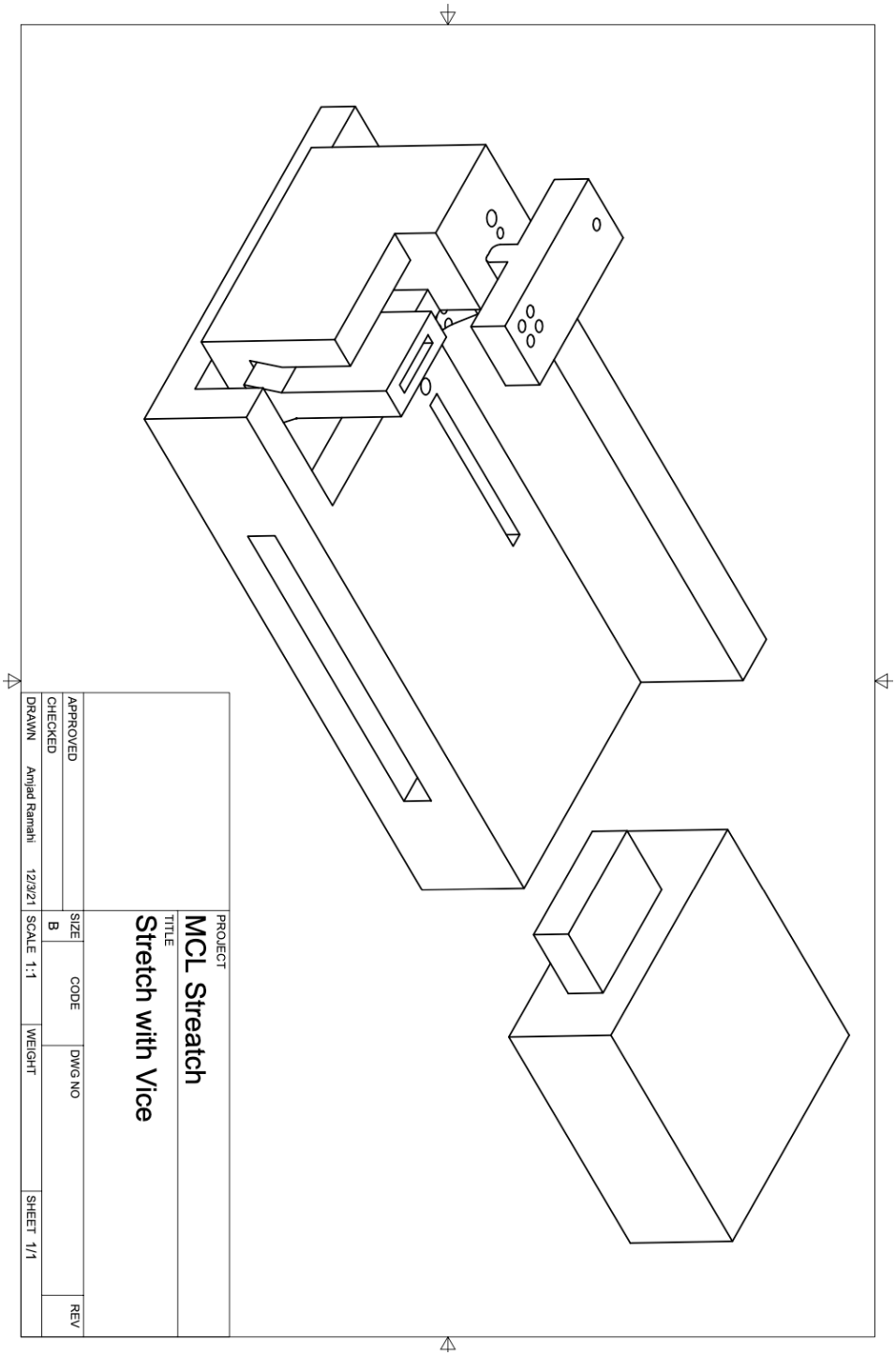


Figure 5-7
Drawing of MCL Stretch Device. The drawing shows an isometric view of all components used in the assembled MCL stretch device.

