Boundary lubrication of articular cartilage: contribution of hyaluronan in health and injury

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BOUNDARY LUBRICATION OF ARTICULAR CARTILAGE: CONTRIBUTION OF HYALURONAN IN HEALTH AND INJURY

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Bioengineering by Jennifer M. Antonacci

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2011
The dissertation of Jennifer M. Antonacci is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2011
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Journal Articles


Selected Abstracts

Antonacci JM, Cai MZ, Beeman SM, Schumacher BL, Chen AC, McIlwraith CW, Temple-Wong MM, Sah RL: Mechanisms of Articular Cartilage Lubrication by


Healthy articular cartilage provides low friction properties to the synovial joint through both fluid film and boundary lubrication. Boundary lubrication, mediated by synovial fluid (SF), is important in reducing articulation-induced shear and wear. SF is composed of molecules including hyaluronan (HA), proteoglycan-4 (PRG4), and surface active phospholipids (SAPL) that have been shown to contribute to boundary lubrication. Joint injury predisposes articular cartilage to deterioration, and the mechanism of this may involve impairment of boundary lubrication function, possibly due to altered concentrations of HA, PRG4, and/or SAPL. However, clear relationships between the composition—boundary lubrication function of SF in health
and injury, particularly at physiological test surfaces, remains to be established. Therefore, the goal of this dissertation was to further the understanding of the contributions of SF macromolecules, with a focus on HA, to the boundary lubrication of articular cartilage under normal and pathophysiologic conditions, and to what extent chemical augmentation of injured SF by HA can restore lubrication function of SF and articular cartilage.

SF lubrication function and composition were altered coordinately after acute joint injury. Steady-state friction coefficients were higher, while HA concentration and molecular weight (MW) were shifted to lower levels in injury SF. Supplementation in vitro of the abnormal SF with HA of physiologic MW and concentration led to a marked recovery of boundary lubrication function. Articular cartilage lubrication by HA was markedly dependent on concentration and MW, approaching the low friction coefficients of native SF particularly at the MW and concentration of HA found in normal SF. The depletion of HA from SF led to a marked increase in friction coefficients for both normal and injury SF. Additionally, lubrication function of the HA-depleted SF was recoverable to normal levels upon repletion with high MW HA.

Collectively, these results elucidate the role of HA as a boundary lubricant for articular cartilage, contributing to the understanding of the altered low-friction properties of cartilage following joint injury, and may also aid in the development of targeted clinical treatments and repair strategies designed to restore failed joint lubrication and prevent further cartilage damage.
CHAPTER 1:

INTRODUCTION

1.1 General Introduction to the Dissertation

Articular cartilage covers the ends of long bones and acts as a low-friction, wear-resistant material that allows joint movement. Synovial fluid (SF) present within the joint acts as a biomechanical lubricant, facilitating joint movement by reducing surface friction and wear [123]. Healthy articular cartilage provides low friction properties to the synovial joint through both fluid film and boundary lubrication. Boundary lubrication, mediated by SF [105], is important in reducing articulation-induced shear and wear, providing protection and maintenance of the articular surface. SF is composed of molecules, including hyaluronan (HA), proteoglycan-4 (PRG4), and surface active phospholipids (SAPL), that have been implicated as boundary lubricants. Unfortunately, joint injury predisposes articular cartilage to deterioration, and the mechanism of this may involve impairment of boundary lubrication function, possibly due to altered concentrations of HA, PRG4, and/or SAPL. Recently, HA has been identified as an effective boundary lubricant at a cartilage-cartilage interface, but a clear relationship between the composition—lubrication function of SF in health and injury, particularly at physiological test surfaces, remains to be established.
Therefore, the aim of this dissertation work was to further the understanding of the contributions of synovial fluid macromolecules, with a focus on HA, to the boundary lubrication of articular cartilage under normal and pathophysiologic conditions, and to what extent chemical augmentation of injured SF by HA can restore lubrication function of SF and articular cartilage. Toward this goal, (1) the concentration and/or quality of HA, PRG4 and SAPL in pathological SF were characterized, (2) the extent to which altered SF lubricant composition reduces the boundary lubricating function of normal SF on articular cartilage was determined, (3) whether addition of the deficient molecules to SF could restore lubrication function was determined, (4) extending on the previous aims, the contributions of HA as a boundary lubricant for articular cartilage due to its local concentration and molecular size between cartilage surfaces was assessed, (5) the dependence of the friction-lowering ability of SF on articular cartilage on the presence of HA in SF was experimentally determined by selective depletion of HA from SF, and (6) the ability to restore the lubrication function of HA-depleted SF was investigated.

This work has contributed to a better understanding of the mechanisms of boundary lubrication of articular cartilage by HA, while elucidating the altered low-friction properties of cartilage and SF following joint injury and in degenerative joint disease. It may also contribute to the motivation for, and development of, clinical treatments and repair strategies designed to target failed joint lubrication in order to prevent further cartilage damage.

This chapter begins with an overview of the structure, function and composition of key components of the synovial joint system. The posited mechanisms of articular cartilage lubrication are then described, followed by a detailed review of the potential molecular contributors to the boundary lubrication of articular cartilage.
by SF. A more in depth review of the structure, function, and composition of HA is then given. Finally, altered lubrication function and SF composition in health, injury, and disease are discussed.

Chapter 2, which has been submitted to *Arthritis and Rheumatism*, assesses the boundary lubricating ability of SF for acute and chronic injured joints, as well as control joints, at a cartilage-cartilage interface. The concentration and/or molecular weight distribution of lubricant molecules HA, PRG4, and SAPL were also determined, and the relationships between lubrication function and composition were examined. Expanding on the contributions of HA to the lubrication of articular cartilage, tests were performed for HA of low, intermediate and high molecular weight (MW) at physiologic and pathophysiologic concentrations, under previously established test conditions for the boundary lubrication regime. Finally, the restoration of lubrication function of equine SF by addition of HA was experimentally determined.

Chapter 3, which has been submitted to *Journal of Bone and Joint Surgery*, extends on the findings from Chapter 2, examining the biochemical composition, including HA and PRG4, and the friction-lowering boundary lubrication function of trauma synovial fluid from humans afflicted with acute tibial plateau fractures compared with normal human synovial fluid. In addition, the possible biomechanical basis for impaired lubricant function was assessed by correlating friction coefficient and lubricant concentration.

Chapter 4 extends on the more detailed examination of the boundary lubrication mechanisms of articular cartilage by HA. Specifically, the effects of molecular weight and sliding velocity on their contributions to HA lubrication at an articular cartilage-cartilage interface were investigated. Tests of low, intermediate and
high MW HA at physiologic concentration were carried out to assess the variation in lubrication function with increasing sliding velocity at physiologic test surfaces. In order to assess the contributions of HA to SF lubrication function under normal and injury conditions, the lubrication function of HA-depleted SF from normal and injured joints was also examined at a cartilage-cartilage interface. SF was first treated with hyaluronidase to ensure the large MW species were reduced to small MW fragments, and the enzyme was then subsequenlty inhibited prior to friction testing. The ability to restore lubrication function of HA-depleted SF by the addition of exogenous HA at physiologic MW and physiologic and supra-physiologic concentrations was then determined.

Finally, Chapter 5 summarizes the major findings of this work and discusses future directions.

1.2 Composition and Function of the Synovial Joint

The synovial joint includes: articular cartilage, a load bearing connective tissue that normally facilitates low-friction and low-wear articulation [117]; synovial fluid (SF), a dialysate of blood plasma, biochemical depot and biomechanical lubricant for articular cartilage; and, the synovium, a thin, flexible lining of the joint composed of cells that synthesize and secrete key molecules that contribute to cartilage lubrication by SF, including HA [33, 113] and PRG4 [47, 107]. Other tissues, including ligaments and tendons, are also present and provide stability to the joint to allow for proper joint motion.

During joint movement, cartilage is subjected to repeated cycles of compressive and shear loading, with the compressive forces supported by cartilage in
the knee reaching several times body weight [3, 83], while the shear forces result from the frictional forces produced during articulation. Articular cartilage is a multiphasic tissue with a microstructure that is essential to its function. Adult articular cartilage is composed of relatively few chondrocytes within a fluid filled extracellular matrix. The fluid phase consists primarily of water (60 – 80% of the wet weight) as well as dissolved electrolytes, and the solid phase is composed mostly of collagen (10 – 20% of the wet weight) and proteoglycans (5 – 10% of the wet weight) [11, 72-74, 85, 86]. The SF, contained within the joint at a volume of 1 – 2 ml in a normal human knee joint [99], is composed of a variety of macromolecules, regulatory cytokines, and other factors, providing nutrients to the articular cartilage while facilitating joint movement.

In studying the lubrication of articular cartilage, it is important to consider its physico-chemical properties, which are mainly defined by the properties of the extracellular matrix [73], with the collagen fibers being capable of resisting high tensile stresses, embedded in a gel of proteoglycans (PG) and water acting as the compression-resisting element of cartilage tissue. The properties of the PG-water gel are what principally govern both fluid and solute transport in cartilage. Due to the fixed negatively charged carboxylate and sulphate groups of the PG (chondroitin and keratan sulphates), this gel can be considered as a polyelectrolyte solution and the ion exchange theory can be used to study the distribution of solutes between cartilage and external solutions [70, 71, 75]. The high density of negatively charged groups of the PG allows for maintenance of tissue hydration even under high applied loads, due to their high osmotic pressure [72, 76, 77, 129].
In the synovial joint, articular cartilage is in contact with synovial fluid, and as such, the solute concentration in cartilage depends on the concentration of the solute in SF and the distribution of solute between SF (or an equivalent solution) and cartilage [73]. The high fixed charge density of cartilage means that there are more ions in cartilage than in SF, and the osmotic pressure difference between cartilage and SF is due to the excess macromolecules and small ions [73]. The distribution of solutes between cartilage and an external solution is molecular weight dependent [71], where the distribution of molecules decrease with increasing MW such that the largest molecules able to penetrate into cartilage are on the order of the size of hemoglobin (MW~65kDa).

Unfortunately, the low-friction, wear-resistant properties of articular cartilage often begin to break down with aging, and also after joint injury. The normal pristine articular surface becomes roughened, eventually eroding down to the underlying subcondral bone, predisposing people to osteoarthritis (OA). OA is a debilitating, painful degenerative joint disease that affects ~20 million Americans, with a substantial economic impact of ~ $60 billion [13], and the need for therapeutic agents for the treatment and prevention of further progression of cartilage deterioration in OA is a major unmet medical challenge.
Figure 1.1: Modes of lubrication proposed for articular cartilage (cartoons were adapted from [5, 136]).
1.3 Mechanisms of Synovial Joint Lubrication

In synovial joints, articular cartilage bears load and slides relative to apposing tissue surfaces, benefiting from a combination of lubrication mechanisms. In fluid mediated lubrication, pressurized fluid, within the tissue and between the surfaces, such as in a fluid film, can bear significant portions of the load. In boundary lubrication, load is supported by surface-to-surface contact [136], mediated by a molecular surface layer or film at the articular surface, which may be composed of a variety of molecules. As such, boundary lubrication is important in reducing articulation-induced shear and wear. Boundary lubrication is mediated by synovial fluid (SF) [105] and postulated to be critical to cartilage homeostasis by maintaining low friction and wear [12].

Individuals typically take ~1-4 million steps each year [112], with joints being subjected to periods of rest and motion with each gait cycle. In the knee, the femoral surface slides and rolls against the tibial surface, maintaining contact during ~60% of the walking cycle [5], often under high contact stresses ranging up to 10 times body weight [1, 7, 81, 84], thus presenting a major biomechanical challenge to the synovial joint. The peak translational speeds between two articulating surfaces, within various joints over a variety of activities, can range from 0.06 to 0.6 m/s [5]. Therefore, the magnitude and duration of joint loading, as well as the relative motion between the articulating surfaces, are all factors that should be considered when discussing the lubrication mechanisms responsible for the mechanical integrity of articular cartilage that is maintained over the lifetime of synovial joints.

There are several possible modes of lubrication that have been proposed to account for the friction and wear resistant characteristics of synovial joints under various loading conditions (Figure 1.1). In fluid lubrication, hydrodynamic lubrication
(Figure 1.1A) occurs when surface separation results from the formation of a thick fluid film due to the kinematics of opposing surfaces. Hydrodynamic lubrication depends on a number of factors that are critical to mechanisms of lubrication, including the macroscopic bearing geometry (curvature effect), the interfacial topography (roughness effect), the normal load (pressure effect), the relative speed (shear rate effect), and fluid film rheological properties (viscosity effect) [5, 24, 136]. A common engineering example of for this lubrication mode is crankshaft bearings of car engines, which are free to move through small eccentrically distributed radial clearances, which forms a film of varying thickness [123]. For cartilage and SF, this mode of lubrication may occur due to the high viscosity of SF and the relative motion of joint surfaces, which can create a thin wedge-shaped fluid layer due to hydrodynamic pressure build up in the SF, which generates lift and forces the two surfaces apart [5, 136]. However, to generate a large enough load-carrying pressure, continuous high-speed relative motion between the two bearing surfaces is required (eg; as may be the case in the shoulder joint of a pitcher). Generally, such high speeds and lighter loads are not often present for most daily activities, which generally involve intermittent and low-speed motions in various joints [5, 14, 15]. In elastohydrodynamic lubrication (Figure 1.1B), pressure in a self-generated hydrodynamic fluid film causes elastic deformation of the confining surfaces, and the film thickness also depends on the elastic properties of the solid surfaces [123, 136]. Including cartilage deformation in the hydrodynamic mode would act to spread the joint load over a larger surface area, reducing the velocity gradient between the two surfaces and thus increasing the film thickness between joint surfaces. However, film thicknesses reported for this lubrication mode are still less than the surface roughness of contacting cartilage surfaces, which may be on the order of $1 - 6 \mu m$ [24].
Hydrostatic, or weeping, lubrication (Figure 1.1C) occurs when a fluid film that separates the opposed surfaces is generated by external pressurization means (e.g., a pump). No external pressure exists in the case for synovial joints, however self pressurization of the joint fluid may be possible due to exudation of interstitial fluid from the articulating joint under compression – sometimes called ‘weeping lubrication’. In boosted lubrication (Figure 1.1D), as articulating surfaces approach, the water component of SF passes into cartilage, which then concentrates the lubricants in SF, and this concentrated pool of lubricants at the articular surface may provide significant friction-reducing properties to the synovial joint.

The transition between the various lubrication regimes depends on the surface roughness and the film thickness, which is a function of the fluid viscosity, sliding speed, and applied normal load (or mean pressure). In boundary lubrication (Figure 1.1E), load is supported by surface-to-surface contact, and relative movement in this regime does not favor the formation of any significant fluid film. The associated frictional properties are predominantly determined by lubricant surface molecules. Friction coefficients being invariant with factors that affect the formation of a fluid film, such as normal load and relative velocity of the articulation surfaces [5, 123, 136], is a defining feature of boundary lubrication. This mode has been proposed to be important for the maintenance of the articular surface because the opposing cartilage layers make contact over ~10% of the total area, and this may be where much of the friction occurs [82]. In the absence of a strongly adsorbed, continuous and self-replenishing boundary layer, prolonged cartilage surface – surface interactions may promote higher friction, wear, and degradation of the articular cartilage. Combinations of fluid and boundary lubrication modes are likely to contribute to the lubrication of synovial joints at certain phases of the gait cycle (Figure 1.1F).
When analyzing mechanisms of boundary lubrication of articular cartilage, it is important to characterize the test configuration, surfaces, and operative mode of lubrication. Accordingly, a protocol, developed recently in our lab [105] based on pioneering studies of Davis [19, 20] and Fung and Malcom [27, 68] to achieve and study the boundary mode of lubrication in an annulus-on-disk rotational test configuration, will be used throughout this proposal. Several combinations of in vitro mechanical test systems and test surfaces, at various scales, have been used to study joint lubrication, each with their advantages and disadvantages. Artificial surfaces, such as latex-on-glass [19, 25, 49, 98], or semi-physiological systems such as cartilage-on-glass [30, 58, 60], can be easily manipulated and may reproduce some, but likely not all, of the molecular interactions that are operative in physiological articulation. Conversely, cartilage-on-cartilage at the whole joint scale [88, 124] retains a realistic, complex geometry, where many modes of lubrication are likely operative. Cartilage-cartilage tests at the tissue scale lack the complexity of joint scale tests, yet retain a physiological test surface. These tests can be performed in a sliding or rotational configuration. In the rotational configuration, plowing friction losses are minimized because the opposed surfaces remain in contact and fluid pressure effects are minimal at relatively slow velocities after the initial pressure dissipates. Also, with the use of an annular geometry, the variation in sliding velocity is reduced. Therefore, the annulus-on-disk rotational test configuration is advantageous for studying boundary lubrication at an articular cartilage-on-cartilage interface. By examining the lubricating ability of healthy and pathological SF on physiological test substrates, certain aspects of naturally articulating surfaces can be mimicked, and thus allows for molecular interactions that occur during physiological articulation [10].
1.4 Molecular Mechanisms of Articular Cartilage Boundary Lubrication by Synovial Fluid

The molecular layer at the articular surface consists of glycosaminoglycans, such as HA [89], proteoglycans, such as PRG4 [121], and surface active phospholipids (SAPL) [109]. PRG4 is secreted into synovial fluid by cells of cartilage [106], synovium [54], and meniscus [108], while HA and SAPL are secreted primarily by type B synoviocytes [23]. These molecules that have been localized at the surface of articular cartilage [37, 47, 107, 137], as would be expected for a boundary lubricant, are also present at high concentrations in SF [78, 79, 95, 102]. The degree to which each of these molecules contributes to the boundary lubrication of articular cartilage has been the subject of debate for decades.

