

Preventing Biological Wear in the Synovial Joint: The Role of Lubricin as a Boundary
Lubricant and Tribosupplement

By

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B.S., Boston University, May 2008

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Curriculum Vitae

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6. **Waller KA**, Darling EM, Jay GD. 2010. AFM Analysis of Cartilage Degradation in a Rat Model Following ACL Transection. *American Society of Biomechanics*. Providence, RI.

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- (PRG4) and Recombinant Lubricin Prevents Cartilage Degeneration and Lowers Urinary CTXII in the Rat ACL Injury Model. Podium Presentation. *Orthopedic Research Society*. New Orleans, LA.
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Preface and Acknowledgements

My graduate school experience has been an incredible journey. As a senior at Boston University, I remember receiving a phone call from Dr. Jay in the early spring of 2008, to schedule an interview to learn about his research at Brown. From that point forward, I was hooked. I moved to Providence and started working in the Emergency Medicine Lab that summer, and I can truly say that my experiences from then forward have enriched my love of biomedical science and provided me with a strong basis for future scientific endeavors.

I must begin by acknowledging my advisor, Dr. Jay. Your mentorship far outreached what I could have imagined for a graduate experience. With your guidance, unwavering optimism and faith in me, I've been able to achieve much more success than I had ever imagined. More importantly, through your example, I have learned what it truly means to be immersed and dedicated to my work. I'm incredibly proud to be a part of your research group. Furthermore, I cannot thank you enough for all of your support and encouragement in my life, both in and outside of the lab.

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with rat surgeries on your single vacation week during residency when you were also very pregnant, and I feel like this experience really sums up how amazing it has been to work with you. Dr. Khaled Elsaid, you welcomed me into the lab at the start of my graduate experience and taught me so many important skills that have been essential to my research. I am honored to have had you as part of my thesis committee and am very grateful for your help and guidance.

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Finally, I must thank my family. I would like to start with my Grandparents. Grandpa Frank and Grandma Trudy, you taught me the importance of knowledge and ambition at a very young age and that hard work pays off. I miss you so much, but I'm

continually inspired by your sacrifice, hard work and perseverance. Grandma Georgia and Peepaw Pete, you taught me so much about hard work and believing in yourself. Grandpa, I miss your humor and am inspired by the determination you showed throughout your life. Grandma Georgia, you continue to amaze me with your kindness, optimism and your ability to find good in everyone. I have been truly blessed with amazing grandparents that held family in the highest regard and loved me my entire life. I hope that my hard work has made you proud.

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success and willingness to “go the distance” for the people you love. Thank you so much for driving hours down to Providence through blizzards, rain and traffic, just to play soccer and hang out with me. Katie, I am so proud of you. I cannot believe how much you’ve grown up and am amazed by your selflessness and kindness. My sisters, you are the best in the world and I love you more than I can say.

Finally, Mom and Dad, I am so proud to be your daughter. Your faith in me and your unconditional love have carried me from the beginning of my life. Thank you for raising me and supporting me through all of my projects and adventures. I love you.

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Chapter 1

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Figure 1.2. Lubricin structure. The N-terminus, which contains two somatomedin B domains and the C-terminus, which contains a hemopexin domain, are connected by a long mucin-like domain, which is located on exon 6 and is heavily glycosylated with O-linked $\beta(1-3)$ -Gal-GalNAc and non-uniformly capped with NeuAc (30). The C-terminus interacts with the cartilage surface.8

Figure 1.3: Pendulum systems for measuring whole joint COF and applying cyclic loading in rodent stifle joints. (left) The joint is fixated on a mounting block. The distal tibial and proximal femur are fitted into brass tubing that is secured to the pendulum base by set screws. The proximal end of the femur tube fits into the pendulum arm, and the knee joint serves as the fulcrum. The mounting block contains a chamber that can be filled with fluid. (center) Passive pendulum. The mounting block is secured to the pendulum base. Four (4) reflective markers are located on the base and on the pendulum arm. The pendulum arm is rotated $\sim 12^\circ$, released and allowed to swing freely until motion ends. (right) Active pendulum. A DC motor drives continuous pendulum motion $\pm 15^\circ$ at a rate of 1.5 Hz. The limb is submerged in cell culture media to prevent desiccation. (adapted from Drewniak, *et al.*, 2012) (77).....14

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Chapter 2

Figure 2.1: A.) Chondrocyte apoptosis in mice with different *Prg4* genotypes. Activated caspase-3 immunodetection in coronal sections of knee joints from 10-wk-old $Prg4^{+/+}$, $Prg4^{+/-}$, and $Prg4^{-/-}$ mice that were not tested using the passive pendulum system. (First column) immunofluorescence detection (red) of active caspase-3. (Second column) same sections fluorescently imaged to reveal DAPI stained (blue) chondrocyte nuclei. (Third column) merged active caspase-3 and DAPI images. Note the red fluorescence at the cartilage surface represents non-specific antibody binding to proteinaceous deposits that normally accumulate on the cartilage surface in $Prg4^{-/-}$ mice. Whereas, the punctate red-fluorescence observed below the cartilage surface overlaps with DAPI stained nuclei indicating chondrocytes undergoing apoptosis. In contrast to $Prg4^{-/-}$ knees, the knees in $Prg4^{+/+}$ and $Prg4^{+/-}$ mice had few chondrocytes with activated caspase-3. (fourth column) TUNEL staining and methyl green counterstaining of cartilage. More apoptotic (brown) chondrocyte nuclei are present in $Prg4^{-/-}$ cartilage compared to $Prg4^{+/+}$ and $Prg4^{+/-}$ cartilage. Scale bars represent 100 μm . (Bottom row) region of interest taken from the image above to show the cells located just below the flattened superficial chondrocytes that are most susceptible to apoptosis. B.) Whole Joint COF in mice with different *Prg4* genotypes. Bar graphs depicting mean + 1 SD COF measurements in knee joints from 10-wk-old wild-type ($Prg4^{+/+}$), heterozygous lubricin knockout ($Prg4^{+/-}$), and homozygous lubricin knockout ($Prg4^{-/-}$) mice. Each individual COF measurement is indicated by an open circle. $Prg4^{-/-}$ knees have significantly higher COF than either $Prg4^{+/+}$ or $Prg4^{+/-}$ knees. * $p < 0.01$ compared to $Prg4^{+/+}$ or $Prg4^{+/-}$ knees.....53

Figure 1.2: COF in bovine cartilage bearings lubricated with different test solutions. Bar graphs depicting mean + 1 SD static (unshaded) and kinetic (shaded) COF measurements in bovine cartilage bearings lubricated with either phosphate buffered saline (PBS), synovial fluid from patients with CACP (CACP-SF), purified human synoviocyte lubricin (HSL), human synovial fluid from normal joints (HSF) and CACP-SF that has been supplemented with HSL (CACP-SF +HSL). Static COF was significantly higher in PBS and CACP-SF lubricated bearings compared to HSL and HSF lubricated bearings. Kinetic COF was significantly higher in bearings lubricated with PBS compared to all other groups.55

Figure 2.3: Apoptosis in bovine cartilage bearings lubricated with different test solutions. The lower explant cartilage bearings were stained for active caspase-3 (red, left column) and nuclei were counterstained with DAPI (blue, middle column) following friction and wear testing. Images were merged to show colocalization of caspase-3 within cells (right column). We observed many apoptotic cells in the superficial and upper middle zones of PBS and CACP-SF lubricated samples, while few apoptotic cells were observed in the unloaded bearings and in bearings lubricated with HSL, HSF or CACP-SF + HSL. Scale bars represent 100 μm57

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Chapter 3

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Table of Abbreviations

AC	Articular Cartilage
ACL	Anterior cruciate ligament
ACLT	Anterior cruciate ligament transection
AFM	Atomic Force Microscopy
BSA	Bovine serum albumin
CACP	Camptodactyly-arthropathy-coxa vara-pericarditis
COF	Coefficient of friction
CTXII	C-telopeptide of type II collagen
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
FAC	Focal Adhesion Complex
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GT	Genetrap allele
GT*	Mosaic genetrap allele, Prg4 recombined
GT ^R	Recombined genetrap allele
HA	Hyaluronic acid or hyaluronan
HSF	Human synovial fluid
HSL	Human synoviocyte lubricin
IP	Intraperitoneal injection
KO	Prg4 null allele
MMP	Matrix metalloproteinase
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PBS	Phosphate buffered saline
rhPRG4	Recombinant PRG4
RT	Room temperature
SF	Synovial Fluid
TJR	Total joint replacement
Tnf- α	tumor necrosis factor- α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Chapter 1

Introduction

1.1 Overview

Articular cartilage (AC) is a smooth, lubricious, load bearing tissue that lines the ends of articulating bones, including the knee. It's characterized by a very low coefficient of friction which is facilitated by the components of synovial fluid, including lubricin and hyaluronic acid. The specialized structure and composition of AC, which is mainly composed of collagen II and proteoglycans, allows it to withstand significant shear and axial stresses that occur during locomotion.

Although healthy AC is able to remain intact despite decades of use, there are a variety of risk factors for cartilage damage, including age, obesity and injury, that result in degenerative joint diseases, including osteoarthritis (OA). OA is characterized by pain, loss of motion and cartilage loss. Many biological and mechanical pathways have been linked to OA, but the exact etiology of the disease is still unknown. While therapies to treat joint pain are available, there are no disease modifying treatments for OA apart from total joint replacement.

Lubricin, a mucinous glycoprotein secreted by cells in the synovium and in the superficial zone of AC, is a boundary lubricant responsible for maintaining low frictional forces at the surface of AC. Lubricin is down-regulated following traumatic joint injury and is absent from joints of patients with a loss-of-function mutation in PRG4, resulting

in a lack of lubricin expression. These patient populations are at high risk for degenerative joint diseases.

Lubricin supplementation of synovial fluid in rodent models of OA have shown efficacy in preventing damage following traumatic injury. Additional studies investigating the mechanisms between lubricin-facilitated friction reduction and chondroprotection in AC should provide insight into the etiology of OA and other degenerative joint diseases.

The long term goal of this study is to develop a tribological lubricin supplement for the prevention of cartilage degeneration in patients at risk for degenerative joint disease. This dissertation investigates the relationship between lubricin-mediated boundary lubrication and its relationship to chondrocyte health and cartilage homeostasis.

1.2 Background

1.2.1 Osteoarthritis

OA involves the degeneration of AC, and is a widespread disease with large societal and healthcare implications. In the United States alone, 27 million people are estimated to be diagnosed with symptomatic OA, which affects 10% of males and 13% of females over 60 (1-4). Up to 20% of OA cases are attributed to a traumatic origin, but a variety of risk factors, including age, gender, ethnicity, trauma and obesity, predispose large portions of the population to degenerative joint disease (2, 4, 5). It is difficult to diagnose OA clinically before the onset of symptoms, such as pain and loss of function, and radiological evidence, which occur in the later stage of the disease (6). Furthermore, besides total joint replacement, there are no disease modifying treatments for OA (5, 7). As a result of treatment and diagnostic limitations, understanding the events that underlie OA initiation are a subject of extensive research.

While healthy AC is smooth and void of fissures or attachments, OA cartilage is characterized by surface fibrillation, loss of chondrocytes and, ultimately, full thickness cartilage loss (6, 8, 9). The early stages of OA are characterized by a loss of SZ chondrocytes, cartilage lesions and fissuring. As OA develops, chondrocytes begin to clone. Following a period of hypercellularity, cartilage cells die and there is matrix degradation. Often a deposit of fibrocartilage occurs on the surface of the cartilage. In advanced OA, there is cellular infiltration and the neovascularization of the cartilage cells by cells in the synovium. Chondrocytes begin to divide within their lacunae, a process recognized as ‘cloning’, in an attempt to replace the lost proteoglycan.

AC has little capacity for renewal, so prevention of the onset of OA is vital to cartilage preservation (10). The etiology of OA is largely unknown, but its onset is characterized by an imbalance between anabolic and catabolic processes that favors matrix degradation. Proteoglycan depletion is followed by the degradation of collagen II, resulting in mechanical failure and cartilage erosion.

1.2.2 Articular Cartilage

AC is a load bearing tissue that lines the ends of long bones in animal joints and provides support during gait and reduces friction and wear in articulating joints. As a result, stress is dissipated in the loaded joint, and the tribological system of cartilage and synovial fluid together enable (1). AC is comprised of chondrocytes surrounded by a dense extracellular matrix made up of proteoglycans, collagen fibers and water. Collagen fibers are arranged in zonal configurations in order to provide structural support during the axial compression and shear stress associated with animal locomotion. Collagen II is most abundant in AC, but it also includes collagen types III, V, VI, IX, X, XI, XII and XIV (11-14). Although the specifics of zonal configurations differ between species, in human, murine and porcine cartilage, broad collagen bundles extend radially from the calcified cartilage above the subchondral zone to form the deep zone, which is the largest zone, then bundles extend in arch-like structures through the middle zone that cross one another, then become parallel to the articular surface at the superficial zone (15, 16) (**Figure 1.1**). In bovine and rat cartilage, collagen bundles are thinner and more columnar in the deep and middle zones, but also become parallel to the surface at the superficial level (15). The perpendicular structure of the large deep zone resists compression, while the parallel fibers in the superficial zone resist shear stress as the joint surfaces move

against each other. Embedded in this collagen meshwork are proteoglycans, mainly the large protein aggrecan, which forms aggregates with hyaluronic acid (HA), and also includes small proteoglycan molecules such as keratin, decorin and lumican. These proteoglycans absorb water due to their high density of fixed negative charges in their anionic glycosaminoglycan side chains, which allows the cartilage to resist the compressive forces associated with load bearing and aids in the recovery of the AC.

Despite occupying a very small portion of the overall volume of AC, chondrocytes maintain various metabolic functions in AC, including the maintenance of the extracellular matrix (17, 18). The arrangement of chondrocytes can also be described in zones, which can differ between species (15). Superficial zone chondrocytes are flattened and disc-shaped and align with the surface of the cartilage. In humans and species described in this thesis, the superficial zone is highest in cellularity. Middle zone and deep chondrocytes are rounded, and the deep zone chondrocytes form columns perpendicular to the articular surface. Individual or groups of chondrocytes are surrounded by a tissue region called the pericellular matrix, which is largely made up of collagen VI, fibronectin and aggrecan 2B6, which together with the enclosed cell or cells are referred to as the 'chondron' (19). The pericellular matrix is thought to regulate both the mechanical and biochemical environment of its enclosed chondrocyte(s) (19-22).

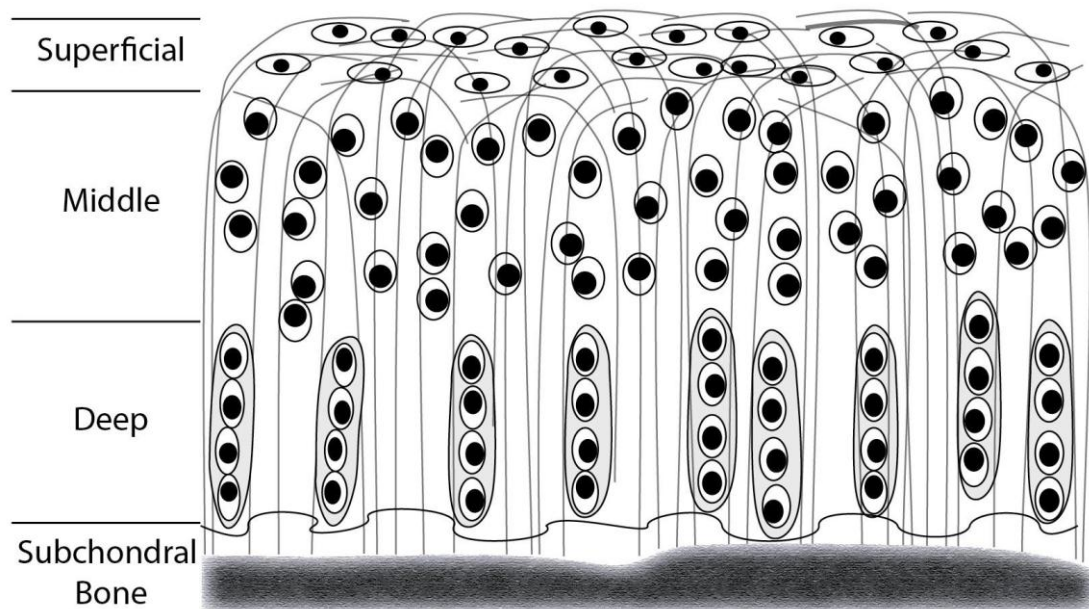


Figure 1.1: Zonal configuration of articular cartilage (AC). Collagen bundles extend radially from the calcified cartilage to form the deep zone, bend in arch-like structures through the middle zone, and form a network parallel to the surface in the superficial zone. The radial fibers resist axial compression and the parallel fibers resist shear stress. Proteoglycans absorb water to provide additional structural support and shock absorption. Superficial zone cells are flattened and align with the surface of the cartilage. Chondrocytes are also arranged in zones. In the middle zone and deep zone, cells are rounded. In the deep zone, cells form columnar chondrons surrounded by pericellular matrix.

1.2.3 Joint Lubrication and Chondroprotection

Synovial fluid (SF) is a multi-component, viscous fluid that provides joint lubrication and chondroprotection in articulating joints, primarily via its lubricating components, lubricin and HA, which work synergistically (23). A number of changes have been reported in the synovial fluid of patients with OA, including changes in the concentrations and structure of HA and lubricin (24-26).

1.2.3.1 Lubricin

Lubricin, also known as megakaryocyte-stimulating factor and superficial zone protein (27), is a surface-active mucinous glycoprotein produced by synovial cells and chondrocytes. Lubricin, comprised of a 1,404 amino acid sequence, has an approximate molecular weight of 227.5 kDa (28) and is present at a concentration of ~250 µg/ml (29). A mucin-like domain is flanked by a C-terminal haemopexin domain and 2 somatomedin domains at the N-terminus (**Figure 1.2**), which are homologous to the C- and N-terminals on vitronectin. The central mucin-like domain contains the repeated motif of KEPAPTT/T, and is subject to 50% (w/w) O-linked $\beta(1-3)$ -Gal-GalNAc glycosylations which are non-uniformly capped with NeuAc. The glycosylations in this domain contribute to boundary lubrication by conferring steric repulsion (29) via strong repulsive hydration forces to the apposed articular surfaces.

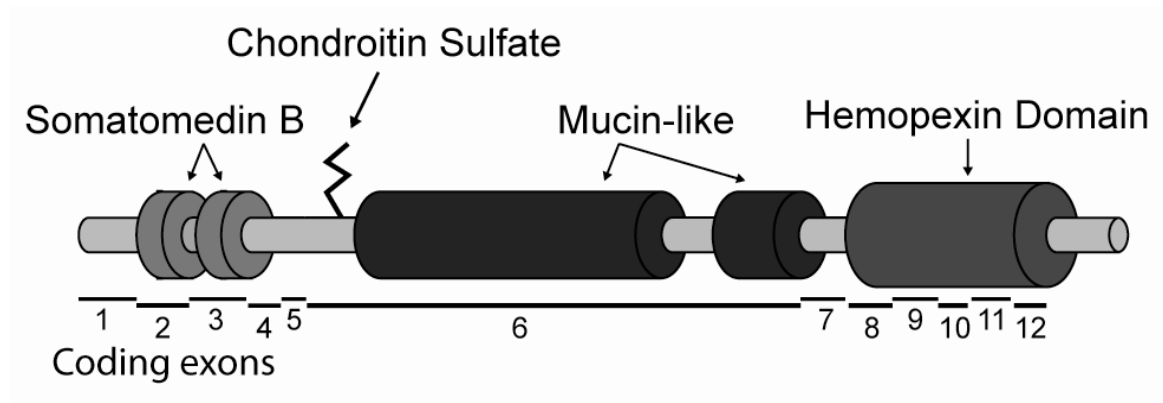


Figure 1.2. Lubricin structure. The N-terminus, which contains two somatomedin B domains and the C-terminus, which contains a hemopexin domain, are connected by a long mucin-like domain, which is located on exon 6 and is heavily glycosylated with O-linked $\beta(1-3)$ -Gal-GalNAc and non-uniformly capped with NeuAc (30). The C-terminus interacts with the cartilage surface.

In healthy articular joints, a layer of lubricin molecules form end-grafted brushes and cover the surface of cartilage, acting as an anti-adhesive and boundary lubricant in situations involving high load and low velocity when cartilage is pressurized during the loaded part of the gait cycle (28, 30-38). Lubricin has matrix binding properties associated with the N-terminal domain, boundary lubricating and chondroprotective properties provided by the mucin-like mid-domains, and matrix binding properties localized within the C-terminal domain. Lubricin molecules, which can also form intramolecular disulfide bridges in the N-terminous and intramolecular disulfide bridges in the C-terminus (39), interact with the thin layer of HA, proteoglycans and lipids that cover the surface collagen of cartilage. Furthermore, a synergistic relationship between HA and lubricin, which enhances boundary lubrication, has also been reported (23, 40-43).

Patients with the autosomal recessive disorder camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome have a truncating mutation resulting in no protein expression from the PRG4 gene (10, 44). These patients present in early childhood with flexion contractures of the phalangeal joints of the fingers and toes (camptodactyly), pain, swelling and restricted range of motion in the large joints (arthropathy), and in some cases thickening of the pericardium, which is not associated with inflammation (44). Synovial hyperplasia, without inflammation, is a hallmark of the disease and plays a role in the deterioration of the joints and cartilage damage (44).

Clinical findings of patients with CACP, relating to joint destruction, have been recapitulated in a murine knockout model (10, 45). Lubricin null ($Prg4^{-/-}$) mice are born with normal cartilage but exhibit cartilage surface roughness and synovial hyperplasia in

their stifle joints at 2-weeks of age (10, 45). Elevated friction has been reported at 1 month of age, with increasing differences in friction between $\text{Prg4}^{-/-}$ mice and lubricin expressing $\text{Prg4}^{+/-}$ and $\text{Prg4}^{+/+}$ littermates (45). At 2 months of age, there is significant synovial hyperplasia, subintimal fibrosis, protein deposits, irregular joint surfaces and endochondral growth plates. Later in life, $\text{Prg4}^{-/-}$ mice experience precocious joint failure, characterized by a joint space filled with hyperplastic synovium and severe cartilage damage and loss (10).

1.2.3.2 Hyaluronic Acid (HA)

HA, also known as hyaluronan, is a high molecular weight glycosaminoglycan that is present throughout articular cartilage, in extracellular matrices and in the synovial fluid of articular joints (46). In synovial fluid, HA has been reported in concentrations of 0.5-4 mg/ml with an approximate molecular weight of 3-4 MDa (46, 47). HA is composed of linear repeating units consisting of β -(1 $\rightarrow$ 4)-linked D-glucopyranuronic acid and beta-(1 $\rightarrow$ 3)-linked 2-acetamido-2-deoxy-D-glucopyranose (48, 49). HA interacts with cell-surface receptors, including CD44 and forms pericellular matrices around chondrocytes (50).

HA is responsible for the viscoelastic behavior of synovial fluid and also aids in shock absorption and hydrodynamic lubrication, which occurs at high velocities and low loads when the fluid layer is wider than the asperities on the articular surface and is dominated by fluid mechanics (51, 52). HA has also been shown to contribute to boundary mode lubrication, particularly in combination with lubricin (23, 30, 40, 53, 54).

1.2.4 Therapeutics

A number of therapies are available to patients with symptomatic OA, including bracing, exercise and non-steroidal anti-inflammatory drugs (NSAIDs), such as naproxen and ibuprofen, for pain in the early stage of OA (7, 55). For patients that do not respond to these treatments, the injection of corticosteroids into the intraarticular space to treat pain has become common in the conservative management of knee OA (56, 57). Ultimately, many patients turn to total joint replacement to regain joint function despite the fact that it requires invasive surgery and carries risks, including implant loosening and infection (5, 55).

1.2.4.1 Viscosupplementation (HA)

An increasing number of patients with symptomatic OA that do not respond to corticosteroids are turning to viscosupplementation, which involve the intraarticular injection of HA in various forms, and serve to correct any changes in synovial fluid or loss of viscoelasticity that has been reported in some patients with OA (58-62). Although the use of HA products to treat OA are somewhat controversial, it remains a common practice for pain management and increased function in symptomatic knee OA. Viscosupplements are available in different molecular weights, including the commonly used high molecular weight, cross-linked form hylan G-F 20 (Synvisc, Genzyme Corp., Cambridge, MA, USA) (63).

Hylan G-F 20 is administered to patients who do not respond well to non-pharmacological treatments, such as weight loss and physical therapy, or simple analgesics. Hylan G-F 20 is an FDA approved high molecular weight (average 6000 kDa)

hyaluronan product with two cross-linked components that originate from chicken combs (63). Either three weekly doses of 2 mL or a single 6 mL dose is administered to patients (63). Prior to injection, arthrocentesis is advised, which may remove important components of the synovial fluid, including lubricin, which is vital to boundary lubrication (10, 45, 63). Replacement of native synovial fluid with hylan G-F 20 could possibly have detrimental effects on cell survival.

Numerous studies have tested the outcome of hylan G-F 20 injection compared to other intraarticular HA treatments, including NSAID and corticosteroid therapies (57, 64). The mode of action of hyaluronan injection in decreasing joint pain in OA affected joints remains unclear. Some studies have reported that loss of viscosity due to HA depletion may play a role in OA progression, although the finding of reduced HA content in OA SF has been challenged (25, 65). Adding a cross-linked HA, such as hylan G-F 20, to SF reinforces non-Newtonian behavior, which is characteristic of healthy SF (61). Alternate hypotheses, including biosynthetic-chondroprotective effects, anti-inflammatory effects, and analgesic effects due to protective action on nociceptive nerve endings, have also been proposed (65).

1.2.4.2 Tribosupplementation (Lubricin)

Intraarticular injections of lubricin in various forms, referred to as tribosupplementation, have shown disease-modifying effects in rodent models of OA (66-69). Patients with anterior cruciate ligament (ACL) injury are at higher risk for secondary OA, and have a decreased lubricin concentration in SF following injury and in the presence of inflammatory cytokines associated with trauma, such as tumor necrosis factor- α (70). Chondroprotection following lubricin treatment in rats following ACL

transection (ACLT), both in the presence and absence of exercise, and following meniscectomy has been observed (66, 68, 69, 71-73). These studies are supported by histological analysis (66, 68, 69, 71-73), gait measurement (69), urine excretion of C-telopeptide of type II collagen (CTXII) excretion (66, 69, 73), radiographically (72), and the presence of apoptotic cells (66, 73).

Lubricin-based therapies, with or without the addition of HA, show promise to prevent OA in patients with joint trauma and those with inflammatory joint diseases where lubricin is depleted, and as a therapy for patients with CACP.

1.2.5 Cartilage Tribology and Wear Measurements

Synovial joint boundary lubrication and wear has been studied *ex vivo* and *in vitro* using a number of modalities. Many *ex vivo* studies utilize whole joint pendulum systems, such as the one in **Figure 1.3**, which allow the joint to remain in its natural configuration and take into account the roles of the synovium, joint capsule, and ligaments (45, 74-77). Many bearing systems involving both tissue and synthetic bearings have also been used, including latex-on-glass (30, 78), cartilage-on-glass (28) and cartilage-on-cartilage (23, 34, 37, 79, 80). Cartilage-on-cartilage bearings (**Figure 1.4**) allow the user to control the cartilage geometry and monitor lubricant-tissue interaction, which is an advantage over other methods using cartilage against a synthetic bearing. A number of physiologically relevant lubricants have been tested using these bearing systems, including of synovial fluid, lubricin, and hyaluronic acid from various sources.

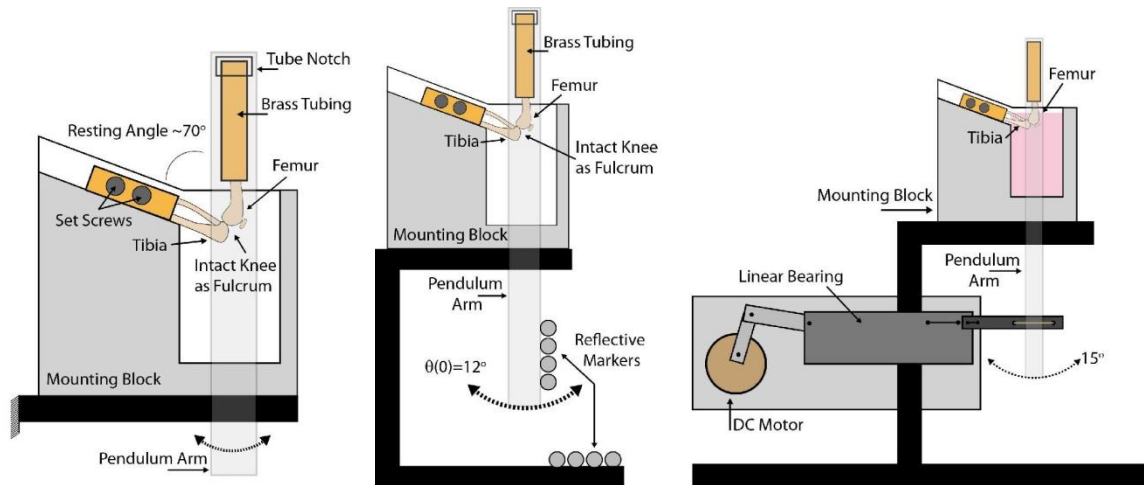


Figure 1.3: Pendulum systems for measuring whole joint COF and applying cyclic loading in rodent stifle joints. (left) The joint is fixated on a mounting block. The distal tibial and proximal femur are fitted into brass tubing that is secured to the pendulum base by set screws. The proximal end of the femur tube fits into the pendulum arm, and the knee joint serves as the fulcrum. The mounting block contains a chamber that can be filled with fluid. (center) Passive pendulum. The mounting block is secured to the pendulum base. Four (4) reflective markers are located on the base and on the pendulum arm. The pendulum arm is rotated $\sim 12^\circ$, released and allowed to swing freely until motion ends. (right) Active pendulum. A DC motor drives continuous pendulum motion $\pm 15^\circ$ at a rate of 1.5 Hz. The limb is submerged in cell culture media to prevent desiccation. (adapted from Drewniak, et al., 2012) (77).

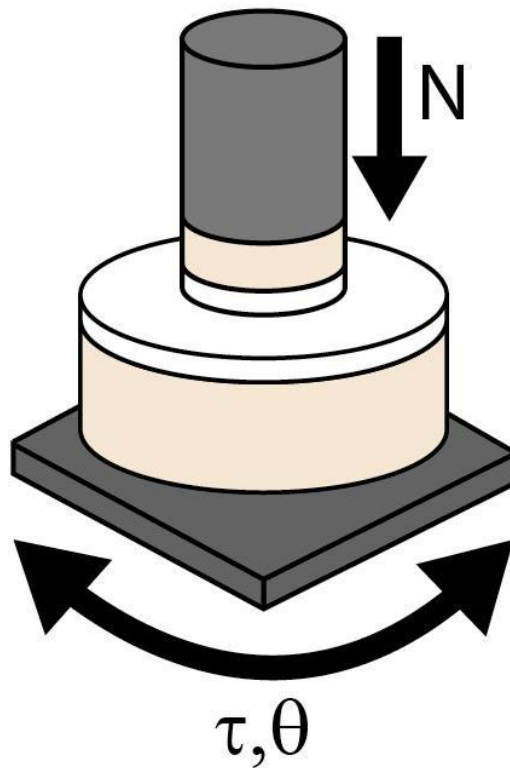


Figure 1.4: Cartilage annulus-on-disc bearing lubrication test. Sample lubricants were tested between the cartilage surfaces of osteochondral bearings placed in apposition, compressed axially and subjected to relative motion. Cartilage plugs were tested in an ELF 3200 with sensors for axial load and torque. Prior to axial compression, test lubricant is added between the surfaces. (adapted from Schmidt, et al., 2007) (79).

While much of OA literature concentrates on the mechanical degradation associated with cartilage damage, it is also important to note the role of biological changes occurring in the chondrocytes and synovial fluid that contribute to the characteristics and progression of cartilage deterioration. Chondrocyte function is altered in OA cartilage, which is manifest in decreased proteoglycan content, protein deposition, including alterations in collagen, and the expression of matrix depleting factors such as matrix metalloproteinases (MMPs) and other catabolic factors (6, 9, 70, 81-86). Chondrocytes also respond to inflammatory cytokines and other factors present in the synovial fluid when a joint is affected by synovitis, which is also a characteristic of OA (87, 88). In an attempt to repair damage, chondrocytes begin to replicate within their lacunae, an abnormal behavior referred to as ‘cloning’ (6). Following a period of hypercellularity, tissue begins to become hypocellular as clones are more susceptible to cell death (6). The change in chondrocyte behavior modifies the structure and composition of AC.

One of the hallmarks of osteoarthritic cartilage is chondrocyte death. Chondrocyte death via apoptotic mechanisms is regulated by numerous factors, including cell-matrix interactions, loss of matrix components and mechanical injury (17). Pro-inflammatory cytokines (84, 89-91), Fas/CD95 (92, 93) and nitric oxide (94-96) play a role in apoptosis regulation both *in vivo* and *in vitro*. Likewise, mechanical loading through shear stress, as well as osteochondral defects and hydrostatic pressure have been shown to increase apoptosis *in vivo*, in cultured chondrocytes, and in cartilage explants (97-102).

A mechanotransduction pathway governing the relationship between joint trauma and apoptosis has been speculated (8, 18, 94, 95, 97, 99, 103-114), but any relationship

between excessive cartilage bearing friction and apoptosis had not been reported. Due its specialized collagen configuration and the flattened structure of superficial zone chondrocytes, the cartilage matrix is able to withstand a considerable amount of shear stress before degradation occurs. Integrin cell surface receptors function as a link between articular chondrocytes and their surrounding extracellular matrix, and regulate many cell-dependent functions including cartilage matrix repair, cell attachment and cell survival (8, 110, 112, 115-118). A number of hypotheses have been proposed based on mechanical stimulation of chondrocytes in monolayer or 3D culture (8, 101, 110, 112, 115-120), but the pathway linking mechanical stimulation via shear stress and chondrocyte apoptosis has not been investigated using explants.

Another important biological change is the attachment of cells and proteins to the superficial zone following joint fibrillation and synovial hyperplasia. These changes are evident in the *Prg4*^{-/-} mouse, where the synovial cells infiltrate the superficial zone and proteinaceous deposits are evident as early as 2 months of age due to the lack of an anti-adhesive lubricin layer (10, 121). Similar changes have been noted in advanced OA, with the replacement of cartilage with fibroblast-like cells and fibrocartilaginous tissue (122, 123). Changes to the synovial membrane, including synovial hyperplasia and cellular infiltration has been observed in OA and CACP syndrome (44, 88, 117, 124), and are detrimental to the mechanics and boundary lubrication of afflicted joints in these conditions.

1.3 Significance

While OA is a widely studied disease, its etiology is still largely unknown and treatment options are very limited. Recent advances in cartilage tribology and the scalable production of lubricin (68, 71) have provided patients with a potential new therapeutic for the treatment and prevention of cartilage damage in patients with OA, CACP, trauma induced arthropathy and other joint diseases. This work investigates the efficacy of lubricin to prevent both mechanical and biological wear in AC, using *in vivo*, *ex vivo* and *in vitro* models. The development of a cartilage bearing lubrication and wear testing regimen allowing for subsequent cell culture and observation of cellular response to friction and wear will facilitate testing of lubricants and therapeutics to evaluate OA treatments and prevention, as well as use of different cartilage bearings to observe lubrication and wear response in compromised tissues. Animal models investigating both intraarticular lubricin and transgenic induction of lubricin production provides valuable insight into the treatment of CACP and OA. Additionally, this work identifies a mechanopathway relating friction and apoptosis, which provides the groundwork for many therapeutic targets in the prevention of biological wear.

1.4 Specific Aims

1.4.1 Aim 1: Investigate friction and apoptosis in the articular joint

1.4.1.A – *In vivo* analysis: Measure friction and observe apoptosis in the knee joints of lubricin-null mice.

The objectives of Aim 1A are to measure the whole joint coefficient of friction in the knees of the *Prg4* mutant mice and evaluate apoptosis in these joints, which we accomplished in Chapter 2 of this dissertation. We measured whole joint coefficient of friction (COF) using a passive modified Stanton pendulum system developed by Drewniak, *et al.* (75), and were able to replicate the results described in their work (77). We observed apoptosis by staining coronal sections of 10-week-old mouse hind limbs for activated caspase-3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). We hypothesized that *Prg4*^{-/-} knees will have a higher COF than *Prg4*^{+/+} or *Prg4*^{+/-} knees, and that elevated friction will be accompanied by apoptosis in *Prg4*^{-/-} joints, but will not be seen in *Prg4*^{+/+} or *Prg4*^{+/-} joints.

1.4.1.B – *In vitro* analysis: Characterize biological wear in a bovine cartilage-on-cartilage bearing using physiologically relevant test lubricants.

The objectives of Aim 1B are to measure coefficient of friction using a cartilage-on-cartilage cartilage bearing system and observe the ability of biologically-relevant test lubricants to provide boundary lubrication and chondroprotection in these bearings. We used a fresh bovine cartilage disc-on-disc bearing system adapted from Schmidt, *et al.*

(79) that was altered to allow subsequent explant culture and observe cellular demise following friction and wear testing. We used this method to complete a three-part study.

In Chapter 2, we compared physiological lubricants in the disc-on-disc bearing system. We tested synovial fluid from patients with CACP (CACP-SF), which lacks lubricin, human synoviocyte lubricin (HSL), and normal human synovial fluid obtained from the contralateral joints of patients undergoing ACL reconstruction, which contains a normal concentration of lubricin. We also observed the ability of HSL to replenish the boundary lubrication and chondroprotective abilities to CACP-SF. PBS served as a negative control for friction and apoptosis, and unloaded control discs provided a positive control for apoptosis measurements. We hypothesized that lubricants containing lubricin (HSL, HSF and CACP-SF + HSL) would have lower coefficients of friction and a lower percentage of apoptotic cells in the superficial and upper middle zone compared to lubricants lacking lubricin (PBS and CACP-SF) (125).

In Chapter 3, we tested the high molecular weight viscosupplement hylan G-F 20 (Synvisc), which is commonly used to treat patients with OA, and compared it to HSF, acquired either from normal patients, post-mortem, or from patients undergoing total joint replacement. We hypothesized that COF and the percentage of apoptotic cells in cartilage bearings lubricated with hylan G-F 20 (Synvisc) will be less than that of PBS and equal to that of HSF (126).

