# FACTORS INFLUENCING CARTILAGE WEAR IN AN ACCELERATED IN VITRO TEST: COLLAGEN FIBER ORIENTATION, ANATOMIC LOCATION, CARTILAGE COMPOSITION, AND PHOTO-CHEMICAL CROSSLINKING

A Thesis

Submitted to the Faculty

of

Purdue University

by

M. Jayed Hossain

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science in Mechanical Engineering

August 2018

Purdue University

Indianapolis, Indiana

# THE PURDUE UNIVERSITY GRADUATE SCHOOL
# STATEMENT OF COMMITTEE APPROVAL

Dr. Diane Wagner, Chair

Department of Mechanical and Energy Engineering

Dr. Alan Jones

Department of Mechanical and Energy Engineering

Dr. Nilsson Holguin

Department of Mechanical and Energy Engineering

**Approved by:**

Dr. Sohel Anwar

Head of the Graduate Program

To my beloved parents, wife and brothers.

# ACKNOWLEDGMENTS

First, I would like to thank Almighty for giving me the strength, ability and opportunity to undertake this research study, to hold on and finish it. I would like to express my sincere gratitude to my thesis advisor, Dr. Diane Wagner, for her proper guidance, cooperation, and support towards the completion of this thesis work. In every step of my research work, I found her as a person of immense knowledge and critical problem-solving abilities. I would like to thank her for keeping faith upon me until the end of this work. I would also like to thank my lab-mates Dr. David Kahn, Dr. Izath Nizeet Aguilar, Amin Joukar, Hessam Noori, and Ghazal Hosseini, for their precious time and support towards my research work. I would like to give special thanks Md. Minal Nahin for his continuous help. I am grateful to my wife and parents for their endless support and for being a source of inspiration for me to pursue my dream and hard work. Without their continuous encouragement, this accomplishment would never come true.

TABLE OF CONTENTS

Page

LIST OF TABLES . . . . . viii

LIST OF FIGURES . . . . . ix

SYMBOLS . . . . . xi

ABBREVIATIONS . . . . . xii

NOMENCLATURE . . . . . xiii

ABSTRACT . . . . . xiv

1 BACKGROUND . . . . . 1

1.1 Cartilage Structure and Function . . . . . 1

1.2 Biphasic Nature of Cartilage . . . . . 4

1.3 Cartilage Diseases and Treatments . . . . . 5

1.4 Cartilage Friction and Wear . . . . . 7

1.5 Cartilage Crosslinks . . . . . 8

2 THE EFFECT OF COLLAGEN FIBER ORIENTATION IN ACCELERATED WEAR TEST OF ARTICULAR CARTILAGE . . . . . 10

2.1 Introduction . . . . . 10

2.2 Methods . . . . . 11

2.3 Results . . . . . 18

2.4 Discussion . . . . . 21

3 EFFECT OF ANATOMIC LOCATION ON CARTILAGE ON WEAR RESISTANCE . . . . . 25

3.1 Introduction . . . . . 25

3.2 Methods . . . . . 26

3.3 Results . . . . . 28

3.3.1 Comparison of Wear Test Results . . . . . 28

Page

3.3.2 Comparison of Compositions in Four Locations . . . . . 28
3.4 Discussion . . . . . 31
4 ANALYSIS OF CARTILAGE COMPOSITION IN RELATION TO WEAR AND FRICTION . . . . . 33
4.1 Introduction . . . . . 33
4.2 Methods . . . . . 34
4.3 Results . . . . . 37
4.3.1 Best Fit Curve Between COF and Time . . . . . 37
4.3.2 Correlation Between Measures of Friction and Wear vs. Cartilage Composition . . . . . 37
4.3.3 Correlation Graphs Between Measures of Friction and Wear . . 39
4.4 Discussion . . . . . 39
5 CROSSLINKING OF CARTILAGE TISSUE TO IMPROVE RESISTANCE DUE TO WEAR . . . . . 44
5.1 Introduction . . . . . 44
5.2 Methods . . . . . 45
5.3 Results . . . . . 47
5.4 Discussion . . . . . 47
6 CONCLUSION AND FUTURE DIRECTIONS . . . . . 53
6.1 Conclusions . . . . . 53
6.2 Future Directions . . . . . 54
REFERENCES . . . . . 55
A Tribolab Wear Test Code . . . . . 62
B Matlab Code for Average COF Calculation . . . . . 67
C Papain Digestion . . . . . 68
C.1 Papain Buffer (PB, can be stored at RT) . . . . . 68
C.2 Activated Papain Solution (make fresh) . . . . . 68
C.3 Papain Digestion . . . . . 69
C.4 Hydroxyprolin Assembly . . . . . 69