Figure 5-8

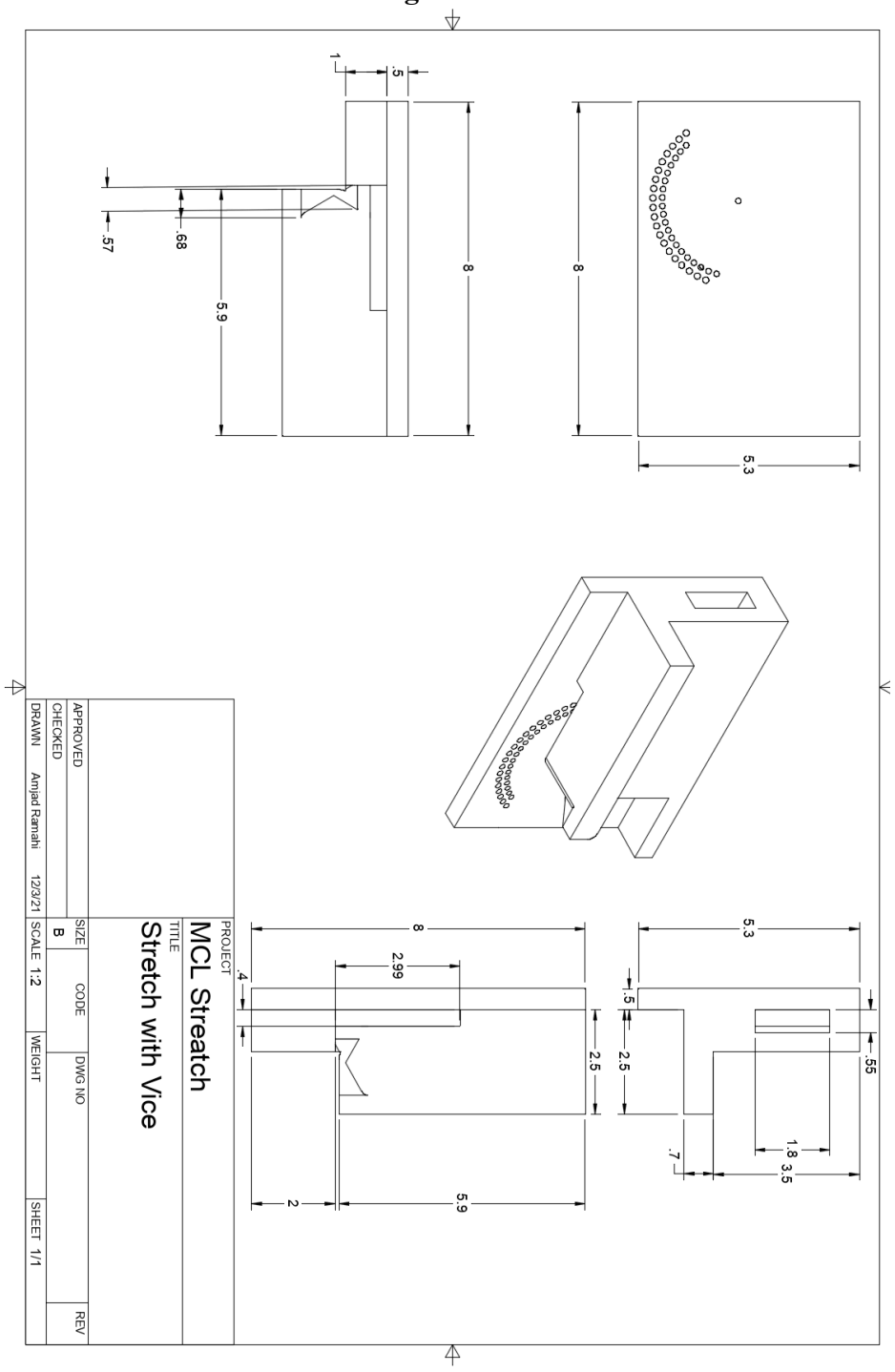


Figure 5-8
Drawing of MCL Stretch Device Base. The drawing shows dimensioned of the base used in the MCL stretch device.