Finally, we compared different sources of synovial fluid and lubricin. We tested HSF from patients with OA undergoing total joint replacement and patients with normal joints, such as contralateral ACL repair joints or post-mortem synovial fluid. We hypothesized that bearings lubricated with HSF from normal joints would have a lower

coefficient of friction compared to synovial fluid aspirated during total joint replacement (OA-SF). We also tested different sources of lubricin, including lubricin purified from synovial fluid aspirated during total joint replacement (PHL), HSL and recombinant human PRG4 (rhPRG4). We hypothesized that PHL would have a higher coefficient of friction and a higher percentage of apoptotic cells than bearings lubricated with HSL or rhPRG4.

1.4.2 Aim 2: Determine the ability of lubricin to rescue a damaged joint

1.4.2.A. Investigate the ability of exogenous lubricin injected into the joint space to reduce cartilage damage and whole joint COF in $\text{Prg4}^{-/-}$ mice

The objective of Aim 2A is to investigate the ability of lubricin to lower whole joint COF in $\text{Prg4}^{-/-}$ mice at different ages and preserve cartilage 2 weeks following an intraarticular injection in vivo. Whole joint COF was measured with the contralateral knee as a control, and histological analysis was used to measure joint damage, presence of lubricin and apoptosis. We hypothesized that HSL will be able to attenuate cartilage damage and lower COF.

1.4.2.B. Investigate the ability of endogenous lubricin to reduce cartilage damage and whole joint COF in Prg4 genetrap mice of different ages.

The objective of Aim 2B is to investigate the lubricin requirements to sustain an effective superficial zone. Using a gene-trap Prg4 mouse, we investigated the ability of endogenous lubricin to protect the cartilage of a mouse born with a lubricin-null phenotype. The Prg4 gene was activated by daily intraperitoneal (IP) injections of Tamoxifen for 10 days, starting at 3 weeks of age (early intervention), when early

degenerative changes are present in the $\text{Prg4}^{-/-}$ mice, and at 2 months of age (late intervention) when mice show a more dramatic phenotype. Mice were evaluated at 2 months of age and 6 months of age for the early and late intervention schemes, respectively. We measured the whole joint COF in conjunction with cyclic loading, observed joint damage histologically at the prescribed evaluation time points. We hypothesized that lubricin activation at 21 days post-natal would protect the cartilage in Prg4 genetrap (GT) mouse better than treatment at 2 months or later, but that lubricin activation at any age would reduce cartilage damage compared to lubricin-null littermates.

1.4.3 Aim 3: Evaluate the Effectiveness of Etanercept and Lubricin to Prevent Cartilage Deterioration in a Rat Anterior Cruciate Ligament Transection Model

The objectives of Aim 3 are to evaluate the changes in the mechanical properties of rat cartilage following ACL transection (ACLT), and the ability of etanercept or intraarticular lubricin injection to preserve mechanical properties and smooth surface characteristics in these joints. In order to address these aims, we used atomic force microscopy (AFM) to measure the elastic modulus at the microscale of rat femoral condyles in joints that have undergone ACLT, with or without etanercept, lubricin or PBS treatment, and of rats that have only undergone capsulotomy as a sham surgical control. We stained the tibial plateaus of these joints with Safranin-O/Fast Green to measure proteoglycan and detect structural damage, and to detect extracellular matrix proteins and active caspase-3. We hypothesized that the mechanical properties of ACLT

joints left untreated or treated with PBS will decrease with time compared to ACL-intact joints that have undergone capsulotomy surgery. By contrast, we hypothesized that ACLT joints treated with either etanercept or lubricin will have similar mechanical properties compared to joints that undergo capsulotomy surgery. We also hypothesized that joints treated with lubricin or etanercept will have less cartilage damage and fewer apoptotic cells compared to untreated and PBS treated ACLT joints.

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Chapter 2

The Role of Lubricin and Boundary Lubrication in the Prevention of Chondrocyte Apoptosis

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2.1 Abstract

Osteoarthritis is a complex disease involving the mechanical breakdown of articular cartilage in the presence of altered joint mechanics and chondrocyte death, but the connection between these factors is not well-established. Lubricin, a mucinous glycoprotein encoded by the *PRG4* gene, provides boundary lubrication in articular joints. Joint friction is elevated and accompanied by accelerated cartilage damage in humans and mice that have genetic deficiency of lubricin. Here, we investigated the relationship between coefficient of friction and chondrocyte death using *ex vivo* and *in vitro* measurements of friction and apoptosis. We observed increases in whole joint friction and cellular apoptosis in lubricin knockout mice compared to wild-type mice. When we used an *in vitro* bovine explant cartilage-on-cartilage bearing system, we observed a direct correlation between coefficient of friction and chondrocyte apoptosis in the superficial layers of cartilage. In the bovine explant system, the addition of lubricin as a test lubricant significantly lowered the static coefficient of friction and number of apoptotic chondrocytes. These results demonstrate a direct connection between lubricin, boundary lubrication, and cell survival and suggest that supplementation of synovial fluid with lubricin may be an effective treatment to prevent cartilage deterioration in patients with genetic or acquired deficiency of lubricin.

2.2 Introduction

Osteoarthritis (OA), which involves the degeneration of articular cartilage, is a prevalent disease with large societal and healthcare implications. Beside total joint replacement, there are no disease modifying treatments for OA (1). Therefore, the identification of factors that can initiate OA is an area of extensive research. One hypothesis is that OA begins following an increase in coefficient of friction due to loss of boundary lubricant in synovial fluid (2). According to this hypothesis, increased friction between cartilage surfaces causes an increase in shear stress at the cartilage surface, which then affects chondrocyte metabolic function and survival. Ultimately, the combination of increased friction, loss of proteoglycan within the cartilage matrix, and chondrocyte death progresses to end-stage OA, characterized by full thickness cartilage loss (3-5). Support for this hypothesis derives from studies demonstrating the critical role of chondrocytes in maintaining the articular cartilage matrix (6, 7) and associating chondrocyte apoptosis with OA pathology (8-10). However, a direct connection between friction and chondrocyte apoptosis has not yet been demonstrated in the context of lubricating synovial fluid components.

Cartilage lubrication in articulating joints is facilitated by synovial fluid (SF), which contains the lubricating factors lubricin and hyaluronic acid (11-13). Lubricin, a mucinous glycoprotein produced by synovial cells and superficial zone chondrocytes (11, 14), provides lubrication in the boundary mode (15). Boundary lubrication dominates during periods of high load and low velocity, when the lubricant film layer is thinner than the surface roughness (16). In healthy articular joints, a layer of lubricin molecules covers

the surface of cartilage and acts as an anti-adhesive and boundary lubricant to prevent cartilage damage as surface asperities move against each other (17-20). Patients with the autosomal recessive disease camptodactyly-arthropathy-coxa vara-pericarditis syndrome (CACP) lack functional lubricin and develop precocious cartilage failure. This disease has been recapitulated in mice in which the lubricin encoding gene, *Prg4*, has been knocked out. Early cartilage changes in mice lacking lubricin include fibrillation at the cartilage surface and loss of superficial zone chondrocytes (14, 21).

To determine whether increased friction is associated with loss of superficial zone chondrocytes, we measured whole joint friction and observed the presence of apoptotic chondrocytes in tibiofemoral joints from lubricin knockout (*Prg4*^{-/-}), heterozygous (*Prg4*^{+/-}), and wild-type (*Prg4*^{+/+}) mice. We further investigated the role of lubricin using an *in vitro* bovine cartilage disc-on-disc bearing system. We lubricated this system with biologically relevant test solutions (e.g., normal human synovial fluid, synovial fluid from patients with CACP, lubricin purified from human synoviocyte culture) and measured friction and numbers of apoptotic chondrocytes. Herein we show that friction and chondrocyte apoptosis are directly correlated, and both are reduced in the presence of lubricin.

2.3 Materials and Methods

2.3.1. Modified Stanton Pendulum

Hind limbs were excised from $\text{Prg4}^{-/-}$ (n=6 limbs, n=5 mice), $\text{Prg4}^{+/-}$ (n=6 limbs, n=5 mice) and $\text{Prg4}^{+/+}$ (n=12 limbs, n=8 mice) male, 10 ± 1 wk-old, mice and tested on a modified Stanton pendulum immediately following harvest to achieve a measure of COF, as previously described (22, 23). The hind limbs were dissected, leaving the joint capsule intact, and the proximal femur and distal tibia were rigidly embedded in square brass tubing (6.25 mm wide) using a urethane potting compound (Smooth-ON, Easton, PA). The pendulum arm was fitted to the tube encasing the femur, and the tube encasing the tibia was fixed to the pendulum base using set screws, with the knee at a resting angle of 70 degrees, which was chosen based on previous studies regarding the mean flexion angle during normal murine ambulation (24, 25). Four (4) reflective markers were attached to the pendulum arm and 4 were attached to the base. Following a static loading time of 8 min where the knee was loaded with the weight of the pendulum (50g, approximately 2 times body weight) and submerged in a well filled with PBS. Following the loading period, PBS was removed from the well, and the pendulum arm was rotated to a flexion angle of ~ 12 degrees and released, allowing the joint to oscillate freely until motion was finished. Four (4) trials were collected for each knee. Motion was tracked with a Qualisys AB system at a rate of 60 Hz. Oscillation data was processed with Visual3D software (C-Motion, Inc., Germantown, MD) and a custom MATLAB (MathWorks Inc, Natick, MA) code was used to determine the peak amplitude of each cycle of oscillation and calculate whole joint COF using a Stanton linear decay model

(22). The generation of these mice has been previously described and they have been backcrossed onto a C57BL6/J background strain (14). All animal research was approved by the Rhode Island Hospital Animal Welfare Committee.

2.3.2. Bovine Cartilage Preparation

Full thickness cartilage plug bearings 6 mm and 12 mm in diameter were cored from the femoral condyle of bovine knees (n=10) collected within 2 h of slaughter. Following harvest, the bearings were rinsed with cell culture media [DMEM supplemented with 5% (vol/vol) FBS] and cultured for 24 h at 37°C.

2.3.3. Test Solutions

Phosphate buffered saline (PBS, 1X) was purchased from Life Technologies Corp. Human synovial fluid (HSF) was aspirated from the uninjured contralateral joints of patients with an anterior cruciate ligament injury (N=4). These samples were obtained without lavage just prior to reconstructive surgery (26). Other human synovial fluids were aspirated from knee joints of post-mortem donors with no osteoarthritis within twelve h of death (2 male donors, ages 29 and 39, NDRI). The samples were inspected for blood contamination then centrifuged following aspiration at $12,000 \times g$ at 4 °C for 10 min, and the aliquot was removed and stored at -80°C until testing. Lyophilized human synoviocyte lubricin (HSL) was obtained from SBH Sciences and has been characterized previously (27, 28). This product was solubilized using distilled water to a concentration of 250 µg/ml, which is the estimated lubricin concentration in synovial fluid (26). Synovial fluid from patients with CACP (CACP-SF) was obtained from patients in the years between 2000 and 2011, following diagnostic knee joint aspiration

(n=4). CACP-SF samples were inspected for blood contamination then centrifuged at $12,000 \times g$ at 4°C for 10 min and stored frozen at -20°C until testing. Lyophilized HSL was solubilized in CACP-SF (CACP-SF+HSL) at $250 \mu\text{g/ml}$ 24 h prior to testing, and stored overnight at 4°C . All lubricants were thawed to room temperature immediately prior to testing. Approval to obtain synovial fluid from patients was granted by the Rhode Island Hospital and Boston Children's Hospital Institutional Review Boards.

2.3.4. Bovine Bearings Friction and Wear Testing

This testing method has been described previously (29). Briefly, the average total cartilage thickness for each bearing pair was calculated ($2.95 \pm 0.47 \text{ mm}$) using caliper measurements in four regions along the circumference before testing. The upper disc diameter ($5.62 \pm 0.47 \text{ mm}$) was also measured using two caliper measurements. Cartilage bearings were loaded in a disc-on-disc configuration (the lower disc diameter was 12 mm) into an EnduraTEC ElectroForce 3200 (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN, USA) with cyanoacrylate glue, and any remaining cell culture medium was rinsed off 3 times with 1 ml washes of PBS. Test lubricant was applied between the surfaces. The bearings were axially loaded to 18% total cartilage thickness, reaching a maximum load of $6.87 \pm 3.6\text{N}$, and held at this displacement for 8 min to allow for fluid depressurization (30, 31). Then, the lower bearing was rotated in torsion + 2 revolutions and reset - 2 revolutions at an effective velocity of 0.3 mm/s , which allows the depressurized cartilage-cartilage interface to remain in the boundary mode of lubrication (30), for 12 continuous cycles. Immediately following testing, lower discs were fixed in formalin. Unloaded control discs, also 12 mm in diameter, were fixed

at the time of testing. All tests were performed between 48 and 72 h of cartilage harvest, and bearings were only tested once.

Static and kinetic coefficients of friction were calculated using Equation 1 (29):

$$\text{COF} = |\text{Torque}| / (1/3 \times \text{Upper Bearing Diameter} \times \text{Axial Force}) \quad (1)$$

To calculate static COF, the absolute maximum value of torque at the initiation of motion was used. To calculate kinetic COF, the average torque, observed during the last 720 degrees of rotation was used. The equilibrium axial force (1.55 ± 0.82 N), which was incurred following the 8 minute depressurization period, and the measured diameter of the upper bearing were used for both measurements (29).

2.3.5. Activated Caspase-3 Staining and Quantification

Mouse knees representing each *Prg4* genotype that were not used to measure COF and bovine femoral cartilage lower bearings that were used to measure COF were processed for active caspase-3 detection. Samples were fixed in 10% formalin and decalcified using a solution of 0.48M EDTA adjusted pH to 7.1 with ammonium hydroxide at 4°C for 48 hr (mouse knees) or 2 wk (cartilage bearings). Thin coronal sections (6µm) were taken for histological analysis of apoptosis of chondrocytes in the femoral condyles and tibial plateaus of mouse limbs, and full thickness sections of large bovine bearings. Sections were heated to 60°C for 30 min, deparaffinized and hydrated in 3 changes of xylene and serial alcohol. Sections were then quenched in endogenous peroxidase in 3% hydrogen peroxide for 10 min and antigen retrieval was performed using a pepsin solution (ThermoScientific). A rabbit polyclonal antibody against active caspase-3 (ab13847, Abcam, Cambridge, MA) at 1:50 dilution was added to the sections and incubated at 4°C overnight. After 3 washes with PBS, the sections were incubated

with Cy3 goat anti-rabbit IgG (Life Technologies, Molecular Probes) at 1:50 dilution for 1 hour at room temperature, protected from light. The sections were washed 5 times using PBS, counterstained using Vectashield mounting media with DAPI (1.5µg/ml, Vector Laboratories Inc, Burlingame, CA). Images were captured with a 10X objective, using a Roper Scientific Photometrics CoolSNAP HQ2 monochrome camera (Roper Scientific, Germany) a Nikon Eclipse 90i microscope (Nikon). Fluorescent images were thresholded uniformly to reduce background auto-fluorescence and to adjust DAPI signal using Adobe Photoshop CS5 software (Adobe Systems Inc). Active caspase-3 was also immunodetected with ab13847 (Abcam, Cambridge, MA) and detected using the Vectastain ABC kit (VECTOR Laboratories, Burlingame, CA, USA). Sections were then counterstained with 0.5% methyl green.

The percentage of apoptotic cells in bovine sections was determined using images captured at 20X with Image-Pro Plus software (Media Cyberkinetics, Bethesda, MD). The number of active caspase-3 positive cells was divided by the total number of cell nuclei in three 100µm thick cartilage layers beginning at the cartilage surface. The total percentage of apoptotic cells was the mean across all three zones.

2.3.6. TUNEL Staining

We used the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, Billerica, MA). Mouse knee and bovine cartilage bearing samples were fixed, decalcified, and embedded as previously described (29). Sections were heated at 60°C for 30 min, deparaffinized in 3 changes of xylene and serial ethanol. Then, sections were pretreated with proteinase K (20µg/ml) for 15 min at room temperature, quenched with endogenous peroxidase in 3% hydrogen peroxide for 5 min, and incubated with equilibration buffer

for 30 seconds. Sections were treated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour in a humidified chamber, washed 3 times in PBS, incubated with anti-digoxigenin conjugate for 30 min at room temperature, and washed in PBS. Peroxidase substrate was applied to sections, which were stained for 4 min, washed in deionized water, and counterstained with 0.5% methyl green. Sections were washed in deionized water again, dehydrated in xylene 3 times, and then mounted with Permount mounting media. Images were captured at 20X with Image-Pro Plus software.

2.3.7. Statistical Analysis

Statistical analysis was performed using a one-way ANOVA on ranks ($\alpha \leq 0.05$) with Dunn's comparison tests for COF in *Prg4* mouse limbs. Statistical analysis for bovine bearing static and kinetic COF between groups lubricated with different test lubricants was performed using a one-way ANOVA with Holm-Sidak multiple comparison *post hoc* tests. A Student's t-test was used to determine the difference between the static COF of bearings lubricated with CACP-SF and CACP-SF+HSL. Linear regression analysis was performed comparing static COF and the percentage of activated caspase-3 positive cells in the top 100 μm layer. Differences between apoptosis in the three zones of bovine cartilage discs were analyzed using a two-way repeated measures ANOVA ($\alpha \leq 0.05$) with Holm-Sidak multiple comparison *post hoc* tests, using Sigmaplot software (Systat Software Inc, Chicago, IL). All other statistics were performed using Prism 6 Statistical Software (GraphPad Software Inc., La Jolla, CA), and all values are reported as mean $\pm$ SD.

2.4 Results

We stained coronal sections of knees from 10-wk-old $\text{Prg4}^{-/-}$, $\text{Prg4}^{+/-}$, and $\text{Prg4}^{+/+}$ mice for active caspase-3, which indicates cells in the execution stage of apoptosis (**Figure 1A**). There were more chondrocytes with active caspase-3 in $\text{Prg4}^{-/-}$ knees compared to $\text{Prg4}^{+/+}$ and $\text{Prg4}^{+/-}$ knees. We also performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), as a second means of measuring programmed cell death in knees from mice with different Prg4 genotypes. There were more TUNEL positive cells in $\text{Prg4}^{-/-}$ knees compared to $\text{Prg4}^{+/+}$ and $\text{Prg4}^{+/-}$ knees (**Figure 2.1A**). Regions of apoptotic chondrocytes in $\text{Prg4}^{-/-}$ knees were most pronounced in the upper middle zone just below the layer of flattened chondrocytes (**Figure 2.1A**).

We measured whole joint COF, which is manifested by contributions of static and dynamic COF, using a passive pendulum system (23) in mouse knees from 10-wk-old $\text{Prg4}^{-/-}$, $\text{Prg4}^{+/-}$, and $\text{Prg4}^{+/+}$ mice. The mean $\pm$ SD COF in $\text{Prg4}^{-/-}$ knees was 0.072 ± 0.02 (n=12 limbs), which was more than double that of $\text{Prg4}^{+/+}$ (0.028 ± 0.004 , n= 6 limbs, $p = 0.0006$) and $\text{Prg4}^{+/-}$ (0.031 ± 0.005 , n=6 limbs, $p = 0.0066$) knees (**Figure 2.1B**).

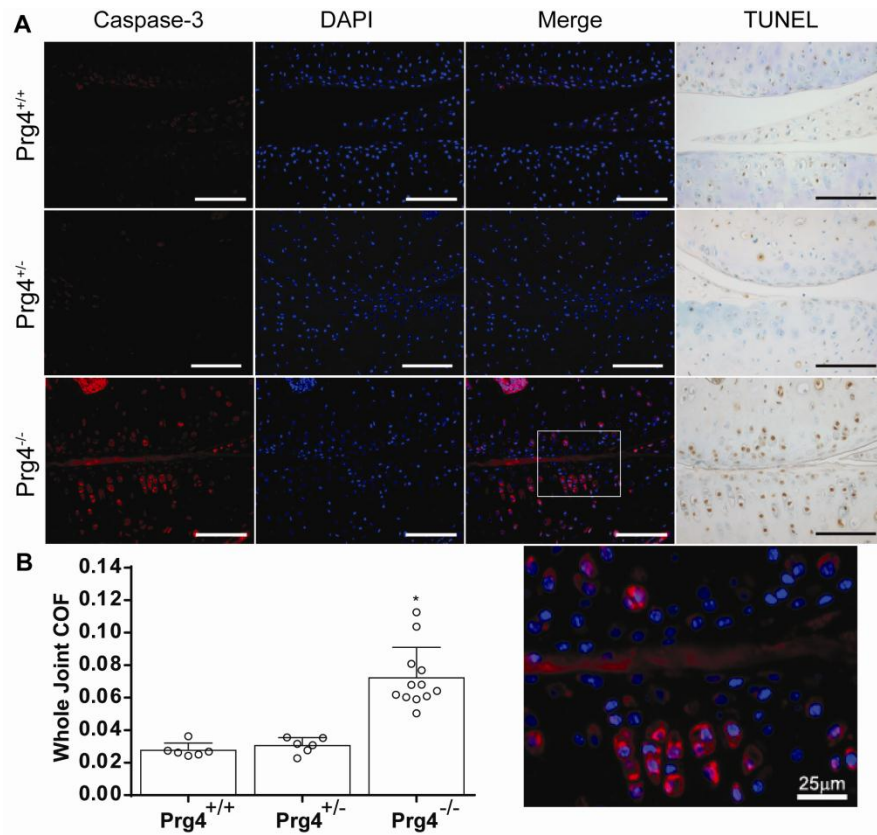


Figure 2.1: A.) Chondrocyte apoptosis in mice with different *Prg4* genotypes. Activated caspase-3 immunodetection in coronal sections of knee joints from 10-wk-old *Prg4*^{+/+}, *Prg4*^{+/-}, and *Prg4*^{-/-} mice that were not tested using the passive pendulum system. (First column) immunofluorescence detection (red) of active caspase-3. (Second column) same sections fluorescently imaged to reveal DAPI stained (blue) chondrocyte nuclei. (Third column) merged active caspase-3 and DAPI images. Note the red fluorescence at the cartilage surface represents non-specific antibody binding to proteinaceous deposits that normally accumulate on the cartilage surface in *Prg4*^{-/-} mice. Whereas, the punctate red fluorescence observed below the cartilage surface overlaps with DAPI stained nuclei indicating chondrocytes undergoing apoptosis. In contrast to *Prg4*^{-/-} knees, the knees in *Prg4*^{+/+} and *Prg4*^{+/-} mice had few chondrocytes with activated caspase-3. (fourth column) TUNEL staining and methyl green counterstaining of cartilage. More apoptotic (brown) chondrocyte nuclei are present in *Prg4*^{-/-} cartilage compared to *Prg4*^{+/+} and *Prg4*^{+/-} cartilage. Scale bars represent 100 μm. (Bottom row) region of interest taken from the image above to show the cells located just below the flattened superficial chondrocytes that are most susceptible to apoptosis. B.) Whole Joint COF in mice with different *Prg4* genotypes. Bar graphs depicting mean + 1 SD COF measurements in knee joints from 10-wk-old wild-type (*Prg4*^{+/+}), heterozygous lubricin knockout (*Prg4*^{+/-}), and homozygous lubricin knockout (*Prg4*^{-/-}) mice. Each individual COF measurement is indicated by an open circle. *Prg4*^{-/-} knees have significantly higher COF than either *Prg4*^{+/+} or *Prg4*^{+/-} knees. * *p* < 0.01 compared to *Prg4*^{+/+} or *Prg4*^{+/-} knees.

We previously described a full-thickness bovine cartilage disc-on-disc bearing system for measuring static and kinetic COF in cartilage explants containing living chondrocytes (29). We lubricated this bearing system with 5 different test solutions: phosphate buffered saline (PBS), wild-type human synovial fluid (HSF), purified human synoviocyte lubricin in distilled water (HSL), human synovial fluid from patients with CACP (CACP-SF), and synovial fluid from patients with CACP to which purified human lubricin has been added (CACP-SF+HSL). CACP-SF is void of lubricin, but otherwise contains normal synovial fluid components (32). The effects of these different test solutions on static and kinetic COF are depicted in **Figure 2.2**.

The highest static COF measurements were observed in bearings tested with PBS as a lubricant. Lubricating bearings with CACP-SF did not reduce static COF compared to PBS, whereas significant reductions in COF were observed when bearings were lubricated with HSF, HSL, or CACP-SF+HSL. Comparing static COF between the lubricants HSL and CACP-SF+HSL, enables us to investigate whether lubricin synergizes with other synovial fluid components, such as hyaluronic acid, to lower friction. We found no difference in static COF between these two lubricants, indicating that lubricin does not require other synovial fluid components to reduce static COF (12, 30) (**Figure 2.2**).

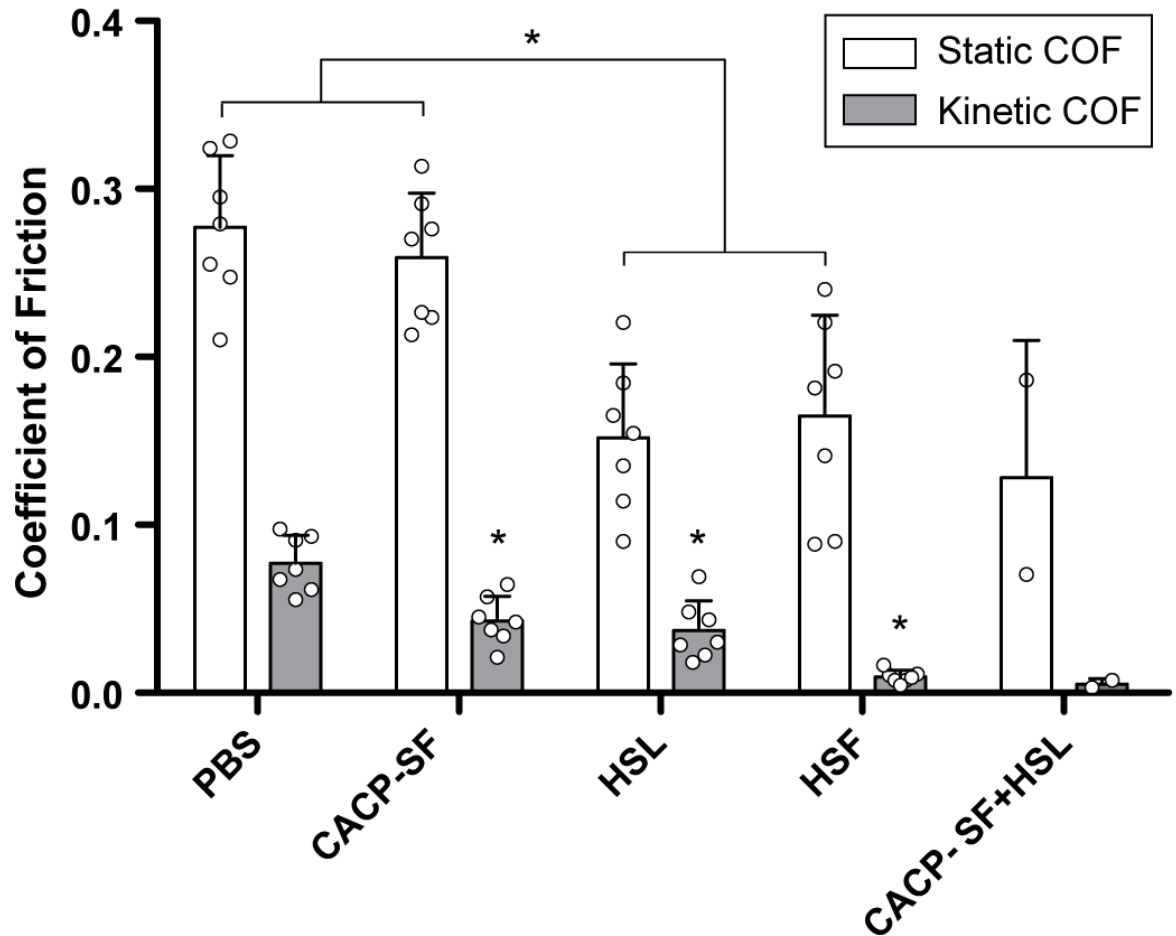


Figure 3.2: COF in bovine cartilage bearings lubricated with different test solutions. Bar graphs depicting mean + 1 SD static (unshaded) and kinetic (shaded) COF measurements in bovine cartilage bearings lubricated with either phosphate buffered saline (PBS), synovial fluid from patients with CACP (CACP-SF), purified human synoviocyte lubricin (HSL), human synovial fluid from normal joints (HSF) and CACP-SF that has been supplemented with HSL (CACP-SF +HSL). Static COF was significantly higher in PBS and CACP-SF lubricated bearings compared to HSL and HSF lubricated bearings. Kinetic COF was significantly higher in bearings lubricated with PBS compared to all other groups.

Different results were obtained when kinetic COF was measured. The highest kinetic COF was obtained with PBS. All other test solutions (i.e., HSF, HSL, CACP-SF, and CACP-SF+HSL) were able to significantly reduce kinetic COF compared to PBS (**Figure 2.2**). However, the greatest reductions in kinetic COF were achieved with HSF and CACP-SF+HSF (**Figure 2.2**), which is consistent with lubricin and other synovial fluid components acting synergistically to lower kinetic COF.

After the cartilage bearings were lubricated with test solution and subjected to a friction and wear regimen, we processed the bearings to look for apoptotic cells by staining for active caspase-3 (**Figure 2.3**). Many more chondrocytes in the bearings lubricated with PBS and CACP-SF contained active caspase-3 compared to chondrocytes in the bearings lubricated with HSF, HSL and CACP-SF+HSL, or compared to bearings that were not subjected to load (i.e., unloaded controls). In bearings that were not lubricated with lubricin, cells located just below the flattened cells at the surface of cartilage appeared to be at highest risk for apoptosis (**Figure 2.3**).

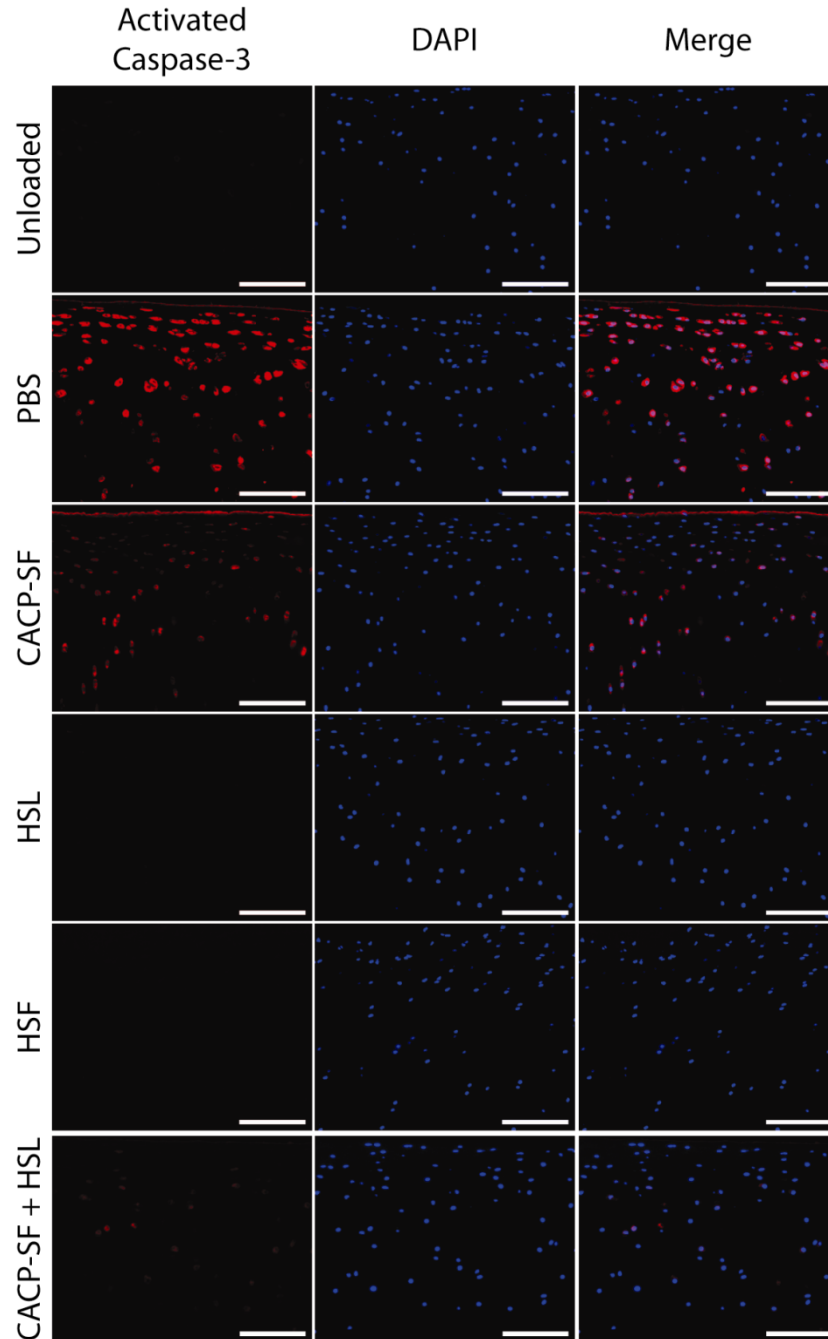


Figure 2.3: Apoptosis in bovine cartilage bearings lubricated with different test solutions. The lower explant cartilage bearings were stained for active caspase-3 (red, left column) and nuclei were counterstained with DAPI (blue, middle column) following friction and wear testing. Images were merged to show colocalization of caspase-3 within cells (right column). We observed many apoptotic cells in the superficial and upper middle zones of PBS and CACP-SF lubricated samples, while few apoptotic cells were observed in the unloaded bearings and in bearings lubricated with HSL, HSF or CACP-SF + HSL. Scale bars represent 100 μm .

We quantified the percentage of apoptotic chondrocytes in three 100 μm deep layers extending radially from the cartilage surface (**Figure 2.4A**). The top layer contains superficial zone chondrocytes and some intermediate zone chondrocytes, whereas the next two layers contain intermediate zone chondrocytes (29). Bearings tested with PBS and CACP-SF had higher mean percentages of apoptotic chondrocytes in the top and middle layers, compared to HSF and HSL lubricated bearings and to unloaded bearings (**Figure 2.4B**). In addition, the more superficial layer of bearings tested with PBS and CACP-SF contained a greater percentage of apoptotic chondrocytes compared to the next layer. For example, in CACP-SF lubricated bearings, the percentage of apoptotic cells in the top layer (Zone A) was greater than that of the middle layer ($p < 0.001$) and the middle layer (Zone B) was greater than that of the lower layer (Zone C) ($p = 0.015$).

Finally, when we graphed static COF and percentage of apoptotic chondrocytes in superficial zone (top layer) independent of the supplemented lubricant, we found a significant correlation between friction and cell death ($r^2=0.41$, $p=0.0007$) (**Figure 2.4C**).

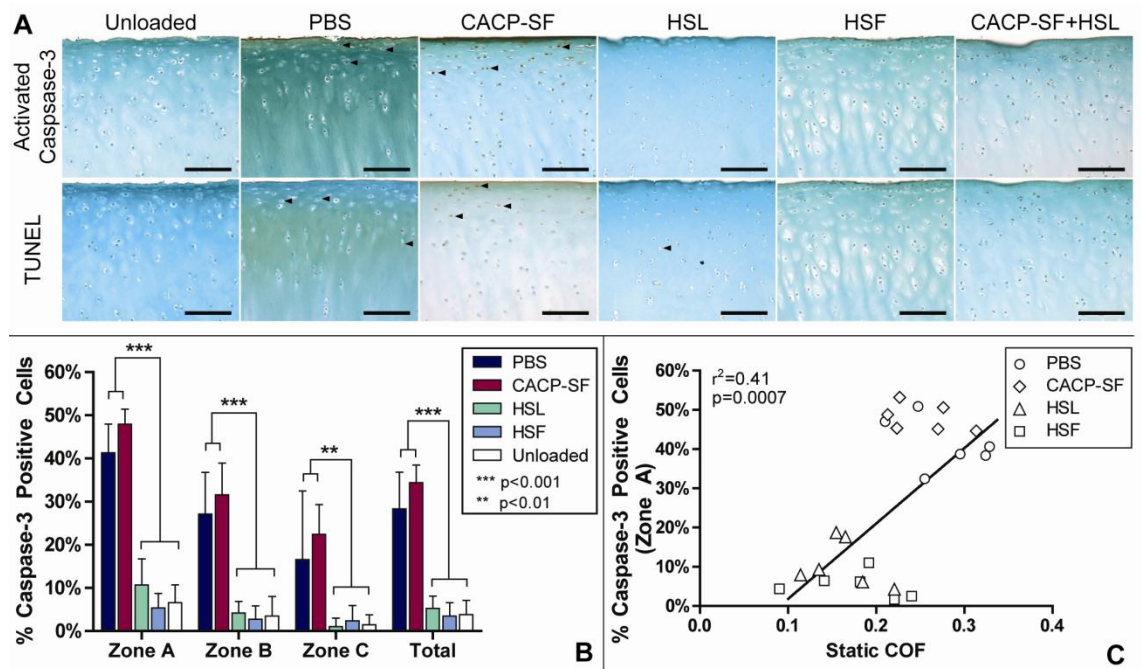


Figure 2.4: A.) Apoptosis in lower bovine cartilage bearings analyzed by zone. Cartilage explant bearings tested with PBS and CACP-SF had a greater number of cells positive for activated caspase-3 (brown, arrowheads) than bearings lubricated with HSL, HSF or CACP-SF+HSL and unloaded bearings. Cells negative for activated caspase-3 are stained blue. TUNEL staining (brown) confirmed apoptosis in bearings lubricated with PBS and CACP-SF (arrowheads), but few cells were TUNEL positive in the bearings lubricated with HSL, HSF or CACP-SF+HSL or in unloaded bearings. Cells negative for TUNEL are stained blue. Scale bars represent 100 μ m. B.) Percentage of activated caspase-3 in lower bovine cartilage bearings. Bearings tested with PBS and CACP-SF had significantly higher percentages of apoptotic cells in the superficial and upper middle zones compared to unloaded bearings and bearings lubricated with HSL and HSF. Error bars indicate SD. C.) Correlation of static COF and activated caspase-3 in zone A. A significant correlation ($r^2=0.41$) between static COF and activated caspase-3 positive cells in Zone A (articular surface-100 μ m) was observed for the mechanically tested bearings, across the different lubricants.