Page

C.5 Hydroxyprolin Stock Solution . . . . . 70
C.6 Citrate Stock Buffer . . . . . 70
C.7 Assay Buffer (per 2 plates) . . . . . 70
C.8 Choloramin T Reagent (per plate): . . . . . 71
C.9 DMBA Reagent (per plate) . . . . . 71
C.10 Hydroxyproline Standards for hydrogels (per 5 plates) . . . . . 71
C.11 Assay Procedure . . . . . 73
D DMMB Assay for Cartilage Tissue . . . . . 74
D.1 Materials . . . . . 74
D.2 Protocol . . . . . 74
E Pico Green Assay . . . . . 77
E.1 Solutions . . . . . 77
E.2 Protocol . . . . . 78

# LIST OF TABLES

Table Page

C.1 Dilution of standard HYP working solution with PB as table below: . . . . 72

D.1 Dilution of standard DMMB working solution with PB as table below: . . 74

E.1 Dilution of standard DNA working solution with TE as table below: . . . . 77

# LIST OF FIGURES


Figure Page

1.1 Diagram of collagen fiber architecture in a sagittal cross section. This figure has been reproduced from ref [2]. . . . . 3

2.1 Picture of knee with marking the anatomic location of cartilage specimens. 12

2.2 Split line analysis shows orientation of fiber. . . . . 13

2.3 Specimen after complete processing and with marking of fiber direction. . . 14

2.4 Wear test of cartilage on plate in the solution bath. . . . . 16

2.5 Schematic diagram of experimental set up. . . . . 17

2.6 a) Split line of specimen or cartilage around the edge, b) cartilage after wear test, c) split line or fiber direction of cartilage in the surface, d) determining the angle of the split lines or fibers. . . . . 19

2.7 Average COF vs Time, a) transverse direction of fiber, b) longitudinal direction of fiber. . . . . 20

2.8 A) Initial COF measurement taken immediately upon onset of the testing, B) Equilibrium COF measured at the end 43hrs testing, C) HYP that was removed from the tissue during wear, D) GAGs that were removed from the tissue during wear. . . . . 22

3.1 Bovine knee with four locations identified. . . . . 26

3.2 A& C) Average amount of wear in terms of HYP & GAGs released in the solution bath for the four locations, B &D) Amount of wear for four locations samples for each donor in terms of HYP and GAGs released to the solution bath. . . . . 29

3.3 HYP content (A-B), GAG content (C-D), DNA content (F-G) and water content (E) in the full thickness (A, C, E, F) or superficial zone (B, C, G) of the cartilage in four specific locations. . . . . 30

4.1 Diagram of correlations among different wear and friction properties of cartilage and cartilage composition. . . . . 35

4.2 A schematic of correlation analysis. . . . . 36

Figure Page

4.3 Diagram of correlation among different friction and wear properties of cartilage. . . . . 37

4.4 Typical best fit curve for COF vs Time data. . . . . 38

4.5 Relationships between cartilage constituent concentrations (y-axis) and friction/wear properties (x-axis). Regression lines and corresponding $R^2$ values are shown where $p < 0.1$. [HYP= Hydroxyproline, GAGs= glycosaminoglycans, FT= Full thickness of the Cartilage, SZ= Superficial zone of the cartilage] . . . . . 40