Figure 5-9

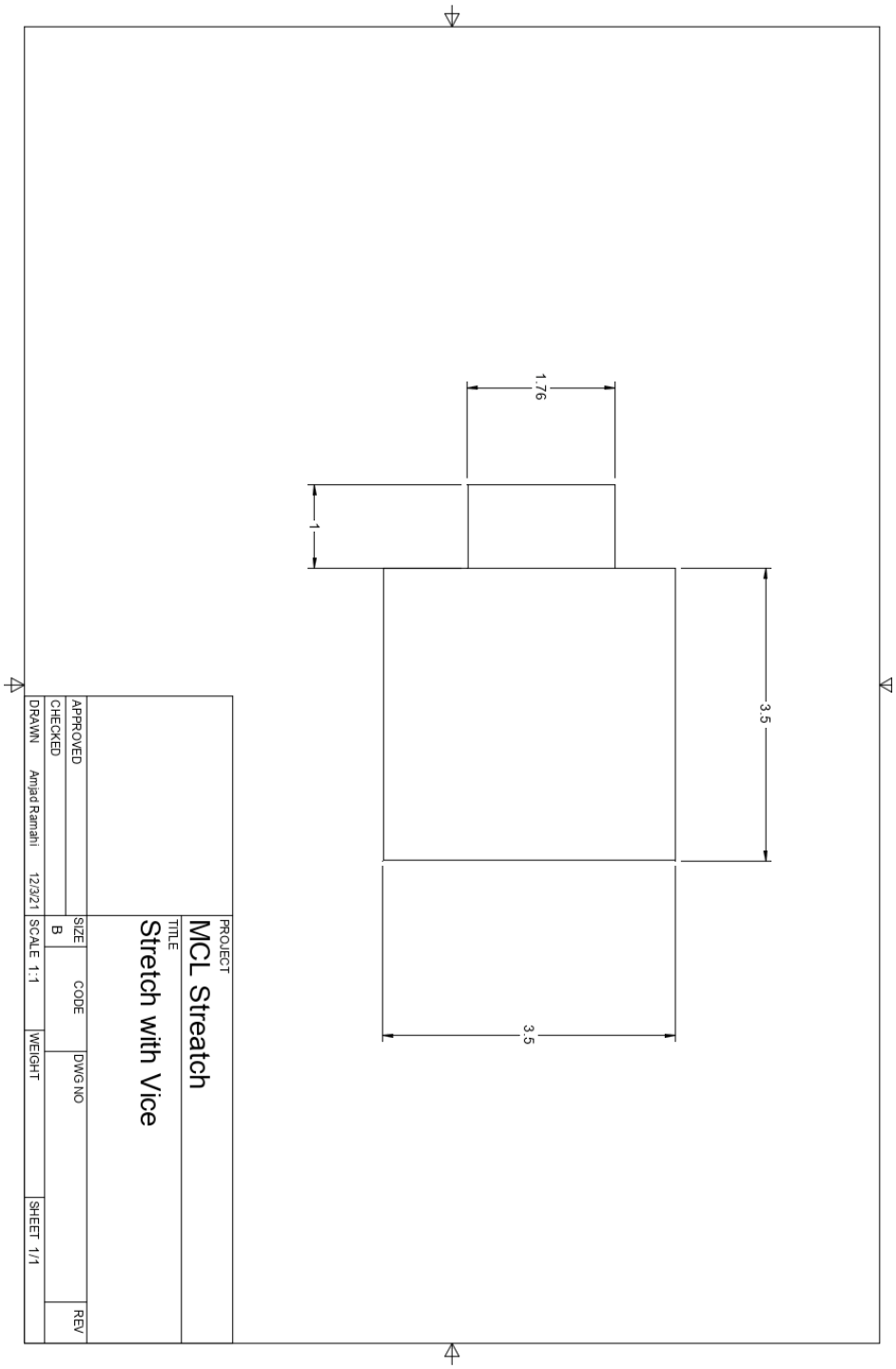


Figure 5-9
Drawing of MCL Stretch Device Head Extension. The drawing shows dimensioned of the head extension used in the MCL stretch device.