2.5 Discussion

Herein we show that increasing static friction causes the death of superficial and upper intermediate zone chondrocytes. Synovial fluid from CACP donors in a cartilage on cartilage bearing system failed to reduce static friction and increased chondrocyte apoptosis when compared to normal human synovial fluid or purified human lubricin. In addition, $\text{Prg4}^{-/-}$ mouse knees had more apoptotic cells and higher whole joint COF compared to $\text{Prg4}^{+/+}$ and $\text{Prg4}^{+/-}$ knees. These results support the hypothesis that increased friction in an articular joint causes chondrocyte apoptosis, which results in progressive loss of extracellular matrix and ultimately joint failure. Consistent with earlier studies (13, 20, 23, 29, 33-36), our study confirms the importance of lubricin in providing boundary lubrication to preserve structural and cellular integrity at the cartilage surface.

Boundary lubrication decreases friction between surface asperities that are pressurized together during periods of high load and low speed (37). This paradigm is particularly relevant in large weight-bearing joints, when the fluid layer normally found on the surface of cartilage is measurably thinner than surface asperities (16, 37, 38). In this study, the axial load is held constant across samples, testing the ability of each test solution to provide boundary lubrication, prevent shear stress, resultant strain and consequential chondrocyte apoptosis. Lack of boundary lubrication causes stick-slip to occur, which results in sub-surface strain (39) and causes surface asperities to deform destructively (32, 40). In contrast to the inability of CACP-SF to reduce static COF, CACP-SF had the same ability to lower kinetic COF as purified human lubricin, yet this reduction in kinetic COF did not prevent apoptosis. It should be noted that kinetic COF values may represent contributions of both mixed mode and boundary modes of

lubrication. Therefore, the chondroprotective properties of lubricin are linked to its ability to lower COF at the *initiation* of motion. Further support for this mechanism derives from the observation that adding purified human synoviocyte lubricin to CACP-SF restored the fluid's ability to lower static COF and to prevent apoptosis.

Pendulum analysis allows the observation of contributions of adherent sliding surfaces that naturally articulate in a roller-slider movement. Elevated whole joint COF in $\text{Prg4}^{-/-}$ mouse joints is directly related to the absence of lubricin in synovial fluid and may also be influenced by cartilage surface damage and protein deposition that characterize the $\text{Prg4}^{-/-}$ phenotype. Interestingly, in our mouse limbs and in the bovine cartilage bearing model, when lubricin was missing, the occurrence of chondrocyte apoptosis was greatest just below the cartilage surface (**Figures 2.1A and 2.3**). Previous work looking at shear deformation and shear strain in cartilage explants from human (39, 41) and neonatal bovine (41, 42) sources found shear deformation was highest in the uppermost layers of cartilage, just below the superficial zone, and did not propagate linearly. Furthermore, under 10% compressive axial strain, cartilage explants experienced the largest deformations in the transitional region between the superficial and intermediate zone (41). Our finding of increased chondrocyte apoptosis at these locations when lubricin is missing is consistent with the predicted sites of maximum tissue deformation, especially around cells just below the superficial zone (42). Previous studies have reported elevated shear strain and cartilage deformation in human cartilage explants when lubricated with PBS as opposed to normal bovine synovial fluid (39). Furthermore, shear strain is exaggerated in degenerative cartilage samples (39). Also of interest are reports

that administering pan-caspase inhibitors to experimental OA models resulted in fewer cartilage lesions by preventing cell death (8, 43).

Lubricin is normally produced in synovial joints by synovial fibroblasts and superficial zone chondrocytes. Shear stress and compression increase lubricin expression in bovine cartilage *when lubricated* (44, 45), so the viability of lubricin-producing cells in the superficial zone and the availability of lubricin in synovial fluid to deposit on the cartilage surface help maintain boundary lubrication (46). The importance of rapid and continuous replenishment of lubricin during joint loading is supported by our studies which found a significant increase in COF after 30 h of *ex vivo* joint oscillation under load in Prg4^{+/-} compared to Prg4^{+/+} mouse limbs (23). Prg4^{+/-} limbs do not exhibit increased COF compared to Prg4^{+/+} limbs in the absence of continuous oscillation.

In a previously normal joint, changes in lubricin abundance following injury may affect the risk for developing cartilage damage. Common traumatic knee injuries, such as anterior cruciate ligament tears, are strong risk factors for the development of OA. Lubricin levels in synovial fluid decrease after anterior cruciate ligament injury and can take up to a year to return to baseline values (26). Several studies have demonstrated a disease-modifying effect for the intra-articular injection of native or recombinantly expressed lubricin in rodent models of post-traumatic OA (27, 28, 35, 36). Our data indicate that lubricin supplementation likely protects damaged joints by preserving boundary lubrication, thereby protecting articular chondrocytes from apoptosis caused by excessive deformation and shear stress.

Due to the ability of lubricin to re-establish boundary lubrication, provide chondroprotection and restore a low COF in the boundary mode, this study suggests that

lubricin could be used as a therapy in patients with transient deficiency of lubricin, as can occur following traumatic joint injury or inflammatory joint disease. Additional work introducing lubricin in lubricin-null mice through intra-articular injection may provide the basis for a future clinical study in patients with CACP, or in patients with acquired arthropathy.

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Chapter 3

Preventing Friction Induced Chondrocyte Apoptosis: A Comparison of Human Synovial Fluid and Hylan G-F 20

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3.1 Abstract

Symptomatic osteoarthritis (OA) is a common painful disease with limited treatment options. A rising number of OA patients have been treated with intraarticular injections of hyaluronic acid, including the high molecular weight hylan G-F 20, which is injected following arthrocentesis. This study investigated the effectiveness of hylan G-F 20 to lower coefficient of friction (COF) and prevent chondrocyte apoptosis *in vitro*. An disc-on-disc bovine cartilage bearing was used to measure the static and kinetic COF when lubricated with hylan G-F 20, human synovial fluid (HSF) and phosphate buffered saline (PBS). Following friction testing, we stained paraffin embedded sections of these cartilage bearings for activated caspase-3, a marker of apoptosis. Bearings lubricated with hylan G-F 20 had kinetic COF values that were similar to bearings lubricated with PBS, but significantly higher than those lubricated with HSF. There were no significant differences in static COF values in bearings lubricated with hylan G-F 20 as compared to PBS or HSF. However, bearings lubricated with HSF had a significantly lower static COF values compared to bearings lubricated with PBS. The mean percentage of caspase-3 positive chondrocytes in the superficial and upper intermediate zones of bearings lubricated with hylan G-F 20 were significantly higher when compared to bearings lubricated with HSF or unloaded controls, but significantly lower than those lubricated with PBS. These findings indicate that joint lubrication may prevent chondrocyte apoptosis by lowering the COF. Furthermore, removal of synovial fluid prior to hylan G-F 20 injection may be detrimental to cartilage health.

3.2 Introduction

Osteoarthritis (OA) is a painful, debilitating disease in articular joints with large societal implications (1, 2). Currently, treatment options for patients with OA are severely limited, and no disease-modifying treatments, apart from total joint replacement, are available. Most patients are treated with nonsteroidal anti-inflammatory drugs or corticosteroid injections to relieve pain. Viscosupplementation, in which various forms of hyaluronic acid, including hylan G-F 20 (Synvisc, Genzyme Corporation), are administered via intraarticular injection, is becoming a popular alternative.

Synovial fluid is a blood plasma dialysate that contains lubricating components, including hyaluronic acid and lubricin, important to joint lubrication and hence chondroprotection to articular joints. Hyaluronic acid is thought to play a number of roles in synovial fluid, including shock absorption and lubrication, and is responsible for the viscoelastic behavior of synovial fluid (3). Joint lubrication occurs in both hydrodynamic and boundary modes (4). Hyaluronic acid is vital to hydrodynamic joint lubrication, which occurs when the fluid layer is wider than the surface asperities and is dominated by fluid mechanics, including viscosity (4). During periods of high load and low velocity, lubricin serves as the primary boundary lubricant in joints, particularly when cartilage is pressurized during gait. However, hyaluronic acid has also been shown to contribute to lubrication in the boundary mode, particularly in concert with lubricin (5-9).

Healthy articular cartilage is smooth and void of fissures or attachments (10). In addition to surface fibrillation and ultimately full thickness cartilage loss, osteoarthritic cartilage experiences a loss of chondrocytes via apoptosis (10-12). It is important to note

that chondrocytes maintain various metabolic functions in articular cartilage, including the maintenance of the extracellular matrix (13, 14), and that OA pathogenesis is mediated in part by apoptotic mechanisms (15-17). Cartilage wear leading to OA and precocious joint failure has been reported in the absence of adequate joint lubrication *in vivo* (18-20), but the biological underpinnings of wear in response to mechanical mechanisms have not been established. Since articular cartilage has little capacity for renewal, preventing apoptosis via supplemental lubrication may be key to counteract the onset of OA and vital to cartilage preservation (20, 21).

This study investigated the effectiveness of using hylan G-F 20 for lubricating cartilage bearings in the boundary mode to prevent chondrocyte apoptosis using a bovine *in vitro* disc-on-disc cartilage bearing (9, 22). We hypothesized that the coefficient of friction using hylan G-F 20 would be less than that of phosphate buffered saline (PBS) and equal to that of human synovial fluid (HSF). We also hypothesized that the percentage of cells stained for activated caspase-3, a marker of chondrocyte apoptosis, of the bearing lubricated with hylan G-F 20 will be less than that of PBS and equal to that of HSF.

3.3 Materials and Methods

3.3.1. Bovine Cartilage Preparation

To evaluate the ability of the lubricants to provide boundary lubrication and prevent chondrocyte apoptosis, we used a bovine cartilage disc-on-disc bearing system (9). Full thickness cartilage plug bearings 6 mm and 12 mm in diameter were cored from the approximate load bearing regions of femoral condyle of bovine stifle joints (n=5) collected within 2 h of slaughter. Following harvest, the bearings were rinsed 3 times with cell culture media (DMEM/5% FBS) and cultured for 24 h at 37°C. Testing was performed on the cultured plugs at room temperature.

3.3.2. Test Lubricants

Hylan G-F 20 (Synvisc, Genzyme Corp, Cambridge, MA) was kept at room temperature away from light until testing. During testing, it was directly applied to cartilage bearing surfaces from the product packaging using a 22 gauge needle. HSF was aspirated from knee joints of post-mortem donors with no history of joint disease within 12 h of death (3 male donors, Ages 25-39, NDRI) or aspirated from patients undergoing total joint replacement were collected in the operating room and pooled together (n=12). All HSF was frozen and stored at -80°C until analysis and testing. Synovial fluids visually contaminated with blood were not used. PBS served as a negative control. All lubricants were tested at room temperature.

3.3.3. Enzyme-linked Immunoabsorbant Assay (ELISA) of HSF

An ELISA using anti-lubricin monoclonal antibody 9G3 was designed and validated. High binding 96-well plates were coated overnight with purified human lubricin in 0.1 M NaH_2PO_4 , Na_2HPO_4 buffer, pH 6.5, at a final concentration of 10 $\mu\text{g}/\text{ml}$. The plates were washed and blocked with 5% milk in phosphate buffered saline and Tween 20 (PBST) for 2 h at room temperature (RT). The plate was subsequently washed with PBST. HSF test samples were added to the plate at a 1:50 dilution, then 9G3 was subsequently added at a 1:5000 dilution, and the plate was incubated for 1 h at RT. After a wash with PBST, goat anti-mouse IgG was added to the plate at 1:2000 dilution and incubated for 1 h at RT. The plate was then washed, and TMB single solution (Invitrogen) was added. 1M HCL was added 30 min later to stop the reaction which was read at 450nm.

3.3.4. Friction and Wear Testing in Bovine Bearings

Prior to testing, the average total cartilage thickness for each bearing pair was calculated (2.84 ± 0.38 mm) from caliper measurements at four regions locations about the circumference of both cartilage bearings. Small bearing diameters (5.45 ± 0.28 mm) were also measured using calipers.

Cartilage bearings were loaded in a disc-on-disc configuration using a material testing system (EnduraTEC 3200; Bose Corporation, Eden Prairie, MN, USA), which was programmed to apply an axial strain while axial rotations were prescribed to the bearing (**Figure 3.1**). This testing paradigm was adapted from Schmidt et al to accommodate cell culture following friction and wear testing (22). The maximum ranges

of the load, torque and displacement transducers of the test system were ± 22 N, ± 0.7 Nmm, and ± 6.5 mm, respectively. The discs were fixed to the testing platform with cyanoacrylate glue, which was applied to the bony surface of the bearing plugs and allowed to dry completely before testing. During this time, cell culture media was added between the joint surfaces to prevent cartilage desiccation. Prior to testing, cell culture media was then rinsed off of the cartilage bearing surfaces three times with PBS. Test lubricant, either PBS, hylan G-F 20 or HSF, was applied between the bearing surfaces (n=8 for all groups). The bearings were axially loaded to 18% of the mean total cartilage thickness, and held at that displacement for 8 min to allow fluid depressurization (9, 22, 23). The disc was then rotated in torsion +2 revolutions and reset -2 revolutions at an effective velocity of 0.3 mm/s (9) for 12 continuous cycles. Unloaded control discs were kept in cell culture media during testing. All tests were performed between 48 and 72 h of harvest, individual bearings were only tested with a single lubricant, and bearings taken from each knee were tested using each test lubricant.

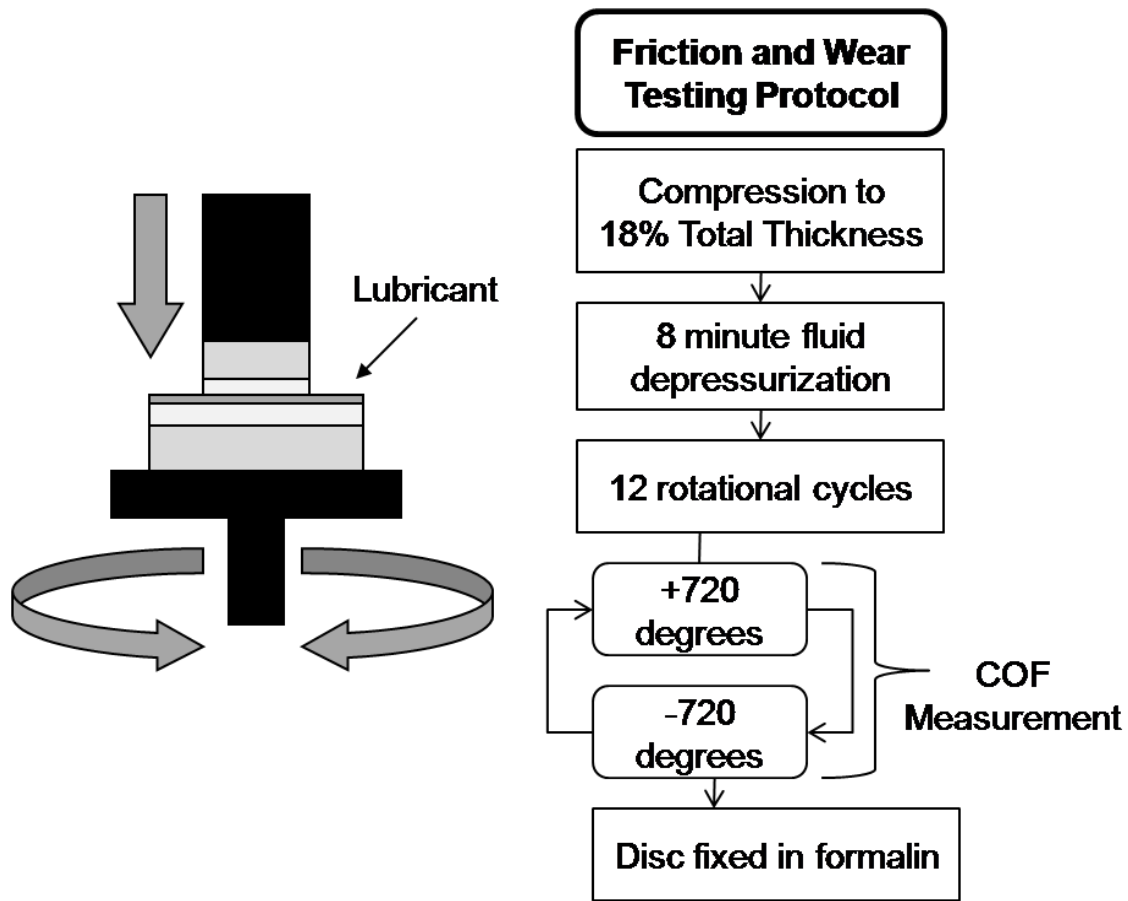


Figure 3.1: Schematic diagram of friction and wear testing system and the experimental protocol. Test lubricant was applied between the bearing surfaces, and the bearings were axially loaded to 19% of the mean total cartilage thickness, and held at that displacement for 8 min to allow for fluid depressurization. The large disc was then rotated in torsion +2 revolutions and reset -2 revolutions at an effective velocity of 0.3 mm/s for 12 continuous cycles. Axial load and torque were measured throughout the testing protocol.

3.3.5. Coefficient of Friction Determination

The static COF (a measure of the stick-slip condition) and the kinetic COF (a measure of the equilibrium COF) were calculated using Equation 1 (22).

$$\text{COF} = \text{Torque} / (1/3 \times \text{Small Disc Diameter} \times \text{Axial Force}) \quad (1)$$

To calculate the static COF, the absolute maximum torque that occurred within the first 10 degrees of rotation and the equilibrium axial force following the 8 min depressurization period were substituted into Equation 1. To calculate the kinetic COF, the average torque observed during the last 720 degrees of rotation and the equilibrium axial force were used.

3.3.6. Activated Caspase-3 Staining and Quantification

To test the efficacy of each lubricant to provided chondroprotection during frictional testing, we stained paraffin-embedded sections of each cartilage bearing disc with an antibody specific for activated caspase-3, which stains chondrocytes primed for apoptosis. Immediately following testing, cartilage discs were fixed in 10% buffered formalin. The unloaded control discs were also fixed in formalin at the time of testing. The discs were paraffin embedded and cut vertically at the center of the disc into thin sections of full cartilage thickness (250 μm). Sections were heated to 60°C for 30 min, deparaffinized and hydrated in xylene and alcohol. Rabbit polyclonal antibody against active caspase-3 (cat#ab13847, Abcam, Cambridge, MA) at 1:50 dilution was added to slides at 40°C overnight according to VectaStain procedures. Following the addition of biotinylated secondary antibody solution and 3,3'-diaminobenzidine (DAB), slides were counterstained with 0.5% Methyl green and cover slip slides fixed with Permount

mounting media (Fisher Scientific, Waltham, MA). Apoptosis quantification was performed at 20X magnification for cells in the superficial and intermediate zones along the articular surface where loading occurred at 3 areas of interest. Images were captured at 20X with Image-Pro Plus software (Media Cyberkinetics, Bethesda, MD), and the percentage of apoptotic cells was determined by counting the number of cells positive activated caspase-3 and the total number of cells for 3 distinct 100µm zones representing areas of the superficial and upper intermediate zones: Zone A (articular surface-100µm deep), Zone B (100-200µm deep), Zone C (200-300µm deep). Total percentage of apoptotic cells refers to the mean across all 3 zones.

3.3.7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining

To confirm apoptosis in the bovine cartilage discs, we performed a TUNEL assay on bovine cartilage disc sections using an ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, Billerica, MA). Sections were heated at 60°C for 30 min, deparaffinized in 3 changes of xylene and serial ethanol, then pretreated with proteinase K (20µg/ml) for 15 min at room temperature, quenched endogenous peroxidase in 3% hydrogen peroxide for 5 min, and incubated with equilibration buffer for 30 seconds. Excess liquid was tapped off and the sections were with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h in a humidified chamber. After the reaction was stopped, sections were washed 3 times in PBS, and incubated with anti-digoxigenin conjugate for 30 min at room temperature and washed in PBS. Peroxidase substrate was applied to sections, which were stained for 4min, washed in deionized H₂O, and counterstained with

0.5% methyl green. Sections were washed in deionized dH₂O again, dehydrated in xylene 3 times, and then mounted with Permount. Images were captured at 20X with Image-Pro Plus software.

3.3.8. Statistical Analysis

Comparisons were made between the COF values for the different test lubricants (PBS, hylan G-F 20, HSF) and unloaded control specimens using Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's multiple comparison *post hoc* tests using the Unistat Statistical Package (Unistat Ltd, London, England). The tests were run separately for the static and kinetic COF values. The percentage of apoptotic cells measured in Zone A, Zone B and Zone C of histological sections were compared using a repeated measures two-way analysis of variance with Holm-Sidak multiple comparison *post hoc* tests using Sigmaplot software (Systat Software Inc, Chicago, IL). Pearson correlation coefficients between the static COF and the percentage of apoptotic cells in Zone A and correlations between kinetic COF and the percentage of apoptotic cells in Zone A were performed, and a linear regression was fitted to each plot, using Sigmaplot software. Goodness of fit and significance of the correlation are reported. For all analyses, statistical significance was set at $\alpha = 0.05$ *a priori* and the two-tailed p-value is reported. All values are presented as the mean $\pm$ standard deviation.

3.4 Results

The mean static COF was significantly lower for HSF lubricated bearings compared to PBS lubricated bearings ($p=0.006$) (**Figure 3.2**). Hylan G-F 20 lubricated bearings had a mean static COF that was not significantly lower than PBS ($p=0.18$) or significantly higher than HSF ($p=0.67$). However, the mean kinetic COF values were significantly lower for the bearings lubricated with HSF when compared to those lubricated with hylan G-F 20 ($p=0.022$) or PBS ($p=0.003$). There was no difference between hylan G-F 20 and PBS. There was no difference between COF from sources of HSF (static COF $p=0.070$ and kinetic COF $p=0.086$), and lubricin concentrations were similar (288 ± 77.7 $\mu\text{g/ml}$ for post-mortem HSF and 416 $\mu\text{g/ml}$ for pooled total joint replacement HSF). The equilibrium axial load was 2.11 ± 0.92 N, which corresponds to 0.09 ± 0.03 MPa, and the maximum load applied to the bearings was 13.51 ± 5.3 N, which corresponds to 0.58 ± 0.23 MPa.

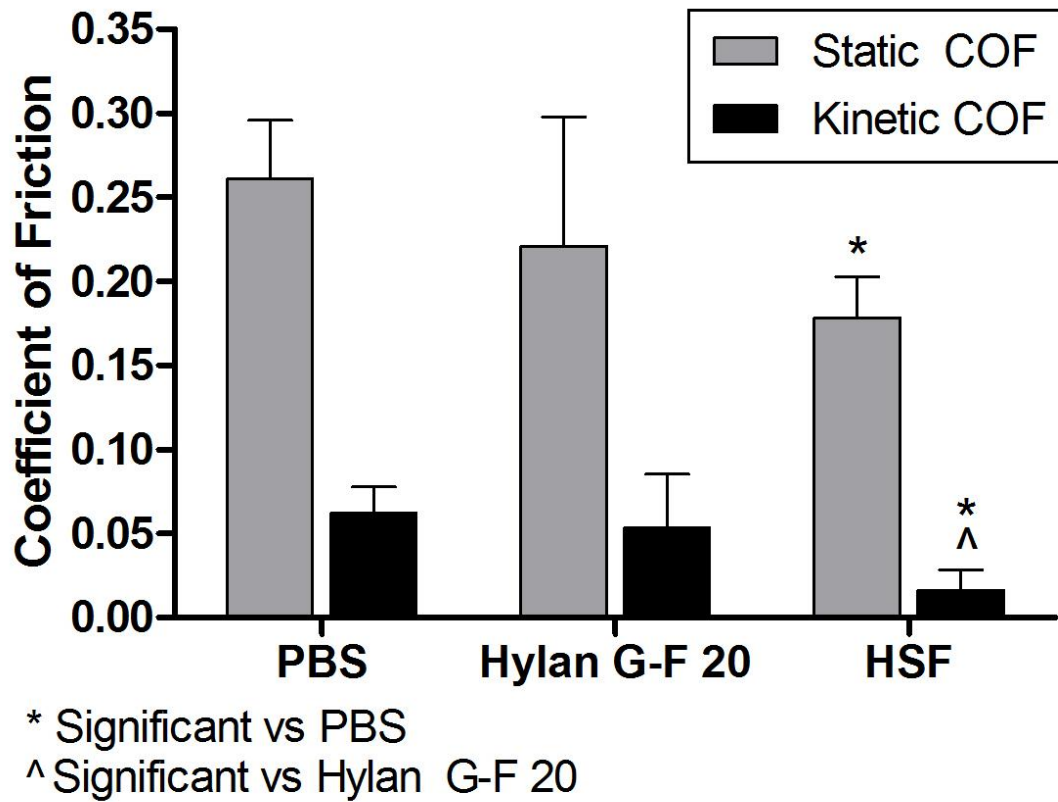


Figure 3.2: COF of bovine osteochondral bearings. Bearings were tested with test lubricants, either PBS, hylan G-F 20 or HSF following the protocol outlined in Figure 1. Lubrication with HSF had the lowest mean static and kinetic COF values. Bearings lubricated with hylan G-F 20 had no significant difference compared to either group in static COF, but had a significantly higher kinetic COF compared to HSF. * $p < 0.05$

A significant interaction ($p < 0.001$) was observed between the lubricant treatment group and zone. Hylan G-F 20 lubricated bearings had significantly higher mean percentage of apoptotic cells in Zone A, Zone B, and when pooled over all 3 zones as compared to HSF lubricated bearings (Zone A $p < 0.001$, Zone B $p = 0.006$, total $p < 0.001$) and unloaded controls (Zone A $p < 0.001$, Zone B $p = 0.004$, total $p < 0.001$), but there was no difference between the groups in Zone C (**Figure 3.3**). A significant increase in the mean percentage of cells staining positive for activated caspase-3 was observed in bearings lubricated with PBS compared to bearings lubricated with HSF (Zone A $p < 0.001$, Zone B $p < 0.001$, Zone C $p = 0.003$, total $p < 0.001$), hylan G-F 20 (Zone A $p < 0.001$, Zone B $p = 0.005$, total $p = 0.001$) and unloaded bearings (Zone A $p < 0.001$, Zone B $p < 0.001$, Zone C $p = 0.005$, total $p < 0.001$). We also observed significant differences between the zones in bearings lubricated with PBS and Hylan G-F 20. For these bearings, Zone A had a significantly higher mean percentage of apoptotic cells in Zone A compared to Zone B (PBS $p < 0.001$, hylan G-F 20 $p = 0.004$), and in Zone B compared to Zone C (PBS $p < 0.001$, hylan G-F 20 $p = 0.009$). HSF also showed a significant difference between Zone A and Zone C ($p = 0.028$). Cells in representative osteochondral plugs staining positive for activated caspase-3 were also TUNEL positive (**Figure 3.4**).

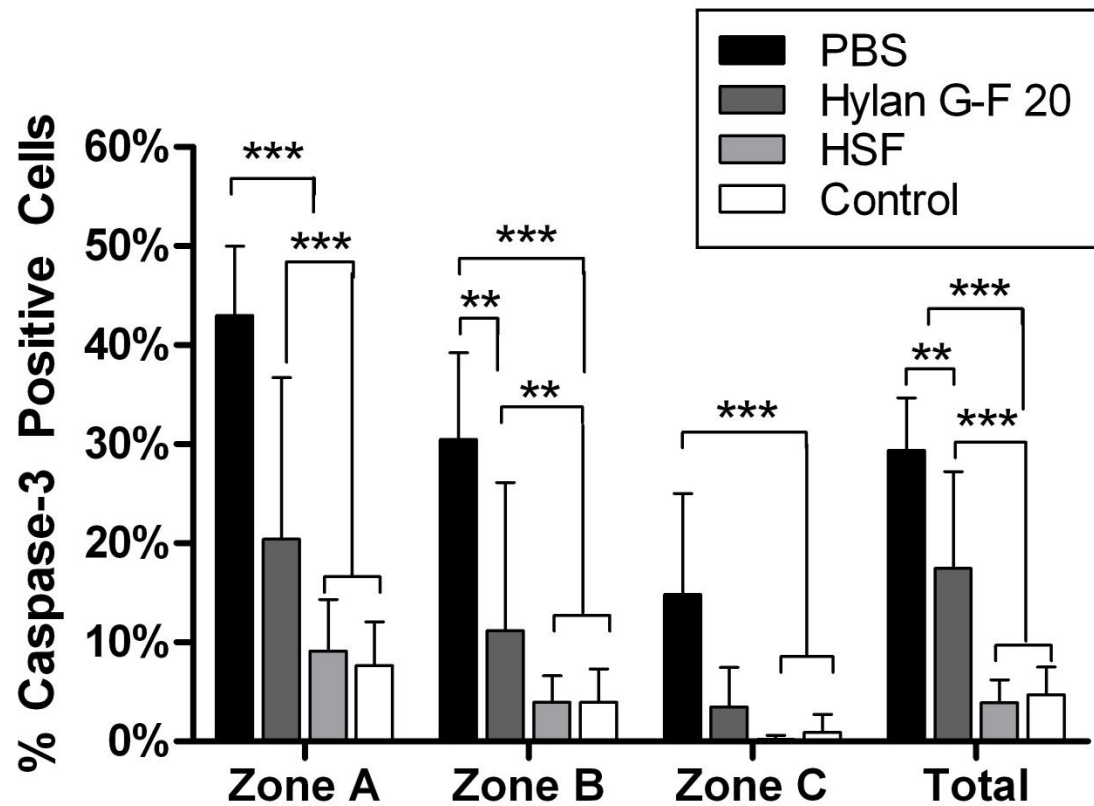


Figure 3.3: Activated caspase-3 in superficial and upper intermediate zone chondrocytes (right). Bearings lubricated with hylan G-F 20 had a significantly higher percentage of cells positive for activated caspase-3 cells in Zone A, Zone B and across all zones compared to unloaded control bearings or bearings lubricated with HSF, indicating that hylan GF-20 is less chondroprotective than HSF. Bearings lubricated with PBS had a significantly higher percentage of apoptotic cells in all zones compared to all other groups. **p<0.01, ***p<0.001

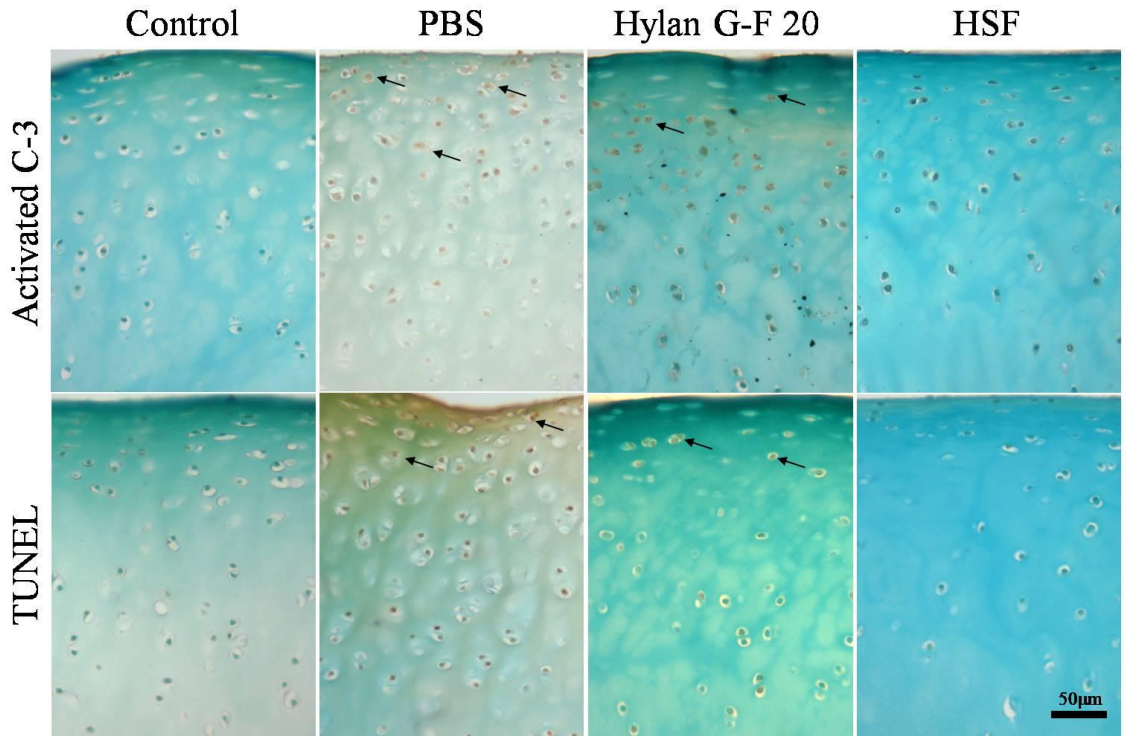


Figure 3.4: Bovine osteochondral bearings stained for activated caspase-3 (top) and TUNEL (bottom). Cells positive for activated caspase-3 and TUNEL are brown. Negative cells are stained blue. We observed more apoptotic cells in the PBS and hylan G-F 20 lubricated bearings compared to HSF lubricated bearings and unloaded controls.

We observed a positive correlation between static COF values and the percentage of apoptotic cells ($p=0.007$) and between kinetic COF values and the percentage of apoptotic cells ($p=0.015$). **Figure 3.5** shows HSF data points clustered near the origin, indicating that low COF values correspond with low percentage of apoptotic cells. In contrast, the PBS data points clustered further up the regression line, indicating that high COF values correspond with high percentage of apoptotic cells. The data points for the bearings lubricated with hylan G-F 20 fell between the high and low clusters, indicating that hylan G-F 20 possesses better chondroprotective and lubricating ability as compared to PBS.

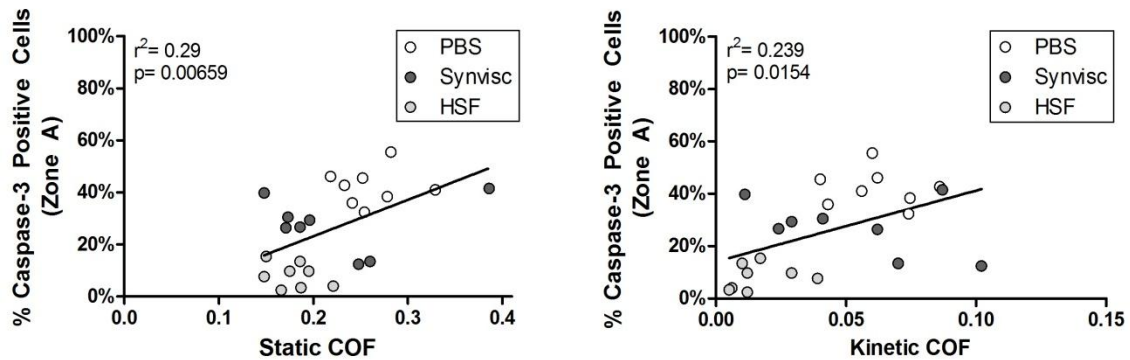


Figure 3.5: Correlation between static COF (left) and kinetic COF (right) and percentage of activated caspase-3 positive cells in Zone A of the superficial zone. We observed a significant correlation between coefficient of friction and percentage of apoptotic cells in Zone A of the cartilage bearings. We observed trends for each lubricant, with the HSF lubricated bearings showing clustering towards the origin, indicating low COF and low percentage of apoptotic cells. In contrast, PBS lubricated bearings clustered in the upper right hand section of the plot, indicating high COF and high percentage of apoptotic cells. Hylan G-F 20 lubricated bearings fell between the two extremes, showing that it has some lubricating and chondroprotective abilities compared to PBS. Note that the x-axis differs between graphs for better visualization of the data points.

3.5 Discussion

We observed a significantly higher mean percentage of caspase-3 positive and a significantly higher mean kinetic COF in bearings lubricated with hylan G-F 20 compared to those lubricated with HSF. While these findings suggest that hylan G-F 20 was able to prevent apoptosis with more efficiency than PBS, apoptosis was more prevalent compared to the HSF treated bearings. Furthermore hylan G-F 20 was unable to significantly lower static or kinetic COF values compared to PBS under these testing conditions. These results suggest that hylan G-F 20 itself may be insufficient as a boundary lubricant in joints, and that this viscosupplement does not provide the same degree of chondroprotection to superficial zone and upper middle zone chondrocytes as native human synovial fluid under these boundary lubrication conditions. The significant correlations between static and kinetic COF and the percentage of apoptotic cells provide further evidence that elevated friction in this bearing system results in an increase in the percentage of apoptotic cells in the superficial 100 μ m of the bovine cartilage explant bearing.

Apoptotic cell death in vivo is mediated by cell-matrix interactions (24, 25), growth factor and cytokine signaling (26), and tissue injury. Cartilage explants and cultured chondrocytes have been used to study apoptosis in response to hydrostatic pressure (27), shear stress and strain (28, 29), and mechanical injury (30, 31). In our study, the elevated friction leading to apoptosis is likely due to an increase in shear stress. Currently, the mechanopathway relating mechanical stress via friction and apoptosis in chondrocytes has not been established but is under investigation.

In this study we observed a zonal dependence on the mean percentage of

apoptotic cells, especially in bearings lubricated with PBS and hylan G-F 20, which exhibited higher percentages of apoptosis compared to bearings lubricated with HSF and unloaded control bearings. These findings indicate that the bulk of the apoptotic response occurs in the uppermost 300 μm of the cartilage where the shear forces and deformation are greatest (29, 32) and the significant correlation between COF and apoptosis indicates that an increase in friction is associated with apoptosis. The zonal differences also suggest that the collagen architecture in the superficial zone can absorb most of the shear stress and protect the deeper zones from deformation, and hence protecting the deeper chondrocytes from apoptosis in the early stages.

Hylan G-F 20 is administered to patients who do not respond well to non-pharmacological treatments, such as weight loss and physical therapy, or simple analgesics. Hylan G-F 20 is an FDA approved high molecular weight (average 6000 kDa) hyaluronan product with two cross-linked components that originate from chicken combs (33). Either 3 weekly doses of 2 mL or a single 6 mL dose is administered to patients diagnosed with OA (33, 34). Prior to injection, arthrocentesis is advised, which may remove important components of the synovial fluid, including lubricin, which is vital to boundary lubrication (19, 21, 35). Therefore, replacement of native synovial fluid with hylan G-F 20 alone could possibly have proximal detrimental effects on cell survival.

There are a number of limitations associated with this model of evaluating the efficacy of hylan G-F 20 to prevent friction and apoptosis. The viscosity of hylan G-F 20 is much higher than synovial fluid, causing interstitial fluid depressurization of cartilage to occur at a lower rate than when the bearings are lubricated with HSF or PBS. Hylan G-F 20 is an elastoviscous fluid with elasticity (storage modulus G') of 111 ± 13 Pa at 2.5 Hz

and a viscosity (loss modulus G'') of 25 ± 2 Pa (33). Normal synovial fluid exhibits a storage modulus (G') of approximately 19.3 ± 3 Pa and a loss modulus of 10 ± 1 Pa (36, 37). This thixotropic behavior may be preventing the cartilage bearings from achieving proper asperity contact with one another, by creating a thick fluid layer, and not allowing lubrication to occur truly in the boundary mode, and deflating static COF. After the disc rotates, the fluid is displaced by motion and kinetic COF is significantly higher for hylan G-F 20 lubricated bearings compared to HSF lubricated bearings.