4.6 Correlation graphs between different measures of friction and wear. Regression lines and corresponding $R^2$ values are shown where $p < 0.1$. [HYP= Hydroxyproline, GAGs= Glycosaminoglycans]. . . . . 41

4.7 Correlation between HYP content in the full thickness(FT) of the cartilage and HYP removed from the tissue during the wear test. . . . . 42

5.1 Laser treatment of the cartilage. . . . . 48

5.2 Cartilage after the wear test a) untreated, b) treated with Crosslinking protocol-1 b) treated with Crosslinking protocol-2. . . . . 49

5.3 A) Initial COF, B) Equilibrium COF, C) HYP due to wear of untreated and, two treatment groups (Crosslink protocol-1 & 2). *Indicates significant difference from untreated. . . . . 50

5.4 Indentation test result for untreated, crosslink protocol-1 & 2. Results are shown as max load after crosslinking divided by max load before crosslinking. [Wagner Lab]. *Indicates significant difference from untreated. . . . . 51

# SYMBOLS

$\tau$ time constant

# ABBREVIATIONS

| | |
|---|---|
| AC | Articular Cartilage |
| ECM | Extracellular Matrix |
| HYP | Hydroxyproline |
| GAGs | Glycosaminoglycans |
| OA | Osteoarthritis |
| PTOA | Post-traumatic Osteoarthritis |
| ACI | Autologous Chondrocytes Implantation |
| MACI | Matrix-induced Autologous Chondrocyte Implantation |
| MMPs | Matrix Metalloproteinase |
| PYD | Pyridinoline |
| COF | Coefficient Of Friction |
| DNA | Deoxyribonucleic Acid |
| FT | Full Thickness |
| SZ | Superficial Zone |

# NOMENCLATURE

| | |
|---|---|
| CASPc | Chloro-Aluminum Phthalocyanine Tetrasulfonic Acid |
| PBS | Phosphate-buffered Saline |

# ABSTRACT

Hossain, M. Jayed. M.S.M.E., Purdue University, August 2018. Factors Influencing Cartilage Wear in an Accelerated In Vitro Test: Collagen Fiber Orientation, Anatomic Location, Cartilage Composition, and Photo-Chemical Crosslinking. Major Professor: Diane Wagner.

Articular cartilage (AC) is a strong but flexible connective tissue that covers and protects the end of the long bones. Although cartilage has excellent friction and wear properties that allow smooth joint function during daily activities, these properties are not fully understood. Many material properties of cartilage are anisotropic and vary with anatomic location and the composition of the tissue, but whether this is also true for cartilage friction and wear has not been previously determined. Furthermore, cartilage disease and injury are major health concerns that affect millions of people, but there are few available treatments to prevent the progression of cartilage degeneration. Collagen crosslinking may be a potential treatment to reduce cartilage wear and slow or prevent the progression of cartilage disease. The objectives of this thesis were to investigate the relationships between the friction/wear characteristics of cartilage and the orientation of the preferred fiber direction, the anatomic location of the tissue, the composition of the tissue, and exogenous photochemical crosslinking.

In the superficial zone, AC has preferential fiber direction which leads to anisotropic material behavior. Therefore, we hypothesized that AC will show anisotropic behavior between longitudinal and transverse direction in an accelerated, in vitro wear test on bovine cartilage in terms of friction and wear. This hypothesis was proven by the quantification of glycosaminoglycans released from the tissue during the wear test, which showed that more glycosaminoglycans were released when the wear direction was transverse to the direction of the fibers. However, the hydroxyproline released

from the tissue during the wear test was not significantly different between the two directions, nor was the coefficient of friction.

The material properties of AC can also vary with anatomic location, perhaps due to differences in how the tissue is loaded in vivo. We hypothesized that cartilage from a higher load bearing site will give better wear resistance than cartilage from lower load bearing regions. However, no differences in friction or wear were observed between the different anatomic locations on the bovine femoral condyles. The concentration of collagen, glycosaminoglycans, cells and water in the tissue was also quantified, but no significant differences in tissue composition were found among the locations that were tested.