HA is a negatively charged unbranched glycosaminoglycan, composed of repeating disaccharides of N-acetylglucosamine and D-glucuronic acid. HA is found in all tissues and body fluids, and it is the macromolecule of high-molecular weight (10^5-10^8 Da) that imparts high viscosity to SF [63]. PRG4, also known as superficial zone protein (SZP) and lubricin [41, 47, 54, 106-108], is encoded by the prg4 gene. It is a glycoprotein with a mucin domain primarily secreted into the SF and localized to the articular cartilage and synovial membrane. The functional importance of PRG4 was elucidated when mutations in the prg4 gene were linked to camptodactyly-arthropathy-coxa vara-pericarditis (CACP) disease syndrome, an autosomal recessive disease that leads to synovial hyperplasia and premature cartilage deterioration [69], indicating the importance of PRG4 to the health of synovial joints. SAPL are composed of a polar head group and fatty acid chains that comprise the non-polar end, reported to exist as an oligolamellar structure at the articular surface [36, 38]. Although dipalmitoyl phosphatidylcholine (DPPC) is the most abundant phospholipid
at the articular surface and in SF [95, 102], other species of phospholipids present include other phosphatidylcholines along with phosphatidylethanolamines (~27%) and sphingomyelins (~32%) [102].

The contribution of HA in the lubrication of articular cartilage has been analyzed using a variety of friction tests. Variations include experiments on whole joints [66, 120] versus small specimen, and different test surfaces such as rubber, latex, glass, and cartilage. Since these studies employed different whole joint test systems in which several modes of lubrication were likely operative, it is not surprising that HA has been reported to be both effective [9, 35, 67] and ineffective [65, 96] as a boundary lubricant. In the cartilage-on-cartilage test configuration, HA does indeed appear to contribute, in a dose-dependent manner, to the boundary lubrication of articular cartilage [104].

Lubricin/SZP/PRG4 (which collectively will be referred to as PRG4 from here on out) has been shown to possess boundary lubrication function at a cartilage-glass interface [103, 119, 122], a latex-glass interface [46, 47, 50-53], and a cartilage-cartilage interface [104], acting as a boundary lubricant under a number of different postulated motifs [10, 34, 40, 51, 109].

Similar to previous studies on the role of HA in the lubricating ability of SF, there have been varying results regarding the contribution of SAPL to the boundary lubrication of articular cartilage. Friction was slightly lowered when DPPC was tested at a cartilage–steel interface [90], considerably reduced when a very high concentration of phosphatidylcholine was tested under boundary-lubricating conditions on a latex–glass substrate [48], and not significantly lowered when a physiologic concentration of DPPC was tested at a cartilage-cartilage interface in the boundary lubrication regime [104]. Although DPPC is the most abundant
phospholipid in SF [95, 102], the role of other species of phospholipids present in SF, such as phosphatidylcholines, phosphatidylethanolamines, and sphingomyelins [102], in the boundary lubrication of articular cartilage remains to be determined.

1.5 Structure, Function, and Composition of Hyaluronan

HA is a multi-functional high MW polysaccharide found in many tissues throughout the body, especially in the extracellular matrix of connective tissues such as cartilage, and also SF. An adult with an average body weight of 70 kg contains ~15 g of HA [131]. HA is a linear polymer built from repeating disaccharide units with the structure [D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]n [133] (Figure 1.2A), where the disaccharide units are linked by a B(1→4) bond [26, 62, 63]. Despite its relatively simple structure, HA is an exceptionally versatile glycosaminoglycan with multiple roles in many biological functions including cell signaling, wound repair and regeneration, morphogenesis, and pathobiology (reviewed in [57, 115, 131]); and, as the topic of this dissertation, a lubricant for lubrication of synovial joints.

Structure and Properties of Hyaluronan

HA is the simplest glycosaminoglycan (GAG), being unbranched, unsulphated, and immunologically inert. And, unlike other GAGs, it is synthesized as a free polysaccharide (ie; not covalently linked to proteins) in the plasma membrane, rather than the endoplasmic reticulum and the Golgi [94], ranging from 2500 – 25,000 repeating disaccharides in length. The molecular weight of HA is usually several hundred thousands to millions, reaching up to a molecular mass of $10^6 – 10^7$ Da [8, 26, 56]. An HA polymer has one carboxyl group per repeating disaccharide unit, therefore
making it a polyelectrolyte with a negative charge at neutral pH. Studies have shown evidence both in favor of and against HA chain-chain interaction in aqueous solutions. Molecular diffusion studies of HA at high concentration (>1.0mg/ml) in aqueous solution indicate HA properties that reflect molecular entanglement with no evidence of chain-chain association [31, 32]. While other studies have found evidence of secondary hydrogen bonds thought to stabilize the HA structure along the chain axis, imparting a stiffened helical configuration and generating hydrophobic patches that permit interaction with other HA chains [110, 111] (Figure 1.2B), which gives HA an overall expanded coil structure in solution [2, 111]. Despite the conflicting evidence of HA chain-chain interaction in aqueous solution, HA chains are very mobile, forming a dynamic network that can restrict the movement of other macromolecules, but is freely permeable to low molecular weight substances [8].

**HA Biosynthesis**

HA is synthesized in the plasma membrane (PM) by membrane-bound enzymes referred to as the HA synthases (HAS) [21, 22]. In eukaryotes, because of its enormous size, HA is coordinately synthesized by three integral membrane HAS enzymes (HAS1, HAS2, HAS3) [43, 45, 132] on the inner surface of the PM and extruded across the membrane out into the extracellular space [92, 93]. The complex regulation of HA biosynthesis is based on substrate concentration, expression of HAS, transfer of two substrates and elongation and secretion of the product [44].

Although each HAS is able to synthesize *de novo* HA chains with identical structures, each HAS enzyme has distinct properties. HAS1 is the least active, responsible for synthesizing a wide size range of HA polymers, from $2 \times 10^5$ to $2 \times 10^6$ Da. HAS2 more actively synthesizes large MW forms HA greater than $2 \times 10^6$ Da, and
is implicated in developmental and repair processes involving tissue expansion and growth. HAS2 is believed to be the enzyme that responds to stress-induced increases in synthesis of HA, as found in inflammation, shock, and after major blood loss [131]. HAS3 is the most active HAS enzyme, responsible for the synthesis of large amounts of HA over a wide range of MW, from $0.2 \times 10^6$ to $2 \times 10^6$ [45] in smaller forms than HAS1 and HAS2.

The distinct expression patterns of the three isoforms of HAS enzymes are in part controlled by growth factors and cytokines [59, 97, 118]. The major HAS enzymes in human synovium are HAS1 and HAS2 [97]. Analysis of the HAS enzymes in synovial fibroblasts in patients with rheumatoid arthritis and OA revealed that HAS1 mRNA is up-regulated by TGF-β while HAS3 is up-regulated by IL-1β and TNF-α. Such regulation may indicate a possible role of altered HA synthesis and/or HA degradation as mechanisms behind the alteration of high MW HA and decreased HA concentrations in SF of patients with OA and inflammatory diseases. A possible shift from HAS1- to HAS3-mediated synthesis may be one explanation for the lower molecular weight and concentration of HA in SF from diseased joints [8].

It is important to note that the exact function of each of the HAS enzymes is unknown and little is known about the regulation of the three eukaryotic HA enzymes.

**HA Degradation by Hyaluronidases (HYALs)**

*In vivo*, HA is degraded by hyaluronidases (HYALs) [17] and by the action of oxygen free radicals (discussed below). The turnover of HA in vertebrate tissues is rapid, with an estimated daily turnover of 5g in a 70kg individual containing ~ 15g of total HA [131]. The half-life of HA in the blood stream is 2 – 5 minutes and ~ 12 hours in SF. HA catabolism is predominately regulated by several hyaluronidases, or
HYALs, classified primarily as endo-β-N-acetylgulcosaminidases according to their hydrolytic mechanisms in mammalian types [42]. The products of HYAL degradation of HA have chemical structures identical to that of the parent polymer, aside from chain length.

The family of human HYAL genes includes HYAL1, HYAL2, HYAL3, HYAL4, HYALP1 and PH-20. HYAL 1 and HYAL2 are considered the major HA-degrading enzymes in somatic tissues [16, 64]. HYAL1 is found in plasma, SF, and urine, in addition to lysosomes where the final step of HA degradation by HYAL1, together with β-D-glucuronidase and β-hexosaminidase, to its constituent monosaccharides occurs [17, 114]. Prior to degradation by HYAL1, it is thought that HYAL2 cleaves high MW HA to fragments of ~20kDa [64]. There is less known about the role of HYAL3, HYAL4, and HYALP1 in humans.

Due to the dynamic, diverse and ubiquitous nature of HA in many physiologic and pathophysiologic conditions, inhibition of HA degradation by hyaluronidases appears critical to homeostasis of many tissue environments. A wide variety of substances have been identified to have at least partial inhibitory function of the various hyaluronidases, in many different forms. These potent regulating agents include proteins, GAGs, polysaccharides, fatty acids, antibiotics, antioxidants, polyphenols, and anti-inflammatory drugs, in addition to many more [29, 80]

**HA Degradation by Non-enzymatic Reactions**

In addition to catabolism by HYALs, HA degradation can occur by a number of non-enzymatic reactions. Degradation by acid or alkaline hydrolysis leads to a random mixture of oligo- and monosaccharides [55, 134], while ultrasonication (US) degrades HA in a non-random fashion [130] to oligo-saccharides of 100kDa or less.
HA, and other GAGs, is also susceptible to degradation by various reactive oxygen species (ROS), via scissions of the glycosidic linkages. Many human diseases are associated with the harmful action of ROS, such as hydrogen peroxide, hydroxyl radicals, and nitric oxide, to name a few. The reduction in HA MW in the SF of patients suffering from rheumatic diseases has led to in vitro studies of HA degradation by ROS (reviewed in [116]), indicating ROS may be detrimental to the quality and quantity of HA present within the synovial joint.

**HA in the Synovial Joint**

The mechanical properties of cartilage rely on an intact collagen network and a high concentration of aggrecan. Aggrecan monomers bind to HA and a small protein, called link protein, forming non-dissociable aggregates that can contain up to 100 aggrecan monomers with a MW of ~ $2 \times 10^6$ Da per monomer. The very large MW of aggrenan aggregates act to limit its loss from cartilage, and this immobilization of aggrecan is an important function of HA [8]. Although the concentration of HA in human articular cartilage increases with age, the MW decreases to ~ $3 \times 10^5 - 6 \times 10^5$ Da [39], while the half-life of HA in cartilage is on the order of 2-3 weeks.

HA in SF is secreted primarily by synoviocytes. The majority (~70%) of HA in normal SF is reported to be at greater than or equal to 4,000 kDa with the remaining HA being distributed at lower MWs in the range of <4000 kDa to ~100-200 kDa [6, 18, 61]. The HA content in SF has been widely studied, with a broad range of values reported for horses (from 0.3 to 1.3 mg/ml HA) [91, 100, 101, 126, 127] and humans (from 1-4 mg/ml HA) [78, 101].
Figure 1.2: Structure of hyaluronan (A) single disaccharide unit and (B) polysaccharide in solution. (adapted from [57] and http://glycoforum.gr.jp/science/hyaluronan.html).
1.6 Altered Lubrication and Synovial Fluid Composition in Health, Injury, and Disease

Joint injury predisposes articular cartilage to deterioration [128], and the mechanism of this may involve impairment of boundary lubrication function [25, 49], possibly due to altered concentrations of HA, PRG4, and/or SAPL. Normal joints may progress to become osteoarthritic after acute injury [28, 135], such as an anterior cruciate ligament tear or an intra-articular fracture. This progression may involve reduction in functional boundary lubrication of articular cartilage that is due to alteration in the SF lubricant composition. Specifically (Table 1.1), the concentrations of PRG4 have been shown to decrease after acute injury [25], and become slightly elevated in OA [87, 103]; while concentrations of HA appear to decrease after joint injury and in OA [4, 78]; and phospholipids are decreased in acute injury and slightly elevated in OA [78, 95]. HA content in SF from horses with traumatic synovitis, were not significantly different from control SF but tended to decrease for acute injury SF (0.47 vs 0.61 mg/ml) [101].

The MW distribution of HA also change with injury and age. HA in SF from patients with rheumatoid arthritis and other joints diseases ranges from 0.3 – 5 x 10^6 Da, while that of normal SF ranges from 2 – 10 x 10^6 [6, 18, 78]. HA MW in normal equine SF ranges from 2 – 3 x 10^6 Da, which does not differ significantly from MW of HA in SF from horses with arthritis (1.5 – 3 x 10^6 Da) [127]. Although the size distribution of HA in pathological SF has been extensively studied, there is little information about the effect of acute or traumatic injury on the MW distribution of HA in SF.

Such alterations in lubricant concentration and quality may manifest as increased friction, as has been demonstrated in acute synovitis in humans [49]. By the
time of development of osteoarthritis (OA), the friction coefficients of SF appear to return to near normal values [49]. Previous studies of the effects of SF lubrication function after injury in humans and other animal models have used either material-on-material, particularly latex-on-glass [19, 25, 49, 98], or whole joint [88, 125] friction test systems. Jay [49] demonstrated that human SF from acutely injured knees exhibited poor boundary lubrication, while SF from human OA knees lubricated test surfaces similar to that of normal human SF. The latter findings for OA SF were also observed by Davis [19]. In a rabbit injury model [25], a decrease in boundary lubrication of injured SF was observed at 2 and 3 weeks post-injury, with friction coefficients ranging from ~0.03 - 0.09 at 1 week post-injury, to ~0.05 - 0.13 and 0.07 - 0.10 at 2 and 3 weeks, respectively. Identifying the contribution of injury SF to boundary lubrication at a cartilage–cartilage interface will extend on these previous results by examining the lubricating ability of pathological SF on physiological test substrates, thus mimicking certain aspects of naturally articulating surfaces and allowing for molecular interactions that occur during physiological articulation [10].
Table 1.1: Synovial fluid lubricant composition in health and disease [4, 25, 78, 87, 95, 103].

<table>
<thead>
<tr>
<th>Synovial Fluid Constituent</th>
<th>Concentration in SF [mg/ml]</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Hyaluronan (HA)</td>
<td>1 – 4</td>
</tr>
<tr>
<td>Proteoglycan4 (PRG4)</td>
<td>0.05 – 0.35</td>
</tr>
<tr>
<td>Phospholipid (SAPL)</td>
<td>0.1</td>
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1.7 References


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CHAPTER 2:

EFFECT OF EQUINE JOINT INJURY
ON BOUNDARY LUBRICATION OF
ARTICULAR CARTILAGE BY SYNOVIAL FLUID:
ROLE OF HYALURONAN

2.1 Abstract

Objective. To compare equine synovial fluid (eSF) from post-injury and control joints for (1) cartilage boundary lubrication function, (2) putative boundary lubricant molecules hyaluronan (HA), proteoglycan-4 (PRG4), and surface-active phospholipids (SAPL), (3) relationships between lubrication function and composition, and (4) lubrication restoration by addition of HA.

Methods. eSF from normal (NL), acute injury (AI), and chronic injury (CI) joints were analyzed for boundary lubrication of normal articular cartilage as kinetic friction coefficient (\( \mu_{\text{kinetic}} \)). eSF were also analyzed for HA, PRG4, and SAPL concentrations and HA molecular weight (MW) distribution. The effect of addition of HA, of different concentrations and MW, to AI- and NL-eSF samples on \( \mu_{\text{kinetic}} \) was determined.
**Results.** The $\mu_{\text{kinetic}}$ of AI-eSF (0.036) was higher (+39%) than that of NL-eSF (0.026). Compared to NL-eSF, AI-eSF had a lower HA concentration (−30%) of lower MW forms, higher PRG4 concentration (+83%), and higher SAPL concentration (+144%). CI-eSF had $\mu_{\text{kinetic}}$, HA, PRG4, and SAPL characteristics intermediate to that of AI-eSF and NL-eSF. Regression analysis revealed that $\mu_{\text{kinetic}}$ decreased with increasing HA concentration in eSF. The friction-reducing properties of HA alone improved with increasing concentration and MW. Addition of high-MW HA (4,000kDa) to AI-eSF reduced $\mu_{\text{kinetic}}$ to a value near that of NL-eSF.