Furthermore, the test protocol used in our study was adapted from the methods cited (9). However, the technique was modified to permit subsequent culture of cartilage explant bearings. The cited methods require cartilage explants to be held while mechanically stressed without culture medium for ~ 2.5 hr (9, 22). By shortening the testing procedure to approximately 20 min, we were able to collect data about boundary lubrication, since the entraining velocity and load are similar to these previous methods (9, 22), while preserving the cellular viability. While the duration of loading was shorter in our method, it still approximates zero-interstitial pore pressure at the beginning of oscillation. Previous studies have shown that after 8 min 85% of pore pressure was likely dissipated and the bearing surfaces were close to the equilibrium COF. It's also important to note that each measurement represents an independent pair of cartilage bearings, as each was tested only a single time in order to observe histological data linked to particular lubricants. The wear protocol following the decompression period was also extended to 12 cycles, as opposed to 2 (6, 22).

Numerous studies have tested the outcome of hylan G-F 20 injection compared to other intraarticular HA treatments, including NSAID and corticosteroid therapies (38).

The mode of action of hyaluronan injection in decreasing joint pain in OA affected joints remains unclear. Some studies have reported that loss of viscosity due to HA depletion may play a role in OA progression, although that finding has been challenged (39, 40). Adding a cross-linked HA, such as hylan G-F 20, to SF reinforces non-Newtonian behavior, which is characteristic of healthy synovial fluid (41). Alternate hypotheses, including biosynthetic-chondroprotective effects, anti-inflammatory effects, and analgesic effects due to protective action on nociceptive nerve endings, have been alternatively proposed (40).

In spite of studies showing safety and efficacy in treating OA pain as a clinical end point, the prevention of further cartilage damage following HA injection has not been established, although a delay of total knee replacement has been demonstrated in some patients with severe OA (42). The present study informs us that joint lubrication is a complex phenomenon and chondroprotection requires more than a low COF but is also related to the prevention of chondrocyte apoptosis. Our results suggest that the resident normal synovial fluid of a weight bearing joint should not be removed in the evaluation and treatment of the symptomatic large joint.

Many studies have indicated a possible synergistic effect of combining HA and lubricin in both the prevention of secondary OA in animal studies (43), as well as decreases in the COF in *in vitro* cartilage bearings (6, 9) and latex-on-glass bearings (8). HA of various lower molecular weights and concentrations have been shown to lower static and kinetic COF in uncultured bearings (6, 9), but the ability of these molecules to prevent chondrocyte apoptosis was not investigated. Based on their ability to lower COF in these studies, there may be value in combining HA and lubricin to develop a

therapeutic for patients with OA or other degenerative joint diseases (6, 9).

This study suggests that the use of hylan G-F 20 following arthrocentesis may not adequately protect cartilage from mechanical wear due to increased friction or biological wear that occurs due to chondrocyte apoptosis. This study also determined that an increase in the COF of articular cartilage is correlated with an increase in chondrocyte apoptosis.

3.6 Acknowledgements

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Chapter 4

Tribosupplementation Lowers Whole Joint COF in Lubricin-null Mouse Joints

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4.1 Abstract

Lubricin is a mucinous glycoprotein found in synovial fluid that provides boundary lubrication, anti-adhesion and chondroprotection to articulating surfaces of synovial joints. In this study, we investigate the efficacy of *ex vivo* and *in vivo* lubricin tribosupplementation to prevent cartilage damage and lower whole joint coefficient of friction (COF) in *Prg4*^{-/-} mouse joints. We hypothesized that tribosupplementation would reduce whole joint coefficient of friction *ex vivo* and *in vivo*, and that *in vivo* tribosupplementation would also reduce the number of apoptotic cells and decrease cartilage damage in lubricin-treated joints compared to contralateral joints and untreated control joints. *Ex vivo* tribosupplementation significantly lowered whole joint coefficient of friction. Two weeks following *in vivo* tribosupplementation, we observed a significant decrease in the percentage of apoptotic cells and less histological joint damage in lubricin-treated joints compared to control joints. Whole joint coefficient of friction tended to be lower in lubricin-treated joints compared to untreated contralateral joints, but these results did not reach significance. These results indicate that tribosupplementation may be a viable treatment option for patients with CACP that present with minor joint pain and that increased frequency of dosage and administration earlier in disease progression may improve these parameters.

4.2 Introduction

Lubricin is a mucinous glycoprotein encoded by the PRG4 gene that plays an important role in the health and homeostasis of articulating joints (1-6). Lubricin is secreted by synovial cells into the synovial fluid (2) and by superficial zone chondrocytes onto the articular surface of cartilage and acts as a boundary lubricant and anti-adhesive to lubricate cartilage and prevent protein deposition and cell adhesion on cartilage surfaces and synovial overgrowth (5). Patients with the disorder camptodactyly-arthritis-coxa vara-pericarditis (CACP) have loss-of-function mutations in lubricin that result in precocious joint failure (6). In the absence of disease-modifying treatments, besides total joint replacement, lubricin delivered by intra-articular injection may be a viable episodic treatment for the joints of lubricin null individuals. This is particularly relevant since patients with CACP have no treatment options other than arthroplasty.

CACP syndrome has been recapitulated in a murine lubricin knockout model, which has been used to study the progression of the disease (4, 5). Joints are normal at birth, but lubricin null ($\text{Prg4}^{-/-}$) mice exhibit significant joint degradation at 8 weeks of age, compared to heterozygous ($\text{Prg4}^{+/-}$) and wild-type ($\text{Prg4}^{+/+}$) littermates, which express lubricin (4, 5).

Intra-articular administration of several forms of lubricin has proven effective in preventing degeneration in a number of trauma-induced preclinical rodent models (7-11), but has not been tested in treating a natural lubricin deficiency that is not caused by inflammatory cytokines.

The objective of this study was to determine if intraarticular lubricin injection could improve cartilage mechanics in lubricin-null mouse joints. In this study, we perform two tribosupplementation experiments. First, we compared whole joint COF in 10-week-old *Prg4*^{-/-} mouse knees before and after *ex vivo* tribosupplementation. We hypothesize that whole joint COF will be reduced following lubricin administration. Second, we perform *in vivo* tribosupplementation on 6-week-old *Prg4*^{-/-} mouse knees. Two weeks following injection, we measure whole joint COF, the percentage of apoptotic cells, and histological scores of *Prg4*^{-/-} mouse limbs that have received lubricin injection and compare them to internal contralateral joints and untreated mouse joints from *Prg4*^{-/-} littermates. We hypothesized that the supplementation of purified human synoviocyte lubricin (HSL) into the joint cavity at 6-weeks of age would lower whole joint coefficient of friction (COF) in *Prg4*^{-/-} mouse knees *in vivo* and prevent chondrocyte apoptosis, compared to contralateral joints 2 weeks following injection and in age-matched littermates that did not receive treatment.

4.3 Methods

4.3.1 *Ex vivo* tribosupplementation of Prg4^{-/-} mouse knees

At 10 weeks of age, Prg4^{-/-} mice were sacrificed and the hind limbs (n=5) were harvested following euthanasia. These mice have been described previously and have been backcrossed onto a C57/BL6 background (5, 12). Baseline whole joint COF was measured as described below. Following whole joint COF measurement, HSL (450 µg/ml in sterile water, 10 µl injection per limb) was injected into the joint space by inserting a 27½G needle into the flexed knee joint just below the patella. Injection of fluid was confirmed by swelling of the joint capsule. Joints were kept hydrated on the bench top for 20 min before COF was re-measured in the treated joints. Previous measurements of untreated COF in Prg4^{-/-} (n=12 limbs) and Prg4^{+/-} (n=6 limbs) knees from male littermates 10 weeks of age were performed in the same manner to provide a baseline of COF for both genotypes (13). All experiments were approved by the Rhode Island Hospital Animal Welfare Committee.

4.3.2. Whole joint COF measurement.

The hind limbs of the mice were isolated, and tissue surrounding the joint capsule was removed, leaving the joint capsule intact. The distal tibia and proximal femur were rigidly mounted in square brass tubing using a urethane compound. Whole joint COF was measured using a modified Stanton pendulum system, as previously described (14, 15). COF measurements were performed with a pendulum load of 50g, which corresponds to 2 times body weight. Limbs were loaded 8 min prior to each set of measurements to

allow cartilage surface apposition and depressurization. Four measurements were taken at each time point per limb.

4.3.3. *In vivo* Tribosupplementation of Prg4^{-/-} Mouse Knees.

Prg4^{-/-} mice 6 weeks of age (n=10) were anesthetized under isoflurane (3-5%). The right hind limb was clipped of hair and the site of injection was prepared with a 3-stage prep involving a povidone iodine scrub, a 70% alcohol wash, and povidone iodine solution. The knee was held in flexion and a 20µl of sterile human synoviocyte lubricin (HSL, 1.6 mg/kg) was injected via a 31G needle through the patellar tendon, which was confirmed by bulging of the joint space. The needle was removed, and the knee flexed and extended 10 times to ensure even distribution throughout the joint cavity. The contralateral knee was not treated. Injection technique was tested on cadaver mice using 10% India ink dye in water.

Two (2) weeks following initial lubricin injection, at 8 weeks of age, injected mice and untreated littermates (n=3) were euthanized by asphyxiation with CO₂, which was confirmed by cervical dislocation. Hind limbs of the mice were removed and coefficient of friction was measured as described above. One Prg4^{-/-} mouse that was injected with lubricin at 6 weeks of age was sacrificed at 8 weeks of age for histological analysis of cartilage without undergoing COF measurement.

4.3.4. Histological Analysis and Scoring.

Following COF measurement or at the time of sacrifice, all knee joints from the *in vivo* study were fixed in 10% formalin and decalcified using a solution of 0.48M EDTA adjusted pH to 7.1 with ammonium hydroxide at 4°C for 48 hr. Thin coronal sections

(6µm) were taken for histological analysis of apoptosis of chondrocytes in the femoral condyles and tibial plateaus. Sections were heated to 60°C for 30 min, deparaffinized and hydrated in 3 changes of xylene and serial alcohol.

Sections were stained with hematoxylin and eosin (H&E). Joint compartments were scored independently by two blinded observers (KW and GJ), using a composite mouse scoring system described in **Table 1** (16-18). This system employs 4 categories: 1) articular cartilage structure (0-4), 2) surface layer morphology (0-3), 3) synovial hyperplasia (0-3), and 4) meniscal changes (0-2). Articular cartilage structure and surface layer morphology were analyzed for tibial plateaus and femoral condyles separately, and summed together. Medial and lateral portions of the tibial plateau and femoral condyles from each joint were averaged together, to obtain a total score of 0-18 for each joint.

Table 4.1: Histological scoring system for the evaluation of murine joint damage.

Histological Scoring System for the Evaluation of Murine Joint Damage	
Osteoarthritic Damage (evaluated separately for the femoral condyle and tibial plateau)	
Description	Grade
Normal	0
Small fibrillations without loss of cartilage	1
Moderate fibrillation without loss of cartilage	1.5
Severe fibrillation without loss of cartilage	1.75
Vertical clefts down to the layer immediate below the superficial layer and some loss of surface lamina	2
Vertical clefts or erosion to the calcified cartilage extending to <25% of the articular surface	3
Vertical clefts or erosion to the calcified cartilage extending to 25-50% of the articular surface	4
Vertical clefts or erosion to the calcified cartilage extending to 50-75% of the articular surface	5
Vertical clefts or erosion to the calcified cartilage extending to >75% of the articular surface	6

Cartilage Surface Layer Morphology (evaluated separately for the femoral condyle and tibial plateau)	
Description	Grade
Smooth surface	0
Some irregularities and/or small amount of protein deposition	1
Moderately roughened or enlarged; moderate protein deposition	2
Severely enlarged and/or cellular infiltrate	3
Protein layer cleaved away	4

Synovial Hyperplasia	
Description	Grade
Normal; synovium 1-2 cell layers thick	0
Mild; hypertrophy of synovial lining 3-4 layers and/or infiltration of the synovium over 0-20% of the articular surface	1
Moderate; marked hypertrophy >4 layers and/or infiltration of the synovium over 20-50% of the articular surface	2
Severe; extensive hypertrophy >5 cell layers and/or infiltration of synovium >50% of the articular surface	3

Chondrocyte Proliferation	
Description	Grade
No evidence of proliferation or cloning	0
Minimal evidence of cloning	1
Substantial evidence of cloning	2

Menisci Architecture	
Description	Grade
Normal	0
Minimal disruption of tissue architecture and/or cell and matrix content and/or minimal proliferation at the synovial-meniscal junction	1
Moderate loss of tissue architecture and/or moderate alteration in cell and matrix content and/or proliferation of cells at the synovial junction and extending into tissue or along surfaces	2
Complete loss of architecture, >50% loss and/or severe loss or disruption of cells, proteoglycan and collagen and/or marked proliferation of cells involving the majority of the remaining tissue	3

4.3.5. Active Caspase-3 Immunohistochemistry and Quantification .

Thin coronal sections were also used for histological analysis of chondrocyte apoptosis in the femoral condyles and tibial plateaus of mouse limbs as described above. Prepared sections were quenched in endogenous peroxidase in 3% hydrogen peroxide for 10 min and antigen retrieval was performed using a pepsin solution (ThermoScientific). A rabbit polyclonal antibody against active caspase-3 (ab13847, Abcam, Cambridge, MA) at 1:50 dilution was added to the sections and incubated at 4°C overnight. After 3 washes with PBS, the sections were incubated with Cy3 goat anti-rabbit IgG (Life Technologies, Molecular Probes) at 1:50 dilution for 1 h at room temperature, protected from light. The sections were washed 5 times using PBS, counterstained using Vectashield mounting media with DAPI (1.5ug/ml, Vector Laboratories Inc, Burlingame, CA). Images were captured with a 10X objective, using a Roper Scientific Photometrics CoolSNAP HQ2 monochrome camera (Roper Scientific, Germany) and a Nikon Eclipse 90i microscope (Nikon Instruments, USA). Fluorescent images were thresholded uniformly to reduce background auto-fluorescence and to adjust DAPI signal using Adobe Photoshop CS5 software (Adobe Systems Inc., Waltham, MA).

Cells positive for active caspase-3, and the total number of cells in each joint compartment were counted manually using ImagePro Plus software (Media Cyberkinetics, Bethesda, MD). Joint compartments were averaged for each knee and the ratio of positive cells to total cell number was reported as a percentage for each joint.

4.3.6. Colorimetric Assay of Lubricin Adhesion to Albumin .

Well plates were coated with 1% bovine serum albumin for 2 hr at room temperature, rinsed 3 times with PBS, followed by 24 hr incubation with 100 µg/ml purified human lubricin (BSA-HSL). Control groups included PBS, lubricin alone (HSL) and BSA alone (BSA).

Colorimetric analysis was performed to detect the presence of lubricin and determine its ability to adhere to the BSA coated surface. The wells were washed three times with PBS, blocked 1 h with 5% nonfat dry milk in PBST. The plates were then incubated with 1:2,000 9G3 anti-lubricin antibody, washed 3 times, then incubated with 1:2000 dilution HRP secondary antibody (goat-anti-rabbit, Santa Cruz Technologies), and washed 4 times with TBST. Finally, lubricin was detected using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and photographed.

4.3.7. Statistical Analysis

Comparisons between whole joint COF of 10-week old $\text{Prg4}^{-/-}$ knees before and after *ex vivo* HSL injection were performed using a two-tailed paired Student's t-test ($\alpha=0.05$).

Comparisons of COF, total histological scores and the percentage of apoptotic cells between HSL injected limbs, contralateral limbs and untreated control limbs were performed using a one-way ANOVA with Tukey's multiple comparison test ($\alpha=0.05$). Comparisons between COF in HSL injected limbs and contralateral limbs of experimental mice and between left and right limbs of untreated control mice were compared using a paired two-tailed Student's t-test ($\alpha=0.05$). All data were reported as

mean $\pm$ standard deviation (SD). Statistical analysis was performed using Prism 6 software (GraphPad, La Jolla, CA).

4.4 Results

4.4.1 *Ex vivo* Tribosupplementation

Whole joint COF was significantly lowered in our *ex vivo* tribosupplementation experiment using 10-week-old mouse limbs from 0.065 ± 0.007 to 0.060 ± 0.006 following HSL injection ($n=5$, $p=0.008$) (**Figure 4.1**). However, the change in whole joint COF was 0.006 ± 0.003 , with the greatest change of 0.008, which is well below the change that could be considered therapeutic.

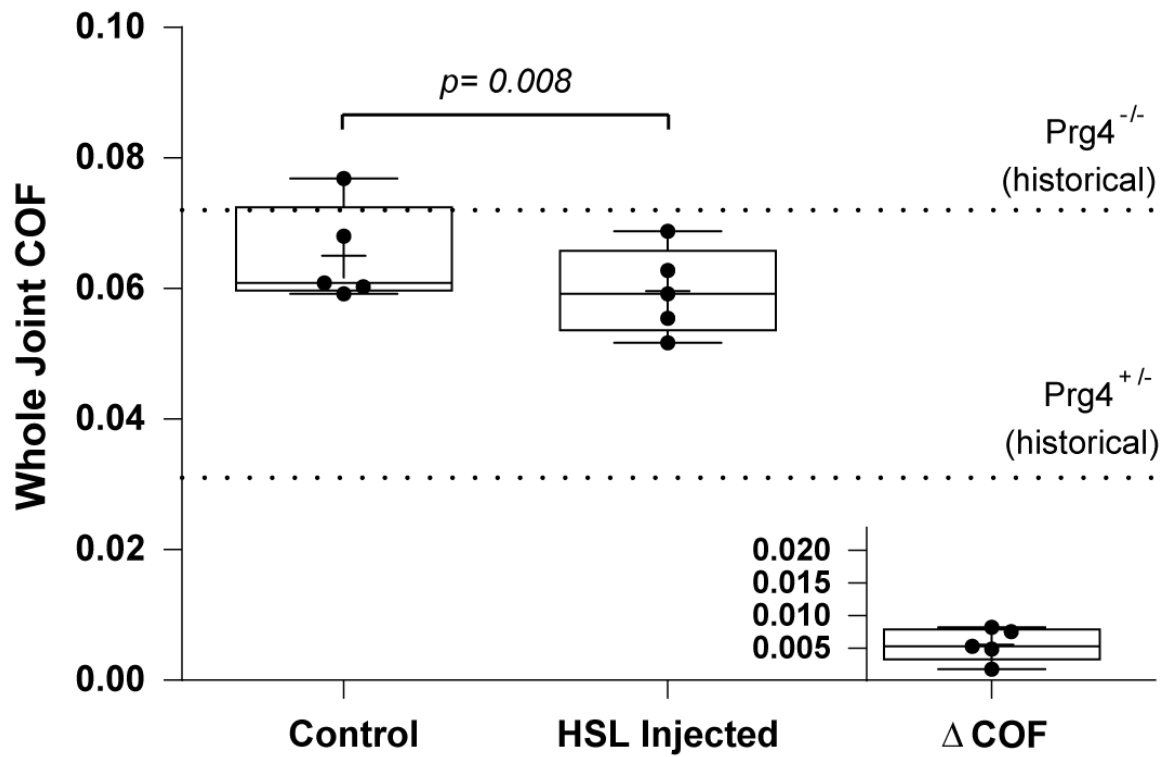


Figure 4.1: Whole joint COF following *ex vivo* tribosupplementation. Tribosupplementation using 250 μ g/ml HSL resulted in significantly lower whole joint COF in 10-week-old $Prg4^{-/-}$ mouse knees. Whiskers represent maximum and minimum values, with lines at the median. + indicates mean. Dotted lines at historical mean values for whole joint COF in 10-week-old $Prg4^{-/-}$ and $Prg4^{+/-}$ mouse knees are provided for reference.

4.4.2 *In vivo* Tribosupplementation

Whole joint COF was lowered between CL controls (COF = 0.070 ± 0.01) and HSL injected (COF = 0.058 ± 0.008) joints, but did not reach significance (n=9 joint pairs, $p=0.059$) (**Figure 4.2**). The mean Δ COF for experimental joint pairs was 0.012 ± 0.016 . Control joint pairs that did not receive injections had no change in COF (Δ COF = 0.000052 ± 0.001) between the left and right limbs (n=3 joint pairs, $p=0.93$). HSL injected knees and knees from control mice (COF = 0.064 ± 0.002) did not have significantly different whole joint COF ($p=0.5$).

Histological staining revealed minor cartilage damage in all knees, especially changes in surface morphology characterized by protein deposition (**Figures 4.3**). Knees that received HSL injection had significantly lower total histological scores compared to knees from untreated control littermates ($p=0.04$) (**Figure 4.4**). However, there was no significant difference between the total histological scores of HSL injected knees compared to paired contralateral controls ($p=0.54$) (**Figure 4.4**).

We observed cells positive for active caspase-3 in all joints (**Figures 4.3**). The percentage of apoptotic chondrocytes was significantly lower in HSL treated knees compared to contralateral knees (**Figure 4.5**, $p=0.048$) and compared to control knees ($p=0.02$). There was no significant difference between the percentage of apoptotic chondrocytes in contralateral knees compared to control knees ($p=0.76$).

HSL was able to successfully adhere to BSA coated and uncoated tissue culture plastic, as qualitatively observed (**Figure 4.6**), indicating that lubricin is able to adhere to protein layers similar to those seen on the surface of *Prg4*^{-/-} mouse cartilage.

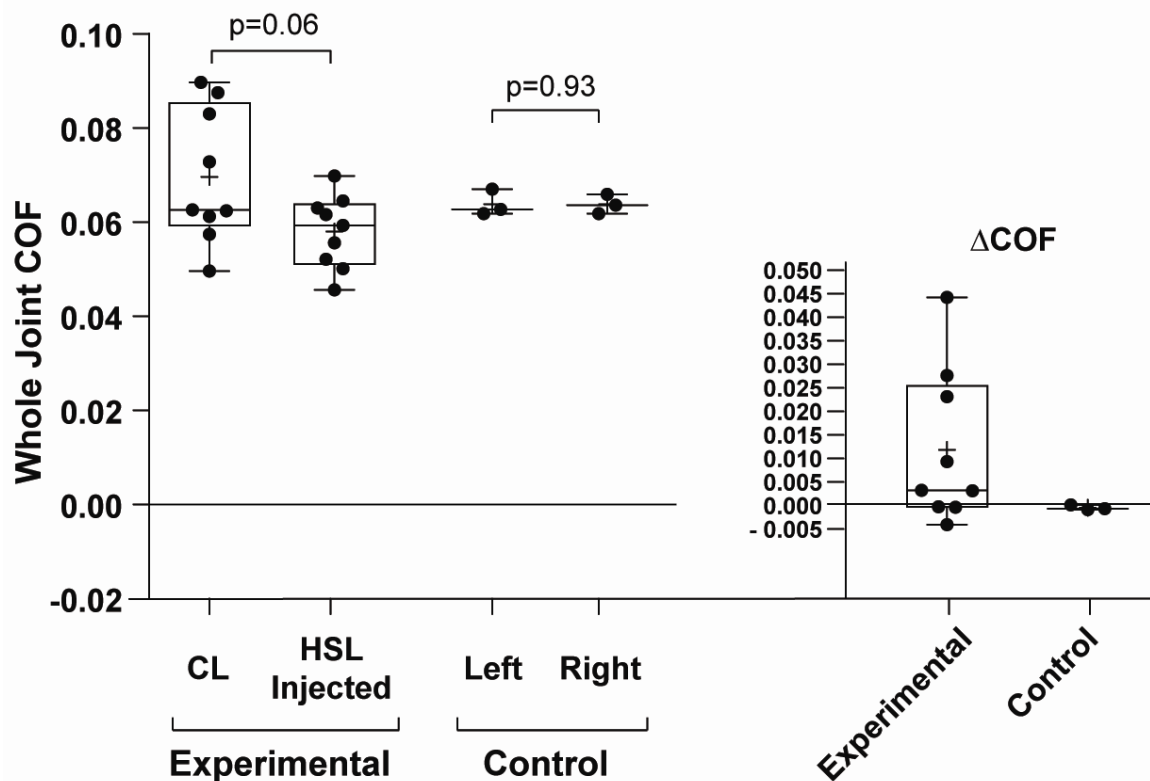


Figure 4.2: In vivo whole joint COF following tribosupplementation. We observed a lower whole joint COF for HSL injected joints compared to contralateral controls, which approached significance. Untreated control mice had no difference between their left and right limbs. Change in whole joint COF (Δ COF) is depicted on the right side for experimental mice that represents the change in whole joint COF between the contralateral knee and the knee that received an HSL injection for the experimental group, and the difference between the left and right limb in the control group, which did not receive any injection. Whiskers represent maximum-minimum values, + indicates mean, and lines represent median values.

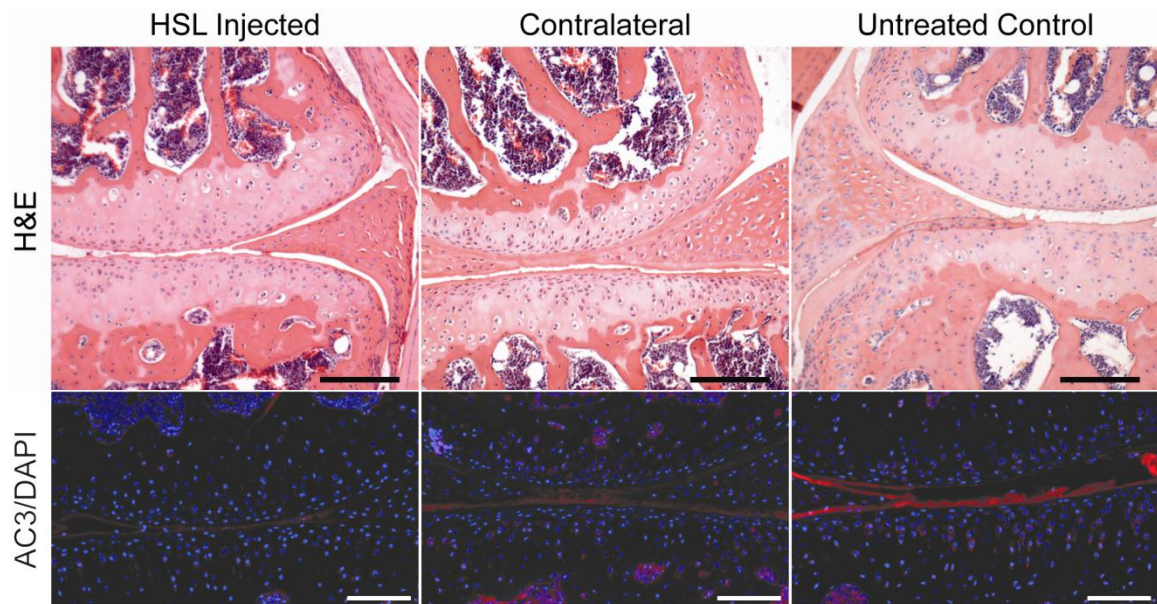


Figure 4.3: Coronal sections of median scored mouse joints stained with H&E (top row) and stained for apoptosis (bottom row). (top row) Joint irregularities were observed in most treated and untreated joints had enlarged cartilage with a protein layer, minor synovitis, cellular cloning and minor meniscal disruption. Mouse joints treated with lubricin (left column) had a smoother joint surface with less protein deposition compared to untreated control joints (right column). (bottom row) Active caspase-3 (red) staining indicated apoptotic cells, which were counterstained nuclear DAPI (blue) staining. HSL injected joints also had fewer apoptotic chondrocytes (white arrows), as observed qualitatively. In active caspase-3 stained joints, a proteinaceous layer at the surface of cartilage stained with red fluorescence, which has been previously observed (13).

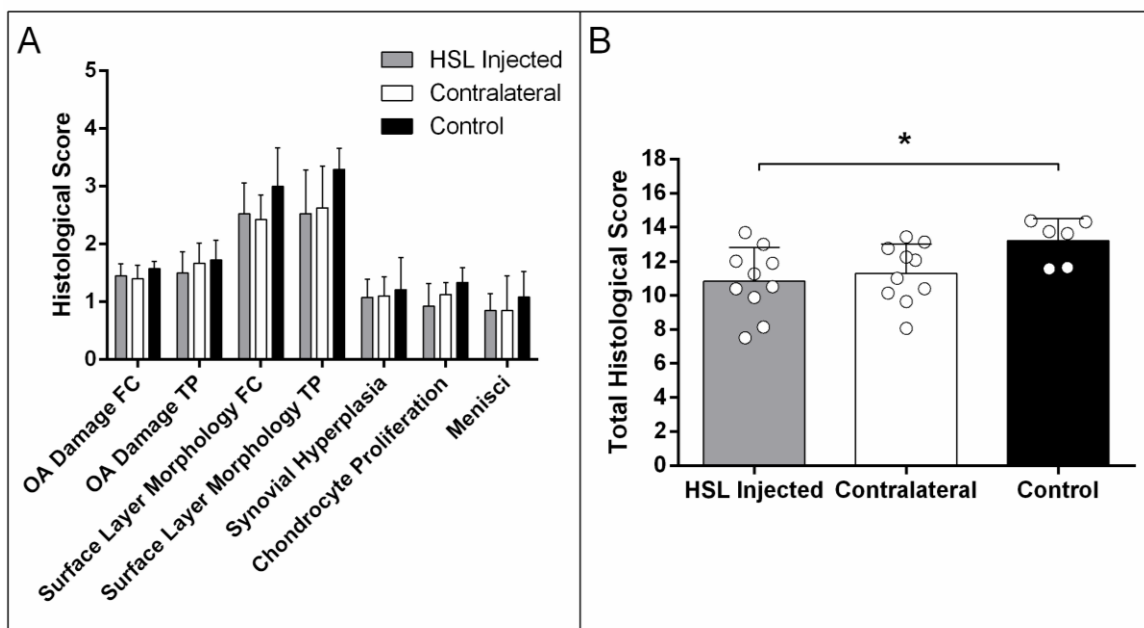


Figure 4.4: Histological scoring results. A.) Individual histological scores for HSL injected, contralateral and untreated control joints from littermates. B.) Total histological scores for HSL injected, contralateral and untreated control joints. We observed significantly higher total scores for control joints compared to HSL injected joints ($p=0.04$). Bar graphs represent mean $\pm$ SD.

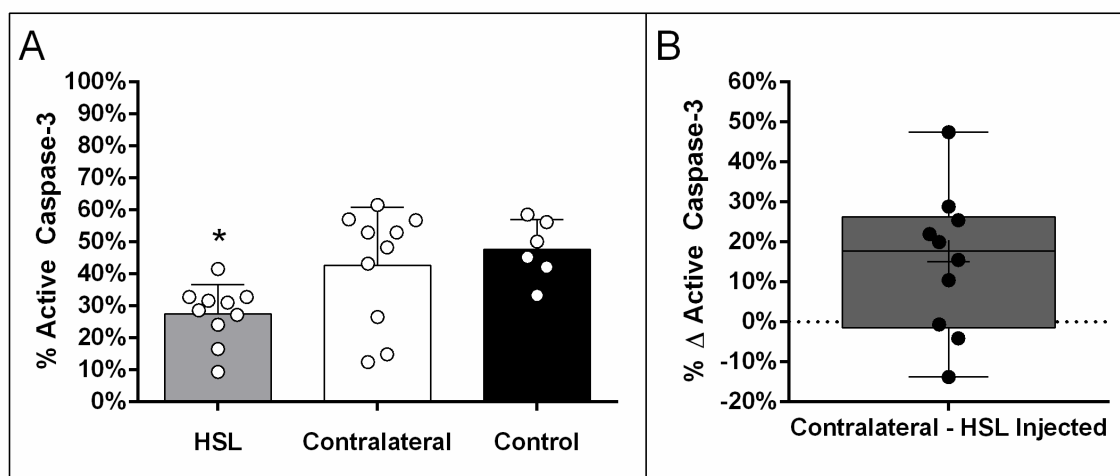


Figure 4.5: Percentage of cells positive for active caspase-3. A.) HSL injected mice had a significantly lower percentage of apoptotic cells compared to contralateral joints ($p=0.048$) and joints from untreated mice ($p=0.02$). Bar graphs represent mean $\pm$ SD. B.) Change in the percentage of cells positive for active caspase-3 between injected and contralateral limbs. In most cases there was a decrease of 10% or more in active caspase-3 between contralateral and HSL injected joints. Whiskers represent maximum and minimum values with line at the median value. + indicates mean value

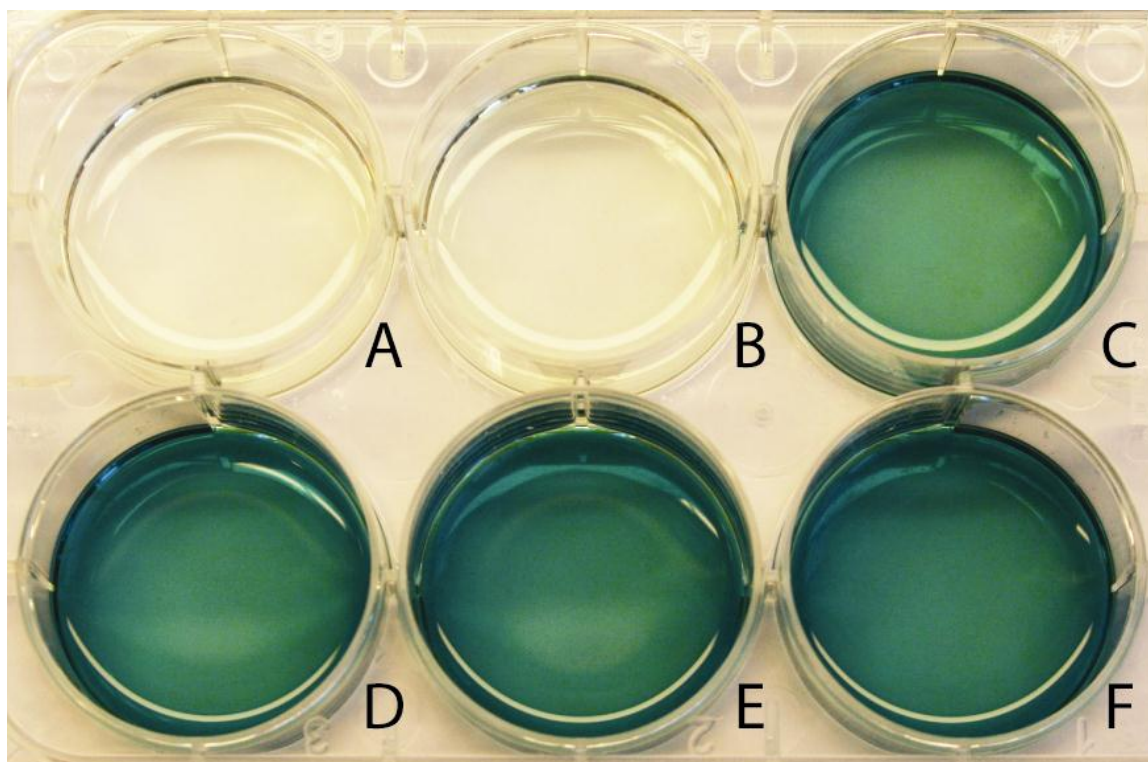


Figure 4.6: Colorimetric Assay. A.) BSA only B.) PBS only C.) HSL only D.)-F.) BSA + HSL. HSL was able to successfully adhere to well plates coated with BSA as well as tissue culture plastic.

4.5 Discussion

In this study, we first investigated the use of tribosupplementation to provide boundary lubrication via supplementation of lubricin *ex vivo*. *Ex vivo* supplementation provides a proof-of-concept for the lowering of whole joint COF by replenishment in the synovial fluid, in a 10-week-old mouse limb with moderate joint damage, including cartilage fibrillation, protein deposits and synovitis. We obtained favorable results, although not necessarily therapeutic, that lubricin may be able to play a lubricating role even in joints with cartilage damage (**Figures 4.2 and 4.3**). This change is likely due to its ability to prevent adhesion of the joint surfaces as the joint is loaded prior to oscillation. However, this *ex vivo* lubrication experiment also stressed the importance of preventing cartilage damage at an earlier time point in order for lubricin to prevent cartilage damage via boundary lubrication. Previous studies investigating the tribological properties and biomechanics of human osteoarthritic cartilage have indicated that friction is increased in joints with cartilage damage (15, 19-21); however coefficient of friction is still decreased by lubricating damaged cartilage with healthy synovial fluid as opposed to PBS (20, 22). The lower whole joint COF that was observed justified an effort to reproduce these results *in vivo*.

In vivo tribosupplementation via a single high dose of HSL at 6 weeks of age resulted in reduction in whole joint COF, which approached significance (**Figure 4.3**). Our findings *in vivo* show that reduction in COF appeared to prevent chondrocyte apoptosis, which is relevant to cartilage health as chondrocytes are responsible for the maintenance of the cartilage matrix. We also observed significantly lower histological

scores, indicating that HSL injected joints have better joint health compared to untreated joints.

Lubricin has shown success as a tribosupplement, even with a single dose, in rodent secondary osteoarthritis models (8-11, 23), and overexpression of Prg4 prevented cartilage damage in a post-traumatic murine osteoarthritis model (24). Previous studies generally use semi-quantitative histological scoring systems that assess joint damage using less stringent criteria than described in this study (8-11, 23, 24), but gait analysis has also been used as an outcome measurement. Coefficient of friction using similar pendulum systems have been used to evaluate treatments to prevent lubricin downregulation following ACLT (7, 9), and lubricin supplementation of synovial fluid from patients with CACP lowered coefficient of friction and reduced chondrocyte apoptosis *in vitro* using idealized cartilage bearing surfaces (13). However, to the knowledge of the authors, this study is the first investigating whole joint coefficient of friction measurement following lubricin tribosupplementation *in vivo*.

The availability of endogenous lubricin following tribosupplementation in rodent secondary OA models is influenced by upregulation of lubricin excretion, which is otherwise lost following the inflammatory cascade in response to injury (9). Since Prg4^{-/-} mice and patients with CACP are unable to produce endogenous lubricin, numerous injections would be required to sustain boundary lubrication and prevent future cartilage damage.