Figure 5-10

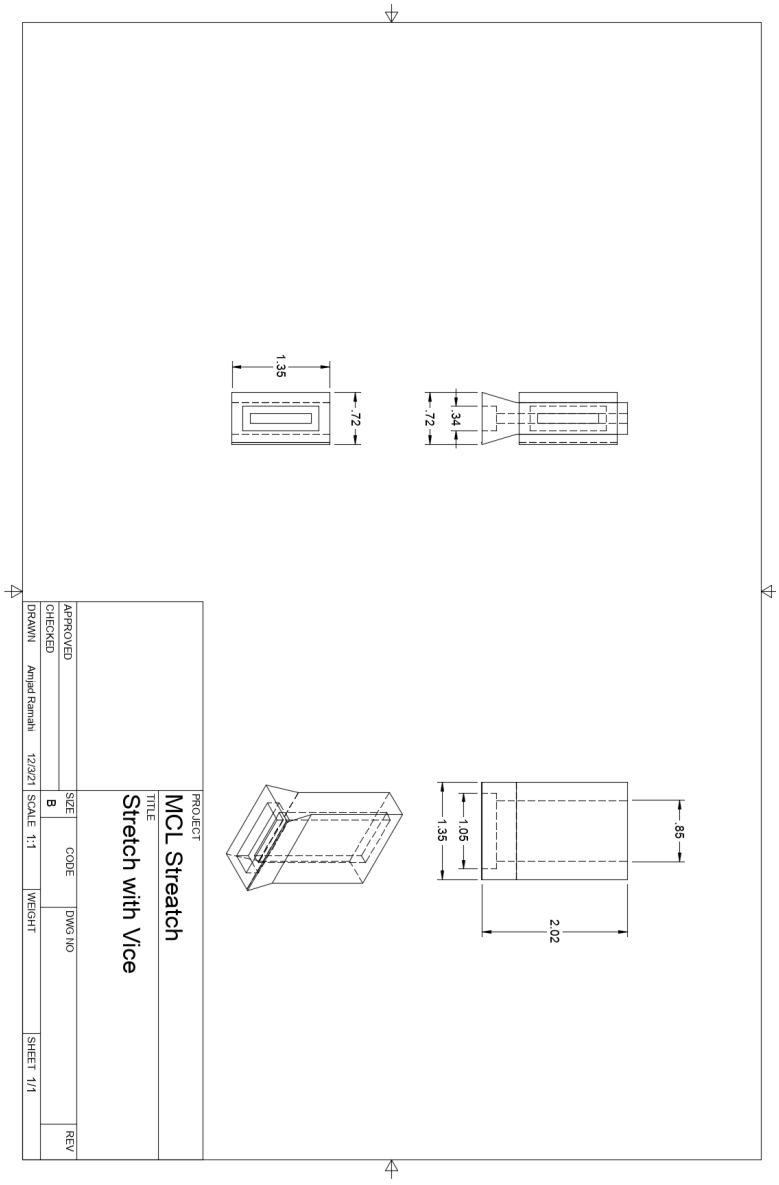


Figure 5-10
Drawing of MCL Stretch Device Vice Component 1. The drawing shows dimensioned of the vice component 1 used in the MCL stretch device.