**Conclusion.** In the acute post-injury stage, eSF exhibits poor boundary lubrication properties as indicated by a high $\mu_{\text{kinetic}}$. HA of diminished concentration and MW may be the basis for this, and adding HA to deficient eSF restored lubrication function.
2.2 Introduction

In synovial joints, articular cartilage bears load and slides relative to apposing tissue surfaces, with friction and wear reduced through a number of biophysical mechanisms including boundary lubrication [2, 43]. Boundary lubrication of articular cartilage is mediated by synovial fluid (SF) components that reduce the interaction of articulating surfaces [15, 18, 38]. Normal SF contains a number of molecules that have been implicated in contributing to the boundary lubrication of articular cartilage. These putative lubricant molecules include hyaluronan (HA) [28], proteoglycan-4 (PRG4) [42], and surface active phospholipids (SAPL) [41]. Each of these molecules is present at high concentrations in synovial fluid [24, 26, 32, 36] and has been localized at the surface of articular cartilage [40, 41, 50], as would be expected for a boundary lubricant.

Alteration of the friction-lowering function of SF may contribute to deterioration of articular cartilage in joint disease and after joint injury [7, 9, 20, 27, 33]. However, the lubrication function of SF varied substantially in these studies, as did the biomechanical test methods and counter-face materials used in the lubrication tests. In general, lubricant solutions exhibit boundary-mode friction that is less for cartilage-on-cartilage than glass-on-rubber [33] and cartilage-on-glass [16, 27], and similar for glass-on-latex [7, 9, 20]. After acute injury [14, 48], such as anterior cruciate ligament rupture, meniscal tear, or intra-articular fracture, synovial joints are pre-disposed to deterioration and premature osteoarthritis (OA). Such deterioration may involve reduction in functional boundary lubrication of articular cartilage due to
alterations in the concentrations of SF lubricant molecules [9, 21, 44]. After acute injury, the diminished lubrication properties of pathological SF have been associated with lower concentrations of PRG4 [9]. In OA, the friction coefficient of SF appears to be normal [20], and the concentration and molecular weight (MW) distribution of HA are shifted to lower levels [3, 6, 13, 45]; however, the concentration and MW distribution of HA has not been associated directly with decreased lubrication. Additionally, the concentration of phospholipids was lower in acute injury compared to SF from uninjured joints, but higher in OA [24, 32]. It remains to be established if SF lubricant dysfunction occurs after different types of joint injury and whether such alterations relate to variations in the concentrations and quality of lubricant molecules.

Race horses are commonly afflicted with osteochondral fractures and OA of the carpal and metacarpophalangeal joints, and thus provide a natural model system for study of joint injury [25]. Horses with joint injuries are often evaluated acutely for treatment of osteochondral chip fragments or slab fractures, and such joints exhibit signs of acute synovitis. In contrast, some horses are evaluated for more chronic joint damage and secondary osteoarthritic changes. The SF of such injured joints may be affected both in lubrication function and lubricant composition.

The objectives of this study were to determine, for equine synovial fluid (eSF) from acutely injured (AI), chronically injured (CI), and normal (NL) joints, (1) the coefficient of friction at a cartilage-cartilage interface in the boundary lubrication regime, (2) the concentrations and/or MW of HA, PRG4, and SAPL, (3) the relationships between lubrication function and composition, (4) the contribution of
HA to cartilage-cartilage lubrication at different MW and concentrations, and (5) if addition of the deficient molecules to eSF could restore lubrication function.
Figure 2.1: Arthroscopic views of a normal (A) and acutely (B) injured metacarpophalangeal joint with a fracture fragment (indicated by the solid arrow head) off the proximal (P) dorsal aspect of the first phalanx (S: synovium, R: radius); (C) normal and (D) chronic injury of the equine antebrachio-carpal joint with a fragment off the intermediate (I) carpal bone and full-thickness erosion of the articular cartilage, as well as chronic synovitis (thickened synovial villi indicated by narrow arrow head).
2.3 Materials and Methods

Materials. Materials for lubrication testing were obtained as described previously [38]. In addition, hyaluronan (HA) was obtained as 6.4, 51, and 780 kDa forms from Lifecore Biomedical (Chaska, MN), ~800 kDa (SupArtz®, Smith and Nephew, Memphis, TN, MW range 620-1,170 kDa, polydispersity index 1.6 [47]), and 4,000 kDa (Healon®, Advanced Medical Optics, Santa Ana). An antibody to PRG4 was anti-Lubricin from AbCam (Cambridge, MA); non-specific rabbit IgG was from Pierce (Rockford, IL); mouse anti-rabbit IgG secondary antibody was from Jackson ImmunoResearch (West Grove, PA). *Streptomyces* hyaluronidase was from Seikagaku (Tokyo, Japan). SeaKem® gold agarose was from Lonza (Rockland, ME). 50X TAE (2M Tris, 0.5M EDTA) electrophoresis buffer was from Life Technologies (Carlsbad, CA). Hybond™-LFP polyvinylidene difluoride (PVDF) membrane for Western blotting was from GE Healthcare (Piscataway, NJ). Stains-All was from Sigma-Aldrich (St. Louis, MO).

Synovial Fluid Samples. Normal bovine SF (NL-bSF) was prepared as described previously [38] with n=5 pools, each from different adult animals.

Equine SF samples were acquired by one of the authors (CWM) during arthroscopic surgery of adult horses (2-4 y.o., n=20). Synovial fluid was aspirated from the injured carpal (n=14) or metacarpophalangeal (n=6) joint, as well as contralateral joints as controls (n=20). The eSF was classified as acute or chronic based on the estimated duration between joint injury and arthroscopic treatment, as well as arthroscopic observations. Synovial fluid samples from twenty horses were
selected to provide ten AI-eSF, ten CI-eSF, and twenty contralateral normal NL-eSF samples. AI-eSF were from horses that presented for surgery within three weeks of clinical diagnosis, often with signs of moderate to severe synovitis. Figure 2.1A shows an arthroscopic view of a normal metacarpophalangeal joint; Figure 2.1B shows a view of the same location in a joint with an acute injury. CI-eSF was from horses that presented for surgery more than three weeks after injury, where articular cartilage degeneration was often observed and synovitis was generally less severe. Figure 2.1C shows a normal proximal intermediate carpal bone articular surface in the medial side of the antebrachiocarpal joint; Figure 2.1D shows a joint with a chronic fragment off the proximal intermediate carpal bone with erosion of articular cartilage on the surface, partial thickness erosion on the distal lateral radius (lower) and thickened synovial villi (black arrow), indicative of chronic synovitis (secondary osteoarthritis).

All eSF samples were clarified of cells and debris by centrifugation (3,000g, 30min) immediately after joint aspiration. The supernatants were then collected and stored at –80°C until subsequent analysis.

**Experimental Design.** *Exp. 1: Variations in eSF.* Biomechanical and biochemical analyses were performed to determine the effect of injury on eSF lubrication function and composition. Portions of NL-bSF (*n*=5), NL-eSF (*n*=20), AI-eSF (*n*=10), and CI-eSF (*n*=10) samples were analyzed by biomechanical lubrication tests for friction-lowering properties as indicated by kinetic steady-state (equilibrium) and static (start-up) coefficients of friction, *μ*<sub>kinetic</sub> and *μ*<sub>static</sub>, respectively. Other portions were analyzed by biochemical assays for the concentrations and/or MW of
putative lubricant molecules, HA, PRG4, and SAPL. Univariate and multivariate regression analyses were performed to assess the relationship between friction coefficient and lubricant composition.

**Exp. 2: Lubrication Properties of HA of Varying Size and Concentration.** Portions of HA preparations were analyzed by biomechanical lubrication tests for friction lowering properties as indicated by $\mu_{\text{kinetic}}$ and $\mu_{\text{static}}$. HA of MWs 6.4, 51, 780, and 4,000 kDa were analyzed at concentrations of 0.33, 1.1, and 3.3 mg/ml, with n=6 for each MW and concentration combination.

**Exp. 3: Restoration and Enhancement of Dysfunctional AI-eSF.** Based on deficient friction-lowering properties and low HA values in Exp. 1, some samples of AI-eSF (n=7) and NL-eSF (n=9) were analyzed further. To portions of AI-eSF, HA in the form of SupArtz® (HA800, n=3) or Healon® (HA4000, n=4) was added such that the final concentration of exogenous HA in the eSF was 1.0 mg/ml, to restore HA concentrations to those similar to levels found within the normal range (0.3-1.3 mg/ml) reported for eSF [30, 34, 35, 45]. Friction tests were then performed on AI-eSF and AI-eSF+HA samples. Similar experiments were carried out on NL-eSF with HA800 (n=5) or HA4000 (n=4). The addition of exogenous HA to all eSF resulted in a slight (10%) dilution of SF.

**Lubrication Test.** Portions of SF and HA samples were analyzed for $\mu_{\text{static}}$ and $\mu_{\text{kinetic}}$ in the boundary lubrication mode on articulating cartilage surfaces as described previously [37, 38]. Intact articular surfaces were in the form of osteochondral cores and annuli from adult bovine knees, stored in phosphate buffered saline (PBS) supplemented with protease inhibitors (PIs) (2 mM Na-EDTA, 1 mM PMSF, 5 mM
Benz-HCL, and 10 mM NEM) at –80°C. Test lubricants were also supplemented with PIs, resulting in a 3% dilution of the sample. Samples were tested with 18% cartilage compression, an effective sliding velocity of 0.3 mm/second, with pre-sliding durations (T_p, the duration the sample is stationary prior to rotation) of 120, 12, and 1.2 seconds. Friction coefficients (µ) were calculated from the torque, τ, and equilibrium axial load, with µ_{static} calculated from the peak |τ|, measured just after (within 10° of) the start of rotation, and µ_{kinetic} calculated from the |τ| averaged during steady-state sliding. Consistent with previous results [37], µ_{kinetic} did not vary substantially with T_p, so µ_{kinetic} data are presented as the average at all T_p. Also consistent were µ_{kinetic} and µ_{static} for PBS (>0.20), so these results were not analyzed further.

**Biochemical Analysis of Boundary Lubricants.** Portions of SF samples were analyzed biochemically for the concentrations of HA, PRG4 and SAPL. **HA concentration** in eSF samples was determined by an ELISA-like assay using HA binding protein [12]. **HA MW distribution** in eSF samples was determined by horizontal electrophoresis through a 1% agarose gel [5, 23]. Briefly, portions of SF samples were digested with proteinase K, a 300 ng portion of HA was run on each lane, and the gel stained with Stains-All. Gel images were digitized and processed to determine HA distribution and concentration in the MW ranges of 0.05–0.25, 0.25–0.5, 0.5–1, 1–2.5, and 2.5–7 MDa. **PRG4 concentration** in eSF samples was quantified after Western Blot using anti-Lubricin antibody [5]. Briefly, portions of eSF samples were treated with *Streptomyces* hyaluronidase, a 0.5 µl portion was run on each lane of a 2% agarose gel, samples were transferred to a PVDF membrane, the membrane
probed with anti-Lubricin antibody or non-specific rabbit IgG, followed by a mouse anti-rabbit light-chain specific secondary antibody conjugated to horseradish peroxidase, and then quantified by ECL-Plus detection and digital scanning with a STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT). Equine PRG4 standards were from the conditioned medium of horse cartilage explants [39] and loaded at 0.1µg and 0.2µg per lane. Densitometric scans were analyzed with ImageQuant software (Molecular Dynamics) to determine PRG4 concentration. SAPL in eSF samples was measured by an assay that detects phospholipase-sensitive activity [24]. To confirm the specificity of the spectrophotometric SAPL assay, absorption profiles of the assay product of SF samples, pooled for each group, were compared to that of SAPL standards.

**Statistical Analysis.** Data are presented as mean ± SEM. The effects of test lubricant on $\mu_{\text{static}}$ (with $T_p$ as a repeated factor), $\mu_{\text{kinetic}}$, and lubricant concentrations were assessed by ANOVA with Tukey post hoc testing. The dependencies of $\mu_{\text{kinetic}}$ and $\mu_{\text{static}}$ on the biochemical constituents (HA, PRG4, and SAPL) were analyzed by univariate regression as well as multivariate regression. Statistical analysis was performed using Systat 10.2 (Systat; Richmond, CA).
2.4 Results

Lubrication function of eSF. The boundary mode friction coefficients varied with test lubricant (p<0.05, Figure 2.2A) and T\textsubscript{ps} (p<0.001, Figure 2.2B). The lubricating abilities of equine and bovine SF on bovine cartilage were similar for normal SF from equine (NL-eSF, \(\mu\text{kinetic} = 0.026\)) and bovine (NL-bSF, \(\mu\text{kinetic} = 0.025\), data not shown) joints (p=0.76). The \(\mu\text{kinetic}\) for eSF from acutely injured joints (AI-eSF, 0.036) was 39% higher than that of NL-eSF (p<0.05), and \(\mu\text{kinetic}\) for eSF from chronic injury joints (CI-eSF, 0.031) tended to be higher than that of NL-eSF (+20%, p=0.15).

While \(\mu\text{static}\) varied with T\textsubscript{ps} (p<0.001), it was not affected by joint injury (p=0.49) and did not show an interaction effect (p=0.76, Figure 2.2B). As T\textsubscript{ps} decreased from 120s to 1.2s, \(\mu\text{static}\) values approached \(\mu\text{kinetic}\) values (Figure 2.2A). Although \(\mu\text{static}\) values were not statistically different between groups, those at the shortest T\textsubscript{ps} of 1.2s were highest for AI-eSF (0.042) followed by that of CI-eSF (0.036) and that of NL-eSF and NL-bSF (both, 0.030), similar to the ordering of groups for \(\mu\text{kinetic}\).

Biochemical analysis of eSF. Analysis of the concentrations of HA, PRG4 and SAPL in eSF revealed variations in lubricant molecule concentrations or MW with joint injury.

HA. The concentration of HA in eSF varied with joint injury (p<0.05, Figure 2.3A). Opposite to the higher friction coefficients for AI-eSF than that of NL-eSF, the
HA concentration of AI-eSF (0.21 mg/ml) was 30% lower (p<0.05) than that of NL-eSF (0.30 mg/ml) and not significantly different for that of CI-eSF (p=0.40).

The MW distribution of HA in eSF varied between NL-, AI-, and CI-eSF (p<0.05), being shifted with injury to lower MW forms (Figure 2.3B and C). Relative to NL-eSF, AI-eSF had HA concentrations that were similar in the lower MW ranges of 0.05—2.5 MDa (p=0.16—0.59), and markedly lower in the highest MW range, 2.5—7 MDa (−63%, p<0.05). CI-eSF was similar to NL-eSF in HA concentrations at all HA MW ranges (p=0.48—0.99).

**PRG4.** The concentration of PRG4 in eSF varied with joint injury (p=0.01). Western Blot analysis of individual NL-, AI-, and CI-eSF samples identified a specific immunoreactive band (Figure 2.4A). PRG4 concentrations were highest for AI-eSF (104 µg/ml, p<0.05 vs NL-eSF), then CI-eSF (95 µg/ml, p=0.066 vs NL-eSF), and then NL-eSF (57 µg/ml) (Figure 2.4B).

**SAPL.** The SAPL concentration in eSF also varied with joint injury (p<0.001, Figure 2.4D). Compared to that of NL-eSF, SAPL concentration was markedly higher in AI-eSF (+144%, p<0.001, Figure 2.4D) and elevated in CI-eSF (+64%, p<0.05). Absorption profiles for the reaction products of SF treated with phospholipase C were similar for all pooled SF samples (Figure 2.4C), supporting the fidelity of the assay.

Regression analysis indicated certain correlations between friction coefficients and eSF lubricant molecule concentrations. Univariate regression showed a significant negative correlation between \( \mu_{\text{kinetic}} \) and HA (slope = −0.031 ml/mg, \( r^2 = 0.195 \), p<0.01) as well as positive correlations between \( \mu_{\text{kinetic}} \) and SAPL (slope = +0.045 ml/mg, \( r^2 = 0.277 \), p<0.005) and between \( \mu_{\text{kinetic}} \) and PRG4 (slope = +0.003 ml/mg, \( r^2 \)
= 0.124, p<0.05). Similar correlation trends were also observed for $\mu_{\text{static}}$ (data not shown). Multivariate regression revealed the independent relationship of $\mu_{\text{kinetic}}$ to HA (mg/ml), PRG4 (mg/ml), and SAPL (mg/ml), $\mu_{\text{kinetic}} = -0.019*HA + 0.032*PRG4 + 0.029*SAPL + 0.029$ with $r^2 = 0.342$, $p=0.001$ (p<0.01 for HA; p<0.05 for PRG4; and p<0.001 for SAPL).