In addition to providing boundary lubrication, lubricin also acts as an anti-adhesive and prevents synovial invasion into the cartilage matrix. Synoviocyte hyperplasia and synovial thickening are observed as early as 15 days after birth and

progress to the intimal and subintimal layers of the synovium. A proteinaceous layer determined the “pseudo-lamina splendens” has been observed on the articular surface of $\text{Prg4}^{-/-}$ joints, and is noticeable as early as 15 days postnatally (5). While lubricin is still able to adhere to a protein layer, as seen in our colorimetric assay, this non-uniform and often fibrillated layer on the surface of cartilage may inhibit the ability of lubricin to work as a boundary lubricant. Previously, incubation with lubricin-containing cell culture media prevented the adhesion of primary $\text{Prg4}^{-/-}$ mouse synoviocytes to petri dish plastic (5). As a result, the presence of lubricin both in synovial fluid and on the articular surface prevents protein deposition and synoviocyte adhesion. In this study, we have shown that lubricin is able to adhere to BSA coated tissue culture plastic, but its ability to remain present and provide boundary lubrication in the presence of shear and axial stresses is not evaluated in our *in vitro* test.

Histological scoring of H&E stained coronal sections revealed minor cartilage damage in all knees, especially changes in surface morphology characterized by protein deposition. Knees that received HSL injection did not have significantly lower histological scores compared to their contralateral joint. However, knees that received HSL injection had significantly lower total histological scores compared to knees from control littermates that did not receive any treatment. This finding indicates that contralateral limbs may see some attenuated damage as a result of improvement in HSL treated knees, and may not provide an accurate control.

HSL injected cartilage had a significantly lower percentage of apoptotic cells compared to paired contralateral knees and knees untreated control littermates, which indicates lubricin provides chondroprotection. This finding is supported by previous

observations indicating that decreased friction results in fewer apoptotic chondrocytes (13). Apoptotic cells were mainly found just below the layer of flattened superficial zone chondrocytes, as previously reported (13). Unlike the case in trauma-induced osteoarthritis or rheumatoid arthritis, inflammatory mediators do not play a role in the etiology of cartilage deterioration in the case of lubricin-null mice or patients with CACP, which indicates the pathway for apoptosis is likely unrelated to inflammatory pathways, such as tumor necrosis factor- α , that may play a pro-apoptotic role in trauma-induced arthropathies that precede secondary osteoarthritis (24).

The combination of whole joint COF, histological scoring, and caspase-3 staining allows for a complete picture of wear that provides an underpinning to the etiology of joint degradation that has not been studied previously in a mouse model of naturally occurring arthropathy.

Patients with CACP generally present in childhood with joint contractures, especially in the fingers and toes, with swollen and painful large joints. These patients lack treatment options other than total joint replacement, which is common in early adulthood. Due to constraints concerning the size of $\text{Prg4}^{-/-}$ mouse knees, we injected at the earliest time point feasible. The postnatal age of 6-weeks approximately correlates to human children 14 years of age (25). By this age, most patients diagnosed with CACP also complain of joint pain (6, 26-30). Lubricin-null mice have a collagen fiber damage observable by SEM as early as 7 days after birth, and already have joint damage at 4 weeks of age, which corresponds to 12.5 human years. As a result, the findings of this study would indicate that intervention via lubricin injection in a human joint would be

performed as early as possible, at the time of diagnosis, in order to prevent continuing damage.

There are a number of limitations that accompany tribosupplementation in $\text{Prg4}^{-/-}$ mouse joints. Due to the size of the joint capsule, injections in mice younger than 6-weeks-of age are difficult to perform, which limits the precision of lubricin injection and limits volume of lubricin that could be administered. Also, unlike in previous successful studies investigating the prevention of rodent secondary OA, $\text{Prg4}^{-/-}$ mice cannot produce endogenous lubricin, so all available lubricin must be administered exogenously. The half-life of recombinant lubricin in a rat study was 10-12 h (31). As a result, we predict that the findings in this study would be improved by increasing the frequency of dosage. Employing a sham saline injection as a control for injection is also necessary to confirm our observations.

The results of this study suggest that tribosupplementation with lubricin may be a viable treatment option to mitigate subsequent cartilage injury in patients with CACP presenting with minor joint pain. We anticipate that intra-articular injection would need to be accomplished at least monthly. These patients have no other definitive medical treatment options. Although this report is based upon a suitable animal model, this study did not incorporate other measures of murine behavior, such as gait, which could be affected.

4.6 Acknowledgements

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Chapter 5

The Genetic Rescue of Lubricin-Null Mouse Knees Mitigates Cartilage Damage and Lowers Whole Joint Coefficient of Friction

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5.1 Abstract

Lubricin, the lubricating glycoprotein product of the PRG4 gene, is responsible for boundary lubrication and chondroprotection in articular joints. In this study, we investigate the ability of endogenous lubricin to restore joint health or prevent subsequent damage following degeneration in a Prg4 genetrap mouse. This mouse was bred onto a Rosa26::creERT2 strain. Following Tamoxifen injection, Prg4 expression is restored. We measured whole joint coefficient of friction following progressive cyclic loading and evaluated the joints histologically for degenerative changes and apoptosis for 2 interventional schemes: Prg4 activation at 3 weeks of age, and evaluation at 2 months of age and Prg4 activation at 2 months of age and evaluation at 6 months of age. We hypothesized that lubricin activation would result in lower whole joint COF, less joint damage and fewer apoptotic cells and that intervention at 3 weeks would provide more joint protection compared to intervention at 2 months. We observed a chondroprotective effect of joints recombined at 3 weeks and evaluated at two months compared to the joints of untreated genetrap mice. In contrast, genetic recombination at 2 months postnatally and evaluated at 6 months was unable to provide additional protection compared to untreated genetrap mouse joints. The findings of this study indicate that genetic intervention with lubricin is a viable treatment option for patients with lubricin deficiency, but that early intervention prior to significant joint damage is vital to treatment success.

5.2 Introduction

Lubricin is a lubricating glycoprotein encoded by the *PRG4* gene that plays an important role in the health and homeostasis of articulating joints. Lubricin, also known as superficial zone protein, is secreted by synovial cells into the synovial fluid, as well as by chondrocytes onto the articular surface of cartilage. It acts as a boundary lubricant and anti-adhesive to lubricate cartilage and prevent protein deposition and cell adhesion on cartilage surface.

Truncating mutations on *PRG4* in humans results in lack of lubricin expression and causes the autosomal recessive disease camptodactyly-arthritis-coxa vara-pericarditis (CACP) syndrome, in which individuals develop joint failure. This syndrome has been recapitulated in a mouse model, which has been used to study the progression of CACP (1-3). A *Prg4* genetrap mouse model was designed to study the effect of endogenous lubricin production in a genetrap mouse that was bred with a *Rosa26::creERT2* strain. These mice are lubricin-null from birth, but following Tamoxifen injection *Prg4* is activated.

The objective of this study was to determine if lubricin-null mouse joints could be rescued following degeneration by the induction of endogenous lubricin production in the cartilage and synovium at two intervention times, 3 weeks of age and 2 months of age. We hypothesized that genetrap mouse knees from both age groups would be protected by the restoration of lubricin after birth, resulting in lower coefficient of friction, fewer apoptotic cells, and less cartilage damage compared to lubricin-null knees. We also

hypothesized that intervention at 3 weeks would provide better chondroprotection compared to intervention at 2 months of age.

5.3 Methods

5.3.1 Prg4 Genetrap Mouse

Prg4 genetrap mice were generated by crossing Prg4^{-/-} mice to B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J mice, resulting in a genetrap mouse with a default genotype of Prg4^{-/-} (**Figure 5.1**). The genetrap cassette, located between two recombination sites, consists of a reporter gene (lacZ) flanked by a splice site (Splice acceptor; SA) and a transcriptional termination sequence (polyadenylation sequence; PA-PA-neo-PA), was inserted between coding exons 1 and 2 on the *Prg4* gene. As a result, the transcript produces a truncated, non-functional form of Prg4. This mouse strain was bred with a Rosa26::creERT2 strain. To initiate the expression of Prg4, intraperitoneal injections of tamoxifen (50 mg/kg in corn oil) were administered to Prg4 GT/GT mice daily for 10 days to initiate the production of lubricin in both the cartilage and synovial tissues, thus changing their genotype to Prg4 GT^R/GT, which is approximately equivalent to a Prg4^{+/-} mutant mouse. All experiments were approved by the Rhode Island Hospital Animal Welfare Committee.

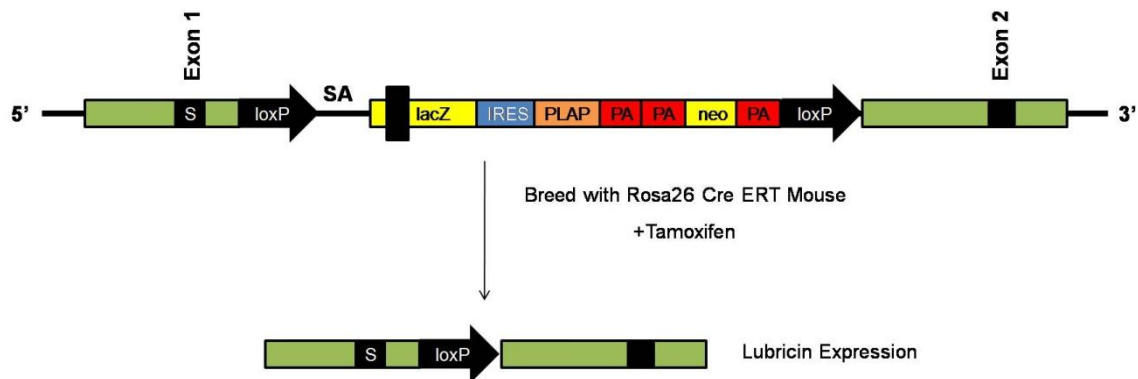


Figure 5.1: Diagram of the genetrap cassette. The genetrap cassette, located between two recombination sites (loxP), consists of a reporter gene (lacZ) flanked by a splice site (Splice acceptor; SA) and a transcriptional termination sequence (polyadenylation sequence; PA-PA-neo-PA), which is inserted between coding exons 1 and 2 on the PRG4 gene. Since transcription is terminated prematurely at the polyadenylation sequence, the transcript produces a truncated, non-functional form of Prg4. This mouse strain has been bred with a Rosa26::creERT2 strain. Following tamoxifen injection, cre-recombinase translocates to the nucleus, allowing recombination at the loxP sites, resulting in Prg4 expression.

5.3.2 Lubricin Intervention

We investigated two time points for genetic activation of *Prg4*. The early intervention scheme involved mice treated with tamoxifen on days 21-31, then sacrificed at 8.5 ± 0.5 weeks (GT^{R3wk}/GT , $n=6$ mice). Three weeks of age was chosen as a time point for early intervention due to the approximate human age of 8.5 years. At two weeks of age in a *Prg4*^{-/-} mouse, minor degenerative changes have been observed, such as surface disruption and proteinaceous biofouling, and small differences in COF at 1 month of age were reported (2). The late intervention group were treated with tamoxifen at 2 months and sacrificed at 6 months (GT^{R2mo}/GT , $n=3$ mice). At 2 months of age, significant protein deposition and surface morphological changes have been reported in *Prg4*^{-/-} mice (4), in addition to elevated friction compared to mice at 1 month of age (2). This time point approximates 16 years of age in a human. We tested untreated *GT/KO* ($n=2$ mice for early intervention, $n=3$ mice for later intervention), which behave most like *Prg4*^{-/-} mice, as negative controls, in addition to *GT/GT* mice ($n=5$ for early intervention and $n=4$ for late intervention). Mosaic mice recombined before birth (GT^*/GT , $n=3$ mice or GT^*/KO , $n=3$ mice) recombined at birth were used as positive controls, for the early and late intervention groups, respectively. GT^* represents one parent that was heterozygous for the recombined genetrap (GT^*/KO), which results in lubricin expression from birth.

5.3.3 Pendulum Systems

At the above outlined time points, the hind limbs were harvested following euthanasia. Tissue surrounding the joint capsule was removed, but the capsule was kept

intact. The distal tibia and proximal femur were rigidly mounted in square brass tubing using a urethane compound (Smooth-on, Easton, PA). A passive modified Stanton pendulum was used to measure whole joint COF, as previously described (1, 5). A cyclic loading regimen using an active pendulum system was used in conjunction with the passive pendulum to monitor progressive increases in COF following simulated exercise (1).

5.3.3.1 Modified Stanton Pendulum

COF measurements were taken at time 0, 15 min, 30 min, 45 min and 1 hour following cyclic loading, which was performed with a load of 50g, which corresponds to 2 times body weight. Limbs were submerged in PBS and statically loaded for 8 minutes prior to each set of measurements to allow the surfaces to pressurize together. Prior to frictional testing, the PBS was removed from the test chamber, the pendulum arm was rotated to place the knee 12° degrees in flexion, and the arm was released. The pendulum arm was allowed to freely swing until stopping. Four measurements were taken at each time point per limb. Reflective markers on the base (4) and pendulum arm (4) were used for motion tracking using a Qualysis AB System at a rate of 60 Hz. Four (4) trials were collected for each knee. Oscillation data was processed with Visual3D software (C-Motion, Inc., Germantown, MD) and a custom MATLAB (MathWorks Inc, Natick, MA) code was used to determine the peak amplitude of each cycle of oscillation and calculate whole joint COF using a Stanton linear decay model (6).

5.3.3.2 Active Pendulum and Cyclic Loading Protocol

Between measurements, the distal tibia limb was secured to the mounting block of the active pendulum with set screws and the proximal femur was inserted into the pendulum arm. To cyclically load the knee, the active pendulum arm was rotated $\pm 15^\circ$ at a rate of 1.5 Hz in increments of 15 minutes at which time coefficient of friction measurements were performed. During cyclic loading, the knee was entirely submerged in Dulbecco's Modified Eagle Medium (DMEM, 1M, Life Technologies) to prevent desiccation. All experiments were carried out at room temperature.

5.3.4 Immunohistochemistry

Mouse knees from each genotype were processed for active caspase-3 staining following friction testing and cyclic loading. Samples were fixed in 10% formalin and decalcified using a solution of 0.48M EDTA adjusted pH to 7.1 with ammonium hydroxide at 4°C for 48 hr. Thin paraffin-embedded coronal sections (5 μ m) were taken for histological analysis of apoptosis of chondrocytes in the femoral condyles and tibial plateaus of mouse limbs. Sections were heated to 60°C for 30 min, deparaffinized and hydrated in 3 changes of xylene and serial alcohol. Sections were then quenched in endogenous peroxidase in 3% hydrogen peroxide for 10 minutes and antigen retrieval was performed using a pepsin solution (ThermoScientific). A rabbit polyclonal antibody against active caspase-3 (ab13847, Abcam, Cambridge, MA) at 1:50 dilution was added to the sections and incubated at 4°C overnight. After 3 washes with PBS, the sections were incubated with Cy3 goat anti-rabbit IgG (Life Technologies, Molecular Probes) at 1:50 dilution for 1 hour at room temperature, protected from light. The sections were washed 5 times using PBS, counterstained using Vectashield mounting media with DAPI

(1.5ug/ml, Vector Laboratories Inc, Burlingame, CA). Images were captured with a 10X objective, using a Roper Scientific Photometrics CoolSNAP HQ2 monochrome camera (Roper Scientific, Germany) connected to a Nikon Eclipse 90i microscope (Nikon). Fluorescent images were thresholded uniformly to reduce background auto-fluorescence and to adjust DAPI signal using Adobe Photoshop CS5 software (Adobe Systems Inc).

5.3.5 Histological Scoring and Analysis

Sections were also stained with H&E, and were imaged at 10X using an Olympus BX51 microscope (Olympus America Inc.) using Image Pro Plus 7.0 software (Media Cyberkinetics, Bethesda, MD). Histological scoring was performed by blinded observers (KW and GJ) using a composite mouse cartilage scoring system outlined in Chapter 4 (3, 7, 8). This system employs four categories: articular cartilage structure (0-4), surface layer morphology (0-3), synovitis (0-3) and meniscal changes (0-2). Articular cartilage structure and surface layer morphology were analyzed for the tibial plateau and femoral condyles separately. Medial and lateral portions of the tibial plateau and femoral condyles from each joint were averaged together, to obtain a total score of 0-18 for each joint.

5.3.6 Statistical Analysis

Statistical analyses for early and late intervention schemes were evaluated separately. Statistical analysis of whole joint COF comparing genotypes and cyclic loading duration was performed using a two-way repeated measures ANOVA ($\alpha=0.05$) with Tukey's multiple comparison test. Comparisons between total histological scores of

genotypes were analyzed using a one-way ANOVA with a Holm-Sidak multiple comparison test. Statistical analysis was performed using Prism 6 Statistical Software (GraphPad Software Inc., La Jolla, CA).

5.4 Results

In the early intervention (3w-2mo) group, we observed significant interaction was observed between the genotype ($p=0.003$) and cyclic loading duration ($p<0.0001$). We did not observe significant differences in whole joint COF between genotypes prior to cyclic loading, but following 15, 30, 45 and 60 minutes of cyclic loading, the mean COF of GT/KO limbs was significantly higher than GT^{R3wk}/GT ($p=0.002$, 0.0004 , 0.003 and 0.0002 , respectively) (**Figure 5.2A**). Following 15, 30, 45 and 60 minutes of cyclic loading, COF across GT/KO limbs were significantly higher than GT*/GT knees, which express lubricin from birth ($p=0.001$, <0.0001 , 0.0001 and <0.0001 , respectively). Following 45 and 60 minutes of cyclic loading, COF across GT/GT limbs were significantly higher than GT*/GT knees ($p=0.03$ and 0.01). There were no significant differences in COF between GT^{R3wk}/GT and GT*/GT knees, even after 60 minutes of cyclic loading.

We observed significant increases in COF with cyclic loading in GT/KO, GT/GT, and GT^{R3wk}/GT mouse knees. GT/KO limbs had a significant increase in COF between $t=0$ minutes and $t=15$ ($p=0.005$), 30 ($p<0.0001$), 45 ($p<0.0001$) and 60 minutes ($p<0.0001$). GT/GT limbs had significantly higher mean COF between $t=0$ minutes and $t=30$ ($p=0.03$), 45 ($p=0.001$) and 60 minutes ($p<0.0001$). GT^{R3wk} limbs had significantly higher mean COF after 45 ($p=0.0004$), and 60 minutes of cyclic loading ($p<0.0001$), compared to $t=0$ minutes. GT*/GT mouse knees, which express lubricin from birth, had no significant changes in COF following cyclic loading ($p>0.96$ for all time points).

In the late intervention (2mo-6mo) group, we observed differences between genotypes following cyclic loading (**Figure 5.2A**). After 15 minutes of cyclic loading, GT/KO had significantly higher whole joint COF compared to GT*/KO joints, which express lubricin from birth ($p=0.005$). Following 30, 45 and 60 minutes of cyclic loading, GT^{R2mo}/GT, GT/GT and GT/KO had significantly higher whole joint COF compared to GT*/KO joints (30 minutes $p=0.02$, 0.01 and 0.003 , 45 minutes $p=0.003$, 0.003 and 0.0004 , 60 minutes $p=0.001$, 0.0008 and 0.0003 for GT^{R2mo}/GT, GT/GT and GT/KO, respectively compared to GT*/KO). There were no differences between GT^{R2mo}/GT, GT/GT and GT/KO for any of the time points.

We observed significant increases in COF with cyclic loading for GT/KO, GT/GT and GT^{R2mo}/GT, which do not express lubricin from birth. GT/KO limbs had a significant increase between t=0 minutes and t=15 ($p=0.02$), 30 ($p=0.006$), 45 ($p<0.0001$) and 60 minutes ($p<0.0001$). GT/GT limbs had significantly higher mean COF between t=0 minutes and 30 ($p=0.001$), 45 ($p<0.0001$) and 60 minutes ($p<0.0001$). GT^{R2mo}/GT limbs had significantly higher mean COF between t=0 and 30 ($p=0.003$), 45 ($p<0.0001$) and 60 ($p<0.0001$). GT*/KO mice, which express lubricin from birth, had no significant change in COF following cyclic loading ($p>0.96$ for all time points).

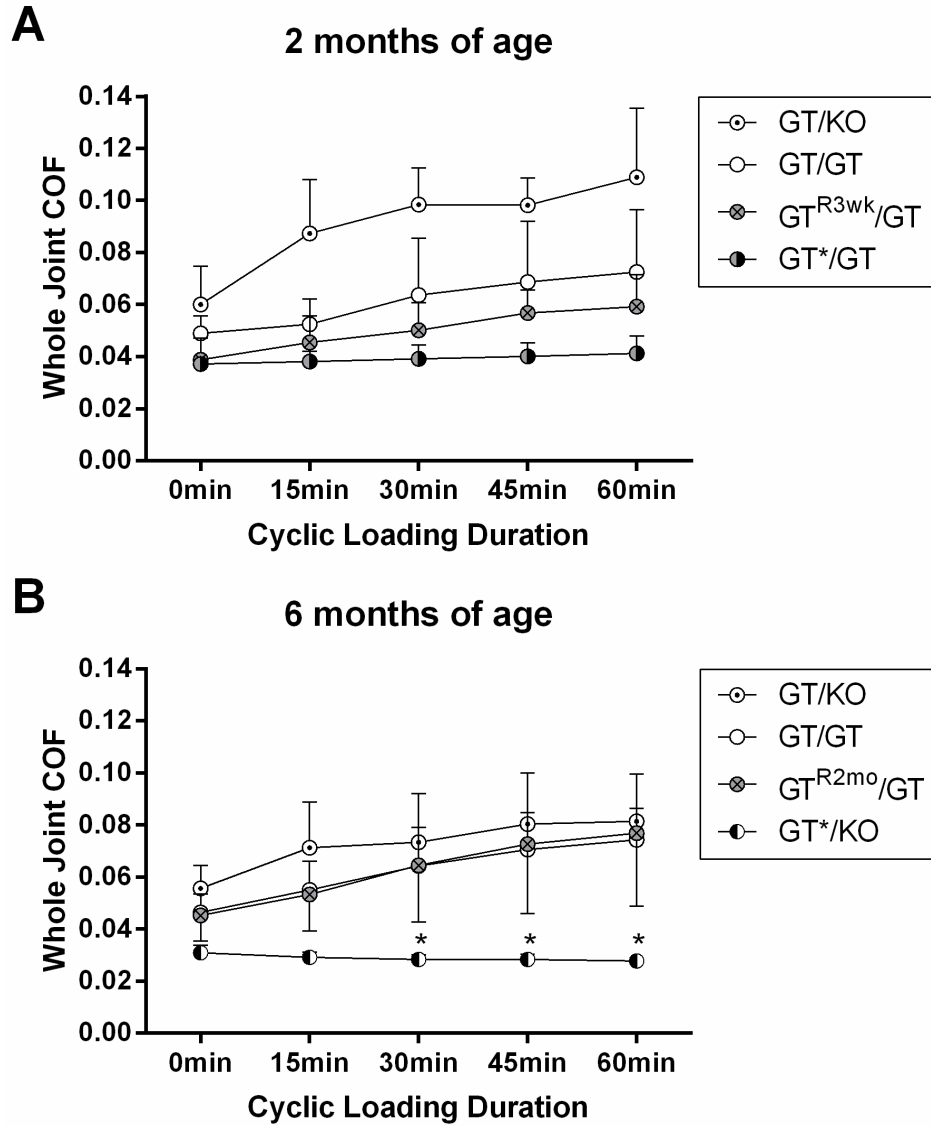


Figure 5.2: (A) Whole joint COF at 2 months of age. The whole joint COF of GT/KO, GT/GT and GT^{R3wk}/GT limbs increased with duration of cyclic loading, whereas GT*/GT limbs did not increase with time. We observed significantly higher whole joint COF for GT/KO compared to GT^{R3wk}/GT and GT*/GT limbs after 15 minutes of cyclic loading. GT^{R3wk}/GT limbs and GT*/GT limbs did not have significantly different whole joint COF. (B) Whole joint COF at 6 months of age. The COF of GT/KO, GT/GT and GT^{R2mo}/GT limbs increased with duration of cyclic loading, whereas GT*/KO limbs did not increase with time. After 15 minutes of cyclic loading, GT/KO limbs had significantly higher COF compared to GT*/KO limbs. GT^{R2mo} mice did not show improvement in COF compared to GT/GT or GT/KO limbs. GT*/KO limbs had significantly lower COF compared to GT/KO after 15 minutes of cyclic loading, and compared to all other groups following after 30 minutes of cyclic loading. Data points and error bars indicate mean $\pm$ SD.

H&E staining revealed some cartilage damage in all joints, with the exception of those expressing lubricin from birth (**Figure 5.3**). Joint damage included protein deposition, chondrocyte cloning, surface fibrillation, synovial hyperplasia and changes in the morphology of the menisci. Histological scoring of 2 month old mice showed significantly lower total scores for GT*/GT joints that expressed lubricin from birth ($p<0.05$) (**Figure 5.4A**). At 6 months of age, cartilage degeneration was increased in all genotypes that do not express lubricin from birth, and was significantly lower in GT*/KO mice compared to other genotypes ($p<0.05$) (**Figure 5.4B**). Furthermore, initiation of Prg4 expression did not prevent subsequent cartilage damage in GT^{R2mo}/GT mice compared to GT/GT and GT/KO littermates.

Active caspase-3 was observed in all joints at 2 months, but was most pronounced in GT/KO and GT/GT compared to GT^{R3wk}/GT and GT*/GT joints, qualitatively (**Figure 5.5**). Likewise, active caspase-3 was observed in all joints at 6 months of age. GT^{R2mo}/GT, GT/GT and GT/KO mice had similar amounts of apoptosis, qualitatively, but there were few apoptotic cells in GT*/KO joints.

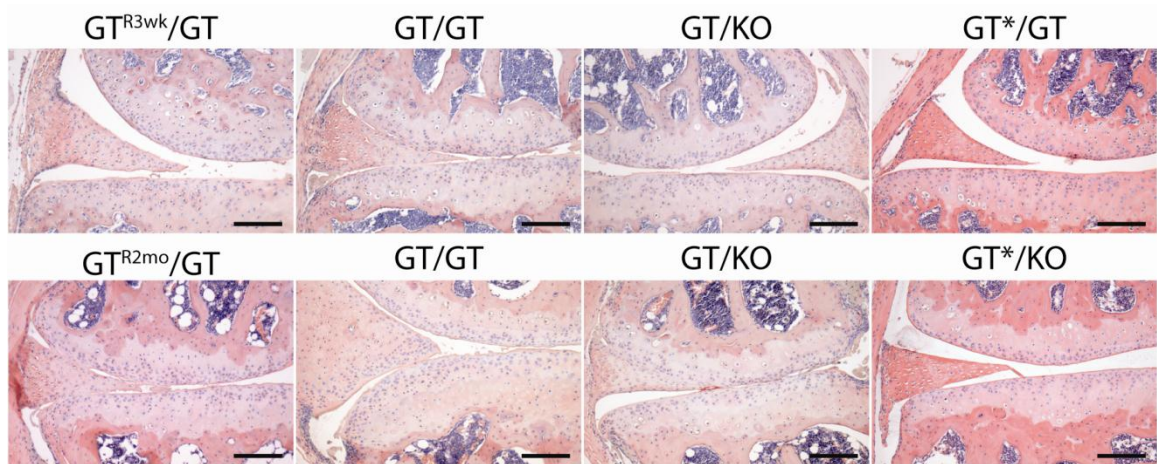


Figure 5.3: H&E staining of coronal sections for genetrapp mouse genotypes. We observed increased joint damage, including protein deposition, chondrocyte cloning, surface fibrillation and clefts and synovial invasion in GT^{R3wk}/GT, GT/KO, GT/GT mice 2 months of age (top row). GT/KO and GT/GT mice 6 months of age (bottom row) showed increased damage compared to 2 month old mice, and damage was not attenuated by lubricin activation at 2 months of age (GT^{R2mo}/GT). GT*/GT and GT*/KO mice that expressed lubricin from birth had very few signs of degenerative changes.

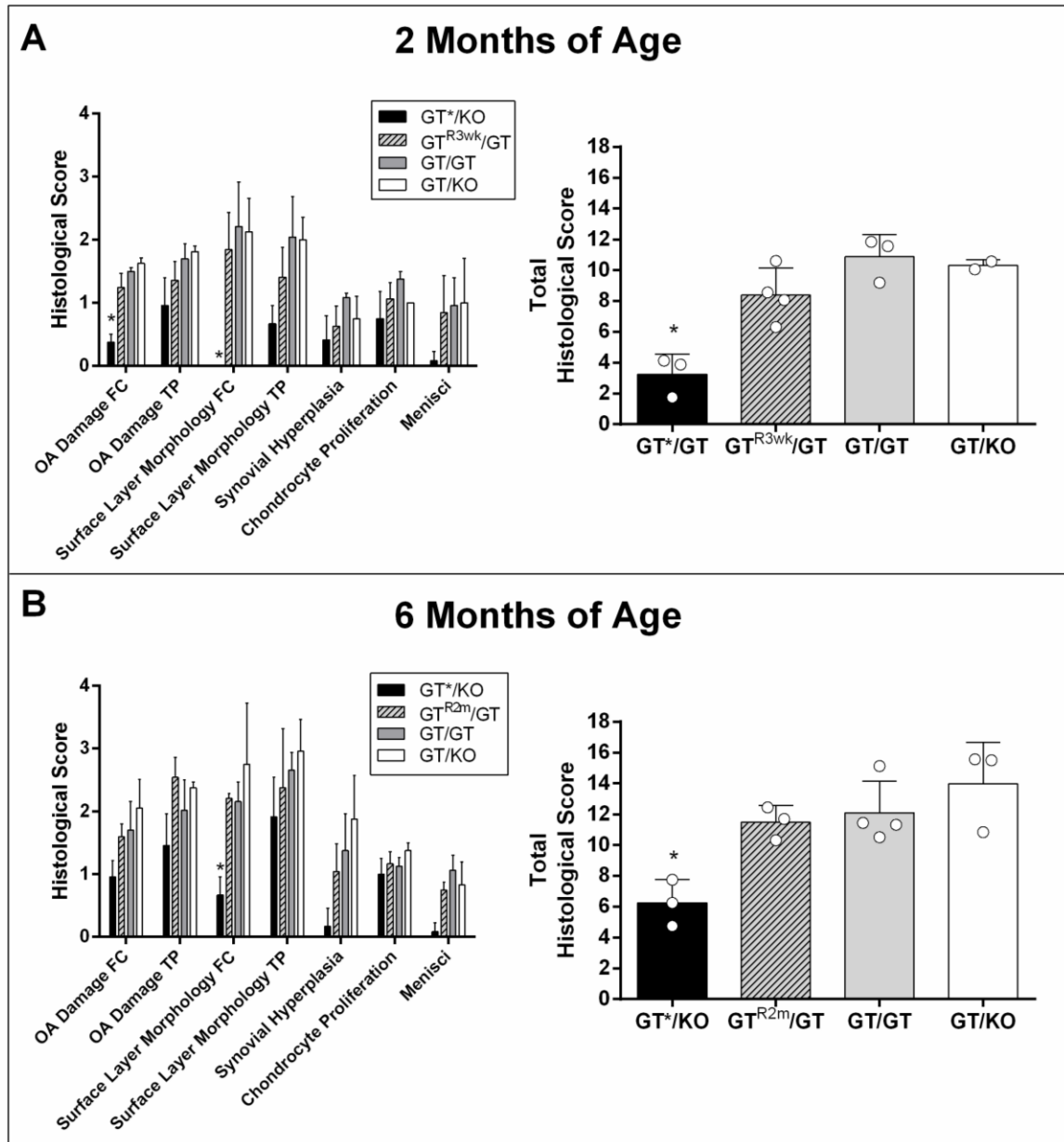


Figure 5.4: Histological Scoring. A.) At 2 months of age, GT*/GT mice had significantly lower total histological scores compared to other genotypes and GT^{R3wk} mice tended to have lower scores compared to GT/GT and GT/KO, but the difference did not reach significance. B.) At 6 months of age, GT*/KO mice had significantly lower total histological scores compared to other genotypes. There were no significant differences in histological scores between GT^{R2m}, GT/GT and GT/KO mouse joints.

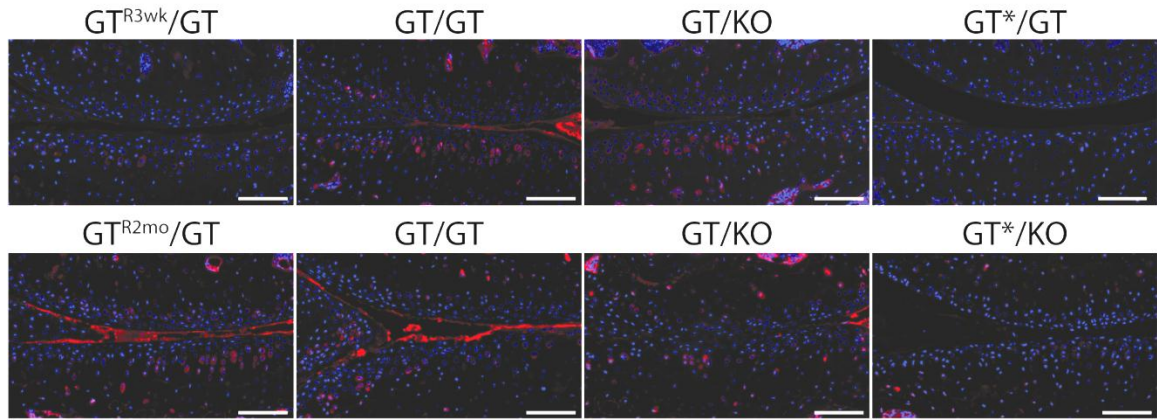


Figure 5.5: Immunofluorescence staining for apoptosis of coronal sections for genetrapp mouse genotypes at 2 months of age (top row) and 6 months of age (bottom row). Active caspase-3 staining (red) indicates apoptotic cells and DAPI (blue) staining labels nuclei. In 2 month old mice, we observed more apoptotic cells in GT/GT and GT/KO mice compared to GT^{R3wk}/GT mice and GT^*/GT mice, indicating that genetic expression of lubricin at 3 weeks was chondroprotective. In 6 month old mice, we observed a similar amount of apoptosis in GT^{R2mo}/GT , GT/GT and GT/KO mouse joints, but few apoptotic cells in GT^*/KO joints. These results indicate that lubricin expression at 2 months of age was unable to prevent chondrocyte apoptosis compared to GT/GT and GT/KO mice. We also observed red staining at the surface of damaged joints, indicating protein deposition. Scale bars indicate 100 μm .

5.5 Discussion

Lubricin-null mice appear normal at birth, but by 2-weeks of age, these joints experience cartilage deterioration and synovial hyperplasia, which eventually results in joint failure (2). In this study, we show that cartilage can be rescued by endogenous lubricin production, as measured by COF, if initiated early enough in the progression of the disease.

Interestingly, at 2 months and 6 months of age, the whole joint COF at $t=0$ was not significantly different between mouse genotypes, which is the case between 10-week-old $\text{Prg4}^{-/-}$ and $\text{Prg4}^{+/-}$ mice (1), but following 30 minutes of cyclic loading, we observe significant trends that indicate lubricin concentration plays a major role in the preservation of cartilage. A comparison between different initiation times for $\text{GT}^{\text{R}}/\text{GT}$ mice indicates that early intervention provides a much better outcome in terms of whole joint COF (**Figure 5.6**). This finding indicates that early intervention with lubricin is crucial to the preservation lubricin-null knee joints and carries clinical implications regarding time of intervention in human patients.

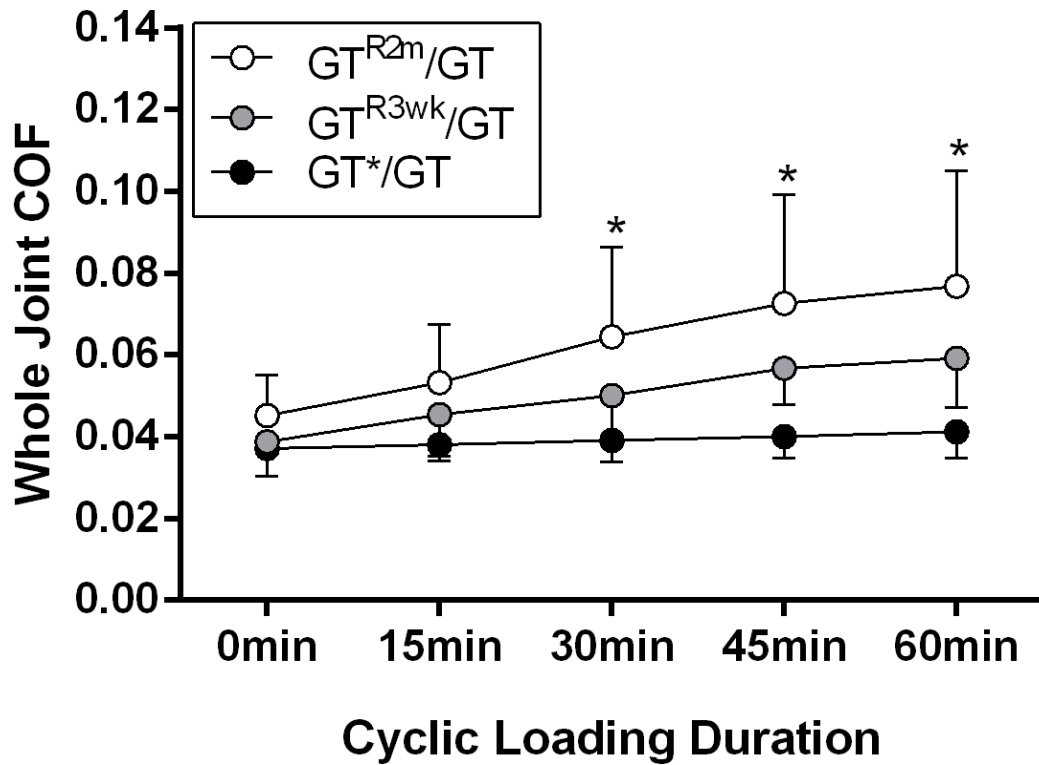


Figure 5.6. Comparison of lubricin initiation of mice according to time. Initiation at birth or 3 weeks reduced COF following 60 minutes of cyclic loading, but Prg4 initiation at 2 months failed to provide chondroprotection. Following 30, 45 and 60 minutes of cyclic loading, GT^{R2m}/GT had significantly higher COF compared to GT^{*}/GT mice, that express lubricin from birth, whereas there was no significant difference in COF between GT^{3wk}/GT and GT^{*}/GT mouse knees.