Figure 5-11

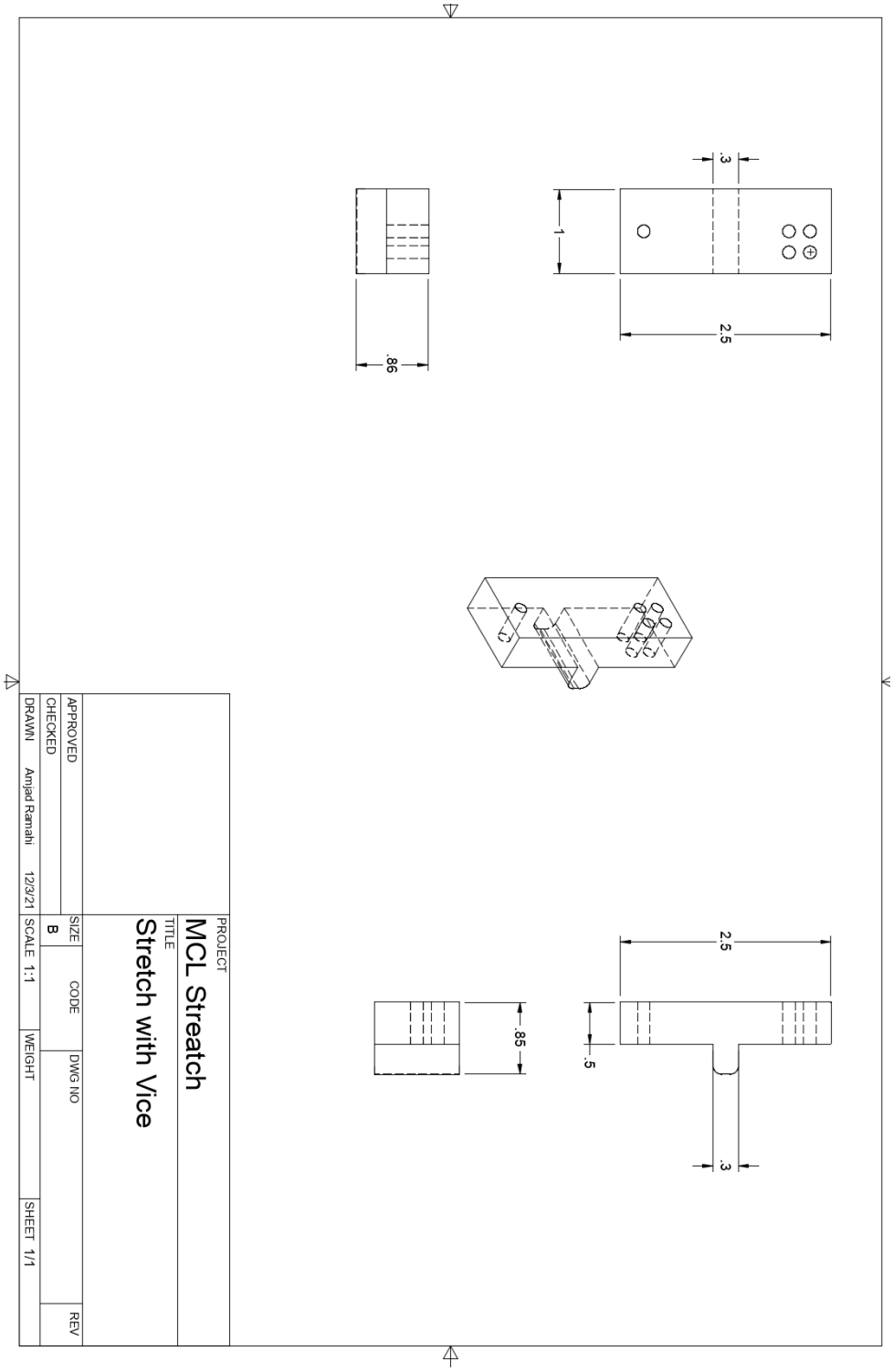


Figure 5-11
Drawing of MCL Stretch Device Vice Component 2. The drawing shows dimensioned of the vice component 2 used in the MCL stretch device.