Lubrication properties of HA. The friction-reducing properties of HA solutions for articular cartilage depended on both HA concentration and MW (Figure 2.5). $\mu_{\text{kinetic}}$ decreased with increasing HA concentration (p<0.001) and HA MW (p<0.001, Figure 2.5A). At 0.33mg/ml HA, $\mu_{\text{kinetic}}$ of 6.4kDa and 51kDa HA were similar to that of PBS (0.255) (p=0.98 and 0.85, respectively), while $\mu_{\text{kinetic}}$ for 4,000 kDa HA was significantly reduced from that of PBS (−64%, p<0.01). Similar trends were observed at 1.1 and 3.3mg/ml HA. For 4,000kDa HA increasing from 0.33 to 3.3mg/ml, $\mu_{\text{kinetic}}$ attained low values, not significantly different than that of bovine SF (p=0.47, Figure 2.5A). $\mu_{\text{static}}$ increased with $T_{\text{ps}}$ (p<0.001) and decreased with increasing HA concentration (p<0.001) and HA MW (p<0.001), with trends similar to those for $\mu_{\text{kinetic}}$ at all $T_{\text{ps}}$. Results for $\mu_{\text{static}}$ at $T_{\text{ps}}$ of 120s are shown in Figure 2.5B, and similar trends were found for $T_{\text{ps}}$ of 1.2 and 12s (data not shown).

Restoration of dysfunctional AI-eSF. The boundary lubrication function of eSF from AI horses was effectively normalized by addition of high MW HA for $\mu_{\text{kinetic}}$ (Figure 2.6A). The average $\mu_{\text{kinetic}}$ of these AI-eSF samples was almost double that of NL-eSF (p=0.001) and lowered by addition of exogenous HA4000, but not HA800, to a value near that of NL-eSF samples. Also, $\mu_{\text{static}}$ of AI-eSF tended to be lowered with addition of HA4000 (p=0.19, Figure 2.6B). Addition of HA to NL-eSF did not lower
friction coefficients any further compared to those of untreated samples (Figure 2.6A,B).
Figure 2.2: Effect of joint injury on the boundary lubrication of articular cartilage by equine SF. (A) Kinetic friction coefficients and (B) static friction coefficients for NL-eSF (n = 20), AI-eSF (n = 10) and CI-eSF (n = 10). Data are mean ± SEM. * indicates p<0.05.
Figure 2.3: Effect of joint injury on hyaluronan (HA) (A) concentration (mg/ml) and (B, C) HA molecular weight (MW) distribution in NL-eSF (n = 19-20), AI-eSF (n = 10) and CI-eSF (n = 10). (B) Electrophoretic separation of typical samples. (C) Concentration of HA in 0.05 – 0.25, 0.25 – 0.5, 0.5 – 1, 1 – 2.5, and 2.5 – 7 MDa ranges. Data are mean ± SEM. * indicates p<0.05.
Figure 2.4: Effect of joint injury on PRG4 (A, B) and SAPL (C, D) in NL-eSF (n=19-20), AI-eSF (n=10), and CI-eSF (n=10) (A) Representative PRG4 Western blot probed with an antibody to Lubricin/PRG4; (B, D) concentrations of PRG4 (µg/ml) and SAPL (mg/ml), respectively; and (C) spectrophotometric absorption profiles for the reaction products of SAPL from pooled NL-eSF, CI-eSF, and AI-eSF. Data are mean ± SEM. * indicates p<0.05, ** indicates p < 0.001.
Figure 2.5: Dependence of (A) kinetic ($\mu_{\text{kinetic}}$) and (B) static ($\mu_{\text{static}}$) friction coefficients on the MW (kDa) and concentration (mg/ml) of HA. n = 6; data are mean ± SEM.
Figure 2.6: Dependence of (A) kinetic ($\mu_{\text{kinetic}}$) friction and (B) static ($\mu_{\text{static}}$) friction (at $T_{ps} = 120s$) of NL- and Al-eSF before and after the addition of exogenous HA of average MW 800kDa (HA800) or 4,000kDa (HA4,000). $n = 3 - 9$; data are mean ± SEM. * indicates $p<0.001$. 
2.5 Discussion

The results of this study indicate that SF lubrication function and composition are altered coordinately after acute joint injury to race horses, and that supplementation in vitro of the abnormal SF with high-MW HA restores boundary lubrication function. In the acute stage, the boundary lubrication function of SF is reduced, as indicated by a friction coefficient that is higher than normal ($\mu_{\text{kinetic}} = 0.036$ vs 0.026, Figure 2.2). This alteration in lubrication function may be due to the diminished concentration and MW of HA in AI-eSF (Figure 2.3), despite the elevated concentrations of PRG4 and SAPL in AI-eSF over NL-eSF (Figure 2.4). The role for HA appears particularly important since the friction-lowering properties of HA were size- and concentration-dependent (Figure 2.5). Also, the addition of HA4000 to AI-eSF restored boundary lubrication function, lowering $\mu_{\text{kinetic}}$ by ~30% to a level indistinguishable from NL-eSF (Figure 2.6). In the later chronic stage after injury, the boundary lubrication function of SF appears to be partially recovered, possibly due to restoration and normalization of HA, PRG4, and SAPL concentrations (Figures 2.3 and 2.4). Articular cartilage may be particularly vulnerable when boundary lubrication is deficient in the acute stage after injury. During this time, addition of lubricant molecules to SF may restore its lubrication function.

A direct comparison of the boundary lubricating ability and lubricant composition of pathological SF to its normal counterpart is important for understanding the molecular basis for altered lubrication function. Both pathological and normal contralateral SF could be obtained fresh in a sufficient quantity and
controlled manner for the experiments performed here. The carpal and metacarpophalangeal joints of the race horses from which SF samples were aspirated are weight-bearing and loaded actively, providing a useful model system to study joint injury. Although equine cartilage was not studied as a substrate for the boundary lubrication tests of eSF, normal eSF tested on adult bovine tissue substrates had a lubricating ability similar to that of bovine SF. Finally, although lubricant molecules may differ between species in concentration and quality, understanding the composition–function relationship of putative lubricant molecules in eSF provides insight into the molecular basis for SF and articular cartilage alteration with injury, and possible sequelae.

The analysis of the boundary lubricating ability of eSF after injury is consistent with and extends previous studies on SF from humans and animal injury models that used non-cartilage substrate friction test systems. At a latex-glass interface, boundary lubrication function of AI-SF was also reduced for that aspirated from humans with knee joint synovitis [20] and from rabbits after ACL and PCL transection [9]. Thus, such SF from injured joints reduces surface interactions between articulating substrates at both cartilage-cartilage and non-cartilage interfaces. The relatively normal lubricating ability of CI-eSF and OA human SF [20] was also observed for SF from patients with degenerative joint disease [7], where friction coefficients similar to those measured here (~0.024) for NL-eSF and NL-bSF were measured for a latex-glass interface, consistent with the ability of SF from joints with certain chronic pathologies to reduce the surface interaction between articulating surfaces similar to that of normal SF. Friction coefficients for PBS at a cartilage-
cartilage interface in the present study (~0.26) were ~3x that (~0.09) for saline controls at a latex-glass interface [7, 9, 20]. The different absolute friction values between previous and current studies may be attributable to differences in the test configurations, substrate materials, and test protocols. Examining the lubricating ability of pathological SF on physiological test substrates mimics certain aspects of naturally articulating surfaces and allows for molecular interactions that occur during physiological articulation [4], expanding on findings for the boundary lubricating ability of SF at a non-cartilage interface. Deficient lubrication by AI-eSF also leads to an altered mechanobiologic environment for cartilage, with elevated shear strains observed during tibio-femoral human cartilage articulation in the presence of AI-eSF [49] further supports the phenomenon of impaired boundary lubrication function of SF after joint injury.

The alterations in SF lubricant composition with injury and disease observed in this study are in general agreement with previous studies on HA concentration and MW distribution in SF. The concentration of HA in eSF from clinically normal horses has been reported to have averages ranging from 0.33–1.3 mg/ml HA [30, 34, 35, 45]. HA concentration in eSF from horses with various arthritides is somewhat less, 0.18–0.3 mg/ml HA for horses with acute traumatic arthritis [45, 46] and 0.18–0.74 mg/ml HA for horses with more chronic injury conditions [13, 45, 46]. Consistent with the current findings, the HA concentration in SF from acutely injured joints from horses [35] and humans [1, 31] were lower than that in control SF. The altered HA concentrations observed in the present studies indicate local changes afflicting a specific joint, rather than a systemic change. Thus, although lowered HA
concentrations with injury are evident, the wide variability of reported HA values suggests possible effects of sample source, analysis method, and species, particular joint and state of health, injury, or disease.

The present results also extend knowledge of the altered MW distribution of HA in SF with joint diseases. Previous studies of SF have generally described the predominant HA MW range rather than quantitative distributions, with that from patients with rheumatoid arthritis and other joint diseases being 0.3–5 MDa compared to normal SF of 2–10 MDa [3, 6, 24], consistent with the AI-eSF findings of the present study. Previously, HA MW in normal eSF (2–3 MDa) was not found to differ significantly from that of SF from horses with established arthritis (1.5–3 MDa) [45], consistent with CI-eSF. Thus, the present studies provide new, quantitative information about the effect of traumatic injury on HA MW distribution in SF.

The in vitro finding that addition of high-MW HA to AI-eSF restores lubricant function suggests that intra-articular supplementation may modulate and restore, to some degree, the boundary lubrication function of SF after injury. Although the concentration of exogenous HA that was added to AI-eSF was higher than the average measured levels in NL-eSF samples, the increase in concentration by the addition of HA (to 1 mg/ml) is within the concentration range of HA in normal eSF reported previously and lower than that of normal human SF. Further analysis of the dose-dependent effects of exogenous HA on the lubricating ability of HA-deficient SF would help to elucidate whether lower concentrations of added high MW HA would also enhance SF lubrication function. HA supplementation is a common clinical treatment for people with osteoarthritis, and postulated to have disease modifying and
chondroprotective effects [17]. Previous studies have focused on clinical outcomes of HA supplementation and biological mediators rather than direct effects of HA, or other putative lubricant molecules, on SF lubricant function. Since HA has a relatively short half-life, a single bolus addition of HA may not provide long lasting enhancement of the lubricant function.

The effects of joint injuries and disease on the concentration of PRG4 appear somewhat variable. The elevation in PRG4 concentration determined here is consistent with the initial transient elevation in biosynthesis and secretion as a response to cartilage injury [22] in vitro. The majority of PRG4 secreted by chondrocytes is released into the synovial fluid rather than retained with the matrix [39]. However, previous studies of injury in animal models [9, 10, 44] and humans [8] have typically found a decrease in PRG4 concentration. These variable PRG4 concentrations may be due to a number of factors including the type of injury, the duration of injury before the SF was analyzed, the sample source, and the analysis method. Previous studies included soft tissue and joint destabilization injuries, while the current study included osteochondral damage. The particular type of injury may affect both the regulated level of PRG4 secretion, as well as pathways of efflux from the joint, both of which affect PRG4 concentration in SF.

The injury-mediated increase in phospholipid concentration appears consistent, although the lubrication consequences are somewhat controversial. With acute and chronic injury phospholipid concentration increased, from 0.1 mg/ml in normal human SF to 0.2-0.3 mg/ml for human OA SF and 0.5 mg/ml for SF from total knee arthroplasties [24]. However, the contribution of SAPL to the boundary lubrication of
articular cartilage has been conflicting [19, 29, 37]. The results of the present study, elevation of both SAPL and friction coefficients, argue against the role of phospholipid as a boundary protectant of the articular cartilage surface after acute injury. Here also, more detailed analysis of the distribution of phospholipids may clarify their role [32, 36].

The current studies provide further evidence that molecular features of lubricant molecules can affect their functional quality in certain states of post-traumatic joint injury, and such features may be important considerations for therapeutic interventions. The approach of analyzing both the concentration and structure of lubricant molecules present in injury SF, together with testing their role independently and as a supplement in cases of deficiency, can be used to screen putative lubricant therapies. Such analyses may also clarify the interaction of chemical factors. The results also suggest that the natural time course of altered lubrication is important. At particular stages following injury, biological or physical treatments to modulate lubrication of HA, as well as PRG4, may help to protect the articular cartilage and joint from damage. Such deficiencies in lubrication may occur not only after injury occurring naturally, but also following arthroscopic procedures including cartilage repair [11].

### 2.6 Acknowledgments

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Schmidt, Lisa A. Serventi, Matthew Z. Cai, Yu Yu L. Shu, Barbara L. Schumacher, and Drs. C. Wayne McIlwraith and Robert L. Sah for their contributions. This work was supported in part by the National Institutes of Health and the National Science Foundation, and by a grant to the University of California, San Diego, in support of Dr. Robert Sah, from the Howard Hughes Medical Institute through the HHMI Professors Program. Additional individual support was received from an NSF Graduate Research Fellowship (JMA) and UCSD Chancellor’s Research Scholarship (LAS).
2.7 References


9. Elsaid KA, Jay GD, Warman ML, Rhee DK, Chichester CO: Association of articular cartilage degradation and loss of boundary-lubricating ability of


CHAPTER 3:

EFFECT OF TIBIAL PLATEAU FRACTURE
ON LUBRICATION FUNCTION AND COMPOSITION
OF SYNOVIAL FLUID

3.1 Abstract

Background: Intra-articular fractures may hasten post-traumatic arthritis in patients who are typically too active and young for joint replacement. Current orthopaedic treatment principles, including recreating anatomic alignment and establishing articular congruity, have not eliminated post-traumatic arthritis. Additional biomechanical and biological factors may contribute to the development of arthritis. The objective of the present study was to evaluate human synovial fluid following tibial plateau fractures for friction-lowering function and the concentrations of putative lubricant molecules.

Methods: Synovial fluid specimens were obtained from the knees of five patients with a tibial plateau fracture, and from the contralateral knees of six patients for control synovial fluid. Each specimen was centrifuged to obtain a fluid sample, separated from a cell pellet, for further analysis. For each fluid sample, the start-up (static) and steady-state (kinetic) friction coefficients in the boundary mode of lubrication were determined from a cartilage-on-cartilage biomechanical test of friction. Also,
concentrations of the putative lubricants, hyaluronan and proteoglycan-4, as well as total protein, were determined for fluid samples.

Results: The injury group of experimental samples (n=5) was from patients 45±13 years old, after a post-injury period of 11±9 days. Start-up and kinetic friction coefficient demonstrated similar trends and dependencies. The kinetic friction coefficients for human plateau fracture synovial fluid were ~100% higher than that of control human synovial fluid. Hyaluronan concentrations were 9-fold lower for plateau fracture synovial fluid compared with the control synovial fluid, whereas proteoglycan-4 concentrations were >2-fold higher in plateau fracture synovial fluid compared with the control synovial fluid. Univariate and multivariate regression analysis indicated that kinetic friction coefficient increased as hyaluronan concentration decreased.

Conclusions: Joints afflicted with a tibial plateau fracture have synovial fluid with decreased lubrication properties in association with a decreased concentration of hyaluronan.

Clinical Relevance: Tibial plateau fractures result in a post-traumatic deficiency in synovial fluid lubrication function.
3.2 Introduction

Traumatic intra-articular fractures, such as those of the tibial plateau, are at risk of joint degeneration and post-traumatic arthritis even when treated according to traditional orthopaedic principles to restore articular congruency and anatomic alignment [7, 12, 19, 21, 22, 28, 39]. The consequences of poor results can be life-changing—painful weight-bearing, restricted activity and lost time in the work force [4, 13, 35, 39]. The costs of post-traumatic arthritis are a component of the estimated $60 billion annual burden of osteoarthritis on the U.S. economy, with 13% of knee osteoarthritis estimated to be of post-trauma etiology; furthermore, patients with post-traumatic arthritis and its chronic consequences tend to be younger with an additional impact on employment [4]. Compared to patients without post-traumatic arthritis, patients with post-traumatic arthritis have worse clinical outcomes after arthrodesis or arthroplasty [5, 40]. Post-traumatic arthritis and its consequences have not been eliminated by employing traditional orthopedic principles, suggesting a possible role for additional biological and biomechanical factors.

The pathogenesis of post-traumatic arthritis is complex and multifactorial. Recent studies have focused on articular chondrocyte death and cartilage damage due to direct trauma, enzyme-mediated cartilage degradation, and the role of reactive oxygen species [10, 20, 23, 38]. Deficient lubrication may also contribute to cartilage deterioration after trauma. Lubrication typically allows articular cartilage to bear load and slide with low friction and low wear [3], and is mediated by high levels of proteoglycan-4 and hyaluronan in synovial fluid [36]. Synovial fluid lubricant
molecules are secreted by chondrocytes and synoviocytes lining the joint, and are concentrated through selective retention by synovium. However, after an intra-articular fracture, soft tissues that normally produce and retain synovial fluid lubricants are damaged, and in addition, blood and cellular components from damaged tissues and bone marrow infiltrate the joint space [6, 10, 11, 14, 29]. Such alteration of synovial fluid may disrupt its lubrication functions.

Alteration in the lubricating function and lubricant composition of synovial fluid appears to be involved in cartilage deterioration after anterior cruciate ligament (ACL) injury, as well as other injuries of the knee joint. After ACL injury of human knees, the level of proteoglycan-4 in synovial fluid was reduced, while levels of degradative enzymes, cartilage matrix degradation products, and inflammatory markers were increased for up to 6-18 months after injury [8, 20]. Deficient lubrication after an injury has similarly been detected in the synovial fluid from acutely injured equine joints, in association with a decreased concentration of hyaluronan [1]. In guinea pig [37] and rat [9, 18] models of knee joint injury, synovial fluid lubrication function and lubricant levels are also diminished. These studies suggest that human knee trauma, and specifically intra-articular tibial plateau fracture, may lead to a deficiency in synovial fluid lubrication.