We observed an increase in whole joint COF over time for all groups that did not have lubricin initiation at birth. The increase in whole joint COF for GT^{R3wk}/GT mice over time, although not as pronounced compared to GT/KO or GT/GT littermates, occurred despite the production and availability of lubricin, which may indicate an insufficient amount of lubricin available to the joint or, alternatively, it could be related to degenerative changes observed in GT^{R3wk}/GT mouse joints, including disruption of the articular surface, which has a detrimental effect on boundary lubrication. The inability of lubricin to lower the whole joint COF of GT^{R2mo}/GT mice compared to GT/GT or GT/KO mice at 6 months of age supports the hypothesis that lubricin is unable to adequately lubricate and protect joints that have significant surface damage.

Although we observed histological damage in GT^{R3wk}/GT mice that was similar to lubricin-null phenotypes at 2 months, we observed fewer apoptotic cells in these joints, which is indicative of chondroprotection. In contrast, GT/GT and GT/KO mice had apoptotic cells that were primarily located just below the flattened superficial chondrocytes, which has been observed in Prg4^{-/-} mouse cartilage (9).

Following cyclic loading, we have observed that the histological phenotype of the GT/GT mouse to be less severe compared to GT/KO littermates, and that the GT/GT mouse COF tended to be lower compared to GT/KO mice. This phenomenon may be linked to “leakiness” in the genetrap which allows a small amount of lubricin expression in GT/GT mouse joints. Two limitations come with this observation: a small amount of lubricin is able to attenuate cartilage damage and provide boundary lubrication and the damage that has accrued at the time of genetrap initiation in GT/GT mice may not be as severe as that seen in Prg4^{-/-} or GT/KO at some selected time point. For example, in the

late intervention group, the whole joint COF overlaps between the GT^{R2mo}/GT and GT/GT cases, indicating that after 2 months, additional lubricin expression was unable to prevent subsequent damage or provide enough boundary lubrication to these joints.

Previously, lubricin supplementation via intraarticular injection (tribosupplementation) has been used to prevent cartilage damage in a number of secondary osteoarthritis models, with favorable results (10-13). Lubricin was administered prior to significant cartilage damage and at a time following a catabolic state in the traumatized joint (14). Furthermore, the rats in secondary osteoarthritis models have the ability to produce endogenous lubricin, and expression is encouraged by tribosupplementation (11, 12). In contrast, patients with CACP and lubricin-null mice are unable to produce endogenous lubricin, so lubricin concentration would be determined solely by injection dosage and half-life, which is on the order of 12 hours (13). The present transgenic approach has an advantage over lubricin-injections because it allows for continuous endogenous production in the superficial zone chondrocytes and synoviocytes onto the cartilage surface and in the synovial fluid, instead of simply introducing exogenous lubricin into the synovial fluid.

At the time of lubricin initiation by Tamoxifen injection, lubricin-null mouse cartilage already shows protein deposition and cartilage damage, which may impede exogenous lubricin from attaching to the cartilage surface (15), thus thwarting its ability to prevent additional cell and protein adhesion and act as a boundary lubricant during locomotion. In contrast, genetically rescued limbs are able to produce lubricin in a consistent manner both onto the cartilage surface via synoviocytes and superficial zone chondrocyte expression. Recently, the overexpression of lubricin via an adenoviral vector

in a mouse traumatic injury model also revealed that Prg4 is highly chondroprotective and preventative of post-traumatic osteoarthritis (16).

A number of limitations accompany this transgenic approach, which were detected via whole joint COF, especially following cyclic loading. We observed that even without gene recombination, GT/GT mice trended towards lower COF and less joint degradation, as determined histologically, compared to GT/KO littermates. Furthermore, 2 month and 6 month GT/GT mice had similar COF as GT/GT mice at 2 months of age (Figure 6). To ensure a suitable negative control, we confirmed that GT/KO mice appear more like Prg4^{-/-} mice at 2 months of age.

Currently the treatment options are limited for patients with CACP and inflammatory conditions in which lubricin is deficient in joints, such as following traumatic joint injury. Gene therapy provides consistent endogenous lubricin production, which is essential for boundary lubrication and chondroprotection in articular joints, and may be a viable therapy for patients deficient in lubricin. The nascent expression of lubricin and re-establishment of chondroprotection also suggests that the re-introduction of recombinant lubricin via intraarticular injection may be a viable temporizing treatment in young patients with CACP who have unmet medical needs.

5.6 Acknowledgements

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Chapter 6

The Effect of TNF- α Inhibition on the Microscale Mechanical Properties of Articular Cartilage in a Rat ACL Transection Model

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6.1 Abstract

Injury to the anterior cruciate ligament (ACL) is a significant risk factor for secondary osteoarthritis (OA), due to a number of factors including loss of lubricin due to cytokine activity during inflammation. The objective of this study is to measure the microscale stiffness of rat cartilage following ACL transection and evaluate the ability of etanercept to prevent loss of cartilage stiffness. We performed unilateral ACLT on Lewis rats. Three (3), 6 and 9 weeks following surgery, the microstiffness of femoral condyles was measured and the tibial plateaus were analyzed histologically to observe macroscale degeneration and matrix components. We hypothesized that etanercept treatment would prevent loss of microstiffness compared to untreated ACLT. We observed significantly lower microstiffness for untreated and etanercept treated ACLT cartilage compared to capsulotomy. These results indicate that microstiffness is lost within 3 weeks of ACLT, prior to significant histological changes. Furthermore, microstiffness may be lost prior to day 7, when etanercept is administered. This investigation prompts additional studies into the use of etanercept as a long-term solution for preventing cartilage degeneration associated with a loss of lubricin following injury.

6.2 Introduction

Injury to the anterior cruciate ligament (ACL) results in altered joint biomechanics (1), inflammation induced changes to lubricating components of synovial fluid, particularly lubricin (2-4), and enzymatic degradation to the articular cartilage (5, 6). As a result, ACL injury is widely accepted as a significant risk factor in the progression of secondary osteoarthritis (OA) (1, 3, 4, 7, 8).

Previously, cartilage damage and joint lubrication have been restored to rodent models by replenishment of lubricin via intraarticular injection or by the inhibition of tumor necrosis factor- α (tnf- α), an inflammatory cytokine released following injury that has been shown to down-regulate lubricin in a traumatized joint (3, 4, 9-11). Tnf- α inhibition via 2 weekly subcuticular injections of etanercept around the area of the joint following ACL transection (ACLT) in a rodent model prevented lubricin down-regulation and preserved cartilage integrity 4-weeks following surgery.

Atomic force microscopy (AFM) allows users to reliably measure stiffness in the surface of the superficial zone (12). Since the superficial zone is the first region to lose proteoglycan staining and become fibrillated following ACLT, changes detected in this region would provide early insight into the etiology of cartilage degeneration. We hypothesize that etanercept injection would attenuate joint damage following ACLT, resulting in the preservation of mechanical stiffness in the superficial zone, in addition to preventing cartilage damage and chondrocyte apoptosis. The results of this study could provide insight into the feasibility of etanercept treatment to prevent lubricin loss following traumatic joint injury.

6.3 Methods

6.3.1 Surgical ACL Transection (ACLT)

Skeletally mature male Lewis rats (8-10 wks) underwent ACLT of the right hind leg (n=24). The rats were anesthetized via an intraperitoneal injection of ketamine and medetomidine and prepared for surgery using the standard operating room procedure. A prophylactic antibiotic (Cefazolin, dose 20 mg/kg) was administered via an intramuscular injection. An incision was made laterally to the right joint and the joint capsule opened through the lateral patellar tendon. The patella was displaced laterally with the leg in extension. The ACL was sectioned using a #11 scalpel with the knee in flexion. A positive anterior drawer test was used to ensure full ACL transection. The patella was relocated and the tendon incision repaired using 5-0 PDS II (Polydioxanone, Ethicon) resorbable suture. The joint capsule and skin incision were sutured closed and sealed with VetBond tissue adhesive (3M). Sham capsulotomy surgeries (n=9) were performed by skin and tendon incision, patellar displacement and replacement, and wound closure. All experimental procedures were approved by the Rhode Island Hospital Animal Welfare Committee.

ACL transected rats were randomly divided into groups that were given etanercept (n=7), or no injection (n=15). In the etanercept group, ACL transected rats were injected with a 0.5 mg/kg subcutaneous dose (total volume 0.05 ml) of etanercept (Enbrel, Immunex Corporation, CA) in the area of the knee joint. This dosing regimen was chosen based on the optimal dose reported previously (3).

At the pre-determined time points of 3, 6 or 9 weeks post-operatively, rats were euthanized by CO₂ asphyxiation, and the right hind limbs were harvested. Three joints that underwent ACLT were analyzed histologically one week post-operatively.

6.3.2 Microstiffness Measurement

Medial and lateral femoral condyles of the right hind limbs were harvested and kept in PBS supplemented with a protease inhibitor cocktail (Roche) at 4°C. Following the isolation and separation of the femoral condyle into medial and lateral compartments, the samples underwent AFM analysis in three compartments using two probe types to analyze microstiffness and map the collagen structure of the cartilage surface. Prior to testing, specimens were mounted on Petri dishes. Samples were kept hydrated in PBS containing protease inhibitor throughout testing, which was concluded within two days of harvest.

Stiffness measurements of articular cartilage were performed by indentation in three areas of the femoral condyle. Load-displacement curves were recorded in contact mode at a trigger point that corresponds to the maximum deflection, of 150 nm, which corresponds to a load of ~800 nN, for the microspheres (SiO₂ Glass Particle 5µm in diameter, mounted on a cantilever, nominal spring constant 7.5 N/m; Novascan, Ames, IA). Elastic moduli were determined by fitting a modified Hertz model to the experimental data in MATLAB software (Mathworks, Natick, MA), as described previously (13, 14). Stiffness measurements for the medial and lateral condyles were averaged and reported as a mean stiffness of each rat knee.

6.3.3 Surface Scanning

Images of the cartilage surface $20\text{ }\mu\text{m}^2$ were taken in contact mode using a sharp, pyramidal probe (115 mm long SiN cantilevers, nominal cantilever spring constant of 0.32 N/m; Veeco, Santa Barbara, CA) at a scan rate of $5\text{ }\mu\text{m/s}$ and an applied force of $\sim 5\text{ nN}$.

6.3.4 Immunohistochemistry

At the time of harvest, tibial plateaus were fixed in 10% formalin, decalcified and paraffin embedded. Thin ($6\text{ }\mu\text{m}$) coronal sections were taken for histological analysis. Sections were heated to 60°C for 30 minutes, deparaffinized, and hydrated in xylene and alcohol. Sections also stained with Safranin-O/Fast Green for assessment of cartilage sGAG content and histological analysis. Additionally, prepared sections were quenched in endogenous peroxidase in 3% hydrogen peroxide for 10 min and antigen retrieval was performed using a pepsin solution (ThermoScientific). Likewise, a rabbit polyclonal antibody against active caspase 3 (catalog no. ab13847; Abcam) at a 1:50 dilution was added to slides at 4°C overnight according to Vectastain procedures, and sections were counterstained with Methyl Green. Likewise, a mouse monoclonal antibody against collagen II (catalog no. MS-235-P; ThermoScientific) at a 1:100 dilution was added to sections at 4°C overnight according to Vectastain procedures.

6.3.5 Statistical Analysis

Comparisons between treatment groups and time points were analyzed using a two-way ANOVA with Tukey's comparison test. Statistical analysis was performed using Prism 6.0 statistical software (GraphPad, La Jolla, CA).

6.4 Results

Three weeks post operatively, microstiffness was significantly lower than capsulotomy (823.8 ± 480 kPa, $n=3$) in both the untreated and etanercept treated joints (328.5 ± 26.2 kPa, $p=0.046$, $n=3$ and 304.2 ± 40.53 kPa, $p=0.018$, $n=2$ respectively) (**Figure 6.1**). Six weeks post-operatively, etanercept treated joints had slightly lower mean microstiffness (267.5 ± 137 kPa, $n=3$), which was significantly lower than sham (749.8 ± 114 , $p=0.0289$, $n=3$), while microstiffness increased slightly in untreated samples (362.1 ± 231.7 kPa, $p=0.052$ compared to capsulotomy, $n=5$). Both untreated and etanercept treated limbs increased in microstiffness (419.8 ± 148 kPa, $n=5$ and 458.3 ± 169 kPa, $n=2$, respectively) at 9-weeks post-operatively. The microstiffness of cartilage was 577.6 ± 85.3 kPa for sham joints. Stiffness of sham cartilage decreased with time, but the changes over time were not significant.

Surface scans taken with the sharp pyramidal probe of the superficial zone of each sample showed a breakdown in cartilage from ACL transected joints, both from the untreated and treated groups (**Figure 6.2**). Cartilage from the capsulotomy group had an even surface with small cartilage bundles. Compared to capsulotomy, ACLT cartilage surfaces appeared broken with observable holes for samples measured 3, 6 and 9 weeks post-operatively from each group. At 9 weeks post-operatively there are visible holes in the matrix and the appearance of collagen fiber thickening, with small fibers surrounding areas missing matrix.

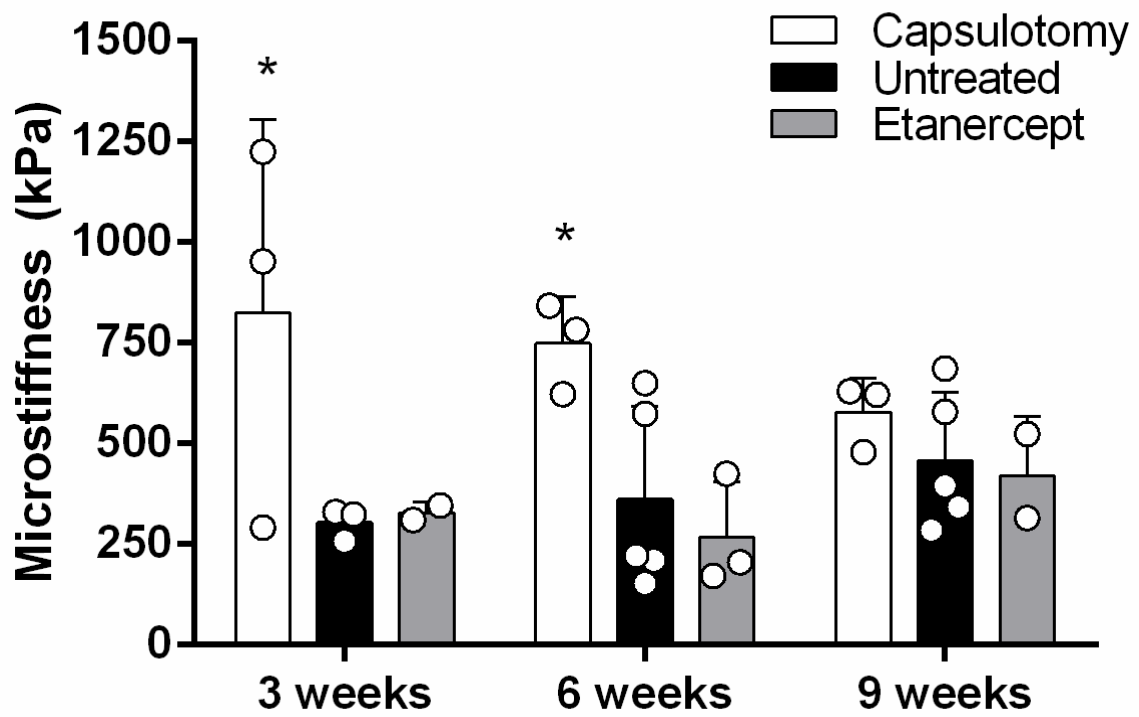


Figure 6.1: Microstiffness of rat joints over time. Three weeks following ACLT, cartilage from the femoral condyles of untreated and etanercept treated joints showed a dramatic decrease in microstiffness compared to capsulotomy cartilage. Six weeks following ACLT, etanercept treated joints had significantly lower stiffness compared to capsulotomy cartilage.

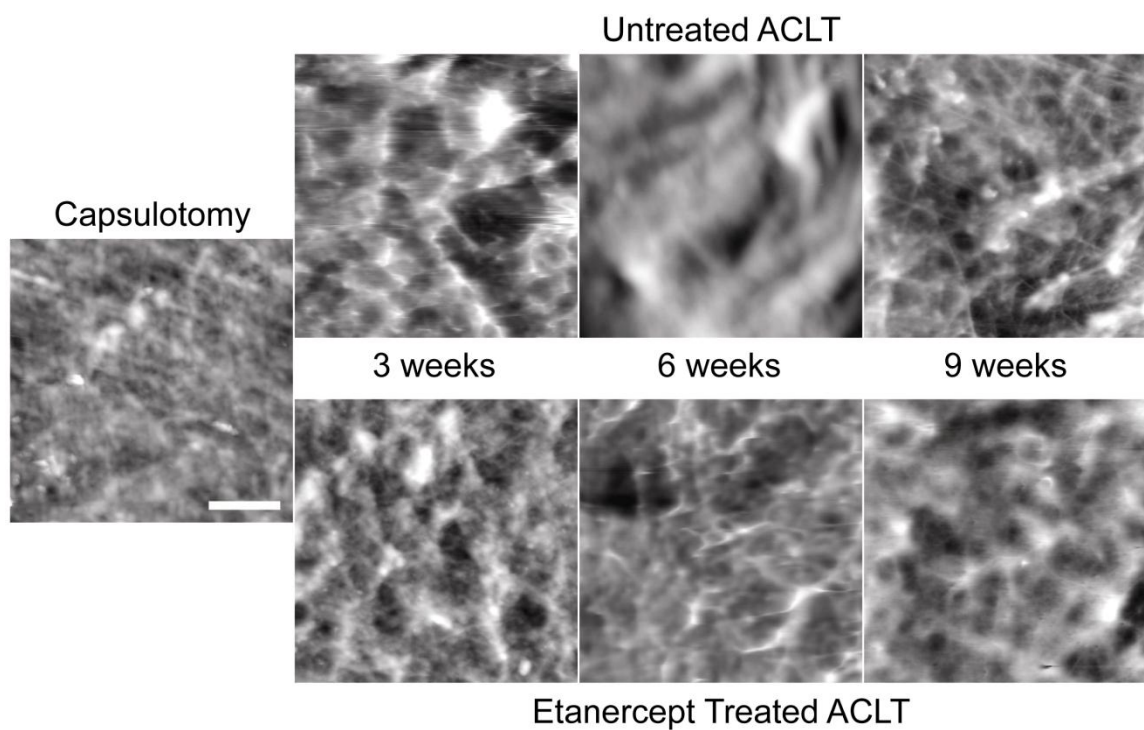


Figure 6.2: Surface scans of rat cartilage from each treatment group. Sham (capsulotomy) cartilage had an even surface with small cartilage bundles uniformly distributed parallel to the cartilage surface. Untreated and etanercept treated ACLT cartilage showed some degenerative changes at 3 weeks, and had thickened collagen bundles with large holes indicating spaces between bundles. Scale bar represents 5 μ m.

The progression of OA was observed in the untreated and etanercept treated joints in Safranin-O/FG stained slides (**Figure 6.3**). Sham joints showed uniform proteoglycan staining and a smooth, intact joint surface and flattened superficial chondrocytes at each time point. We observed a loss of staining and surface roughness in the untreated joints 3 weeks post operatively, as compared to etanercept treated joints and capsulotomy joints. Six weeks post-operatively, there were areas of hypercellularity in untreated and etanercept treated limbs. The untreated group also showed superficial zone fissures and a loss of superficial chondrocytes 6 weeks post-operatively. At 9 weeks, there was significant loss of stain in the superficial zone and into the middle zones of the untreated and etanercept groups as well as fissuring. An acellular substance characteristic of fibrocartilage was observed in the superficial zone of both treatment groups. There was also a loss of superficial chondrocytes in both the untreated and etanercept treated groups 9 weeks post-operatively.

We observed chondrocytes positive for active caspase-3, which indicates apoptosis, in untreated and etanercept treated ACLT cartilage (**Figure 6.4**). The majority of apoptotic cells were located in the superficial and upper middle zone. Capsulotomy treated joints had very few apoptotic cells.

Collagen II staining showed increased staining in capsulotomy treated joints with time (**Figure 6.5**). Untreated limbs showed some staining 3 weeks post operatively, but less staining 6 and 9 weeks post-operatively. Etanercept treated joints had staining 3 weeks post operatively and heavy staining 9 weeks post-operatively, especially surrounding cell clusters.

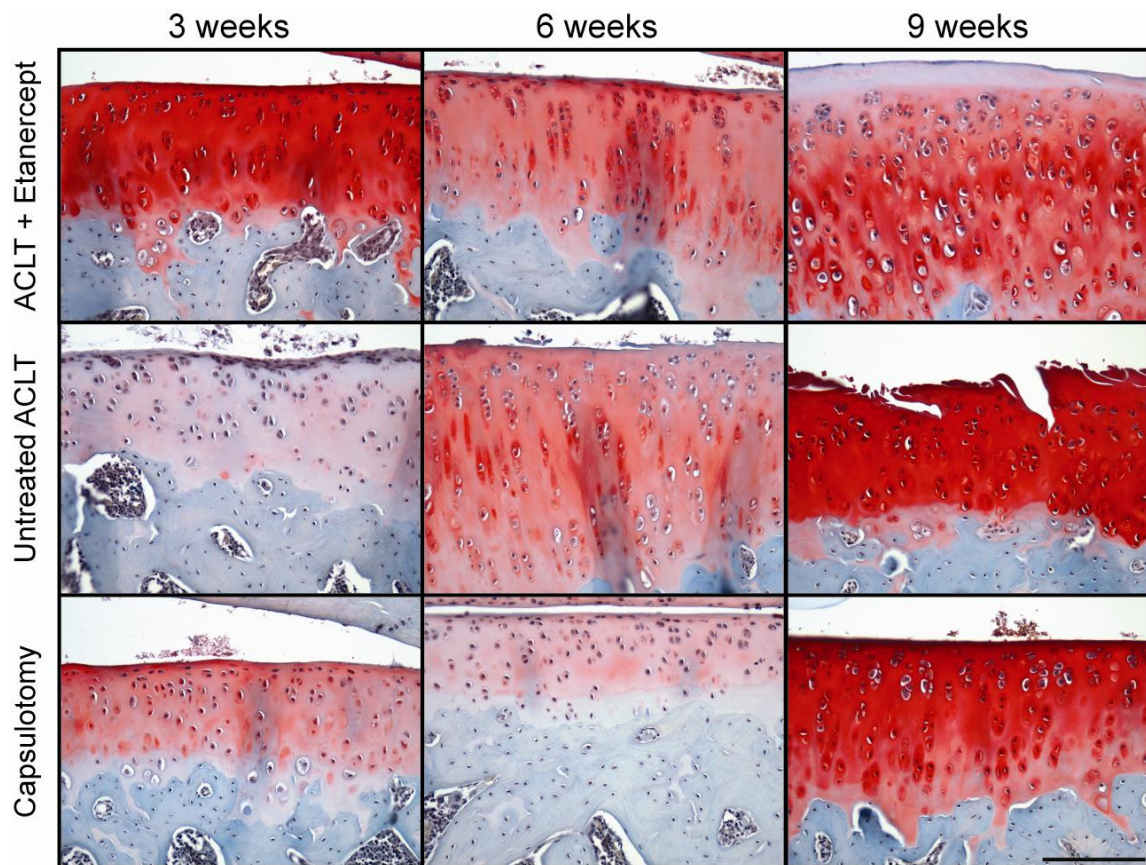


Figure 6.3: Safranin-O/Fast Green stained tibial plateaus of median samples based on histological damage. We observed joint degradation over time in etanercept and untreated ACLT joints, which was characterized by loss of surface architecture, chondrocyte cloning, protein deposition and surface disruption. We also observed synovial invasion in untreated ACLT joints. Capsulotomy treated joints had intact cartilage surfaces and uniform proteoglycan staining. Scale bar indicates 100 μ m.

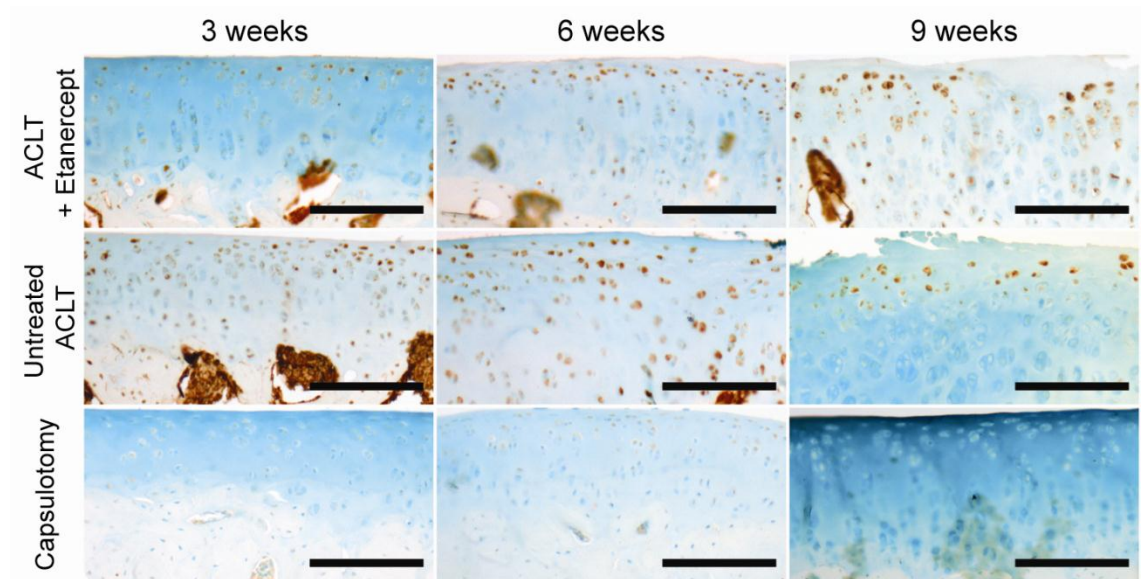


Figure 6.4: Apoptosis in rat cartilage. We observed cells positive for active caspase-3 (brown) in etanercept and untreated ACLT cartilage. Generally, apoptosis occurred in the superficial and upper middle zone at 3-weeks post operatively, and extended deeper into the cartilage with time following injury. Capsulotomy treated joints had very few apoptotic cells. Scale bars indicate 100 μ m.

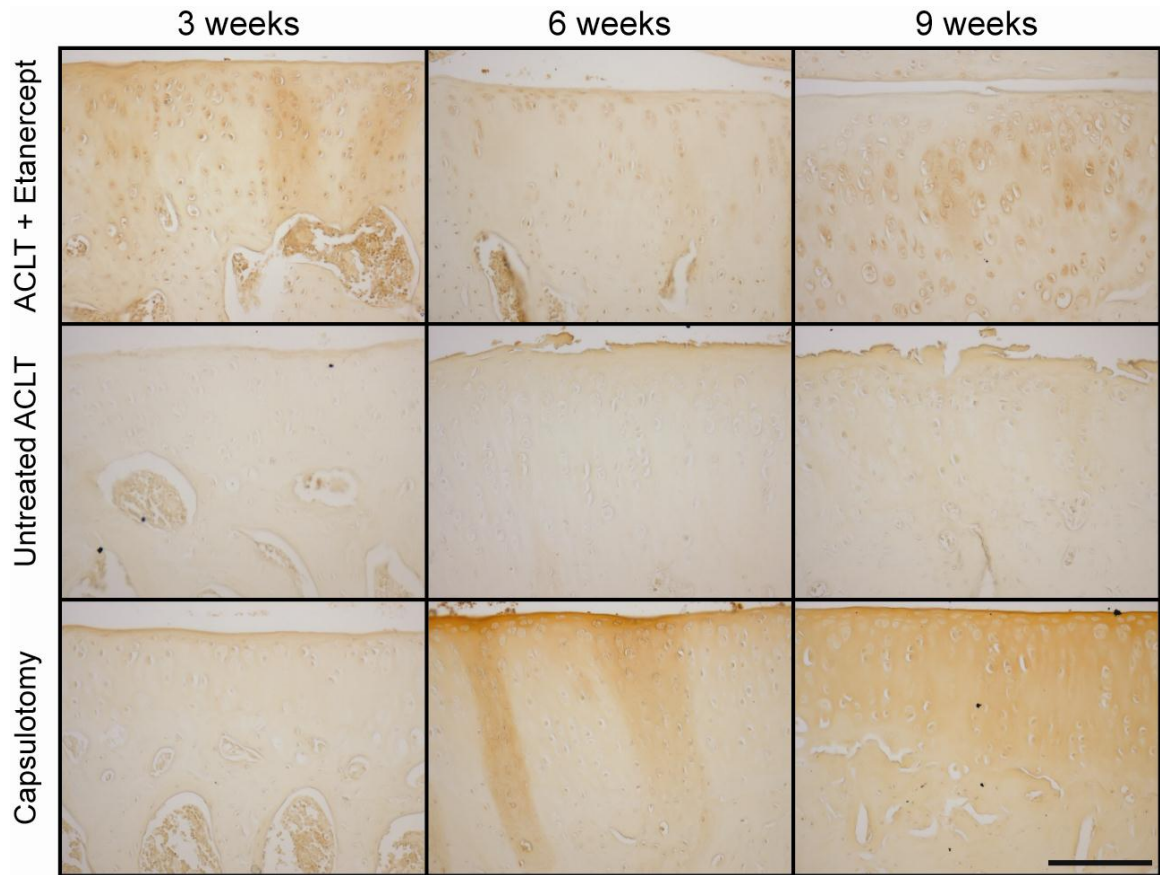


Figure 6.4: Collagen II staining of tibial plateaus. We observed an increase in collagen II staining in etanercept treated joints 3 weeks post-operatively, but an overall decrease in staining over time. Untreated ACLT joints had decreased staining compared to capsulotomy treated joints as well as etanercept treated ACLT. Capsulotomy joints had uniform collagen II staining at all time points, indicating normal chondrocyte function. Scale bar indicates 100 μm .

6.5 Discussion

ACL injury results in an influx of inflammatory cytokines, including $\text{tnf-}\alpha$ and $\text{IL-1}\beta$ (15), which contribute to a loss of lubrication in the knee joint following downregulation of lubricin (2-4) and cartilage matrix breakdown (5, 6, 16). Etanercept is a $\text{tnf-}\alpha$ receptor that competes with cell-surface receptors, acting as an antagonist against the metabolic effects of $\text{tnf-}\alpha$. As a result, etanercept has been shown to attenuate the downregulation of lubricin that occurs following injury, thus preserving some lubricin concentration in the synovial fluid and on the cartilage surface (3).

In this study, we investigated the changes in microstiffness of the superficial zone in rats that underwent ACLT and the ability of etanercept treatment to prevent a loss of mechanical properties in rat cartilage following ACLT. Based on histological evidence, our study coincides with the findings of Elsaid, et al. at 4 weeks post-operatively, where etanercept treated joints appeared healthier than untreated joints (3), but we did observe a decrease in microstiffness in both untreated and etanercept treated joints 3 weeks post-operatively. In the short term, joints treated with etanercept appear to retain the appearance of healthy cartilage as seen histologically 3 weeks post-operatively.

Six months post-operatively, the etanercept treated knees showed more dramatic decreases in microstiffness than untreated ACLT knees. As injury progressed to 6 and 9 weeks post-operatively, the cartilage in the etanercept treated group degraded on a similar timeline as untreated cartilage. AFM measurements and surface scans detected damage to the cartilage before there was visible loss of sGAG in the matrix.

A progressive decrease in microstiffness due to a disease state has been observed in murine and canine studies of degenerative joint diseases (12, 17, 18), and coincides with loss of superficial zone cells (12) and loss of cartilage structure (12, 17, 18), which have also been observed in rats that underwent ACLT in this study. We observed a large number of apoptotic cells in rats that underwent ACLT at all time points, especially in the superficial and upper middle zones of cartilage (**Figure 6.4**), which coincides to previous findings in rodent models of degenerative joint diseases (6, 10, 19). Apoptotic cell death may have resulted directly from cytokine activation via cell death receptors (6, 20), production of nitric oxide (6, 16, 21), or due to elevated shear forces and deformation resulting from lack of lubrication (3, 4, 10, 19). This loss of cells coincides with a loss of collagen II staining, which has been previously observed in the rat ACLT model (5), and may show a link between cellular apoptosis and integrity of the cartilage matrix structure. Since chondrocytes are the only cell type in articular cartilage, they are solely responsible for maintenance of the cartilage matrix. In a disease state, chondrocytes produce catabolic factors, including matrix metalloproteinases, which break down the cartilage matrix (6). As a result, changes in chondrocyte metabolism, apoptosis and subsequent loss of cells contribute to an overall loss of mechanical properties in the rat cartilage following ACLT.

Atomic force microscopy is a surface analysis technique that has been used to measure indentation stiffness at the microscale (12, 17, 22, 23). This technique is useful for quantifying stiffness of cartilage from joints that are too small for conventional indentation methods, including small rodents. The small probe size used in these studies dictates that microstiffness represents the very upper layers of the superficial zone, where loss of proteoglycan and surface architecture occur first in the progression of OA

following traumatic injury, and some loss of superficial zone chondrocytes, cartilage swelling, and minor fibrillation, and increased concentration of C-terminal telopeptide of type II collagen have been observed 7 days following ACLT (5, 24). We detected active caspase-3 and cloning 7 days post-operatively (**Figure 6.6**), which appeared to be coupled with increased proteoglycan production, cellular damage and loss of homeostasis that may have occurred prior to etanercept administration, resulting in joint degeneration prior to the completion of treatment, 14-days post-operatively.

Using AFM, cartilage can be imaged with high resolution in a more native condition compared to SEM imaging. AFM analysis, however, comes with a number of restrictions related to the height limitations of the cantilever and probe. As a result, very uneven or compliant surfaces are difficult to scan. “Sticky” protein deposits can also cause large changes in height along damaged cartilage topography, which was apparent in this study. In addition to these limitations, the maximum area that can be scanned at one time is 90 μ m x 90 μ m, which is a very small proportion of the cartilage surface. Load bearing areas of the femoral condyles were analyzed in order to minimize differences between scanning areas, but unfortunately, we were unable to scan the same area twice using each type of probe.

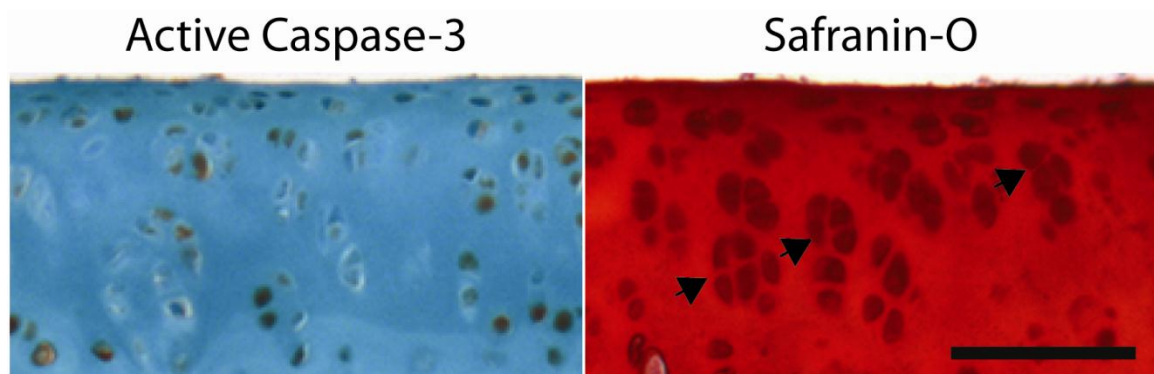


Figure 6.6: Tibial plateau of rat cartilage 7 days following ACLT. Active caspase-3 staining revealed apoptotic cells (brown). Safranin-O staining revealed normal proteoglycan staining and smooth articular surface, but we also observed extensive chondrocyte cloning (arrows), indicating a cellular response following ACLT. Scale bar indicates 50 μ m.

While OA is thought to be mainly associated with gradual degradation due to aging, acute trauma to the joint is a distinct epidemiological risk factor that will accelerate its progression. ACL injury is a common traumatic sports injury (4, 25). Although many patients undergo ACL reconstruction following injury, these patients are still at high risk for developing OA, due to the mechanical and biological changes in the joint in the period preceding surgical repair (7, 26-29). Previous animal studies have shown that ACLT will induce OA-like histopathological changes if left untreated. However, interventions involving supplementation (9-11, 19, 30, 31) or overexpression of lubricin (32) restores boundary lubrication and protects cartilage surfaces from wear and cell loss.

Treatment with subcutaneous etanercept provides a more appealing and patient-friendly option compared to intraarticular lubricin injection, and is already available for the treatment of inflammatory diseases, including rheumatoid arthritis. Self-administration eliminates the need for a visit to a physician, in contrast to procedures that involve entering the joint space, such as arthrocentesis and viscosupplementation. However, intra-articular administration of etanercept may also provide a more targeted approach to decreasing $\text{tnf-}\alpha$, without systemic effects (3).

This study investigates microstiffness of femoral condyles in order to further assess the damage to rat cartilage following ACLT. We saw that cartilage stiffness decreases even 3 weeks post-operatively, when loss of proteoglycan was not detected in all samples. Etanercept treatment at days 7 and 14, while able to provide chondroprotection 4 weeks post-operatively (3), may not be adequate to prevent long term damage without additional administration. Further studies would be required to

determine the extent of chondroprotection provided by as a treatment to prevent chondropathy prior to ACL repair.

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Chapter 7

Discussion and Conclusions

7.1 Discussion

The major findings of this work are found in Chapters 2-7, but additional observations were made over the course of these studies that were not included in the previous chapters and will be discussed in the present chapter.

7.1.1 Mechanotransduction pathway relating friction and apoptosis

In Appendix A, we investigate the mechanopathway that connects the deformation resulting from elevated friction to a cellular response resulting in apoptosis. We used the bovine bearing system described in Aim 1 combined with analysis of mRNA to investigate the role of integrins ($\alpha 1\beta 1$ and $\alpha 5\beta 1$), focal adhesion kinase (FAK), Erk1, p53 and bcl-2 in the preservation of cells or the induction of apoptosis following friction and wear testing. We hypothesized that integrin-related pathways play a vital role in the prevention or induction of friction-induced apoptosis. The altered interaction between integrins and the extracellular matrix (ECM) could lead to cellular dysfunction following deformation of superficial zone chondrocyte in the presence of high friction. Surface and sub-surface strain could translate to cellular strain and tear away ECM from integrin receptors in the event that ECM is torn away from integrin receptors. This event could initiate an intracellular cascade resulting in apoptosis.