Although wear did not vary with anatomic location, variation in the wear measurements were relatively high. One potential source of variation is the composition of the cartilage. To determine whether cartilage composition influences friction and wear, a correlation analysis was conducted. An accelerated, in vitro wear test was conducted on cartilage from bovine femoral condyles, and the tissue adjacent to the wear test specimens was analyzed for collagen, glycosaminoglycan, cell, and water content. Because wear occurs on the cartilage surface, the superficial zone of the cartilage might play an important role in wear test. Therefore, composition of the adjacent cartilage was determined in both the superficial zone and the full thickness of the tissue. A significant negative correlation was found between wear and collagen content in the full thickness of the tissue, and between the initial coefficient of friction and the collagen content in the superficial zone. This correlation suggests that variation in the collagen content in the full thickness of the cartilage partially explains differences in amount of wear between specimens.

The wear resistance of cartilage can be improved with exogenous crosslinking agents, but the use of photochemical crosslinking to improve wear resistance is not well understood. Two photochemical crosslinking protocols were analyzed to improve the wear resistance of the cartilage by using chloro-aluminum phthalocyanine tetrasulfonic acid (CASPc) and 670nm laser light. The cartilage treated with the two crosslinking

protocols had lower wear than the non-treated group without changing the friction properties of the cartilage.

# 1. BACKGROUND

## 1.1 Cartilage Structure and Function

Cartilage is a strong but flexible connective tissue that covers and protects the ends of the long bones and is a structural component of ribs, nose, ears and many other body components. Depending on its structure and function, cartilage is classified as articular cartilage, fibrocartilage or elastic cartilage. Elastic cartilage is responsible for the shape of ear, trachea, etc. Articular cartilage (AC) is found in load bearing surfaces at the end of the bones. It is a resilient load-bearing material in diarthrodial joints, and provides joints with the excellent friction, lubrication and wear resistance characteristics during sliding motion. It also absorbs shock load and spreads the applied load to the supporting bone. Fibrocartilage is a strong and tough tissue found predominantly in intervertebral disks, menisci, and at the insertion site of ligaments and tendons. This is a type of fibrous tissue containing cartilage ground substance and chondrocytes.

Load bearing joints like the hip or knee consist of articular cartilage (AC), synovial fluid, and synovial membrane and are enclosed by a joint capsule [1]. Synovial fluid acts as a lubricant for the AC. The thickness of the AC of femoral condyles of the knee is on average 2.21mm in humans [1]. Normal AC is white, with a smooth surface, and no blood supply.

In normal AC, water content is 65-80% of its total wet weight [2]. The remaining weights are divided in two major classes of extracellular matrix (ECM) with chondrocytes distributed through the tissue. Chondrocytes generate the ECM, and these cells are responsible for the maintenance of the matrix. These cells are metabolically active and respond to variety of stimuli [3]. The major components of ECM are proteoglycans, collagens and water with other lower amount proteins and glycoproteins.

The AC matrix contains different types of collagen like type II, VI, IX, and XI, but type II collagen is 90-95% of all collagens [2]. The main function of collagen is to provide tensile properties of the tissue and to immobilize the proteoglycan within the ECM.

The AC is divided into four zones: superficial zone (12-20% of total thickness), middle zone (40-60%), deep zone (30%) and calcified zone (Fig:1.1) [2]. In the superficial zone the collagen fibers are oriented parallel to the joint surface, in the middle zone the collagens are randomly oriented, in the deep zone the collagen fibers are perpendicular to the joint surfaces, and the calcified zone is the boundary between cartilage and subchondral bone [4]. The superficial zone is responsible for withstanding shear force during sliding motion, the middle zone is responsible for carrying compressive loads and the deep zone also provides resistance to compressive forces [5]. The superficial zone is rich in collagen and relatively poor in proteoglycans. In the middle zone, concentration of proteoglycan increases and collagen content decreases [2, 4].