The hypothesis tested in the present study was that tibial plateau fractures impair the friction-lowering lubrication function of human synovial fluid in association with changes in the concentrations of proteoglycan-4 and hyaluronan lubricant molecules. The objectives of this study were to compare the trauma synovial fluid from joints afflicted with acute tibial plateau fractures with normal synovial fluid
in terms of 1) friction-lowering boundary lubrication function and 2) biochemical composition, including hyaluronan and proteoglycan-4 concentrations. In addition, the possible biomechanical basis for impaired lubricant function was assessed by correlating friction coefficient and lubricant concentration.

Table 3.1: Summary of donor demographics. Tibial plateau fractures are categorized according to the Schatzker and OTA classification systems. Samples were obtained during either external fixation (ex fix) or open reduction, internal fixation (ORIF) of a fracture.

<table>
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<th>Trauma Sample #</th>
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<th>Post-injury Day</th>
<th>Procedure # of Total # of Procedures</th>
<th>Procedure Type</th>
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<td>ORIF</td>
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<td>M</td>
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<td>ATV accident</td>
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</table>
3.3 Materials and Methods

Materials. Materials for lubrication testing were obtained as previously described[33]. The antibody to proteoglycan-4 was that to Lubricin from AbCam (Cambridge, MA); Streptomyces hyaluronidase was from Seikagaku (Tokyo, Japan); SeaKem gold agarose (Lonza) was from Fisher Scientific (Pittsburg, PA) and SDS-horizontal agarose gel electrophoresis and Western blot materials were from Life Technologies (Carlsbad, CA). EDTA-treated adult human blood was purchased from Golden West Biologicals (Temecula, CA). EDTA-treated bovine blood was from Animal Technologies (Tyler, TX).

Patient Samples and Fracture Classification. Samples of human synovial fluid (hSF) were aspirated from consented patients at a level one trauma center under the auspices of an IRB-approved research plan. Patients 21 years and older, scheduled for surgery after sustaining a closed tibial plateau fracture, were asked to be included in the research study prior to the procedure by an orthopaedic surgeon, and consented. Exclusion criteria consisted of open fractures, and vulnerable groups including minors, known cognitively impaired or institutionalized individuals, patients with known HIV or HCV or HBV infections, and patients unable to provide consent.

Radiographs, and CT scans if available based on routine clinical care, were graded by an attending orthopaedic surgeon using the Schatzker classification system for tibial plateau fractures [31].

A total of 11 synovial fluid (SF) samples collected from 8 patients were used in the present study. Attempts were made in all consented patients for aspiration from
both the injured joint and contralateral knee. Synovial fluid samples of sufficient volume (see below) were successfully withdrawn from both the injured knee (Plat-hSF) and contralateral control knee (Ctrl-hSF) of three donors with an acute tibial plateau fracture. For two other patients, Plat-hSF was obtained, but Ctrl-hSF were not of sufficient volume. Thus, three other Ctrl-hSF samples were used from other patients. Radiographs of the contralateral knee were evaluated by radiologists and orthopaedists and confirmed to not have an acute injury. To obtain fluid, following sterilization of skin overlying each joint, a standard 18 gauge hollow bore spinal needle from Becton-Dickinson attached to a 60cc syringe barrel from Becton-Dickinson (Laguna Hills, CA) was introduced into the joint space and SF was aspirated. The site of needle introduction into the knee joint was at the level of the joint line, 1 cm medial or lateral to the patellar ligament on the anterior aspect of the knee [26].

**Experimental Design.** *Gross Analysis of hSF Samples.* Samples were initially studied by gross examination for color and clarity. Samples were noted to be straw-colored or bloody. Clarity was judged as the ability to read markings through a ~1cm distance (through the tube), and also by the degree of opacity (translucent or opaque). Samples were then centrifuged to obtain discrete fractions. The relative volume of fractions was then estimated directly from the centrifuged tubes. Fluid samples were photographed, centrifuged (3,000g for 30 minutes at 4°C), and then photographed again. Following centrifugation, the supernatant and pellet were separated and stored in aliquots at -80°C. Light microscopy analysis of selected supernatants and pellets indicated that the processing was sufficient to separate cells such that they were
present in the pellet and absent from the supernatant (data not shown). Samples with adequate volume (1 ml) after processing were selected for use in the study.

**Assay Validation for Biochemical Analysis of Mixtures of SF and Blood.** An *in vitro* model of mixtures of human blood and human synovial fluid was developed and used to validate the assays for protein, proteoglycan-4, and hyaluronan in solutions containing SF and blood, in order to mimic the trauma SF obtained in this study. First, the macroscopic appearance of mixtures of synovial fluid and blood (SF:Blood) of varying proportions were examined to identify variations after mixing and separation by centrifugation (3,000g for 30 minutes at 4°C). Similar mixtures were made using bovine blood and bovine synovial fluid and photographed with a D40 digital camera (Nikon, Melville, NY). Portions of the human SF:Blood mixture prior to centrifugation (Mixture), and supernatant and pellet portions of the SF:Blood after centrifugation were then assayed for the concentrations of total protein, hyaluronan and proteoglycan-4, relative to the initial mixture volume.

**Biomechanical and Biochemical Analyses of Human Synovial Fluid Samples.** Portions of human synovial fluid samples were analyzed by biomechanical lubrication tests for friction lowering properties, in addition to analysis by biochemical assays for the concentrations of total protein, hyaluronan, and proteoglycan-4.

**Statistical Analysis.** The effects of joint injury (trauma versus control) on SF properties ($\mu_{\text{kinetic}}$ and the concentration of lubricants) were assessed by ANOVA. The effects of trauma on synovial fluid static friction ($\mu_{\text{static}}$) were assessed by repeated measures ANOVA with $T_{ps}$ (1.2, 12, and 120s) considered as a repeated factor. Planned comparisons for $\mu_{\text{static}}$ were performed between trauma and control groups at
each $T_{ps}$ value. The dependencies of friction coefficients on the concentrations of putative lubricants (hyaluronan and proteoglycan-4) were analyzed by univariate and also multivariate regression, with both the absolute and log10 value of hyaluronan concentrations (since it varied by several orders of magnitude). Statistical analysis was performed using Systat 10.2 (Systat; Richmond, CA).

**Analytical Methods**

**Lubrication Test.** Portions of SF samples were analyzed for static and kinetic friction coefficients in the boundary lubrication mode on articulating cartilage surfaces as described previously[32, 33]. Intact articular surfaces were in the form of osteochondral cores and annuli from adult bovine knees, and stored in phosphate buffered saline (PBS) supplemented with protease inhibitors (PIs; 2 mM Na-EDTA, 1 mM PMSF, 5 mM Benz-HCL, and 10 mM NEM) at –80°C. For lubrication testing, cartilage samples were thawed at 4°C, and then bathed in ~0.5 ml of the subsequent test lubricant supplemented with PIs with the cartilage completely immersed for 16-24 hours at 4°C prior to lubrication testing at room temperature. The lubricant sample and cartilage were then tested at room temperature by preconditioning, compressing to 18% of the total cartilage thickness, and allowing 30 minutes for stress relaxation and interstitial fluid depressurization. Then samples were rotated at an effective velocity of 0.3 mm/second with pre-sliding durations ($T_{ps}$; the duration the sample is stationary prior to rotation) of 120, 12, and 1.2 seconds. Friction coefficients ($\mu$) were calculated from the expression $\mu = \tau/(R_{eff} \times N_{eq})$, where $\tau$ is torque, $N_{eq}$ is the equilibrium axial load after 30 minutes of stress relaxation, and $R_{eff}$ is the effective radius of the
cartilage, as described previously [32, 33]. Briefly, a static friction coefficient ($\mu_{\text{static}}$) was calculated using the peak $|\tau|$, measured immediately after (within 10° of) the start of rotation, and $N_{\text{eq}}$. A kinetic friction coefficient ($\mu_{\text{kinetic}}$) was calculated using both the $|\tau|$ averaged during the second complete revolution of the test sample and also $N_{\text{eq}}$.

Consistent with previous results[32] comparing friction coefficients with increasing $T_{ps}$, all test lubricants demonstrated little variation in kinetic friction coefficients with values at $T_{ps} = 1.2$ seconds remaining within 11% of values at $T_{ps} = 120$ seconds; therefore, for brevity and clarity $\mu_{\text{kinetic}}$ data are presented as the average at all pre-sliding durations. As expected, the mean static and kinetic friction coefficients measured for PBS were greater than 0.20 for all $T_{ps}$, consistent with previous results [32], so PBS data were not analyzed further.

**Biochemical Analyses.** Portions of synovial fluid samples were analyzed biochemically for absolute concentrations of lubricant molecules hyaluronan and proteoglycan-4, as well as for total protein. *Hyaluronan.* The concentration of hyaluronan in SF samples was determined by an ELISA-like assay using hyaluronan binding protein (Corgenix). *Proteoglycan-4 (PRG4).* The concentration of PRG4 in hSF samples was quantified by Western Blot using antibody to Lubricin after SDS-horizontal agarose gel electrophoresis on 2% 3mm thick agarose gels and transfer to PVDF membrane (100mA, overnight). The immunoreactive proteins were visualized by ECL-Plus detection and digital scanning with a STORM 840 Imaging System (Molecular Dynamics, Fairfield, CT). ImageQuant (Molecular Dynamics) was used to generate densitometric scans. The PRG4 in hSF was quantified using standards of
PRG4 that were purified[34] from conditioned medium of human cartilage explants and run on the same gels.

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3.4 Results

Patient Demographics. A total of eleven synovial fluid samples from eight patients were used in the present study (Table 3.1). For these patients, the tibial plateau fracture types ranged from Schatzker type II to type VI and from OTA class 41B2.2 to 41C3.3. The control group of normal samples (n=6) was from patients 42±16 years old (mean±SD), with a post-injury period before synovial fluid acquisition of 11±7 days. The injury group of experimental samples (n=5) was from patients 45±13 years old, with a post-injury period of 11±9 days. Of the eight patients, six were male, and two were female; both control and experimental synovial fluid samples were successfully obtained from the two female patients.

Gross Appearance of hSF Samples. The synovial fluid samples from the control and injured joints appeared markedly different. Before centrifugation, the control synovial fluid showed only traces of blood (Fig. 3.1-A-i), whereas the injury synovial fluid was grossly bloody (Fig. 3.1-A-ii). After centrifugation, the supernatant of control synovial fluid samples was clarified and appeared clear or straw-colored (Fig. 3.1-B-i). In contrast, the injury synovial fluid samples separated into a yellow supernatant and a dark red pellet of varying relative volumes (Fig. 3.1-B-ii).

Assay Validation for Biochemical Analysis of Mixtures of SF and Blood. The in vitro mixtures of bovine synovial fluid and bovine blood appeared similar to the range of control and injury human synovial fluid samples. Before centrifugation,
mixtures with increasing proportion of blood were of an increasingly darker shade of red (Fig. 3.1-C). After centrifugation, samples separated into a supernatant that was increasingly yellow and a pellet that was increasing in size as the proportion of blood increased (Fig. 3.1-D). Mixtures of human synovial fluid and human blood appeared to separate similarly, although they were more difficult to visualize due to the small volumes available.

Biochemical analysis of the mixtures of human synovial fluid and blood, as well as the supernatants and pellets, revealed that the hyaluronan and proteoglycan-4 constituents, when present, separated primarily into the supernatant fraction (Fig. 3.2). Hyaluronan and proteoglycan-4 separated primarily in the supernatant (Fig. 3.2-A and B), indicating they were maintained in the fluid phase even when synovial fluid was mixed with blood. In contrast, protein separated between the pellet and supernatant according to the concentrations of protein in the plasma and cells of whole human blood, and the concentration of protein in human synovial fluid (Fig. 3.2-C). The biochemical assays were confirmed to be sensitive and specific to the target components by mixture and spiking studies (data not shown). Consistent with this, the total amount of protein, hyaluronan, and proteoglycan-4 in the supernatant and pellet fractions were similar to that of the corresponding mixtures before centrifugation as well as the proportionate amounts from the pure SF and blood preparations. Thus, supernatant fractions could be readily analyzed biochemically for the putative lubricants, hyaluronan and proteoglycan-4.

**Lubrication Function of Human Synovial Fluid.** The boundary mode friction coefficients were higher for the fracture synovial fluid than the control
synovial fluid (Fig. 3.3-A and B). The $\mu_{\text{static}}$ varied with test lubricant ($p<0.001$) and pre-spin duration ($p < 0.001$) without an interaction effect ($p=0.25$, Fig. 3.3-A). The average $\mu_{\text{static}}$ values were 49-120% higher for fracture synovial fluid than control synovial fluid, with the percentage difference increasing as the pre-spin duration decreased from 120s to 1.2s. At the short pre-spin duration of 1.2s, the $\mu_{\text{static}}$ values approached the $\mu_{\text{kinetic}}$ values (Fig. 3.3-A and B). The average $\mu_{\text{kinetic}}$ value for fracture synovial fluid was double that of control synovial fluid (0.044 versus 0.022, $p<0.001$, Fig. 3.3-B).

**Biochemical Analysis of Human Synovial Fluid.** The concentrations of lubricant molecules were different for fracture synovial fluid and control synovial fluid (Fig. 3.4). The concentration of hyaluronan was markedly lower in fracture synovial fluid than control synovial fluid (0.27 mg/ml versus 2.53 mg/ml, −87%, $p<0.001$, Fig. 3.4-A). Based on Western Blot analysis which identified a specific immunoreactive band at >460 kDa in synovial fluid samples (Fig. 3.4-B), the concentration of proteoglycan-4 was markedly higher in fracture synovial fluid than control synovial fluid (356 versus 139 µg/ml, +156%, $p<0.05$, Fig. 3.4-C). The concentration of protein was somewhat higher in fracture synovial fluid than control synovial fluid (38 mg/ml versus 22 mg/ml, +72%, $p<0.001$, Fig. 3.4-D).

**Regression Analysis of Human SF Lubrication Properties with Biochemical Constituents.** Regression analysis indicated certain correlations between friction coefficients and concentrations of lubricant molecules. Univariate regression showed a significant negative correlation between $\mu_{\text{kinetic}}$ and hyaluronan concentration (in mg/ml) (slope = $-0.0067$, $r^2 = 0.44$, $p<0.05$) as well as between
μ_{kinetic} and ln(hyaluronan concentration in mg/ml) (slope = −0.0081, \( r^2 = 0.63 \), p<0.005, Fig. 5-A), and no significant correlation between \( \mu_{kinetic} \) and proteoglycan-4 concentration (in mg/ml) (slope = 0.027, \( r^2 = 0.13 \), p = 0.28, Fig 3.5-B). Similar correlation trends were also observed for static friction coefficients (data not shown). Multivariate regression yielded similar results (elimination of variation due to proteoglycan-4, leaving only correlation with hyaluronan concentration).
Figure 3.1-A-i through D-vii: Gross images of (A and B) synovial fluid collected following tibial plateau fracture and (C and D) mixtures of bovine synovial fluid and blood. Human synovial fluid was collected from either a normal, non-injured knee (Ai and Bi) or a knee with a tibial plateau fracture (Aii and Bii). Bovine synovial fluid and blood were mixed in designated proportions (Ci-vii and Di-vii). Images are shown for fluids before (Ai,ii and Ci-vii) and after (Bi,ii and Di-vii) centrifugation.
Figure 3.2: Effect of mixing human synovial fluid and blood and subsequent centrifugation on the distribution of (A) hyaluronan, (B) proteoglycan-4, and (C) protein. Samples were analyzed as mixtures before centrifugation, and as supernatants and pellets after centrifugation. Proteoglycan-4 was only detected in supernatants. Linear regression fits are indicated.
Figure 3.3: Effect of tibial plateau fracture on the boundary lubrication of articular cartilage by human SF. (A) Static and (B) kinetic friction coefficients for synovial fluid from control (Ctrl-hSF, n=6) or fractured (Plat-hSF, n=5) knees. Data are Mean±SEM. * indicates p<0.05 and ** indicates p<0.001.
Figure 3.4: Effect of tibial plateau fracture on (A) hyaluronan, (B,C) proteoglycan-4, and (D) protein. (A,C,D) Concentrations were determined for synovial fluid from control (Ctrl-hSF, n=6) or fractured (Plat-hSF, n=5) knees. Data are Mean±SEM. * indicates p<0.05 and ** indicates p<0.001. (B) Representative Western blots of Ctrl-hSF and Plat-hSF samples probed for proteoglycan-4.
Figure 3.5: Correlation of kinetic friction coefficients with concentrations of (A) hyaluronan and (B) proteoglycan-4 in control (green diamonds) and injury (red diamonds) human SF.
3.5 Discussion

The present study identifies marked alterations in the lubrication function and lubricant composition of SF from patients with intra-articular tibial plateau fractures in the initial stages of treatment. Compared with control human synovial fluid, synovial fluid from knees with intra-articular fractures had markedly decreased lubrication ability (+100% increase in $\mu_{\text{kinetic}}$, 0.022 versus 0.044) in the acute post-injury time period that was studied. Concomitantly, tibial plateau fractures led to changes in the concentration of putative lubricant molecules, with a decrease in hyaluronan (−87%, 2.53 mg/ml to 0.27 mg/ml) and an increase in proteoglycan-4 (+156%, 139 µg/ml to 356 µg/ml). Regression analysis indicated that poorly lubricating synovial fluid was associated with diminished hyaluronan concentration.