Preliminary studies indicate an mRNA upregulation of bcl-2 coupled with a downregulation of p53 in lubricin-lubricated bearings, whereas an upregulation of p53 and downregulation of bcl-2 was observed in CACP-SF and PBS lubricated bearings, which had many apoptotic cells. We also measured mRNA levels of Erk1, which indicates cell survival, and observed its elevation in HSL lubricated samples compared to control, but down regulated in PBS and CACP-SF lubricated samples, indicating a loss of survival signaling following the friction and wear testing.

We developed a hypothesis outlining an integrin-mediated pathway relating friction and chondrocyte apoptosis. In future studies, we plan to employ RNAseq, qRT-PCR and Western blot to identify key factors relating friction and chondrocyte apoptosis in bovine cartilage bearings following friction and wear testing. These findings may provide insight into the importance of boundary lubrication in terms of cell survival and may also identify additional targets to prevent cellular loss in cases of genetic or acquired deficiency of lubricin in articular joints.

7.1.2 Time course of chondropathy

These studies have collectively proposed a timeline in our understanding of chondropathy following loss of lubricin due to genetic deficiency or following trauma. In the absence of lubricin, we observed elevated coefficient of friction *in vivo* and *in vitro*, especially at the initiation of motion (Chapters 2 and 3). Elevated friction and adhesion between cartilage surfaces results in an increase in shear strain and deformation that propagates through the cartilage matrix, deforming the extracellular matrix and cells of the superficial and upper middle zones (Chapters 2 and 3) (1-5). We observed cellular

apoptosis that was directly related to high static COF in the superficial and upper middle zone chondrocytes, which occurred within the 20 minute duration of friction and wear testing (Chapters 2 and 3) (1, 2). Down-regulation of lubricin following surgical transection of the ACL (ACLT) in a rat model (6-8) resulted in a cellular response of both chondrocyte cloning and apoptosis 7 days post-operatively (Chapter 6). Three weeks following ACLT, we also observed a decrease in microstiffness of the superficial zone that was associated with a loss of sulfated glycosaminoglycans and increased surface roughness, compared to joints that underwent capsulotomy. At later time points (6, 9 and 10 weeks post-operatively), we observed an increased loss of proteoglycans, collagen II and loss of cells in joints that underwent ACLT (Chapter 6 and Appendix C). In the knee joints of 8-week-old mice that have a genetic deficiency of lubricin (*Prg4*^{-/-}, GT/GT and GT/KO), we observed elevated friction that was accompanied by protein deposition, synovial hyperplasia, irregular articular surfaces, cellular apoptosis and a loss of superficial zone cells (Chapters 2, 4 and 5) (2). The severity of joint damage increased with age, especially in terms of loss of cells, cartilage fibrillation, synovial hyperplasia and protein deposition. Loss of boundary lubrication due to a lack of lubricin leads to a shared response by both the lubricin-null mouse model and the ACLT rat model. Chondrocyte apoptosis was observed *in vivo* in the *Prg4* null mouse model, the *Prg4* genetrap mouse model and the rat ACLT secondary OA model and in the *in vitro* bovine cartilage model when lubricin was absent as a boundary lubricant. This mechanism provides an explanation as to why the number of chondrocytes is decreased in OA, and would also explain why the cartilage tissue cannot synthesize new ECM presumably due

to the loss of chondrocytes. Consequentially, the softening and fibrillation of articular cartilage, which are hallmarks of OA, then appear in concert with clinical symptoms.

7.2 Conclusions

7.2.1 Aim 1: Investigate friction and apoptosis in the articular joint

7.2.1.A – *In vivo* analysis: Measure friction and observe apoptosis in the knee joints of lubricin-null mice.

The initial part of the first aim in this dissertation was addressed in Chapter 1. We measured whole joint COF and apoptosis in $\text{Prg4}^{+/+}$, $\text{Prg4}^{+/-}$ and $\text{Prg4}^{-/-}$ mouse knees, and observed that in lubricin null $\text{Prg4}^{-/-}$ mice there was elevated friction and an increase in the number of apoptotic cells, as had been hypothesized. Furthermore, as predicted, $\text{Prg4}^{+/+}$ or $\text{Prg4}^{+/-}$ joints had few apoptotic cells.

7.2.1.B – *In vitro* analysis: Characterize biological wear in a bovine cartilage-on-cartilage bearing using physiologically relevant test lubricants.

The objectives of Aim 1B were also addressed in Chapters 1 and 2 of this dissertation. Using a bovine cartilage-on-cartilage friction and wear testing protocol lubricated with various test solutions, we measured coefficient of friction and observed the ability of the test lubricants to provide boundary lubrication and chondroprotection in these bearings.

The first part of this study (Chapter 2) compared physiological lubricants. We tested synovial fluid from patients with CACP (CACP-SF), which lacks lubricin, human synoviocyte lubricin (HSL), and normal human synovial obtained from the contralateral joints of patients undergoing ACL reconstruction, which contains a normal concentration of lubricin. We also observed the ability of HSL to replenish the boundary lubrication

and chondroprotective abilities to CACP-SF. PBS served as a negative control for friction and apoptosis, and unloaded control discs provided a positive control for apoptosis measurements. As hypothesized, lubricants containing lubricin (HSL, HSF and CACP-SF + HSL) had significantly lower static coefficients of friction compared to lubricants lacking lubricin (PBS and CACP-SF), but the kinetic coefficient of friction was maintained in CACP-SF, which provided a significantly lower kinetic coefficient of friction than PBS.

Chondroprotection was provided by lubricants containing lubricin (HSF, HSL and CACP-SF+HSL), which was observed by staining for activated caspase-3 and TUNEL in the superficial 300µm of cartilage. The static coefficient of friction had a direct relationship to the percentage of apoptotic cells in the superficial 100µm of the cartilage bearings.

For the second part of our investigation (Chapter 3), we tested the high molecular weight viscosupplement hylan G-F 20 (Synvisc), which is commonly used to treat patients with OA, and compared it to HSF, acquired either from normal patients, post-mortem, or from patients undergoing total joint replacement. We observed that kinetic COF and the percentage of apoptotic cells in cartilage bearings lubricated with hylan G-F 20 (Synvisc) was significantly higher than HSF acquired from either source, but hylan G-F 20 did not have a significantly different static COF compared to either PBS or HSF.

Finally, we compared different sources of synovial fluid and lubricin (Appendix B). We tested HSF from patients with OA undergoing total joint replacement and patients with normal joints, such as joints contralateral to ACL repaired joints or post-mortem synovial fluid. We hypothesized that bearings lubricated with HSF from normal joints

would have a lower coefficient of friction compared to synovial fluid aspirated during total joint replacement (OA-SF). We also tested different sources of lubricin, including lubricin purified from synovial fluid aspirated during total joint replacement (PHL), HSL and recombinant form of PRG4 (rhPRG4). Finally, we compared the ability of PHL and HSL supplementation of CACP-SF to lower COF and prevent chondrocyte apoptosis. The results of our study indicated that lubricin and HSF from various sources, which contain normal levels of lubricin, were able to lubricate cartilage-on-cartilage bearings with similar effectiveness. Furthermore, the supplementation of CACP-SF with HSL was able to provide additional chondroprotection compared to CACP-SF alone.

In Chapters 2 and 3, we observed a direct relationship between static coefficient of friction and apoptosis in chondrocytes located in the superficial 100 μm of cartilage following friction and wear testing. The maximum axial forces on the cartilage bearings approximately 12N (~ 0.5 MPa), which is much lower than the axial force required to induce apoptosis in *in vitro* impact (14 MPa) cyclic loading (5 MPa) experiments (9, 10) as well as measured peak local contact pressures that have been observed *in vivo* (11). These forces were incurred when the bearings were axially compressed to 18% total thickness, and the friction and wear tests had an approximate axial force of 2N (~ 0.1 MPa), which is non-destructive. This study suggests that elevated shear forces due to adhesion and lack of boundary lubrication and result in shear deformation that is destructive to chondrocytes.

The overall finding of this aim indicate the importance of lubricin to provide boundary lubrication in articular joints in order to prevent mechanical and biological wear. Furthermore, the reduction in COF and apoptotic cells following lubricin

supplementation of CACP-SF indicates that lubricin injection may be a viable treatment option for the restoration of boundary lubrication in a lubricin-null joint, which may occur genetically or acquired following trauma.

7.2.2 Aim 2: Determine the ability of lubricin to rescue a damaged joint

7.2.2.A. Investigate the ability of exogenous lubricin injected into the joint space to reduce cartilage damage and whole joint COF in $\text{Prg4}^{-/-}$ mice.

Aim 2A is addressed in Chapter 5 of this dissertation. The objective of this aim was to investigate the ability of lubricin to lower whole joint COF in $\text{Prg4}^{-/-}$ mice and preserve cartilage following an intraarticular administration. First, we provided a proof-of-concept for intraarticular injection by measuring the whole joint COF of $\text{Prg4}^{-/-}$ mice with established phenotypes (aged 10 weeks) before and after injection with human synoviocyte lubricin (HSL). While the results were not dramatic enough to be considered therapeutic, HSL significantly lowered whole joint COF in these knees.

We proceeded with an *in vivo* study. One (1) high dose (1.6 mg/kg) HSL was administered via intraarticular injection to the right knee of $\text{Prg4}^{-/-}$ mice 6 weeks of age. Two weeks later, whole joint COF was measured comparing the injected and contralateral knees. We also compared injected knees to joints of age-matched littermates that did not receive treatment. We observed a therapeutic decrease ($\Delta\text{COF} > 0.02$) in some knees, but the difference between injected and contralateral control failed to reach significance. Joint damage was found in all joints, as assessed histologically using H&E staining. By contrast, HSL injected joints had significantly lower total scores, surface morphology of the tibial plateau scores and cellularity scores, compared to the limbs of

untreated mice. Apoptosis was also observed in these joints by staining coronal sections for active caspase-3. There were significantly lower percentages of apoptotic cells in HSL injected joints compared to their matching contralateral joint and compared to cartilage of untreated animals.

In future studies, we plan to increase the availability of lubricin by moving to two intraarticular injections (1.6 mg/kg) administered a week apart. Since *Prg4*^{-/-} mice cannot produce their own lubricin, replenishment of available lubricin in the synovial fluid would be facilitated by additional injection. In addition, we will administer sham injections of saline to provide an additional injection control.

Finally, we plan to evaluate a recombinant form of lubricin in this model that is produced by transfected Chinese Hamster Ovary (CHO) cells and purified from conditioned cell culture media. This recombinant protein, which has shown efficiency in providing boundary lubrication and preventing apoptosis in a cartilage-on-cartilage bearing, is a likely candidate for use in future preclinical and clinical studies.

7.2.2.B. Investigate the ability of endogenous lubricin to reduce cartilage damage and whole joint COF in *Prg4* genetrap mice of different ages.

The objective of Aim 2B was to determine if lubricin-null mouse joints could be rescued following degeneration by the induction of endogenous lubricin production in the cartilage and synovium, using a transgenic approach. We hypothesized that genetrap mouse knees could be protected by the restoration of lubricin after birth, and, as a result, have lower coefficient of friction, fewer apoptotic cells, and less cartilage damage compared to lubricin-null knees. The *Prg4* gene was activated in GT/GT mice by daily IP injections of Tamoxifen for 10 days, starting 3 weeks postnatally, when minor

degenerative changes are observed in the $\text{Prg4}^{-/-}$ mice, and also at 2 months post-natal, after mice show a more dramatic phenotype. The joints were loaded cyclically in the active pendulum over an hour, with whole joint COF measurements taken at $t = 0, 15, 30, 45$ and 60 minutes of cyclic loading. We hypothesized that lubricin activation at 21 days post-natal will protect the cartilage in GT/GT mouse, but that activation at 2 months of age would not be adequate to prevent cartilage damage.

We compared the whole joint COF of 2 month old $\text{GT}^{\text{R3wk}}/\text{GT}$, which are GT/GT mice that had Prg4 activated at 3 weeks of age, GT/GT, GT/KO and $\text{GT}^{\text{R0}}/\text{GT}$, which are mice that expressed lubricin from birth. Mice that did not express lubricin from birth had progressive increases in whole joint COF over the course of cyclic loading. GT/KO mice had the highest mean whole joint COF, which was significantly higher compared to $\text{GT}^{\text{R3wk}}/\text{GT}$ following 30 minutes of cyclic loading and onward and compared to $\text{GT}^{\text{R0}}/\text{GT}$ following 15 minutes of cyclic loading and onward.

We also compared whole joint COF of 6 month old $\text{GT}^{\text{R2mo}}/\text{GT}$, which were GT/GT mice that had Prg4 activated at 2 months of age, GT/GT, GT/KO and GT^*/GT , which were mice that had lubricin activation from birth. Unlike in the case of $\text{GT}^{\text{R3wk}}/\text{GT}$ mice, there was no difference in whole joint COF between $\text{GT}^{\text{R2mo}}/\text{GT}$ and GT/GT or GT/KO mice.

The findings of this study indicate that genetic intervention with lubricin is a viable treatment option for patients with lubricin deficiency. However, these findings also show that it is imperative that lubricin be introduced early in disease progression in order to alleviate subsequent joint damage and prevent elevation in whole joint COF. Further studies into the long term status of genetrap joints following recombination of Prg4 3

weeks postnatally will provide additional insight into the long-term success of this time course.

7.2.3 Aim 3: Evaluate the Effectiveness of Etanercept and Lubricin to Prevent Cartilage Deterioration in a Rat Anterior Cruciate Ligament Transection Model

The purpose of Aim 3 was to evaluate changes in mechanical properties of rat cartilage following ACLT, and the ability of etanercept or lubricin injection to maintain normal cartilage stiffness. We addressed these aims in Chapter 6 and Appendix C.

We hypothesized that the mechanical properties of ACLT joints left untreated or treated with PBS would become impaired with time compared to ACL-intact joints that have undergone sham surgery, but ACLT joints that are treated with either etanercept or lubricin will have similar mechanical properties compared to joints that undergo sham surgery. We also hypothesize that joints treated with lubricin or etanercept will have less damage and more surface lubricin and lubricin expression, fewer apoptotic cells compared to untreated and PBS treated ACLT.

In the first part of this aim (Chapter 6), we measured microstiffness of femoral condyles from rats that underwent ACLT, ACLT followed by etanercept treatment and rats that underwent capsulotomy at 3, 6 and 9 weeks, post-operatively. We observed a lower microstiffness 3 weeks following ACLT in both untreated rats and those treated with etanercept compared to capsulotomy, and it remained low at 6 and 9 weeks post-operatively. We also observed progressive histological damage in the tibial plateaus of both ACLT untreated and HSL treated joints.

We also used atomic force microscopy (AFM) to measure the elastic modulus on the microscale of rat femoral condyles in joints that have undergone ACLT, with or without etanercept, lubricin or PBS treatment, and of rats that received capsulotomy (Appendix C). We also stained the tibial plateaus of these joints for surface lubricin, endogenous expression of lubricin, Safranin-O/FG to measure proteoglycan and detect structural damage, extracellular matrix proteins and for active caspase-3.

The findings of these studies indicate that loss of stiffness in the superficial zone occurs within prior to significant histological changes, and may occur within one week of injury, which was the time of treatment for both etanercept and HSL injections.

7.2.5 Summary

The work presented in this dissertation served to provide insight into the ability of lubricin to prevent both mechanical and biological wear of articular cartilage using *in vivo*, *ex vivo* and *in vitro* models, and its efficacy as a tribosupplement. We also investigated the relationship between boundary lubrication and cellular response. Our major findings support the further investigation of lubricin as a tribosupplement in large animal preclinical models and in patients with genetic or acquired lubricin deficiency, who have unmet medical needs. Additionally, this work provides insight into the mechanopathway relating friction and apoptosis, which provides the groundwork for many therapeutic targets in the prevention of biological wear, vis-a-vis the restoration of the tribome, which is primarily mediated by lubricin.

7.2.6 References

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Appendix A

The Role of Integrins in Friction-induced Chondrocyte Apoptosis

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A1 Abstract

Integrin cell surface receptors function as a link between articular chondrocytes and their extracellular matrix, and regulate many cell-dependent functions including cartilage matrix repair, cell attachment and cell survival. Previously, we've observed chondrocyte apoptosis in the superficial and upper middle zone of cartilage that has undergone friction and wear testing in the presence of inadequate test lubricants. We posited that the strain experienced by surface and subsurface chondrocytes under conditions of excessive surface friction would separate the extracellular matrix ligands from associated integrins within chondrocyte cell membranes. In this study, we use this cartilage-on-cartilage bearing system investigate the role of integrins in friction-induced apoptosis. Following friction and wear testing, we observed changes in mRNA and protein expression in chondrocytes. We observed an increase in the expression of integrins and pro-apoptotic factors in bearings lubricated with PBS and lubricin-null synovial fluid, and a loss of survival signals. In contrast, bearings that were lubricated with lubricin and normal synovial fluid had increased expression of survival signals and no increase in integrin expression. The results of this study indicate that integrins play an important role governing the fate of chondrocytes in situations of elevated cartilage friction.

A2 Introduction

Integrin cell surface receptors are heterodimer transmembrane glycoproteins that function as a link between articular chondrocytes and their extracellular matrix, and regulate many cell-dependent functions including cartilage matrix repair, cell attachment and cell survival (1-3). Integrins consist of an α and β subunit combinations that determine the ligand specificity of each receptor (**Table A1**). Several members of the integrin family, including $\alpha 1\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$, serve as receptors for collagen types II and VI, fibronectin, vitronectin and osteopontin (4-9).

Table A1: Integrins expressed by chondrocytes and their associated ligands (8-14).

Integrin	Ligand
$\alpha 1\beta 1$	Collagen II, VI
$\alpha 2\beta 1$	Collagen II, VI
$\alpha 3\beta 1$	Collagen II and VI, Fibronectin
$\alpha 5\beta 1$	Fibronectin, Collagen II
$\alpha 6\beta 1, \alpha 7\beta 1$	Laminin
$\alpha 10\beta 1$	Collagen II, VI
$\alpha v\beta 3$	Vitronectin
$\alpha v\beta 5$	Vitronectin, Osteopontin

Once an integrin binds to its ECM ligand, integrin clustering and kinase recruitment to the tail of the β integrin promotes association with the actin cytoskeleton (1). Adaptor proteins form a complex link between the β integrin and actin cytoskeleton which leads to the formation of actin stress fibers (1). A complex known as the focal adhesion complex (FAC) forms as a result of integrin clustering and association with the cytoskeleton, which in turn promotes further integrin clustering and matrix organization (15). Focal adhesion kinase (FAK) is among the first to be recruited, and the formation of FAK and paxillin can occur within 1 minute of initiation of a 0.33-Hz cyclical pressure induced strain (16).

Inhibition of integrin receptors results in loss of cell attachment that can result in apoptosis (12, 13, 17-20), which predisposes cartilage tissue to osteoarthritis (OA). Osteoarthritic chondrocytes showed altered cellular responses to mechanical stimulation, with altered expression of integrin receptors and pericellular matrix proteins, which may influence integrin-mediated signaling (21). Furthermore, chondrocyte dedifferentiation into fibro-blast like cells OA and in chondrocyte culture due to alterations in the extracellular environment (22-26). In addition, the fibronectin content changes in OA cartilage, which may also cause integrin-mediated cell dysfunction (27).

Mitochondrial factors antiapoptotic factor B-cell lymphoma 2 (Bcl-2) and proapoptotic factor Bcl-2-associated X protein (Bax) are part of the BCL family which regulate cellular activities, including apoptosis, in part by regulating the release of cytochrome-c by the mitochondria, which initiates apoptosis (28). Furthermore, in osteoarthritic cartilage, the surviving cells showed an increased ratio of Bcl-2 to

proapoptotic factor p53 (29), which is activated in response to cellular damage (30), indicating a sustained metabolic boost in chondrocytes.

Integrin-mediated chondrocyte-ECM interactions have been studied both *in vivo* and on cells *in vitro*, but matrix-chondrocyte interactions have not been studied *in vitro* in whole cartilage explants, to the knowledge of the authors. Previously, we've shown that elevated coefficient of friction in a cartilage-on-cartilage bearing system occurs due to lack of boundary lubrication and results in cellular apoptosis (31, 32), which we hypothesize occurs due to shear strain in the superficial and upper middle zone of cartilage. In contrast, chondrocytes in bearings that are lubricated by lubricants containing lubricin are preserved.

In this study we investigate the pathway governing the apoptotic response following elevated shear strain due to loss of lubrication in a bovine cartilage-on-cartilage bearing system. We hypothesize that in the absence of boundary lubrication, cells are deformed, altering integrin-bonding to the extracellular matrix, which results in a loss of survival signals associated with the focal adhesion complex, and resulting in an unbalanced ratio of proapoptotic to antiapoptotic factors (**Figure A1**).

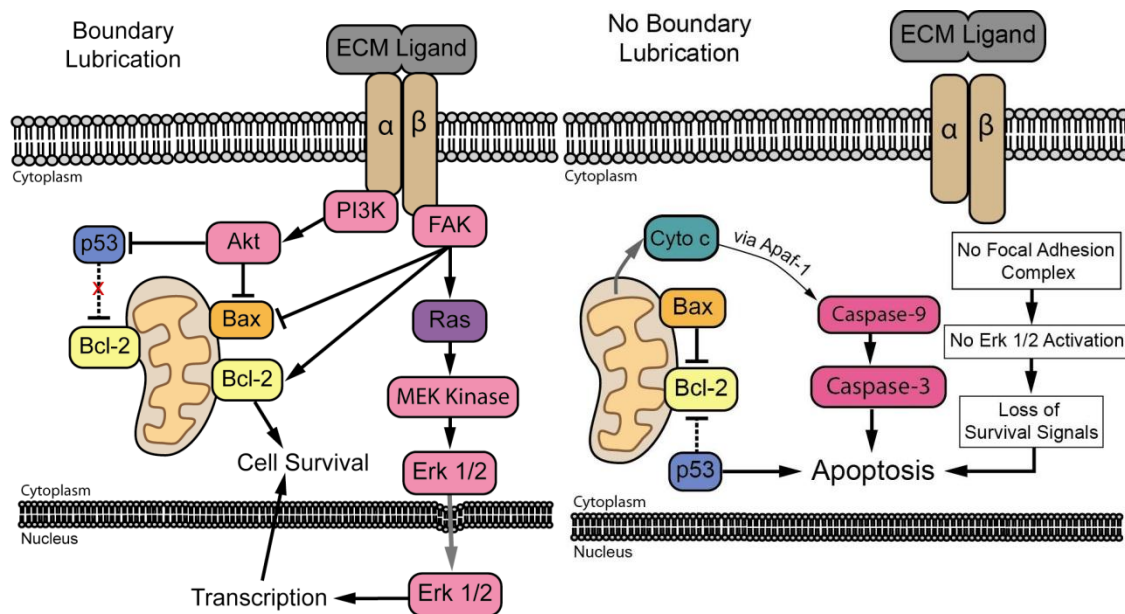


Figure A1: Proposed pathway relating friction and apoptosis in chondrocytes. In the presence of boundary lubrication (left), integrins form bonds with ligands in the extracellular matrix, which results in the recruitment of the focal adhesion complex, involving FAK. This complex promotes cell survival signals via the PI3K/Akt pathway, which downregulates pro apoptotic factors p53 and Bax. FAK activates the Ras/MAPK pathway, which is implicated in transcription, cell survival, and extracellular matrix production. FAK also promotes anti-apoptotic factor Bcl-2. In the absence of boundary lubrication (right), integrins may lose contact with the ECM and are upregulated. Without ligand attachment, survival signals are downregulated and proapoptotic signals may be increased the ratio of p53 and Bax to Bcl-2 becomes positive, which activates the mitochondrial apoptotic pathway via Caspases-9 and -3, resulting in cellular apoptosis. Furthermore, damage to the cell due to shear stress increases expression of the proapoptotic factor p53. Arrows indicate activation, lines with bars indicate inhibition, solid lines indicate direct interaction, dotted lines indicate indirect interaction, gray arrows indicate translocation into the nucleus.

A3 Methods

A3.1. Bovine Cartilage Preparation

Full thickness cartilage plug bearings 6 mm and 12 mm in diameter were cored from the femoral condyle of bovine knees collected within 2 h of slaughter. Following harvest, the bearings were rinsed with cell culture media [DMEM supplemented with 5% (vol/vol) FBS] and cultured for 24 h at 37°C.

A3.2. Test Solutions

Phosphate buffered saline (PBS, 1X) was purchased from Life Technologies Corp. Human synovial fluid (HSF) was aspirated from the uninjured contralateral joints of patients with an anterior cruciate ligament injury (n=4). These samples were obtained without lavage just prior to reconstructive surgery (33). Other human synovial fluids were aspirated in the operating from joints of patients undergoing total joint replacement (n=12) and pooled together. The samples were inspected for blood contamination then centrifuged following aspiration at $12,000 \times g$ at 4 °C for 10 min, and the aliquot was removed and stored at -80°C until testing. Lyophilized human synoviocyte lubricin (HSL) was obtained from SBH Sciences and has been characterized previously (34, 35). This product was solubilized using distilled water to a concentration of 250 µg/ml, which is the estimated lubricin concentration in synovial fluid (33). Synovial fluid from patients with CACP (CACP-SF) was obtained from patients in the years between 2000 and 2011, following diagnostic knee joint aspiration (n=4). CACP-SF samples were inspected for blood contamination then centrifuged at $12,000 \times g$ at 4 °C for 10 min and stored frozen at -20°C until testing. CACP-SF supplemented with HSL at a physiologically relevant

concentration of 250 µg/ml (CACP+HSL) was also tested. All lubricants were thawed to room temperature immediately prior to testing. Approval to obtain synovial fluid from patients was granted by the Rhode Island Hospital and Boston Children's Hospital Institutional Review Boards.

A3.3 Bovine Bearings Friction and Wear Testing

This testing method has been described previously (23). Cartilage bearings were loaded in a disc-on-disc configuration into an EnduraTEC ElectroForce 3200 (Bose Corporation, ElectroForce Systems Group, Eden Prairie, MN, USA) with cyanoacrylate glue, and any remaining cell culture medium was rinsed off 3 times with 1 ml washes of PBS. Test lubricant was applied between the surfaces. The bearings were axially loaded to 18% total cartilage thickness, and held at this displacement for 8 min to allow for fluid depressurization (25, 46). Then, the lower bearing was rotated in torsion + 2 revolutions and reset - 2 revolutions at an effective velocity of 0.3 mm/s, which allows the depressurized cartilage-cartilage interface to remain in the boundary mode of lubrication (25), for 12 continuous cycles. Immediately following testing, lower discs were fixed in formalin. Unloaded control discs, also 12 mm in diameter, were fixed at the time of testing. All tests were performed between 48 and 72 h of cartilage harvest, and bearings were only tested once. Following testing, large diameter bearings were fixed in 10% formalin for histological analysis and small diameter bearings were submerged in RNAlater (Ambion, Inc., Austin, TX), frozen and stored at -80°C.

A3.4 Quantitative Real Time-PCR

Small bearings were thawed and total RNA was extracted from the cartilage tissue using a Qiagen RNeasy Mini Kit (Qiagen, Inc) according to manufacturer instructions. Tissue was disrupted and homogenized using a rotor-stator homogenizer in 1ml of Buffer RLT and centrifuged for 3 minutes at full speed. Supernatant (lysate) was carefully transferred to a microcentrifuge tube, and 1 volume of 70% ethanol was added to the lysate, and mixed by pipetting. Sample was transferred into an RNAeasy spin column in a 2 ml collection tube and centrifuged for 15s at 8000 x g. Flow through was discarded. 700 µl of Buffer RW1 was added to the column, the column was centrifuged for 15s at 8000 x g, and flow through was discarded. 500 µl of Buffer RPE was added to the column, the column was centrifuged for 15s at 8000 x g, and the flow through was discarded. The previous step was performed twice. Finally the spin column was placed in a new collection tube, and 50 µl of Rnase-free water was added directly to the spin column membrane. The column was centrifuged for 1 minute at 800 x g to elute RNA. RNA concentration was evaluated using a Nanodrop 2000c (Thermoscientific).

Three µg of RNA from each group were reverse transcribed into cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Two µg of cDNA from the reverse transcriptase reaction were added to 25 µl mixture of 2×SYBR Mix (with 4 mM Mg^{2+}), 2 µl forward primer (10 µM), 2 µl reverse primers (10 µM), and deionized water (20 µl total volume). The optimized parameters for the thermal cycler were as follows: activation at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 sec, 55 °C for 15 sec and 70 °C for 20 sec, with the final step being that the temperature was increased gradually (0.5 °C/sec) to 95 °C to determine the melting curve. GAPDH was

used as an endogenous reference. Signals were detected with a Mastercycler EP Realplex 4 (Eppendorf). The relative expression level was determined by the $2^{-\Delta\Delta CT}$ method (36) and normalized against GAPDH (p53, Bcl-2, caspase-3), or against expression in unloaded control cartilage (Erk1, Erk2, integrins $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$). Primers are described in **Table A2**.

Table A2: Primer pairs used for qRT-PCR

Target	Forward Primer (5' → 3')	Reverse Primer (3' → 5')
GAPDH	CCCACGGCAAGTTCAACGGC	TGGGGGCATCGGCAGAAGGT
bcl-2	AAGCTGTGCGCAGCGGCCGTA	CGGTGAAGGGCGTCAGGTGC
p53	TGGAGCCCCCTCTGAGTCAGG	AGGTCATCCACGGGTGCGGA
PRG4	ACTCCACCACCTCCACCTCGG	GCTGAACGCTGCCACCTCTC
Erk1	AAAGGGGCAGCCGTTTCGACG	TCTCGCAATGTGCGCTGGCA
Erk2	CGTCCCCGGCTCTCCCGAG	TAGGCGCCCTCGCCGATGTA
Integrin $\alpha 1$	TGTTCTGCGCTTCTGTGTGTCA	GGCCCACATGCCAGAAACCCT
Integrin $\alpha 3$	AACCCGGATCGGAGGCGACT	CATGCCGAGCAACGGGCGTA
Integrin $\alpha 5$	CTGGGGCGAGGGAAGTGGAGA	TCCCGGCTGAGCCCTCCCC

A3.5 Immunohistochemistry

Bovine femoral cartilage lower bearings were decalcified and thin sections (6 μ m) of full thickness cartilage were taken for histological analysis. Sections were heated to 60°C for 30 min, deparaffinized and hydrated in 3 changes of xylene and serial alcohol. Sections were then quenched in endogenous peroxidase in 3% hydrogen peroxide for 10 min and antigen retrieval was performed using a pepsin solution (ThermoScientific). Sections were stained for p44/42 MAPK (Erk1/2) using a rabbit polyclonal antibody (#9102, Cell Signaling Technology, Danvers, MA). Sections were also stained for integrin α 5 β 1 using a rabbit polyclonal antibody (orb13515, Byorbt). Sections were counterstained with 0.5% methyl green.

A4 Results

We observed a negative ratio of Bcl-2/p53 mRNA expression in bearings tested with PBS and CACP-SF, indicating loss of survival signaling in articular chondrocytes (**Figure A2A**). HSL and HSF had elevated Bcl-2/p53 signaling, which indicates survival signaling and a boost in metabolic metabolism.

Relative mRNA bovine PRG4 expression was increased in HSL and HSF lubricated bearings, but downregulated in PBS tested bearings compared to unloaded control cartilage (**Figure A2B**). CACP-SF tested bearings had similar PRG4 expression to unloaded cartilage.

The relative mRNA expression of integrins $\alpha 1$ and $\alpha 5$ was increased in bearings lubricated with PBS and CACP-SF (**Figure A3**). Integrin $\alpha 5\beta 1$ staining in bovine cartilage bearings showed many cells positive for open $\alpha 5\beta 1$ receptors PBS and CACP-SF lubricated bearings (**Figure A4**), which coincides with elevated apoptosis previously observed in these samples (32). Bearings lubricated with HSL and HSF had integrin $\alpha 1$ and $\alpha 5$ mRNA expression levels similar to unloaded control bearings and very few cells that stained positive for $\alpha 5\beta 1$. We observed no significant upregulation in integrin $\alpha 3$ for any samples. We observed a slight downregulation of integrin $\beta 1$ in HSL and HSF lubricated samples, and no change in PBS or CACP-SF tested bearings compared to unloaded cartilage.

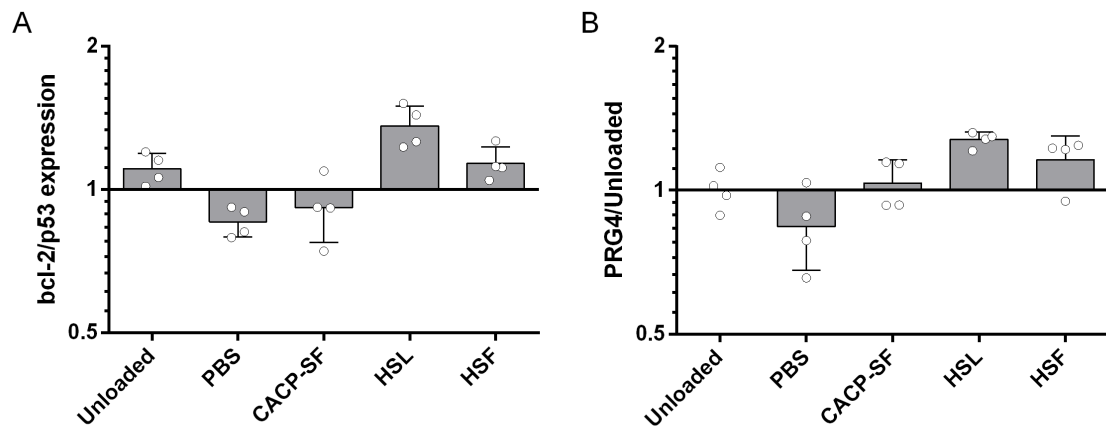


Figure A2: A.) Ratio of Bcl-2/p53 expression in articular cartilage bearings tested with relevant test lubricants. PBS and CACP-SF had a negative ratio of Bcl-2/p53 indicating loss of survival signaling in articular chondrocytes. HSL, HSF and CACP-SF+HSL had elevated Bcl-2/p53 signaling, which indicates survival signaling and a boost in metabolic metabolism. B.) Relative mRNA bovine PRG4 expression, normalized to unloaded control cartilage levels. We observed an increase in lubricin expression in HSL and HSF lubricated bearings, with a downregulation in PBS tested bearings. Error bars indicate SD.

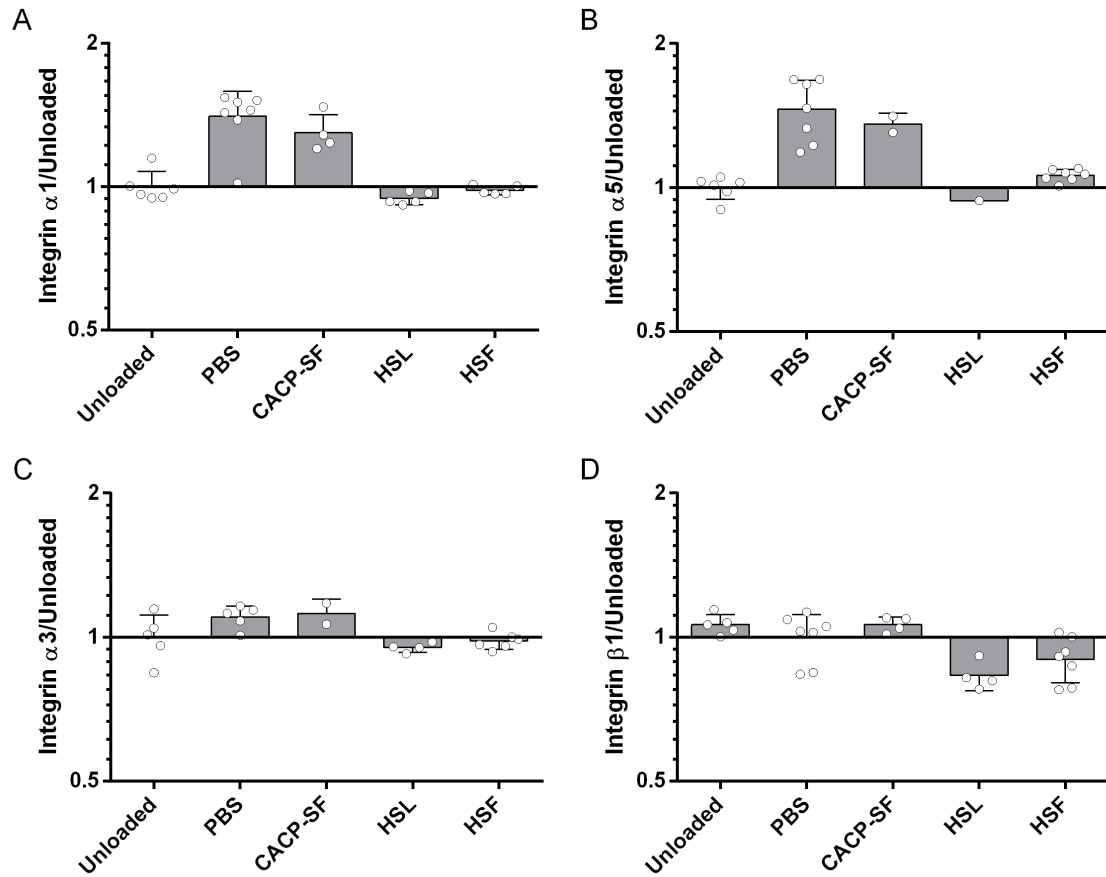


Figure A3: Relative mRNA expression of integrins in bearings lubricated with PBS, CACP-SF, HSL and HSF, normalized to unloaded control bearings. A.) We observed an upregulation in integrin $\alpha 1$ in PBS and CACP-SF tested bearings. B.) Likewise, we observed an upregulation of integrin $\alpha 5$ in PBS and CACP-SF tested bearings. C.) We observed no significant upregulation in integrin $\alpha 3$ for any samples. D.) We observed a slight downregulation of integrin $\beta 1$ in HSL and HSF lubricated samples. Error bars indicate SD.