Collagen fibers are thin (10-100nm) and crosslink to other fibers to create a continuous a collagen network [2]. Collagen fibril structure is a triple helix composed of three polypeptide chains. The amino acid composition of these chains includes large quantities of glycine and proline. Due to the proline content, the individual chains exhibit characteristics of left handed helical configuration and the assembled triple helix winds around a common axis in a right handed configuration; this unique structure resist tensile forces. Amino acids hydroxyproline (HYP), hydroxylysine, and glycosylated hydroxyproline are also present [2]. Hydroxyproline is responsible for the stability of the collagen helix at body temperature due to the formation of intramolecular hydrogen bonds along the length of the molecule. Hydroxylysine is involved in the covalent crosslink formation which mainly stabilize collagen fibril assemble.

Proteoglycans are complex macromolecules and consist of a protein core linked with extended polysaccharides. Proteoglycans in cartilage are 80-90% large, aggregating type known as aggrecan. The aggrecan monomer consist of a large extended

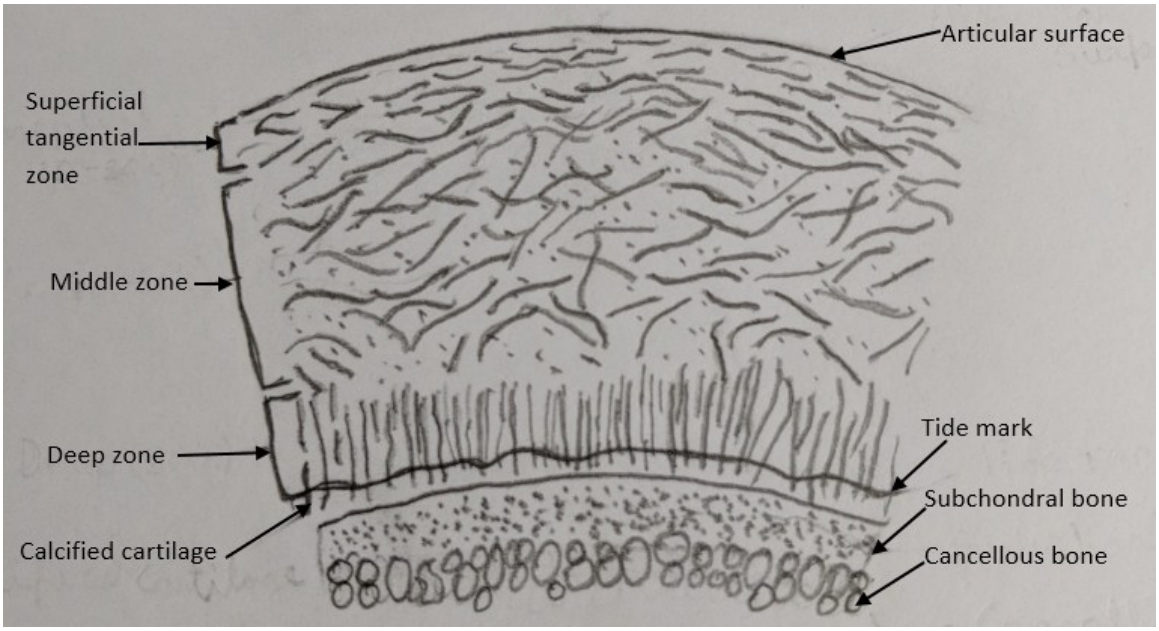


Fig. 1.1. Diagram of collagen fiber architecture in a sagittal cross section. This figure has been reproduced from ref [2].