The experimental approach of the present study involved a number of considerations. Lubrication testing of the human synovial fluid samples was performed with normal adult bovine cartilage surfaces, minimizing effects of variation in cartilage surface properties. The test configuration facilitates boundary lubrication by apposed articular cartilage surfaces, maintaining possible interactions between synovial fluid and the articular surfaces, and also minimizing confounding factors such as ploughing [33]. Adult bovine cartilage appears to be lubricated similarly by normal bovine synovial fluid ($\mu_{\text{kinetic}}=0.025$) [32] and by control human synovial fluid ($\mu_{\text{kinetic}}=0.022$, Fig. 3.3). In addition, human synovial fluid was obtained only during surgical procedures under general anesthesia. These procedures occurred at various
time points up to 30 days after the injury, so the long-term effects of tibial plateau fracture on synovial fluid properties were not determined.

The present study expands upon previous research on the effects of human knee injuries on synovial fluid lubricant properties by focusing on synovial fluid after tibial plateau fracture. In a previous study, synovial fluid aspirated from Emergency Department patients presenting with mono-articular knee effusions and no radiographic abnormalities (e.g., without intra-articular fracture) also exhibited poor lubrication relative to saline ($\Delta \mu = -0.045$) compared with human control synovial fluid relative to saline ($\Delta \mu = -0.095$)[17]. Though the lubrication test methods were different, with the former study using a glass-on-latex arthrotripsometer versus articulating cartilage in the present study, the synovial fluid from intra-articular tibial plateau fractures also resulted in a kinetic coefficient of friction approximately double that of the controls (Fig. 3.3). Thus, the lubrication properties of human knee synovial fluid may be disrupted in a variety of scenarios of acute injury or inflammation.

The present study is also consistent with and adds to information on the concentration of lubricant molecules in joint synovial fluid from humans and animals. The decreased concentration of hyaluronan is consistent with effects of disease and injury of human knees [2, 27]. At the time of total knee arthroplasty, the hyaluronan concentration of synovial fluid from osteoarthritic knees was 1.3 mg/ml [24]. Compared to these values and the control value of 2.53 mg/ml in the present study, hyaluronan concentrations in synovial fluid from knees with tibial plateau fractures was markedly lower at 0.27 mg/ml. In horses, joint injury was also associated with a decreased concentration of hyaluronan [1, 30]. Studies of SF lubrication in
experimental animal injury models have focused on proteoglycan-4 [18, 37] but not hyaluronan. The correlation between decreased lubrication properties and decreased HA concentration in the present study suggests the importance of diminished HA concentration in post-traumatic human synovial fluid.

The reported effects of injury and osteoarthritis on PRG4 concentration in humans and animal models are quite varied, with some studies indicating an injury-associated decrease in concentration of PRG4 [8, 17, 20] and others indicating an increase [1, 25]. In osteoarthritic knees, increasing PRG4 concentration correlated with worsening lubrication and OA severity [25]. Differences in the concentration of PRG4 in synovial fluid of human osteoarthritis (151 µg/ml) and human trauma (356 µg/ml), may be due a difference in analysis methods including standards, or a difference in patient populations. Total protein levels were also markedly different with 27 mg/ml for synovial fluid from end-stage osteoarthritic knees at the time of total knee arthroplasty [24], compared to 38 mg/ml in trauma knees and 22 mg/ml in control knees in the present study. This may be due to differences in the pathologic processes, since after trauma the joint space is compromised and SF may be diluted by extra-articular contents and infiltrated by protein in blood.

The collective results here indicate that lubricant molecules in synovial fluid after a tibial plateau fracture are acutely altered with diminished hyaluronan concentration, and elevated concentrations of proteoglycan-4 and total protein compared with controls. The correlation of impaired lubrication of trauma SF with diminished hyaluronan and elevated proteoglycan-4 remains to be evaluated further. The proteoglycan-4 that is elevated may not be functional alone, or functional in a
manner dependent on high concentrations of hyaluronan. The presence of blood in the knee joint may lead to prolonged derangement of the articular cartilage permanently [14-16]. Together, these post-traumatic changes may lead to prolonged changes in synovial fluid biomechanical function as well as biological function [20]. In view of these results, correction of lubrication and other pathologic changes may offer an opportunity to protect and preserve cartilage, facilitate lubrication for early range of motion activities, and ultimately modulate the development of post-traumatic arthritis.

3.6 Acknowledgments

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3.7 References


CHAPTER 4:

MECHANISMS OF ARITCULAR CARTILAGE LUBRICATION

BY HYALURONAN

4.1 Abstract

Objective. To elucidate the biophysical mechanisms for the contribution to the lubrication of articulating cartilage of hyaluronan (HA) of different molecular weights in synovial fluid (SF) of normal (NL-eSF) and acutely-injured (AI-eSF) equine joints.

Methods. Boundary lubrication tests of normal articular cartilage were performed to determine friction coefficients at start-up ($\mu_{\text{static}}$) and steady-state ($\mu_{\text{kinetic}}$) as well as rotation of start-up at peak torque ($\theta_{\text{static}}$) for (1) HA solutions of 130, 780, and 4,000 kDa and at sliding velocities ($v_{\text{eff}}$) of 0.1–3 mm/sec (2) for NL-eSF and AI-eSF (a) before and (b) after depletion by Hyaluronidase (eSF+HAase), and (c) after repletion with HA (eSF+HAase+HA).

Results. (1) 780 and 4,000 kDa HA, but not 130 kDa HA, had friction-lowering characteristics ($\mu_{\text{static}}$, $\theta_{\text{static}}$, and $\mu_{\text{kinetic}}$) approaching that of normal SF over the range of $v_{\text{eff}}$. (2) Depletion of HA from NL-eSF and AI- eSF (eSF+HAase) worsened lubrication function with marked elevations in $\mu_{\text{static}}$, $\theta_{\text{static}}$, and $\mu_{\text{kinetic}}$ (+150–390%).
Repletion with 4,000 kDa HA (eSF+HAase+HA) restored all lubrication properties for NL-eSF but not $\mu_{\text{static}}$ for AI-eSF.

**Conclusion.** Collectively, these results indicate that high MW HA plays a key and reversible role in the friction-lowering properties of SF from healthy joints. However, for SF of injured joints, start-up friction may be irreversibly impaired by depletion of HA. The contributions of HA to SF lubrication function at the initiation of motion and during established motion may involve independent mechanisms and variable interactions with the articular surface and other molecules present in SF.
4.2 Introduction

Healthy synovial joints contain articular cartilage that bears load and slides relative to an apposing tissue surface with remarkably low friction and wear. These properties in part are due to complex interactions between articular cartilage and synovial fluid (SF). SF is a dialysate of blood plasma, a biochemical depot and a biomechanical lubricant for articular cartilage, acting to reduce the interaction of articulating surfaces [15, 20, 37]. Certain macromolecules in SF appear critical to joint homeostasis by binding to and protecting the articular surface. However, the specific role of these putative lubricants, including hyaluronan (HA), remains to be fully established, particularly at physiologic test surfaces.

Hyaluronan is a multi-functional high molecular weight polysaccharide found in many tissues throughout the body, especially in the extracellular matrix of connective tissues such as cartilage, and also in SF. It is the macromolecule of high-molecular weight (10^5-10^8 Da) that imparts high viscosity to SF [25]. HA is a linear polymer built from repeating disaccharide units with the structure [D-glucuronic acid (1-β-3) N-acetyl-D-glucosamine (1-β-4)]n [45], where the disaccharide units are linked by a B(1→4) bond [14, 25, 26]. Despite its relatively simple structure, HA is an exceptionally versatile glycosaminoglycan with multiple roles in many biological functions including cell signaling, wound repair and regeneration, morphogenesis, and pathobiology (reviewed in[38, 43]); and, as a lubricant for lubrication of synovial joints.
There are several possible modes of lubrication that have been proposed to account for the friction and wear resistant characteristics of synovial joints under various loading conditions. Hydrodynamic lubrication may be dominant for cartilage and SF, when the relative speed of articulation is high enough to generate lift between the bearing surfaces and the two surfaces are forced apart [4, 46]. However, such high speeds and lighter loads are not often present for most daily activities, involving intermittent and low-speed motions in various joints [4, 10, 11, 15]. Hydrostatic or weeping lubrication may occur when interstitial fluid within cartilage becomes pressurized, due to the biphasic nature of the tissue, and is exuded from the tissue. In boosted lubrication, as articulating surfaces approach the water component of SF may pass into cartilage, which then concentrates the lubricants in SF, and this concentrated pool of lubricants at the articular surface may provide significant friction-reducing properties to the synovial joint [44].

When the pressurization of interstitial fluid within the joint subsides and surface asperities come into contact, a combination of lubrication regimes may be present. As asperity – asperity contact area increases, a boundary mode of lubrication may dominate [4, 13, 46]. In boundary lubrication, load is supported by surface-to-surface contact, and relative movement in this regime does not favor the formation of any significant fluid film. The associated frictional properties are predominantly determined by lubricant surface molecules. Friction coefficients being invariant with factors that affect the formation of a fluid film, such as normal load and relative velocity of the articulating surfaces, are defining features of boundary lubrication [4, 42, 46]. This mode has been proposed to be important for the maintenance of the
articulated surface because the opposing cartilage layers make contact over ~10% of the total area, and this may be where much of the friction occurs [33]. In the absence of a strongly adsorbed, continuous and self-replenishing boundary layer, prolonged cartilage surface–surface interactions may promote higher friction, wear, and degradation of the articular cartilage.

Although results for studies that have established a cartilage-on-cartilage friction test maintaining the boundary mode of lubrication [36, 37] indicate that an HA solution can facilitate boundary lubrication of cartilage, suggesting that HA in SF contributes to its boundary lubrication function, studies that digest or deplete native SF of HA have led to discordant results using a variety of lubrication tests [19, 30, 34, 35, 41]. And, although the contribution of HA in the lubricating ability of bovine SF has been researched throughout the past several decades [21, 35, 39], a relationship between the lubricating ability of HA-depleted SF and the ability to restore lubricant deficient SF to normal composition and function at a cartilage-cartilage interface has not been fully elucidated. The ability to inhibit hyaluronidase (HAase) activity after HAase digestion of SF also remains to be established, as does the lubrication properties of HA at physiologic tests surfaces under varying parameters such as sliding speed and viscosity.

Therefore, the objectives of this study were to determine the (1) lubricating ability of HA solutions of low, intermediate, and high molecular weight (MW) at a cartilage–cartilage interface as a function of sliding velocity, (2) ability to inhibit enzyme activity of HAase-treated equine SF (eSF), (3) boundary lubrication function
of eSF before and after treatment with HAase (and subsequent inhibition), and (4) ability to restore lubrication function of eSF after HAase digestion.
4.3 Materials and Methods

Materials. Materials for lubrication testing were obtained as described previously [37]. In addition, HA was obtained as 130k and 780kDa forms from Lifecore Biomedical (Chaska, MN) and commercial HA was also obtained as 4,000kDa (Healon® at 10mg/ml). Hyaluronidase was from MP Biomedical (Solon, OH) at 8,130 U/mg, according to manufacturer specifications; SeaKem® gold agarose was from Lonza (Rockland, ME), 50X TAE (2M Tris, 0.5M EDTA) for horizontal agarose gel electrophoresis running buffer was from Life Technologies (Carlsbad, CA); ascorbic acid 6-palmitate (Vcpal), dimethyl sulfoxide (DMSO), and Stainsall were from Sigma-Aldrich (St. Louis, MO).

Synovial Fluid Samples. Equine SF samples were acquired by one of the authors (CWM) during arthroscopic surgery of adult horses (2-4 y.o., n=20). Synovial fluid was aspirated from the injured carpal (n=16) or metacarpophalangeal (n=6) joint, as well as contralateral joints as controls (n=22). The eSF was classified as acute or chronic based on the estimated duration between joint injury and arthroscopic treatment and observations at which time eSF was collected. AI-eSF were from horses that presented for surgery within three weeks of clinical diagnosis, often with signs of moderate to severe synovitis. All eSF samples were clarified of cells and debris by centrifugation (3,000g, 30min) immediately after joint aspiration, and then supernatants were collected and stored at –80°C until subsequent analysis.

Experimental Design
Exp. 1: Effect of sliding velocity on HA lubrication function. Portions of HA preparations were analyzed by biomechanical lubrication tests for lubrication properties as indicated by steady-state equilibrium and static (start-up) coefficients of friction at effective sliding velocities \( (v_{\text{eff}}) \) of 3, 1, 0.3, and 0.1 mm/sec. HA of MWs 130, 780, and 4,000kDa were analyzed \((n=4–5)\) at 3.3 mg/ml.

Exp. 2: HAase digestion and inhibition in eSF. Portions of NL-eSF \((n=4, \ 4 – 6 \ \text{horses/pool})\) and AI-SF \((n=4, \ 4 – 6 \ \text{horses/pool})\) were pooled to create samples for further analysis of the role of the HA (HAase-sensitive) component of eSF. Prior to biomechanical testing for lubrication function, portions of eSF pools were treated with HAase and then Vcpal for subsequent inhibition. Horizontal agarose gel electrophoresis and staining with Stains-all was performed to confirm HA digestion to low MW forms and also inhibition by Vcpal.

Exp. 3: Restoration and enhancement of HA-depleted eSF. After inhibition with Vcpal, high MW HA in the form of Healon® was added to portions of these HA-depleted eSF such that the final concentration of exogenous HA in the eSF was 1.0 mg/ml (HA1) or 3.0 mg/ml (HA3). Friction tests were then performed on eSF+HAase, eSF+HAase+HA1, and eSF+HAase+HA3 samples. Separate friction tests were performed on portions of the untreated eSF pools. All test lubricants were supplemented with protease inhibitors.

Methods

HA Solutions. 130kDa and 780kDa HA were prepared just prior to friction testing by re-suspending in phosphate buffered saline (PBS) supplemented with
protease inhibitors (PIs) (2 mM Na-EDTA, 1 mM PMSF, 5 mM Benz-HCL, and 10 mM NEM). A 10mg/ml stock of Healon® (MW~4,000kDa) was diluted to 3.3mg/ml in PBS+PIs.

**Synovial Fluid Samples.** Portions of eSF pools were treated with 1 U/ml HAase in buffer (0.02M NaAc, 0.015M NaCl, 0.005M EDTA, pH 6.0) supplemented with PIs, for 36 hours at 25°C. Prior to friction testing, 1mM VcPal in DMSO was added to the HAase-treated eSF and incubated at 37°C for 1 hour [8]. After HAase inhibition, samples were supplemented with PIs prior to friction testing. Separate portions of eSF pools treated as above were supplemented with stock Healon® (10 mg/ml) to a final concentration of 1.0 mg/ml (HA1) or 3.0 mg/ml (HA3) additional HA. The addition of HAase and HA1 to the eSF resulted in a 10% dilution of the eSF by volume. The addition of HA3 resulted in a 30% dilution of the eSF by volume. All samples were subsequently stored at 4°C until lubrication testing.

**Lubrication Tests.** Portions of SF and HA samples were analyzed for static and kinetic friction coefficients in the boundary lubrication mode on articulating cartilage surfaces, essentially as described previously [36,37]. Intact articular surfaces were in the form of osteochondral cores and annuli from adult bovine knees, and were stored in PBS supplemented with PIs at −80°C. For lubrication testing, cartilage samples were thawed at 4°C, and then bathed in ~0.5 ml of the subsequent test lubricant supplemented with PIs, with the cartilage completely immersed for 16-24 hours at 4°C prior to lubrication testing. **Effect of Sliding Velocity.** The lubricant sample and cartilage substrates were compressed to 18% of the total cartilage thickness and allowed to stress relax for 30 minutes. Samples were then tested by
rotating +2 revolutions, immediately followed by –2 reset revolutions at $v_{eff}$ of 3, 1, 0.3, 0.1, and then 3 mm/s, with a pre-sliding duration ($T_{ps}$, the duration the sample is stationary prior to rotation) of 120 seconds between each $v_{eff}$. The test sequence was then repeated in the opposite direction of rotation. Friction coefficients ($\mu$) were calculated from the expression $\mu = \tau/(R_{eff}N_{eq})$, where $\tau$ is torque, $N_{eq}$ is the equilibrium axial load after the 30-minutes stress relaxation, and $R_{eff}$ is the effective radius of the cartilage, as described previously [36, 37]. Briefly, a static friction coefficient, $\mu_{static}$, was calculated using the peak $|\tau|$, measured just after the start of rotation. A kinetic friction coefficient, $\mu_{kinetic}$, was calculated using the $|\tau|$ averaged during the second complete revolution of the test sample. Effect of HAase. The lubricant sample and cartilage were tested at 10°C by preconditioning, compressing to 18% of the total cartilage thickness, and allowing 30 minutes for stress relaxation and interstitial fluid depressurization. Then, samples were rotated at an effective velocity of 0.3 mm/s with $T_{ps}$ of 120, 12, and 1.2 seconds. Friction coefficients, $\mu_{static}$ and $\mu_{kinetic}$, were calculated as above.