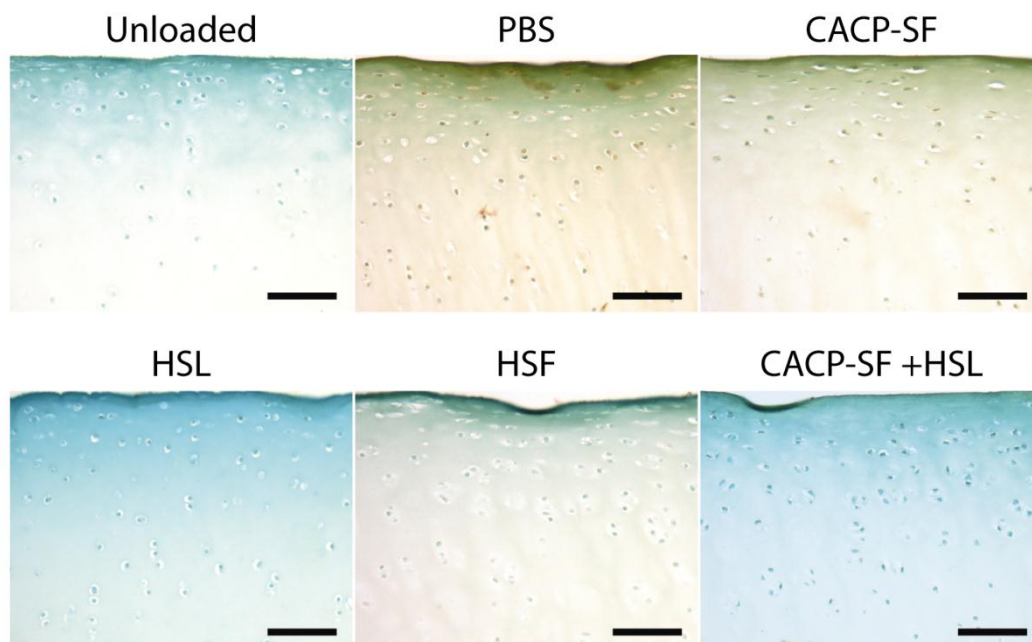


Figure A4: Integrin $\alpha 5\beta 1$ staining in bovine cartilage bearings. We observed many cells positive for open $\alpha 5\beta 1$ receptors (brown) in PBS and CACP-SF, which coincides with elevated apoptosis previously observed in these samples. Sections were counterstained with methyl green (blue). Scale bars indicate 50 μ m.

We observed a downregulation of Erk1 in PBS and CACP-SF tested samples, and slight upregulation in HSL and HSF lubricated bearings (**Figure A5**). We observed no difference between groups in Erk 2 mRNA expression. We observed cells positive for phosphorylated Erk 1/2 (brown) in HSL, HSF, and CACP+HSL lubricated bearings, with some staining in unloaded cartilage (**Figure A6**). In contrast, PBS and CACP-SF tested bearings had very few cells positive for pErk1/2, indicating a loss of survival signals in their superficial and upper middle zone chondrocytes.

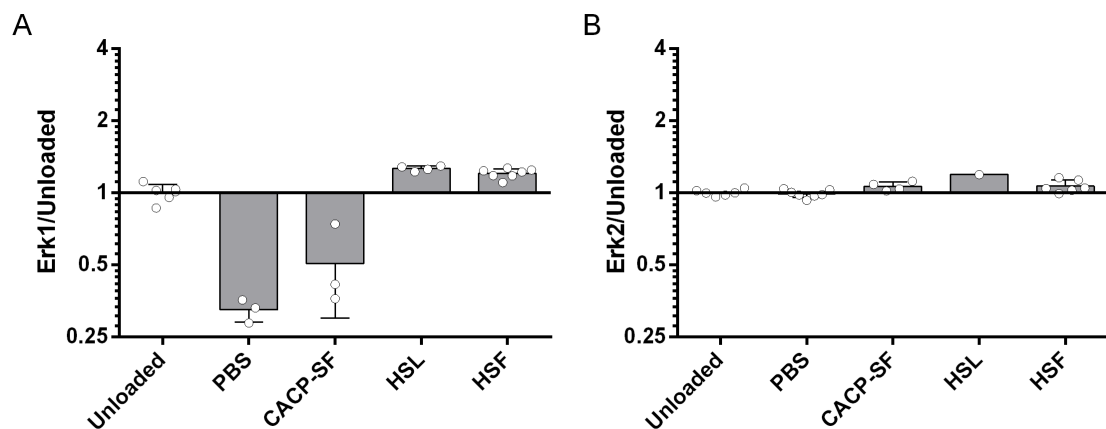


Figure A5: Relative mRNA expression of Erk 1 and 2 in chondrocytes, normalized to unloaded control cartilage, which indicate cell survival. A.) We observed a downregulation of Erk1 in PBS and CACP-SF tested samples, and slight upregulation in HSL and HSF lubricated bearings. B.) We observed no difference between groups in Erk2 mRNA expression. Error bars indicate SD.

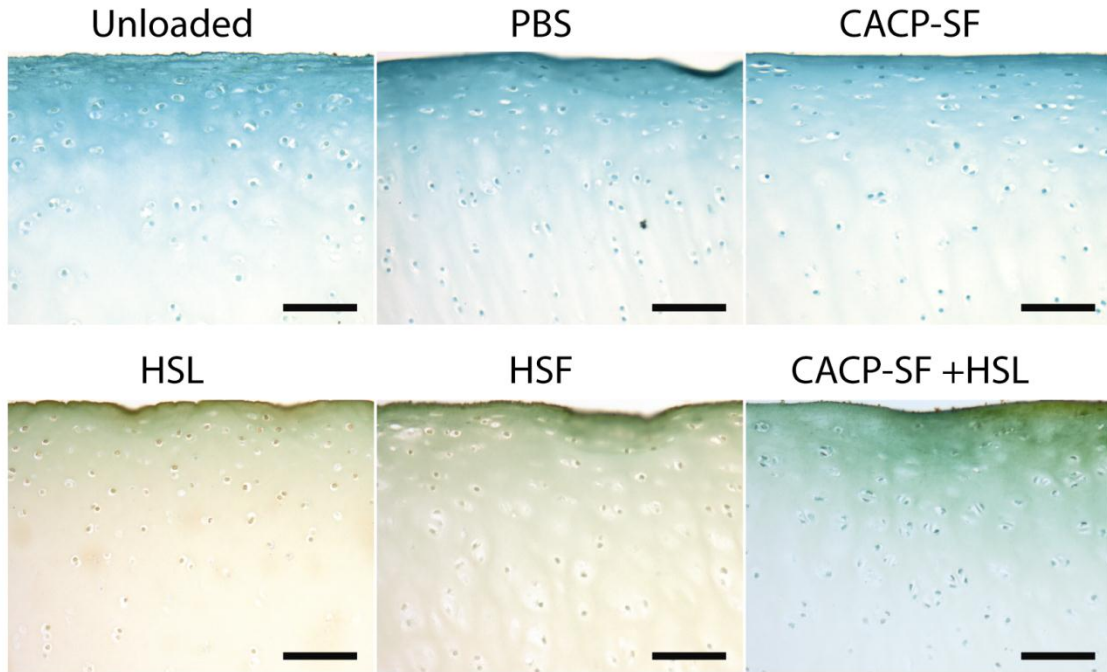


Figure A6: Phosphorylated Erk1/2 staining for bovine cartilage bearings. We observed cells positive for phosphorylated Erk $\frac{1}{2}$ (brown) in HSL, HSF, and CACP+HSL lubricated bearings, with some staining in unloaded cartilage. In contrast, PBS and CACP-SF tested bearings had very few cells positive for pErk1/2, indicating a loss of survival signals in their superficial and upper middle zone chondrocytes. Scale bars indicate 50 μ m.

A5 Discussion

Articular cartilage is subjected to a number of mechanical stimuli *in vivo*, including stretch, shear and compressive force. Previously, we've observed apoptosis in chondrocytes of bovine bearings that experienced elevated coefficient of friction due to lack of adequate boundary lubrication provided by lubricin (31, 32). In order to respond to these stimuli, chondrocytes employ a range of mechanoreceptors, including integrins (4). Integrins provide a linkage between the cytoskeleton and the extracellular matrix (37, 38), which is vital to chondrocyte survival and cartilage homeostasis (7, 12, 20, 29, 38-42).

The preliminary PCR findings support our hypothesis for integrin-mediated apoptosis due to lack of boundary lubrication. We observed a positive Bcl-2/p53 ratio in HSL and HSF lubricated bearings, but we also observed a negative Bcl-2/p53 ratio in PBS and CACP-SF tested samples, which also had a large percentage of apoptotic cells in the superficial and upper middle zone chondrocytes (32). This ratio has been implicated in the survival of cells in cartilage (29). Bcl-2 is an antiapoptotic factor that inhibits release of cytochrome-c from the mitochondria in order to prevent the initiation of the caspase cascade (43). Upregulation of Bcl-2 as a result of $\alpha 5 \beta 1$ activation, and subsequent cell survival, has also been observed (30). P53 plays a proapoptotic role in response to chondrocyte damage, initiating apoptotic signaling, and indirectly interacts with Bcl-2 via Bax (29, 30, 44-50). Shear strain applied directly to chondrocytes resulted in apoptosis mediated by p53 (45). Previously, we've observed that chondrocytes in the areas of cartilage that undergo the greatest shear deformation, located just below the superficial chondrocytes (51, 52), are most susceptible to chondrocyte apoptosis (32).

Integrin analysis via PCR revealed a slight upregulation in integrins $\alpha 1$ and $\alpha 5$ in PBS and CACP-SF tested samples, but no difference between samples for $\alpha 3$ and $\beta 1$. We also immunostained for integrin $\alpha 5\beta 1$ at the protein level, and observed many more positive cells in PBS and CACP-SF tested cartilage compared to other samples, which indicates an increase in the number of binding sites in these samples, compared to control, HSL, HSF or CACP-SF+HSL lubricated cartilage bearings.

The role of integrins in cell survival has been studied in human articular chondrocytes from normal and osteoarthritic donors (10, 12, 13, 50), and lack of integrin $\alpha 1\beta 1$ expression resulted in osteoarthritis in a murine model (41). Cartilage immunostained for integrins $\alpha 1$, $\alpha 3$, $\alpha 5\beta 1$ had an increased number of cells and staining intensity that coincided with an increase in severity of osteoarthritis in cartilage (10). There is also evidence that some integrins may also play a degenerative role in the pathogenesis of osteoarthritis. Integrin $\alpha 5\beta 1$ has been implicated in the breakdown of cartilage via upregulation of matrix metalloproteinases (MMPs) in response to activation via fragments of fibronectin (53), but this response is not seen in reactions with full-length fibronectin. Upregulation of $\alpha 5\beta 1$ in chondrocytes has also been reported in response to inflammatory synovial fluid, which may also be due to fragments of fibronectin (54). Upregulation of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ has also been reported in osteoarthritic cartilage, but this upregulation may be a response to matrix damage (13). Integrin suppression has also been observed in cells that express the survival signals Erk1/2, Akt and Ras (55-57).

Histological analysis of phosphorylated Erk1/2, which is indicative of cell survival and transcription, revealed more positive cells in samples lubricated with HSL,

HSF and CACP-SF+HSL, compared to PBS and CACP-SF lubricated bearings, which had very few cells positive for Erk1/2. MAPK cascades are activated via FAK and the complex associated with it following integrin binding to ECM (58), and blocking Erk1/2 expression in chondrocytes results in cellular apoptosis (59). In addition, high shear stress has been implicated in apoptosis via JNK2-mediated COX-2 expression, which suppresses PI3K and Bcl-2 and results in chondrocyte death via the mitochondrial apoptotic pathway involving caspase-9 (60-62). On the other hand, low shear and hydrostatic pressure elevate survival signals (63-65).

Although the trends presented in this study are encouraging, the fold-change between samples remained low. This low expression may be due to the use of full thickness cartilage, which includes cells below the area that would be affected by the shear strains of this experiment, according to previous studies (31, 32, 51, 52). Furthermore, mRNA expression differs in chondrocytes located in different zones within the cartilage, and contributions of the lower middle zone and deep zone chondrocytes may be diluting the signaling in the upper middle and superficial zone chondrocytes. In future studies, we plan to use only the superficial 250 μm of cartilage bearings.

While this study focuses on integrin-mediated mechanotransduction, a number of other mechanoreceptors also play important roles in communication between the cell and extracellular matrix in cartilage and cartilage homeostasis, including hyaluronan receptor CD44 (13, 66), mechanosensitive ion channels (14, 16, 18, 42, 67-71). These receptors work alongside integrin surface receptors to maintain cartilage metabolism and respond to mechanical stimuli. Likewise, a number of apoptotic pathways in OA have been

observed *in vivo* and *in vitro*, including the death receptor pathways involving NO (47, 61, 72-74), Fas Ligand (72, 75-77) and tumor necrosis factor- α (47, 78-80).

While still largely unknown, integrin function in articular cartilage appears to play a role in the homeostasis and metabolism of cartilage tissue (4, 13). These preliminary findings warrant further exploration into this posited pathway. Additional targets of interest include proapoptotic factors Bax, which directly inhibits Bcl-2 by forming a dimer (47, 49, 81-83), caspase-9, which is located upstream from caspase-3, and cell survival factors including FAK, Akt, also known as protein kinase B, and phosphatidylinositide 3-kinase (PI3K).

RNAseq analysis would allow for the observation of all gene targets simultaneously, and would be a logical next step in mRNA analysis. Furthermore, protein analysis via Western blot and immunohistochemical targeting the phosphorylated versions of these factors would greatly benefit this study. We feel that the findings in this study may identify key therapeutic targets for the prevention and treatment of friction-induced chondrocyte apoptosis.

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Appendix B

Comparison of Sources of Synovial Fluids and Lubricin

As an extension of the work described in Chapters 2 and 3, we also compared the coefficient of friction of human synovial fluid and lubricin from different sources using the friction and wear test described in Chapters 2 and 3. We tested HSF from patients with OA undergoing total joint replacement (TJR SF) and patients with normal joints, such as the contralateral joints of patients undergoing ACL repair (CL-ACL SF) or post-mortem synovial fluid (PM SF). We hypothesized that bearings lubricated with HSF from normal joints would have a lower coefficient of friction compared to synovial fluid aspirated during total joint replacement. We compared these lubricants to PBS and CACP-SF as negative controls.

Synovial fluid samples were tested for lubricin concentration using an ELISA assay using anti-lubricin monoclonal antibody 9G3. CL-ACL SF (n=4) had a lubricin concentration of 220 ± 96 $\mu\text{g/ml}$. PM SF was aspirated from knee joints of post-mortem donors within 12 h of death with no history of joint disease 3 male donors, Ages 25-39, NDRI, lubricin concentration 288 ± 77.7 $\mu\text{g/ml}$). TJR SF was aspirated from joints in the operating room from patients undergoing total joint replacement surgery and pooled together (n=12 per pool, n=2 pooled TJR SF for testing, lubricin concentration 443 ± 37 $\mu\text{g/ml}$).

We also tested different sources of lubricin, including lubricin purified from synovial fluid aspirated during total joint replacement (PHL), HSL and recombinant form of PRG4 (rhPRG4), which is purified from conditioned media of Chinese hamster ovary cells programmed to produce a full-length recombinant form of lubricin (1). Sources of lubricin were tested at a concentration of 250 µg/ml.

The findings of our study showed that the static COF was not different for bearings lubricated with any source of synovial fluid, which were all significantly lower compared to PBS ($p < 0.001$, 0.005 , and 0.001 for CL-ACL SF, PM SF and TJR SF, respectively) and CACP-SF lubricated bearings ($p = 0.0009$, 0.007 , and 0.02 for CL SF, PM SF and TJR SF, respectively) (**Figure B1**). PBS and CACP-SF lubricated bearings were not different ($p > 0.99$). Likewise, kinetic COF was significantly lower for all sources of SF compared to PBS ($p < 0.0001$ for all sources of SF). CACP-SF lubricated bearings had significantly higher kinetic COF compared to ($p = 0.03$), as well as significantly lower kinetic COF compared to PBS ($p = 0.001$).

The static COF of bearings lubricated with all sources of lubricin were not different between samples, but were significantly lower than PBS ($p < 0.0001$, 0.002 , and 0.02 for HSL, PHL and rhPRG4, respectively). Similarly, all sources of lubricin had significantly lower kinetic COF compared to PBS ($p = 0.002$, 0.0005 , and < 0.0001 for HSL, PHL and rhPRG4, respectively).

The findings of this study indicate that OA SF and the lubricin purified from this material are able to lubricate a healthy bovine bearing system. Furthermore, PHL, which is purified from TJR SF, was also able to lubricate this bearing system as well as HSL and rhPRG4. In contrast to our idealized system, patients with OA, especially those with

OA severe enough to require total joint replacement, have altered joint surfaces with early eburnation, which is detrimental to the proper function of boundary lubricants. As a result, tribosupplementation may not be appropriate for patients with severe cartilage damage.

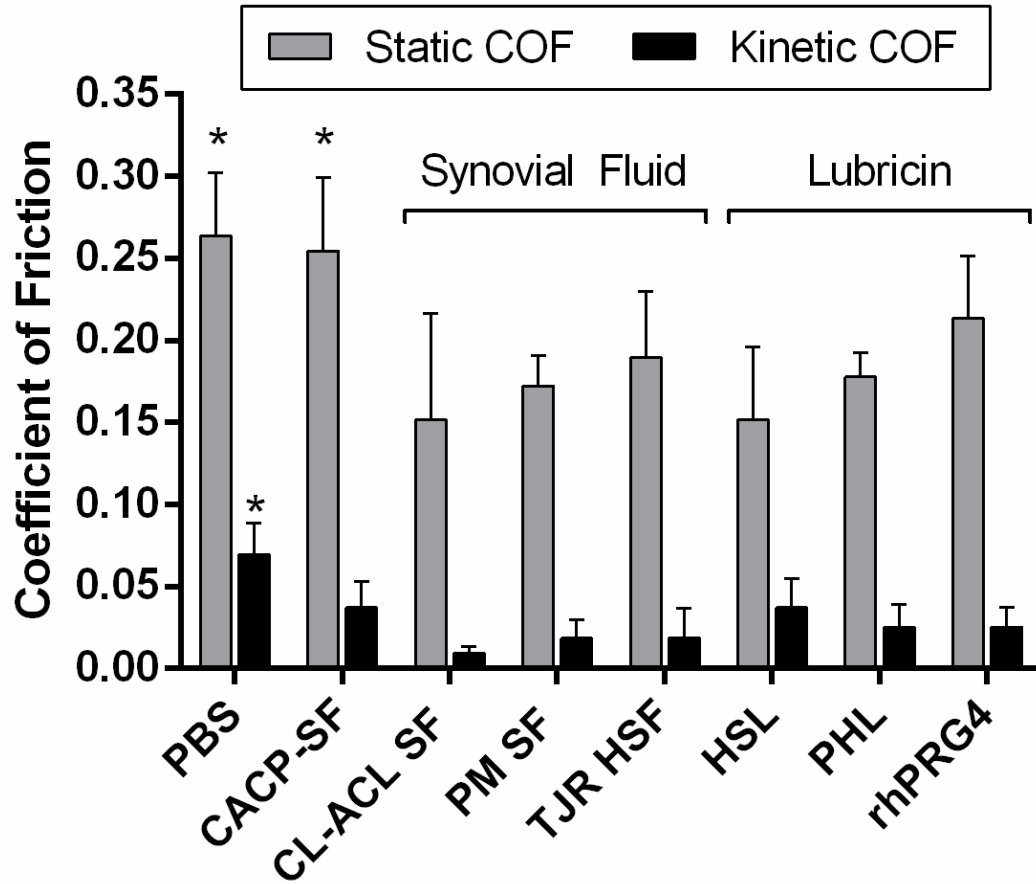


Figure B1: Comparison of synovial fluids and lubricin from different sources. Synovial fluids from all sources and lubricin had comparable static COF, which were lower than CACP-SF and PBS. CL-ACL, PM and TJR HSF had lower kinetic COF compared to CACP-SF, indicating a synergistic relationship between lubricin and hyaluronan. All sources of SF and lubricin had lower kinetic COF compared to PBS.

In vivo, lubricin forms a network of disulfide bonded molecules which were identified previously through alkylation and reduction of PHL, whereas HSL and rhPRG4 are monomer-dimers (1). The results of this comparison study indicate the availability of lubricin in monomer-dimer form is adequate for boundary lubrication and that the presence of networked molecules may not provide any further advantage in terms of reducing static and kinetic COF, in this study, but additional investigation into the molecular structure of PHL, HSL and rhPRG4 would be required to evaluate this finding. All tested sources of HSF and lubricin have shown proficiency in preventing chondrocyte apoptosis (**Figure B2**).

In a rat study of secondary OA, HSL, PHL and rhPRG4 were all able to mitigate cartilage damage in rat joints following the induction of osteoarthritis, but HSL showed the most promising results (1). Furthermore, HSL supplemented CACP-SF also successfully lowered COF of bearings compared to CACP-SF alone (N=5, static COF = 0.19 ± 0.08 , $p=0.01$, kinetic COF = 0.016 ± 0.01 , $p=0.04$), as well as prevent chondrocyte apoptosis (2). In contrast PHL was unable to decrease the COF of CACP-SF (N=3, static COF = 0.25 ± 0.008 , kinetic COF = 0.033 ± 0.006), which may have been due to its inability to properly combine with CACP-SF as a result of the lubricin network. PHL supplementation of CACP was less successful than HSL in preventing chondrocyte apoptosis, which is linked to its inability to lower COF. In addition, the networked conformation of PHL may have prevented it from properly mixing with CACP-SF, resulting in its inability to increase the capacity for lubrication by CACP-SF.

Recombinant PRG4 is a promising candidate for clinical tribosupplementation, due to its capacity for scalable cell-based production and purification (3, 4). These

findings warrant additional studies into the ability of rhPRG4 to supplement synovial fluid in a lubricin-null mouse model and other synovial fluids deficient in lubricin.

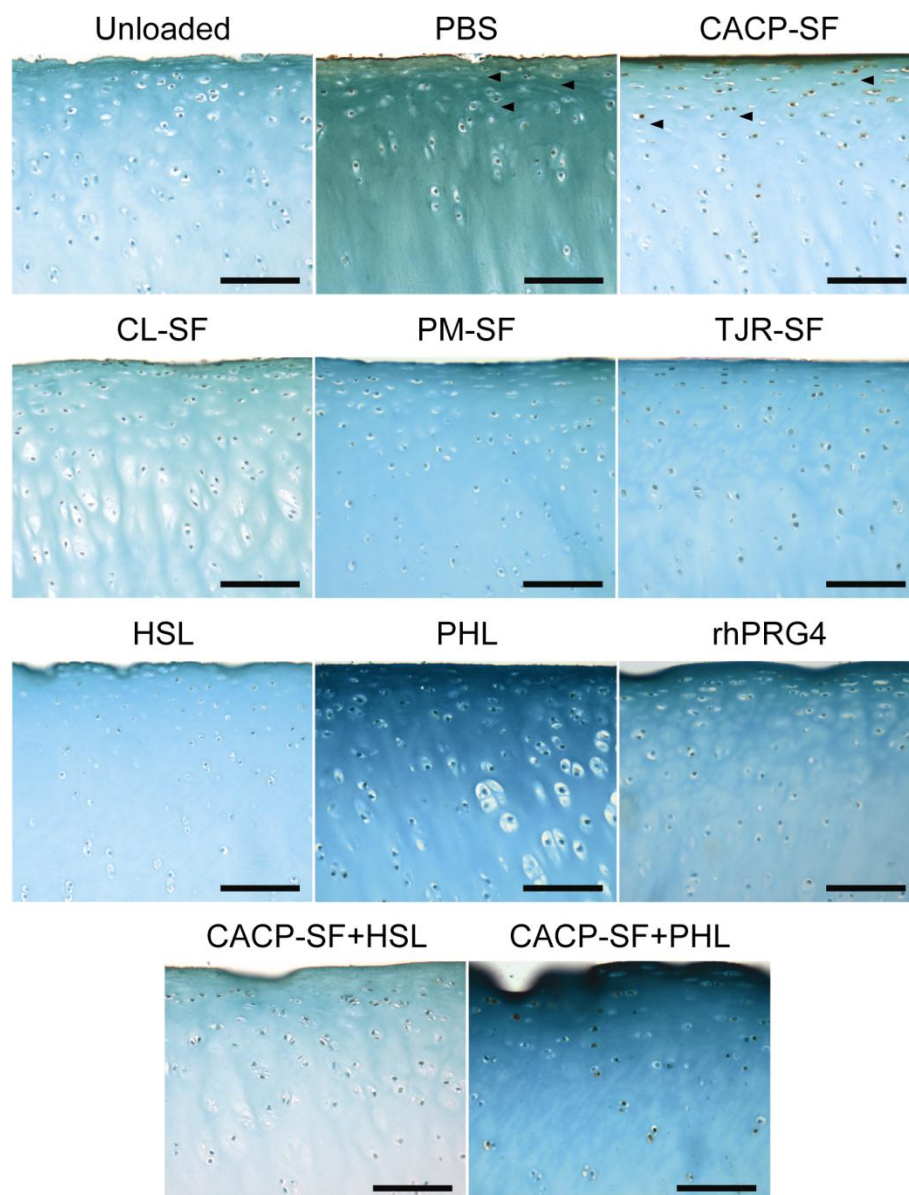


Figure B2: Active caspase-3 staining (brown) for sources of synovial fluid and lubricin. PBS and CACP-SF tested samples had many cells positive for apoptosis compared to samples lubricated with synovial fluid and lubricin, which had very few apoptotic cells. Bearings lubricated with CACP-SF supplemented with HSL (CACP-SF+HSL) had few apoptotic cells, but PHL supplemented CACP-SF (CACP-SF+PHL) was less proficient in preventing chondrocyte apoptosis, which may be due to the networked conformation of PHL and its inability to distribute within CACP-SF.

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Appendix C

The Effect of Intra-articular Lubricin on Cartilage Compliance in a Rat Model of Osteoarthritis

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C1 Introduction

Traumatic injury to the ACL is a significant risk factor for secondary osteoarthritis due to a number of risk factors including alteration of biomechanics and biopathways within the synovial joint. Tumor necrosis factor- α (tnf- α) and Interleukin 1 β (IL-1 β) are released following traumatic injury, resulting in downregulation of PRG4, and subsequent loss of lubricin protein in the synovial fluid and cartilage surface (1). In Chapter 5, we described the effect of tnf- α inhibition via etanercept following ACLT.

C2 Methods

Rats underwent unilateral ACLT or capsulotomy of the right limb as previously described (2). Seven days following surgery, rats that underwent ACLT received intra-articular injections (50 μ l/injection) of human synoviocyte lubricin (HSL; 1.6 mg/ml, n=5), phosphate buffered saline (PBS; n=3) or were not treated (untreated; n=4). Animals (n=3) that underwent capsulotomy received no additional treatments. Ten weeks post-operatively, rats were sacrificed. Tibial plateaus were fixed and processed for histological

staining and analysis, and medial femoral condyles were removed and prepared for AFM measurement of microstiffness, as described in Chapter 6.

Microstiffness was measured using a spherical probe 5 microns in diameter attached to a cantilever of 7.5 N/m stiffness (Novascan, Ames, IA), and a trigger deflection of 150nm, corresponding to approximately 800 nN. Three 90 x 90 micron areas were indented in load bearing areas to yield 256 indentation curves. Each approach indentation curve was fitted to a modified Hertz model, as previously described, to determine elastic moduli using custom Matlab code (Mathworks, Natick, MA) (3, 4). A threshold was applied to discard force-indentation curves that had more than 1 μm of indentation for 10nN or less of force, or more than 2 μm , and represented unreliable indentation curves.

We also approximated surface roughness using the height measurement based on contact point determinations described previously (4). Root mean squared (R_{rms}) and average surface roughness (R_{avg}) were calculated using the height measurements at each point where elastic modulus was calculated to measure elastic modulus, using the following equations:

$$R_{\text{avg}} = \sum_{n=1}^N \frac{|Z_n - \bar{Z}|}{N} \quad (1)$$

$$R_{\text{rms}} = \sqrt{\sum_{n=1}^N \frac{(Z_n - \bar{Z})^2}{N}} \quad (2)$$

C3 Results

We observed significantly lower microstiffness in all knees that underwent ACL transection compared to sham, which had a microstiffness of 877.7 ± 35.9 kPa ($p=0.0039$, 0.0031 and 0.48) compared to HSL, PBS and Untreated ACLT, respectively (**Figure C1**). HSL treated ACLT cartilage had a stiffness of $(310.2 \pm 183$ kPa), which was not significantly different than PBS treated (222.9 ± 37.3 kPa, $p=0.89$) or untreated (482.2 ± 246 kPa, $p=0.46$) joints.

Surface roughness measurements showed increased roughness in ACLT joints compared to sham, with significantly higher average and RMS roughness in HSL treated ACLT joints compared to sham joints ($p=0.01$) (**Figure C2**). Roughness measures in PBS and untreated ACLT cartilage trended higher than sham, but did not reach significance ($p=0.10$ and 0.20 for PBS and untreated cartilage, respectively, for both average and RMS roughness).

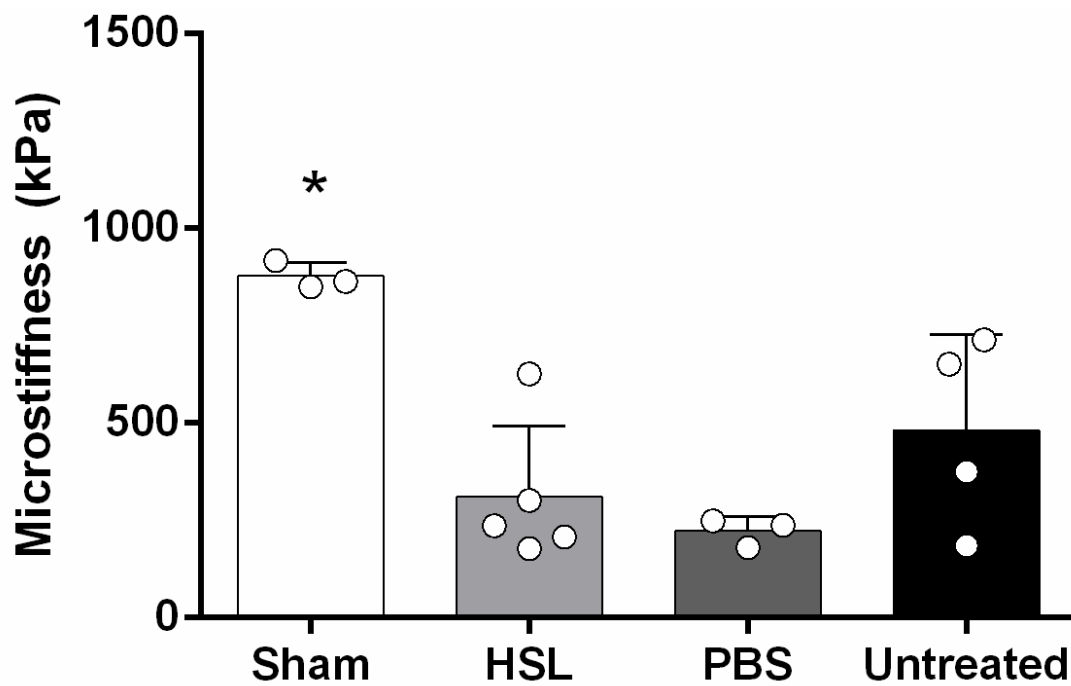


Figure C1: Microstiffness of medial femoral condyles following capsulotomy (sham) or ACLT treated with HSL, PBS or untreated. Sham cartilage was significantly stiffer compared to all groups that underwent ACLT.

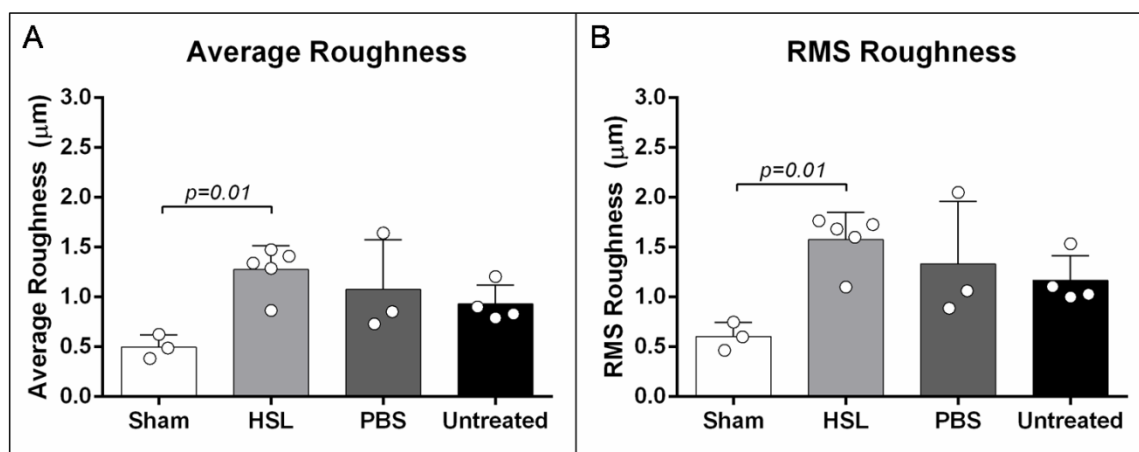


Figure C2: HSL had significantly higher average and root mean squared surface roughness compared to sham treated limbs.

C4 Discussion

Tribosupplementation via HSL injection has shown promising results in preventing cartilage damage following ACLT (5-9). Other measures of animals in this experiment (6) that were treated with HSL also had significantly lower histological scores, less c-telopeptide of type II collagen (CTXII) excretion in the urine corrected for creatinine, and more symmetric gait, as measured in the hind limb, compared to untreated and PBS treated joints (**Figure C3**). Despite improvements in these areas, HSL injection failed to prevent decreased microstiffness in the femoral condyles. Previous measurements of ACLT outlined in chapter 6 indicate that microstiffness decreases occur within 3 weeks of ACLT, and chondrocyte apoptosis occurs as soon as 1 week following ACLT. These rats were treated with HSL 7 days post-operatively and few apoptotic cells were observed compared to PBS and untreated ACLT rat cartilage. However, it is important to note that while HSL treated joints showed improvements over ACLT untreated or treated with PBS, sham joints had no presence of apoptosis, joint damage and significantly lower CTXII measurements compared to HSL injected joints (6).

Changes in AFM measurements of microstiffness changes occur only on the very superficial layer of the femoral condyle, which is the area to first lose proteoglycan and show matrix damage histologically. As a result, microstiffness is able to measure properties specific to the superficial zone and lamina splendens, and is a useful tool to measure very minute changes to the cartilage surface.

These results indicate that while HSL injection prevents much of the macroscopic and cellular degeneration of cartilage that follows ACLT, it is unable to prevent decreases in microstiffness in the superficial zone 70 days following surgical induction of OA.

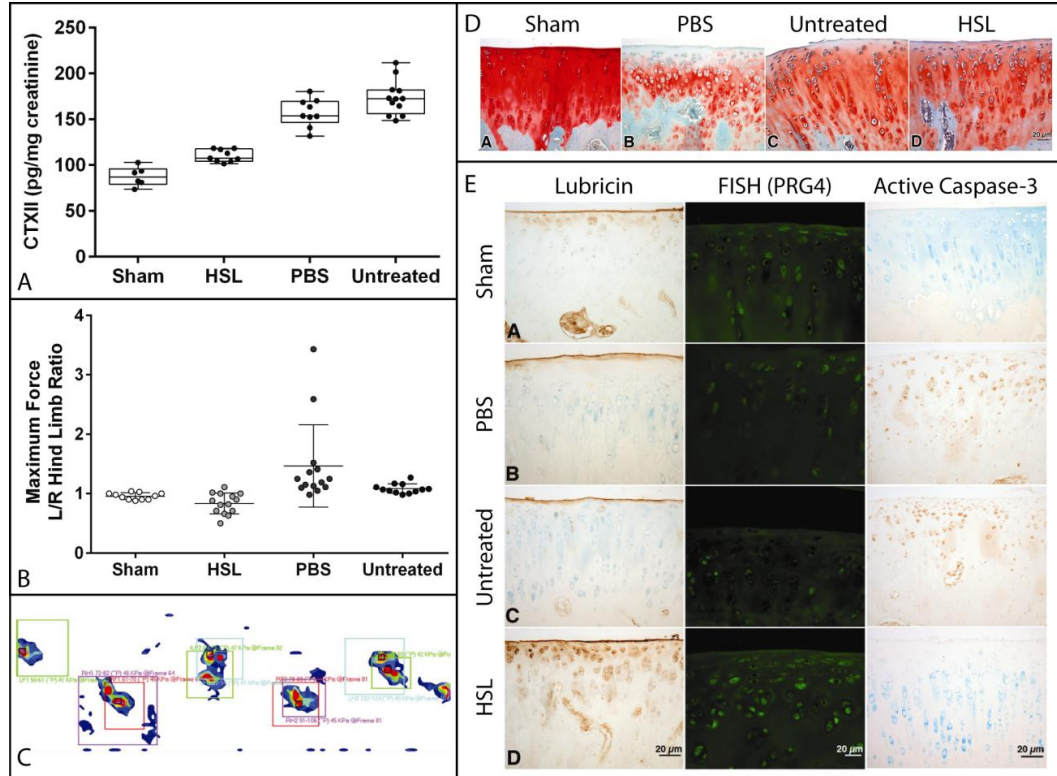


Figure C3: A.) Urinary C-telopeptide of type II collagen (CTX-II) levels normalized to creatinine levels, in control rats with capsulotomy, untreated rats with ACLT, rats with ACLT treated with PBS on day 7 after surgery, and rats with ACLT treated with human synoviocyte lubricin on day 7 after surgery. CTX-II levels were significantly lower in HSL treated animals compared to PBS treated or untreated ACLT ($p < 0.001$). B.) Ratio of average maximum paw strike force of the left hind limb to average maximum paw strike force of the right hind limb. Rats that had PBS-treated and untreated ACLT had unequal hind limb weight distribution during gait and favored their contralateral knee. In contrast, rats treated with HSL following ACLT and rats that underwent sham surgery had equal weight distribution, with some HSL treated animals favoring their surgical knee. C.) Representative rat gait with paw strikes identified over 2 gait cycles. The central maximum pressure within each strike box and its frame number are shown. D.) Safranin-O/Fast green stained tibial plateau cartilage. E.) Immunostaining of tibial plateau cartilage for lubricin using monoclonal antibody 9G3, fluorescence *in situ* hybridization (FISH) for lubricin mRNA and active caspase-3. Increased immunostaining for lubricin and upregulation of lubricin on the mRNA level was observed in the superficial zone and on the surface of cartilage was observed in lubricin-treated and control animals but not in PBS-treated or untreated animals with ACLT. There were more apoptotic cells in the superficial and upper intermediate zones of PBS treated and untreated ACLT cartilage compared to HSL treated or sham cartilage. Figure and caption were adapted from Jay, et al. (6).

C5 Acknowledgements

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