protein core with around 100 chondroitin sulfate and 50 keratan sulfate glycosaminoglycans (GAGs) chains. Many aggrecan monomers (up to 200) can bind to a single chain of hyaluronate to create a proteoglycan aggregate. Because of their size, aggrecan molecules are thought to become immobilized within the collagenous network of cartilage. They are not homogenously distributed in cartilage. The superficial zone is rich in collagen and poor in proteoglycans; the concentration of proteoglycans increases in the middle zone. The distribution and concentration of GAGs in cartilage will change with age and diseases. GAGs have negative charges which attracts water and positive ions through physiochemical mechanisms such as Donnan osmotic pressure caused by the free positive ion like $Ca^{+2}$ and $Na^{+}$ and through electrostatic repulsive force developed between the fixed negative charges. In the AC, the amount of hydration is determined by the balance of total swelling pressure exerted by the GAGs and the constraining forces developed within the collagen network surrounding the trapped proteoglycans. When water interacts with these macromolecules, a cohesive and strong gel is formed that allows the tissue to hold water. Water flows through the tissue and across the articular surfaces to promote nutrient transportation and lubrication of the joint.

## 1.2 Biphasic Nature of Cartilage

Articular cartilage in diarthrodial joints is subjected to high static, cyclic and repetitive loads. Therefore, the structure of the molecules like collagen, proteoglycan etc. are organized into a strong, fatigue-resistant and tough tissue capable of absorbing these loads. The solid matrix is permeable, porous and very soft. Water passes through the porous-permeable solid matrix when a pressure gradient is applied. The behavior of cartilage is naturally biphasic as it is composed of both water and solid matrix [2, 6]. An applied load to the cartilage surface is initially carried primarily by interstitial fluid pressure, the interstitial fluid begins to flow and exudation occurs due to the load. After the external load is removed, proteoglycans attract water and

pull the fluid back into the cartilage, like a sponge soaking up water, in preparation for the next load.

Articular cartilage responds like a viscoelastic material during loading and deformation [2, 6]. During a constant compressive load, deformation increases with time, creeping until an equilibrium strain is reached. Similarly, when a constant strain is applied, stress relaxation occurs [2, 6]. Compressive loads generate interstitial fluid pressure; as creep continues under a constant load, the fluid phase load support gradually transfers to the solid phase. In normal cartilage, the equilibrium process takes 2.78 to 5.56 hrs [2]. In equilibrium, maximum load is supported by the collagen-proteoglycan solid matrix. Because of this long equilibrium time and always-dynamic loading condition, cartilage rarely comes to equilibrium under physiologic conditions. The solid matrix of cartilage is very soft with very low permeability ($10^{-15}$ to $10^{-16}$ $m^4/Ns$) which is responsible for this unique behavior [2].

### 1.3 Cartilage Diseases and Treatments

Osteoarthritis(OA) is the most common orthopedic disease; approximately 27 million adults in the United States have clinical OA (up from the estimate of 21 million for 1995) [7]. It is a slowly progressive disorder of unknown etiology. Although OA is commonly associated with aging, younger people who sustain joint injury are also susceptible to this disease. Mammalian AC has the ability to respond to functional demands, can withstand single or multiple, moderate or high impact loads. However, repetitive or excessing load can cause damage to the cartilage. Although injury normally causes a localized or focal cartilage defect, this can lead to OA if left untreated. This progression to a debilitating joint disease from an overload injury is called post-traumatic osteoarthritis (PTOA) and is responsible for 12% of all OA cases [8, 9]. In the progression of PTOA, cartilage becomes weakened due to both the mechanical and biological responses and makes the tissue more susceptible to wear and degeneration. When the impact overload or excessive repetitive load occurs, the

chondrocytes die, the matrix is damaged, the collagen fiber network breaks down, fissures are created at the articular surface, and the underlying bone is injured [10]. As a result, changes in the composition and micro structure of cartilage could initiate the progression towards OA.

Biochemical degradation is also responsible for cartilage diseases. It is promoted by different cytokines, like as IL1 (interleukin-1) and TNF (tumor necrosis factor-). The synthesis of the structural macromolecules is hindered by the cytokines activation through the emission of proteinases from the chondrocytes [11]. Cartilage regeneration and biochemical degeneration occurs simultaneously. With aging, the regeneration rate becomes lower than the degeneration rate and OA develops.