Biochemical Analysis of eSF. Portions of the eSF samples were analyzed biochemically for the concentration and molecular weight distribution of HA. HA concentration in NL- and AI-eSF pools was determined by an ELISA-like assay using HA binding protein [1]. HA molecular weight distribution in NL- and AI-eSF pools with and without treatment with HAase, Vcpal, and Healon® was assessed by horizontal agarose gel electrophoresis (150V, 45 – 55 minutes) with a running buffer of 40mM Tris-acetate, 1mM EDTA, pH 8.2 followed by overnight staining with 0.001% Stains-all [7]. Samples with HA mass of 300ng were applied to 1% agarose
gels. Samples were not treated with proteinase K prior to gel electrophoresis in order to observe the migration of HA in chemically unmodified eSF. Gel images were digitized with a D40 digital camera (Nikon, Melville, NY).

**Statistical Analysis.** Data are presented as the mean ± SEM. Friction coefficient data was log-transformed because of the large variation between groups. The effect of HA MW on $\mu_{\text{static}}$ and $\mu_{\text{kinetic}}$ (with $v_{\text{eff}}$ as a repeated factor) were assessed by ANOVA. The effect of test lubricant on $\mu_{\text{static}}$ and $\mu_{\text{kinetic}}$ at each $v_{\text{eff}}$ was assessed by ANOVA and Tukey post hoc testing. The effects of HAase digestion and HA supplementation of eSF test lubricants on $\mu_{\text{static}}$ (with $T_{\text{ps}}$ as a repeated factor) and on $\mu_{\text{kinetic}}$ were assessed by ANOVA. The effect of HAase digestion and HA supplementation of eSF test lubricants on $\mu_{\text{kinetic}}$ averaged over all $T_{\text{ps}}$ was assessed by ANOVA and Tukey post hoc testing for comparison between experimental and control groups. Statistical analysis was performed using Systat 10.2 (Systat; Richmond, CA).
4.4 Results

Lubrication properties of HA at varying $v_{\text{eff}}$. The torque and axial load varied with $v_{\text{eff}}$ (Figure 4.1). In all test lubricants, torque and axial load increased qualitatively with $v_{\text{eff}}$. Axial load was $\sim$40% lower in 130kDa HA than SF and 4,000kDa HA test lubricants (Figure 4.1A, D, G), while torque was $\sim$50% greater in 130kDa HA compared to SF and 4,000kDa HA (Figure 4.1 B, C, E, F, G, H). Axial load and torque were similar for SF and 4,000kDa HA. The peak torque (Figure 4.1C, F, G) dissipated to approximately steady-state values by 360°, particularly at slower $v_{\text{eff}}$. SF and 4,000kDa lubricants reached similar steady-state torque values at all $v_{\text{eff}}$, while steady-state torque values for 130kDa HA increased with $v_{\text{eff}}$. The axial load was cyclical, peaking at approximately 180° and 540° (Figure 4.1A, D, G), as observed previously [37].

Friction was modulated by test lubricant and $v_{\text{eff}}$ (Figure 4.2). $\mu_{\text{kinetic}}$ varied with test lubricant ($p<0.05$) but not significantly with $v_{\text{eff}}$ ($p = 0.90$), with an interaction effect between test lubricant and $v_{\text{eff}}$ ($p<0.001$, Figure 4.2A), and $\mu_{\text{static}}$ varied with test lubricant ($p<0.05$) and $v_{\text{eff}}$ ($p<0.001$), with no interaction effects ($p = 0.17$, Figure 4.2B).

The values of $\mu_{\text{kinetic}}$ ranged from 0.138±0.033 to 0.248±0.099 for 130kDa HA, 0.065±0.013 to 0.091±0.016 for 780kDa HA, and 0.054±0.005 to 0.062±0.009 for 4,000kDa HA (Figure 4.2A). $\mu_{\text{kinetic}}$ for samples tested in 130kDa HA were $\sim$200 – 400% higher than samples tested in 4,000kDa HA at $v_{\text{eff}}$ greater than 0.1mm/sec.
(p<0.05), and ~200% and 300% higher than samples tested in 780kDa at 1 and 3mm/sec, respectively (p<0.05). The values for $\mu_{\text{kinetic}}$ tended to be higher for samples tested in 780kDa HA than 4,000kDa HA at slower $v_{\text{eff}}$, and approached $\mu_{\text{kinetic}}$ of 4,000kDa at 1 and 3mm/sec (p=0.86 and 0.94, respectively).

The values of $\mu_{\text{static}}$ increased with increasing $v_{\text{eff}}$ for all test lubricants, ranging from 0.181±0.039 to 0.520±0.136 for 130kDa HA, 0.122±0.020 to 0.270±0.047 for 780kDa HA, and 0.081±0.007 to 0.220±0.012 for 4,000kDa HA (Figure 4.2B). $\mu_{\text{static}}$ for samples tested in 130kDa HA were ~130% higher than samples tested in 4,000kDa HA at all $v_{\text{eff}}$ (p<0.05), and tended to be higher than samples tested in 780kDa. The values for $\mu_{\text{static}}$ tended to be higher for samples tested in 780kDa HA than 4,000kDa HA at all $v_{\text{eff}}$. In addition, the degree of rotation at which peak torque was reached varied with test lubricant (p<0.05) and tended to vary with $v_{\text{eff}}$ (p=0.061), with no interaction effect between test lubricant and $v_{\text{eff}}$ (p=0.45, Figure 4.2C). Peak torque was reached within the first 10 degrees of rotation for 780kDa HA, 4,000kDa HA, and SF. The degree of peak torque for 130kDa HA was ~100 – 500% higher than the other test lubricants at all $v_{\text{eff}}$ (p<0.01).

**Biochemical analysis of eSF and confirmation of HAase inhibition.** The concentration of HA in eSF tended to vary with joint injury (p=0.17, Figure 4.3A). Qualitatively, the MW distribution of HA in NL- and AI-eSF before HAase digestion or HA supplementation consisted of high MW HA at or near the 4,000kDa standard in the HA MW ladder (Figure 4.3B). Treatment of eSF with HAase resulted in a shift to very low MW to around 30kDa HA in all samples (Figure 4.3C and D, lane 1). Subsequent inhibition of HAase was confirmed after addition of 4,000kDa HA to HA-
depleted eSF, both in samples before (Figure 4.3C and D, lane 2) and after (Figure 4.3C and D, lane 3) friction testing, as indicated by the specific staining of HA in the high MW region near the 4,000kDa standard in the HA MW ladder.

**Lubrication function and restoration of HA-depleted eSF.** The torque varied with lubricant group while the axial load was similar for all NL- and AI-eSF lubricant groups tested at \( v_{\text{eff}} \) of 0.3 mm/s and \( T_{\text{ps}} \) of 120s (Figure 4.4). Torque increased qualitatively with addition of HAase (Figure 4.4B, C, E, F), and was normalized by addition of HA for steady-state conditions. The peak torque (Figure 4.4C, F) dissipated to approximately steady-state values by 360°, as observed previously. NL- and AI-eSF reached similar steady-state torque values before addition of HAase and after addition of HAase plus exogenous HA, while peak and steady-state torque values were higher for NL- and AI-eSF treated with HAase only. Although steady-state torque values were normalized by addition of exogenous HA, peak torque values were not reduced to initial values by the addition of HA.

The friction-reducing properties of eSF depended on the presence of high MW HA (Figure 4.5). \( \mu_{\text{kinetic}} \) was higher after HAase treatment (p<0.001, Figure 4.5A) and lowered by the addition of exogenous HA (p<0.001). The \( \mu_{\text{kinetic}} \) was 224% higher for NL-eSF+HAase (0.059) than that for NL-eSF alone (p<0.001), and 151% higher for AI-eSF+HAase (0.062) than that of AI-eSF alone (p<0.001). The boundary lubrication function of HA-depleted NL- and AI-eSF was effectively normalized by addition of high MW HA for \( \mu_{\text{kinetic}} \) (Figure 4.5A). The \( \mu_{\text{kinetic}} \) for NL-eSF+HAase+HA1 (0.027) was 54% lower than that for NL-eSF+HAase (p=0.004), and similar to that for NL-eSF (p=0.315). The \( \mu_{\text{kinetic}} \) for AI-eSF+HAase+HA1 (0.028) was also 54% lower than
that for AI-eSF+HAase (p=0.003), and indistinguishable from that for AI-eSF (p=0.968). Similar results were observed for eSF+HAase+HA3 test lubricants, with μ\text{kinetic} being reduced even further for NL-eSF+HAase+HA3 (0.019) to values indistinguishable from NL-eSF (p=1.00). μ\text{kinetic} for AI-eSF+HAase+HA3 (0.027) remained indistinguishable from that for AI-eSF (p=1.00), but was not reduced any further from AI-eSF+HAase+HA1.

μ\text{static} varied with test lubricant (p<0.001) and T_{ps} (p<0.001), with an interaction effect between test lubricant and T_{ps} (p<0.001). μ\text{static} increased with T_{ps} (p<0.001). At T_{ps} = 1.2s, μ\text{static} (\textbf{Figure 4.5B}) for NL-eSF+HAase (0.095) was 391% higher than that for NL-eSF alone (p<0.001), and 158% higher for AI-eSF+HAase (0.079) than that of AI-eSF alone (p<0.05). μ\text{static} of HA-depleted NL- and AI-eSF at T_{ps}=1.2s was effectively normalized by addition of high MW HA a 1.0mg/ml (eSF+HAase+HA1) (\textbf{Figure 4.5B}), but did not return to the μ\text{static} values of the untreated eSF, as was the case for μ\text{kinetic}. Addition of exogenous HA to 3.0mg/ml did not lower μ\text{static} for either NL- or AI-eSF any further.

In addition, the degree of rotation at which peak torque was reached varied with test lubricant (p<0.05, \textbf{Figure 4.5C}). Peak torque was reached within the first 1 degree of rotation for NL- and AI-eSF before addition of HAase, while the degree of peak torque after addition of HAase was ~100 – 400% higher (p<0.05). The degree of peak torque was normalized by addition of exogenous HA for both NL- and AI-eSF to values similar to those before treatment with HAase (\textbf{Figure 4.5C}).
Figure 4.1: Axial Load (A, D, G) and torque (B, C, E, F, H, I) measurements versus rotation for test solutions of bovine SF (n = 3) and HA of 130k (n=5) and 4,000k Da at 3.3mg/ml, at 18% compression after 30 minutes stress relaxation duration, at effective sliding velocities (v_{eff}) of 3, 1, 0.3, and 0.1 mm/s with 120s pre-spin duration. Average±SEM.
**Figure 4.2:** Effect of HA MW (kDa) and effective velocity (mm/s) on (A) static and (B) kinetic friction coefficients and (C) degree of rotation at which peak torque was reached. All HA solutions were tested at 3.3 mg/ml. Data are mean±SEM, n = 3 – 5. * indicates p<0.05 vs 4,000kDa HA; ♦ indicates p<0.05 vs 780kDa HA.
Figure 4.3: (A) HA concentration (mg/ml) in pooled NL- and AI-eSF (n = 4) and (B-D) typical electrophoretic separation of HA in pooled NL-eSF (B, C) and AI-eSF (B, D) before (B) and after (C, D) treatment with hyaluronidase (+HAase) and addition of exogenous HA (+HA) after HAase inhibition. Data are mean±SEM. Samples in lanes 2 and 3 of (C) and (D) are from portions of SF taken before and after friction testing, respectively. MW: HA molecular weight ladder.
Figure 4.4: Axial load (A, D) and torque (B, C, E, F) measurements versus rotation for test solutions of NL-eSF (A – C) and AI-eSF (D – E) before and after treatment with hyaluronidase (+HAase) and the addition of exogenous HA of average molecular weight 4,000kDa to 1 3 mg/ml (+HA) after HAase digestion, at 18% compression after 30 minutes stress relaxation duration, at v_{eff} of 0.3 mm/s with 120s pre-spin duration. n = 4; data are mean ± SEM.
Figure 4.5: Dependence of (A) kinetic ($\mu_{\text{kinetic}}$) and (B) static ($\mu_{\text{static}}$) (at $T_{ps} = 120s$) friction coefficients, and (C) degree of rotation at which peak torque was reached, for NL- and Al-eSF before and after treatment with hyaluronidase (+HAase) and the addition of exogenous HA (+HA) of average molecular weight 4,000kDa to 1 or 3 mg/ml after HAase digestion. $n = 4$; data are mean $\pm$ SEM. ** indicates $p<0.001$; * indicates $p<0.05$. 
Figure 4.6: Proposed mechanism of articular cartilage lubrication by hyaluronan.
4.5 Discussion

The results described here indicate that HA acts as an effective boundary, both alone in solution and in eSF, with $\mu_{\text{kinetic}}$ being invariant with $v_{\text{eff}}$ for high MW HA solutions, while tending to increase with increasing $v_{\text{eff}}$ for low MW HA at the same concentration (Figure 4.2). For eSF, lubrication function was markedly elevated upon depletion of native high MW HA to small MW fragments, and restoration in vitro with exogenous HA of physiologic high MW was indicated by a marked recovery of SF boundary lubrication function at a cartilage – cartilage interface. Digestion of eSF with hyaluronidase was shown to be effectively inhibited by Vcpal (Figure 4.3) and HA-depletion of eSF resulted in an increase in steady-state ($\mu_{\text{kinetic}} \sim 0.059$ and $0.062$, NL and AI) and start-up ($\mu_{\text{static}} \sim 0.095$ and $0.079$, NL and AI, Figure 4.5) friction coefficients to similar levels for both NL- and AI-eSF. Collectively, these results indicate the presence of high MW HA in eSF is particularly important to its boundary lubrication function since the addition of HA to HA-depleted eSF fully restored $\mu_{\text{kinetic}}$ to levels indistinguishable from untreated eSF, for both normal and injury fluids (Figure 4.5A). Start-up friction coefficients were not fully restored upon repletion with high MW HA (Figure 4.5B), indicating the contributions of HA to SF lubrication function upon the initiation of motion and at steady-state may involve independent mechanisms and variable interactions with the articular surface. Articular cartilage may be particularly vulnerable when boundary lubrication is deficient, as is common in the acute stage after injury, since this may be when the friction and potential wear
are highest. During this time, addition of lubricant molecules, particularly HA of physiologic normal MW, to SF may aid in the restoration of its lubrication function.

The MWs of the HA preparations used in the present study were representative of those in native SF under physiologic and pathophysiologic conditions. Approximately 70% of the HA in normal SF is reported to be ≥4,000kDa, and the remaining ~30% is distributed in the range of <4,000kDa to ~100-200kDa, with the MW of HA in pathological SF being shifted to the levels lower than 4,000kDa [3, 5, 12, 27]. Therefore, the use of HA of average MWs 130k, 780k, and 4,000kDa is reasonable for studying the size-dependent effects of the boundary lubricating ability of HA. HA preparations were tested at a physiologic normal concentration [3, 5, 12, 27] in order to elucidate the MW dependent effects with varying v_{eff}. Additional studies examining how the concentration – function relationship of HA of different MWs at lower concentrations varies with v_{eff} may further delineate the lubrication function of HA under normal and pathologic states.

The ability to inhibit hyaluronidase activity in a controlled manner after effective depletion of high MW HA in eSF is of pivotal importance in clearly elucidating the molecular contributions of HA to synovial fluid lubrication at physiologic test surfaces. If enzyme activity was not effectively inhibited prior to and during tests of boundary lubrication function, the active HAase could potentially alter the articulating cartilage substrates, leading to confounding results that would be difficult to interpret. Several titration studies revealed that inhibition of HAase by Vcpal was markedly enhanced at lower temperatures (data not shown). Therefore, in order to ensure adequate inhibition of the HAase during lubrication testing, friction
tests were carried out at ~10°C. Additional pilot friction tests performed at ~10°C and then again at ~25°C on bovine cartilage substrates with PBS and bovine SF test lubricants resulted in similar friction coefficients within each lubricant group at both temperatures (data not shown).