Figure 5-12

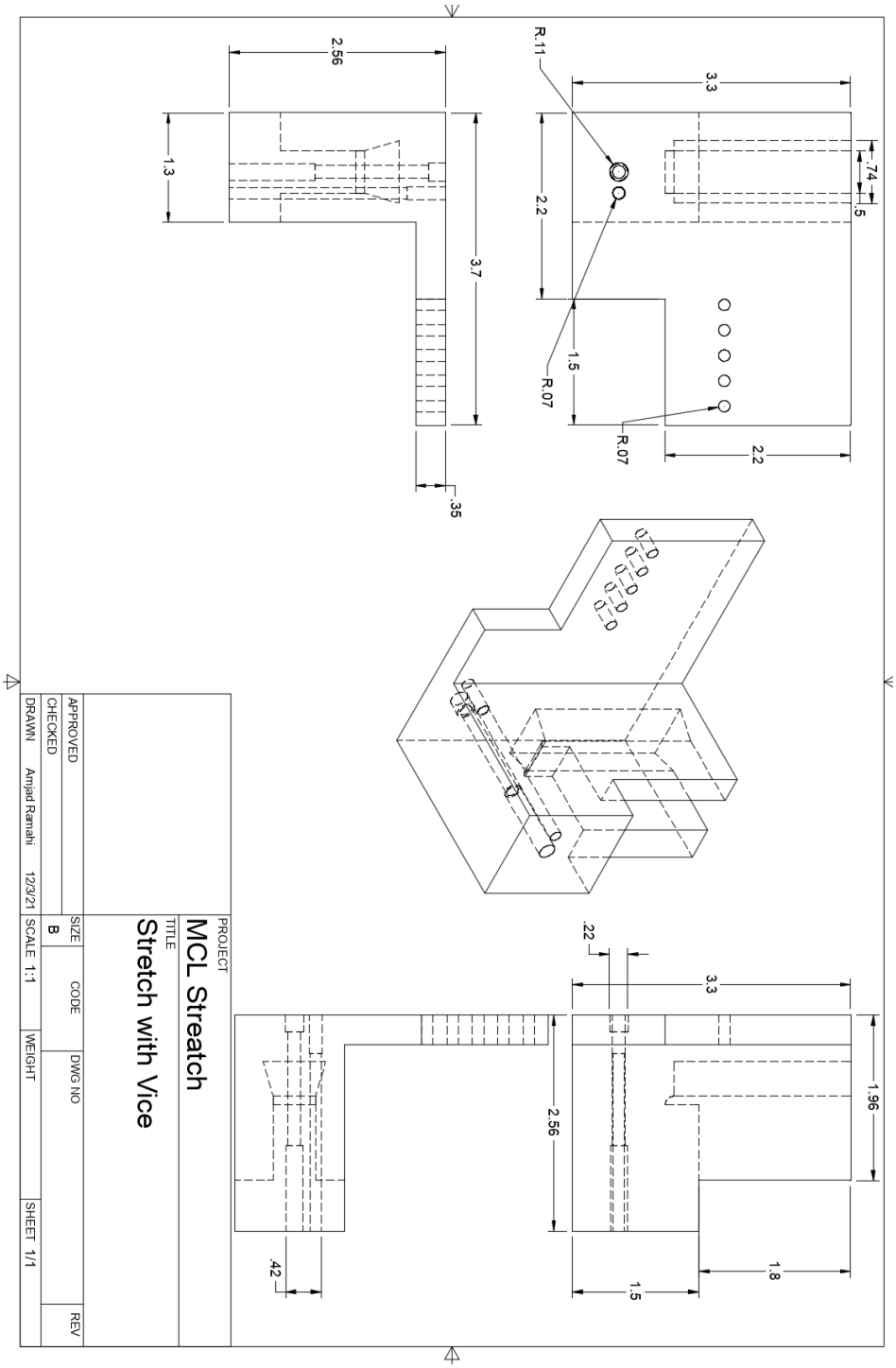


Figure 5-12
Drawing of MCL Stretch Device Vice Component 3. The drawing shows dimensioned of the vice component 3 used in the MCL stretch device.

Figure 5-13

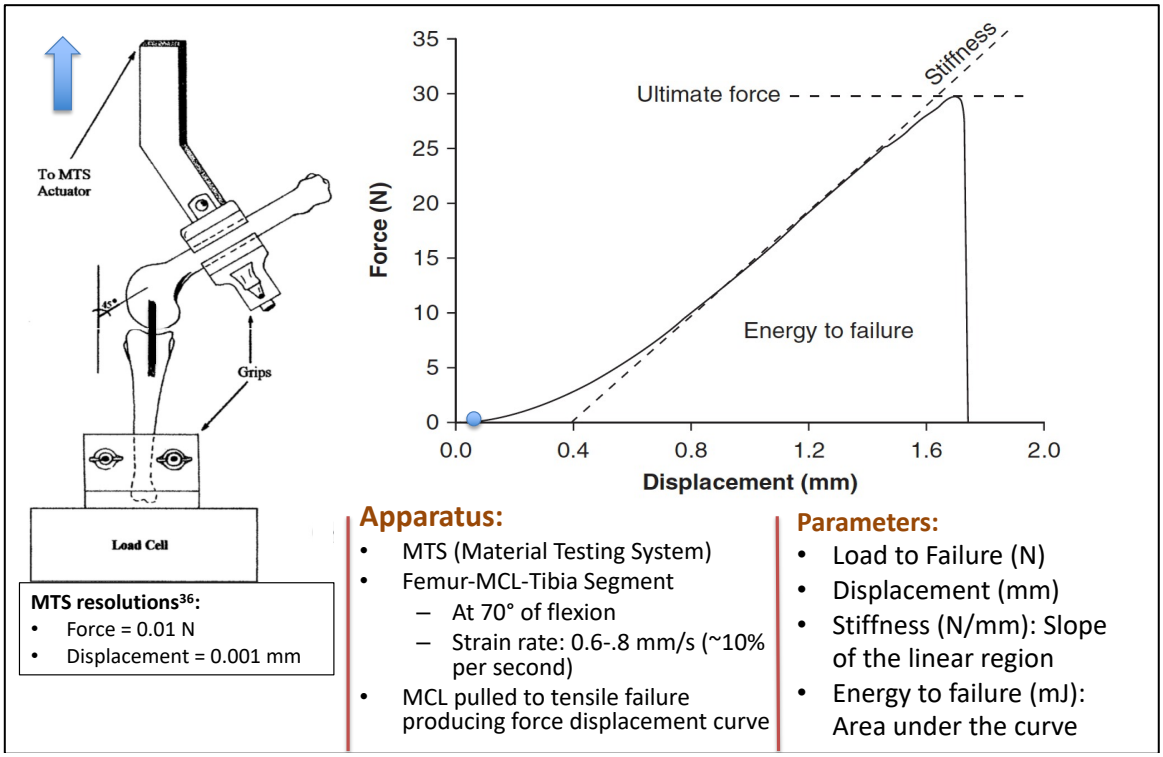


Figure 5-13
Mechanical Testing Method. The drawing shows a diagram of the mechanical testing apparatus as well as the expected force-displacement figure. Details of the apparatus and parameters used are depicted.

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