The most common treatment for severe OA, including PTOA, is to replace the joint with an artificial implant; around $9 billion per year have been spent for hip or knee replacement in the US alone [12]. Different techniques have been developed to repair the cartilage tissue instead of replacing the entire joint, such as osteochondral allograft and autograft transplantation, microfracture, ACI (Autologous Chondrocytes implantation), MACI (Matrix-induced Autologous Chondrocyte Implantation), MMP (matrix metalloproteinase) inhibitors and tissue engineering [13]. Most of these treatments have advantages and limitations [14]. Autograft involves the transfer of AC from a healthy region to the defect to rapidly restore load bearing capacity and cartilage structure. The limitation of this procedure is the lack of healthy cartilage in donor sites and donor site morbidity. Allografts are similar to autografts, but the donor tissue is from a different individual than the host. The limitation of this process is insufficient integration between donor and host cartilage, and more significantly the immune response [13]. ACI is the injection of chondrocytes in suspension under a periosteal flap; this is encouraging for smaller defects and low load bearing sites. This treatment uses adult human chondrocytes, perhaps from OA cartilage, which possess a restricted capacity to form healthy tissue. MACI is similar to ACI but uses a scaffold which is cut to the defect shape and seeded with autologous chondrocytes and secured in the defect using a fibrin glue [15]. Chronic inflammation

occurs in OA, which is associated with a feedback cycle that augments degenerative pathways that activate MMPs. Using MMP inhibitors after the injury can reduce the degenerative process [13]. In different tissue engineering strategies, cells are used in combination with scaffolds to generate a cartilage plug in vitro for implantation in the joint [16]. Finally, biological based experimental treatments such as interleukin antagonist may be isolated from each patients blood and delivered locally into the joint to treat OA [17]. One limitation of these biological treatments is that they are not effective in repairing mechanical damage or preventing the damage from progressing to OA. Therefore, researchers are investigating exogenous crosslinking treatments which increase the mechanical properties of the cartilage tissue.

## 1.4 Cartilage Friction and Wear

Synovial fluid inside the synovial membrane performs three main functions. First, it carries nutrients to the cartilage, second, it removes the waste products and finally, it provides lubrication in the AC joints [18]. During walking or running, high loads occur for a shorter period, while low loads occur for a longer period [19]. These performance characteristics require an efficient lubrication process. The properties of AC and lubrication by synovial fluid serve together to attain this lubrication process. The dynamic friction coefficient was found to be 0.002 to 0.02 in diarthrodial joints of humans with lubricants [18, 19]. Many in vitro frictional analyses have been done; some of them use cartilage on cartilage, cartilage on glass, or cartilage on steel [20]. As a lubricant, some studies have used synovial fluid or phosphate buffered saline (PBS) [21, 22]. When a load is applied, at first it is carried by the fluid inside the cartilage via hydrostatic pressure. In this case, fluid pressure provides significant component of load support and minimizes stress acting on the solid matrix. With time, the amount of fluid will reduce in the AC until an equilibrium condition is reached. At that time, the load carried by the solid matrix will be at its maximum. Fluid is not responsible for frictional forces. So, during loading the fluid moves out

from the cartilage, load is transferred to solid matrix, and consequently the friction increases. In the equilibrium condition, frictional force will be maximum. As a result wear takes place.

Wear is the material removal from the contact surfaces. Once wear starts, the collagen fibers may break and proteoglycans may be released from the cartilage tissue. The amount of wear has been measured by different biochemical methods, physical measurements and microscopy [22]. MRI or magnetic resonance imaging has been used to measure the wear of cartilage [23]. Biochemical markers can also measure the presence of cartilage degradation products in the joint fluid [24–26]. The amount of collagen that is worn from the tissue can be determined through a hydroxyproline (HYP) assay [27], because HYP is an amino acid constituent that is found almost exclusively in collagen; the amount of HYP wear is proportional to the amount of collagen wear. Glycosaminoglycans (GAGs) are a constituent of proteoglycans. So, by quantifying the GAGs that are released in the solution bath during the wear test, wear also can be measured as the amount of this component of the extracellular matrix that has been removed from the tissue.