The contribution of HA to the boundary lubrication of articulating cartilage surfaces at physiologic concentration reported here is consistent with and extends on previous studies examining the lubricating ability of HA at various tests surfaces and configurations under boundary lubrication conditions. Schmidt et al [36] demonstrated that SUPARTZ, a purified sodium hyaluronate extracted from rooster combs with an average MW of 800kDa, functioned as an effective boundary lubricant in a dose-dependent manner, in the same test system used in the current study. However, in that study, HA solutions were tested at a single $v_{\text{eff}}$, 0.3 mm/s, using the protocol previously established for boundary lubrication [37]. The current study extends on these findings by testing HA solutions at a similar MW (780kDa) as well as at lower (130kDa) and higher (4,000kDa) MWs over a range of $v_{\text{eff}}$. The $\mu_{\text{static}}$ (0.141) and $\mu_{\text{kinetic}}$ (0.082) values for the 780kDa HA solution at 0.3 mm/s (and a $T_{\text{ps}}$ of 120s) reported here are in general agreement with the $\mu$ reported for ~800kDa HA in the previous study (0.22 and 0.12 for $\mu_{\text{static}}$ and $\mu_{\text{kinetic}}$ at 3.3mg/ml HA, respectively). The absolute values for friction coefficients in the current study are slightly lower, possibly due to the different test protocol and HA preparation used. The boundary lubrication function of 4,000kDa HA (as Healon) at ~3.3mg/ml was also demonstrated at a latex-glass interface [22], with a friction coefficient being lowered from ~0.14 for PBS to ~0.07 for Healon, indicating the ability of high MW HA at physiologic
concentration to reduce surface interactions between both physiologic and non-physiologic articulating surfaces.

The contribution of HA in the lubrication of articular cartilage has been analyzed using a variety of friction tests. Variations include experiments on whole joints [31, 40] versus small specimen, and different test surfaces such as rubber, latex, glass, and cartilage. Since these studies employed different whole joint test systems in which several modes of lubrication were likely operative, it is not surprising that HA has been reported to be both effective [6, 18, 32] and ineffective [29, 35] as a boundary lubricant. In the cartilage-on-cartilage test configuration used in the previous and current study, HA does indeed appear to contribute to the boundary lubrication of articular cartilage in a dose-dependent [36] and size-dependent manner. The current results may also indicate that below a certain MW the ability of HA to reduce surface interactions is impaired, which may lead to higher friction, and possibly higher wear, as was demonstrated for cartilage articulating on glass after hyaluronidase digestion of the cartilage surface [17].

The significant alterations in SF lubricant function with depletion of high MW HA, and the ability to restored altered lubrication function by addition of high MW HA, extend on previous studies while providing additional insight into the molecular basis, importance, and significance of HA as a physiologic boundary lubricant for articular cartilage. Bovine SF treated with bovine testicular hyaluronidase for 1 hour at 23°C did not result in a higher friction coefficient compared to untreated SF in a whole joint system ($\mu \sim 0.0037$ for both), in which several modes of lubrication were likely operative [28]. The much lower values for $\mu$, compared to the current study,
may be attributed to the whole joint test system and different test protocol used. Although the authors reported a reduction in viscosity of the SF after enzyme treatment, the reduction in HA MW to low MW fragments was not confirmed by biochemical analysis, nor was the enzyme activity inhibited prior to or during lubrication testing. Therefore, it is not clear whether the HA was sufficiently depleted from the SF or whether the articular cartilage was unaltered by the presence of hyaluronidase in the test solution. A series of studies carried out by Radin et al led the authors to conclude that HA acted as a good lubricant when the synovial membrane was articulated against glass [34], but not essential for lubrication of articular cartilage in a whole joint test system [35]. HA of MW reported to be ~20 million in the former study resulted in µ ranging from 0.025 – 0.049, which was similar to µ for bovine SF (0.024 – 0.047), and treatment of SF with hyaluronidase led to µ ~ 0.056 – 0.089, values that are similar to the results of the current study. Again, one of the factors contributing to the differences in the effectiveness of HA as a lubricant between the two studies may be the variation in test systems and protocols between that of the synovial membrane – glass system, where boundary lubrication was likely dominant, and the whole joint system, where multiple lubrication modes were likely operable.

A reduction in friction coefficients of HA-depleted eSF after addition of exogenous HA presented here suggests lubricant supplementation may modulate and restore the boundary lubrication function of SF after biochemical alteration in vivo. Although the concentration of exogenous HA that was added to the eSF was higher than the average measured levels in NL-eSF samples, the increase in concentration by the addition of HA to 1.0 mg/ml is within the concentration range of HA in normal
equine SF reported previously. Supplementation to 3.0 mg/ml HA was carried to determine if supra-physiologic amounts of HA would lead to further enhancement of lubrication function. Additional analysis of the ability of lower concentrations of exogenous HA, and also of more poly-disperse MW solutions mimicking the HA distribution in normal and pathologic SF [2], on the lubricating ability of HA-depleted SF would help to further elucidate the molecular basis of HA lubrication function. In the current study, adding 3.0 mg/ml of HA to SF resulted in a ~30% dilution of the SF, which under normal conditions does not lower $\mu_{\text{static}}$ and $\mu_{\text{kinetic}}$ compared to that of full strength SF [36]. These results were observed for NL-eSF in the current study. However, friction coefficients were not reduced any further when AI-eSF was supplemented with 3.0 mg/ml HA, suggesting a possible dilution effect on molecules other than HA in SF that may contribute to its boundary lubrication function. One such molecule found in SF and localized to the articular surface is proteoglycan-4 (PRG4) [36, 23, 24]. The PRG4 content in the eSF pools analyzed in the current study, before and after treatment with HAase and HA, was not analyzed. Doing so may elucidate further the molecular characteristics of the eSF lubricants and the interaction of HA and PRG4 in native eSF and HA-repleted eSF.

Currently, HA supplementation is a common clinical treatment for people with osteoarthritis, and postulated to have disease modifying and chondroprotective effects [9, 16]. Although several studies have been performed to determine clinical outcomes of HA supplementation, fewer studies have sought to elucidate the efficacy of supplementation with HA, particularly after controlled depletion of HA, to specifically restore SF lubricant function. The collective results of this study provide insight into
the nature of the boundary lubrication of articular cartilage by HA, supporting the concept that at particular stages following injury, biological or physical treatments to modulate lubrication of HA may help to protect the articular cartilage and joint from damage.

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4.7 References


CHAPTER 5:

CONCLUSIONS

5.1 Summary of Findings

The overall motivation of this dissertation work was to further the understanding of the contributions of synovial fluid macromolecules, with a focus on HA, to the boundary lubrication of articular cartilage under normal and pathophysiologic conditions, and to what extent chemical augmentation of injured SF by HA can restore lubrication function of SF and articular cartilage. The major findings were:

1. SF lubrication function and composition are altered coordinately after acute joint injury to race horses, and supplementation in vitro of the abnormal SF with HA leads to a marked recovery of boundary lubrication function.
   a. In the acute stage, the boundary lubrication function of SF is reduced, as indicated by $\mu_{\text{kinetic}}$ that is higher than normal (0.036 vs 0.026). Later, in the chronic stage, the boundary lubrication function of SF appears to be partially recovered.
b. AI-eSF had a lower HA concentration (~30%), a higher PRG4 concentration (+83%), and a higher SAPL concentration (+144%) compared to NL-eSF.

c. The MW distribution of HA in eSF varied between NL-, CI-, and AI-eSF, being shifted with injury to lower MW forms.

d. The role for HA appears particularly important since the addition of HA to AI-eSF restored boundary lubrication function by lowering $\mu_{\text{kinetic}}$ by ~30% to a level indistinguishable from NL-eSF.

2. Marked alterations in the lubrication function and lubricant composition of SF were also observed in SF from patients with intra-articular tibial plateau fractures in the initial stages of treatment.

   a. SF from knees with intra-articular fractures had markedly decreased lubrication ability (+100% increase in $\mu_{\text{kinetic}}$) compared to control SF.

   b. Concomitantly, tibial plateau fractures led to changes in the concentration of putative lubricant molecules, with a decrease in HA (~87%) and an increase in PRG4 (+156%).

   c. Poorly lubricating SF was associated with diminished HA concentration as well as elevated PRG4 and total protein concentrations.

3. Articular cartilage lubrication by HA is markedly dependent on concentration and MW, with the friction reducing properties of HA being dependent on its high MW.

   a. Friction coefficients for 6.4kDa and 51kDa HA were only reduced slightly from those of PBS.
b. For 4,000kDa HA increasing from 0.33 to 3.3mg/ml, friction coefficients were at low values, similar to those of bovine SF.

4. HA acts as an effective boundary, both alone in solution and in eSF
   a. $\mu_{\text{kinetic}}$ was invariant with $v_{\text{eff}}$ for 780k and 4,000kDa HA solutions, and tended to increase with increasing $v_{\text{eff}}$ for 130kDa HA at 3.3mg/ml.
   b. Digestion of eSF with HAase was shown to be effectively inhibited by Vcpal, allowing for the examination of the lubricating ability of HA-depleted SF at a cartilage – cartilage interface.
   c. For eSF, lubrication function was markedly elevated (+ ~150 – 390%) upon depletion of native high MW HA, resulting in an increase in $\mu_{\text{kinetic}}$ to 0.059 and 0.062 (NL- and AI-eSF, respectively) and $\mu_{\text{static}}$ to 0.095 and 0.079 (NL- and AI-eSF, respectively).

5. Restoration in vitro with exogenous HA of physiologic high MW led by a marked recovery of eSF boundary lubrication function to values indistinguishable from untreated eSF.

### 5.2 Discussion and Future Directions

The contribution of HA in synovial fluid to the low-friction, wear-resistant properties of articular cartilage, particularly as a boundary lubricant, has been the subject of much debate over the past several decades. Collectively, the findings of this dissertation further the understanding of the contributions of synovial fluid macromolecules, with a focus on HA, to the boundary lubrication of articular cartilage under normal and pathophysiologic conditions, and to what extent chemical
augmentation of injured SF by HA can restore lubrication function of SF and articular cartilage.

The findings of Chapters 2 and 3 provide insight into the consequences of joint injury of varying severity on synovial fluid lubrication function and lubricant composition. The observed correlation of lower HA concentration and higher PRG4 concentration in injury SF with higher friction coefficients helps to clarify the molecular basis for altered lubrication function after joint injury, suggesting other factors in addition to lubricant molecule concentration play a role in SF lubrication function. Such factors may include the quality of the molecules, such as molecular weight and chemical intactness, and their ability to interact with the articular surface. The shift in both concentration and MW of HA in SF to lower levels after joint injury, along with the clear concentration and MW dependence of the lubricating ability of HA solutions, indicate the importance of both concentration and quality of lubricant molecules on their normal lubrication function. An increase in PRG4 concentration after injury was unexpected, since previous studies on PRG4 concentration in injury SF indicated a reduction in PRG4 content to occur in both animal injury models [9, 10, 23] and human injury [8]. Such differences may be due to factors including the type of injury (ie; soft tissue versus osteochondral injuries), the duration of injury before the SF was analyzed, the sample source, and the analysis method. Previous studies included soft tissue and joint destabilization injuries, while the current study includes osteochondral types of injury. In addition, SF PRG4 concentration may change dynamically after joint injury, and delineating such changes would further clarify the molecular basis of altered lubrication function of SF. Further analysis of the specific molecular and structural properties of the PRG4 in normal and injury SF, in addition to concentration, would also help to further elucidate the alterations in SF
lubricant composition after injury. Analysis of the mRNA expression and potential presence of splice variants of the PRG4 [7] would allow for further interpretation of the increase in PRG4 content with joint injury reported here.

Several studies support the idea of PRG4 as an effective boundary lubricant. However, fewer studies provide evidence in support of HA as a boundary lubricant. The results in Chapter 2 indicate HA of physiologic high MW and concentration alone in solution at physiologic test surfaces can replicate nearly all of the lubrication function of SF, under the test protocol used here, indeed acting as an effective lubricant for articulation between two cartilage surfaces. Previous studies from our lab that combined 800kDa HA with PRG4, both at physiologic concentrations, reported friction coefficient values approaching those of SF, but that did not reach values of SF [21]. Additionally, the combination of HA and PRG4 was more effective at reducing friction than either molecule alone. The synergistic effects that were observed between HA and PRG4 in that study provide further evidence that HA, and PRG4, are critical to proper lubrication function by SF. It will be important to assess the ability of the high MW HA (4,000kDa) used in the current study in combination with physiologic levels of PRG4 to fully recapitulate the lubrication function of SF at a cartilage – cartilage interface. HA may also be retained at or between the articular surfaces acting to facilitate sliding [2] and distribution of shear forces, particularly upon interactions with PRG4 [15]. Based on the current and previous findings, it is expected that certain combinations of HA and PRG4 will indeed replicate the low friction properties of native SF. Such studies may further clarify interactions between HA and PRG4 and provide insight for the physico-chemical therapies currently used in the treatment of OA.
Chapter 3 expands on the results from Chapter 2 by studying the effects of severe trauma on cartilage lubrication by injury SF in humans. Although several studied have investigated the effects of blood introduced into the joint on articular cartilage metabolism and homeostasis, few studies have been carried out to examine the effects of blood in the joint on cartilage mechanics after injury [1, 17]. Blood is introduced into the joint space in a number of clinical situations, ranging from sports-related injuries of vascularized joint tissues such as knee ligaments and meniscus, to trauma with an intra-articular fracture, cartilage repair therapies, and other surgical procedures involving the joint. Even a single incidence of joint bleeding has been shown to lead to irreversible joint damage [18, 19, 22]. As with such pathologies as OA, blood induced arthropathy is initiated several years before evidence of clinical manifestations [11, 24]. The mixture of blood with SF may be harmful to synovial joints through a number of mechanisms. For example, blood components are deleterious to articular cartilage metabolism [13, 14]. Studies of cartilage exposure to blood in vitro have indicated a decrease in PG synthesis, increase in GAG release, increase in MMP activity, and decrease in GAG content, suggesting a possible role of blood in the alteration of levels of SF constituents and lubricating function following acute traumatic injury. Blood may affect SF lubrication of cartilage by modulating the fluid lubricant components of SF and by introducing blood cells, as hypothesized in preliminary studies on the effects of in vitro mixtures of blood and SF on the boundary lubrication of articular cartilage [1]. It is clear that intra-synovial bleeding is harmful to articular cartilage, and the latter study indicates such bleeding is also harmful the boundary lubrication of articular cartilage by SF. Elucidating the mechanical impact of blood introduced into the joint space under a number of clinical situations would help in the development of more effective therapies intended to restore failed joint lubrication.
The results reported in Chapter 4 provide further evidence for the importance of the presence of high MW HA on the normal lubrication function of SF, since depleting SF of high MW HA led to a marked increase in static and kinetic friction coefficients for both NL- and AI-eSF. The inability of exogenous HA to further reduce the friction coefficients of eSF under certain conditions, as reported in Chapter 2 for intact NL-eSF supplemented to 1mg/ml exogenous HA and in Chapter 4 for AI-eSF supplemented to 3mg/ml exogenous HA, may suggest that once the surface of articular cartilage is appropriately coated with HA, an increase in the amount of high MW HA present in the lubricant solution does not enhance its boundary lubricating ability any further. These findings are consistent with studies that point toward lubricant molecules being present in excess in normal SF, as indicated by the lubricating ability of normal bovine SF at a cartilage–cartilage interface being similar between full strength SF and SF after a 3-fold dilution [21]. To that end, the ability to restore lubrication function of HA-deficient SF, both in intact injury SF (Chapter 2) and HAase-treated SF (Chapter 4), provides insight into the effectiveness of tribo-supplementation of HA on the restoration of the lubrication function of injury SF on articular cartilage. Currently, HA supplementation is a common clinical treatment for people with OA, and postulated to have disease modifying and chondroprotective effects [5, 12]. Although several studies have been performed to determine clinical outcomes of HA supplementation, fewer studies have sought to elucidate the efficacy of supplementation with HA, particularly after controlled depletion of HA, to specifically restore SF lubricant function. The challenge will be to identify the time-dependent effects of HA supplementation of SF on its enhanced function, and the ability to retain injected HA within the SF and joint cavity. In injury and OA of the knee, the synovium permeability may be altered [3, 6, 16, 20], and if permeability is increased the likelihood of HA retention may be reduced. However, the chemical
augmentation of dysfunctional SF indicates biomechanical lubricants injected intra-articularly as a therapeutic treatment may be promising. The efficacy of potential lubricants may be investigated by translational studies of animal injury models that examine the mechanical and chondroprotective effects of therapeutic solutions engineered to replicate healthy synovial fluid within the joint cavity. Additionally, the development of predictive models for the lubricating function of HA of a particular MW distribution alone and in combination with PRG4 would be useful for the targeted therapies designed to restore failed lubrication function. Incorporating such a model with one designed to predict SF composition in health, injury and disease, as recently developed by Blewis et al [4], may provide great foresight for the development of such therapies.

Collectively, the work reported here indicates that addition of lubricant molecules to SF can restore its lubrication function and appears to be mechanically beneficial to cartilage. This may be particularly important in the acute stage after injury when the articular cartilage may be particularly vulnerable to consequences of poor boundary lubrication. The impaired ability of SF from joints with osteochondral types of injuries to reduce the surface interactions between articulating surfaces may be deleterious following injury, while supplementation with HA may help to restore SF function and reduce friction demands on the joint to a safe range follow addition of HA. By reducing surface interactions at the articular cartilage–SF interface by addition of HA, the degeneration of cartilage may possibly be prevented following acute injury.
5.3 References