### 1.5 Cartilage Crosslinks

Cartilage crosslinks are covalent bonds between individual collagen molecules [28]. The integrity and tensile properties of AC are attributed to this collagen network and the strength of the bond. Two types of crosslinks are native and exogenous crosslinks. Native crosslinking is the process where crosslink occurs without external treatment; this is a biochemical process. Pyridinoline (PYD) is an example of a native crosslink that increases in concentration from fetal to adult age [29].

Exogenous crosslinking can be used to increase the strength of collagen network [30–32]. One type of crosslinking was formed from advance glycation end products (AGEs) and resulted in increased tissue stiffness and decreased cartilage degeneration by matrix metalloproteinases (MMP) [27,33]. Glutaraldehyde and formaldehyde

fixation via crosslinking has also been shown to increase the wear resistance of cartilage in vitro [30–32]. Gluteraldehyde fixation of healthy bovine cartilage increased the stiffness by 28% and decreased the wear by 20% [30]. From another study, it was found that the equilibrium compressive modulus can be increased approximately 50% to 120% in native cartilage by using genipin [34].

Chemical crosslinking cannot be applied to a localized position. For this crosslinking, full AC specimens such as knee joints have been submerged in the crosslinking solution; it is difficult to soak a particular area only. For that reason, another crosslinking method is being tested, which is known as photo-initiated crosslinking. In this process, both a photo-reactive chemical and light are used. Because the application of light can be delivered to a particular location, just the impacted site can be crosslinked. Photo-initiated crosslinking can occur through two processes or reactions. In a Type-I process, electron/hydrogen transfer directly from the substrate to oxidate the protein side chains. In aType-II process, singlet oxygen is formed by the photo oxidation of the substrate, and protein oxidation is a secondary event [35].

Cartilage crosslinking can be used to adhere implanted cartilage to the surrounding host tissue. This integration is required for nutrient transport, molecular deposition to enhance initial integration, and to transmit stress between the tissues [35].

# 2. THE EFFECT OF COLLAGEN FIBER ORIENTATION IN ACCELERATED WEAR TEST OF ARTICULAR CARTILAGE

## 2.1 Introduction

Cartilage is an anisotropic material with a continuous fiber network and matrix [36]. Collagen fibers are similar to the fiber in a composite material, and the proteoglycans and other materials are similar to the matrix . In the superficial zone, the fiber is parallel to the surfaces, and in the middle zone fibers are in random directions and in the deep zone fibers are perpendicular to the surface (Fig:1.1). In the superficial zone, fibers have a preferential direction [4], which can be found through a split line analysis. In a split line analysis, the AC surface is pierced by a pin and using India ink, a split line indicating the direction of the fibers at that location is revealed.

This preferential direction leads to anisotropic properties related to stiffness and strength characteristics of the tissue [4]. In the superficial zone, cartilage has higher tensile strength, and stiffness in the direction parallel (longitudinal) to the split line than perpendicular (transverse) to the split line [37–39]. However, it has not been determined yet whether the AC is anisotropic or isotropic in friction or wear. We hypothesized that cartilage will wear less in the direction parallel to the preferential fiber orientation or split line direction than the transverse direction in an accelerated wear test. We also hypothesized that the coefficient of friction (COF) could also depend on the relative orientation of the fibers with respect to the direction of motion.

## 2.2 Methods

Bovine stifles were collected from a local abattoir (The Old Farm, Colfax IN) and stored at $-20^0$C before further use. Osteochondral specimen with 9.52mm diameter and length of 18mm were collected from the femoral condyles such that articular surface was perpendicular to the coring axis. Depending on the size of the stifles, 3-5 specimens were collected from each condyle. Locations of the sample harvest sites were tracked by using India ink and taking proper photos (Fig:2.1). A band saw (Skymsen, Manufacturer: Simens LTDA) was used for cutting the stifles. PBS (Phosphate-buffered Saline) solution was applied frequently from the time the knee was opened to the completion of the process, to keep the cartilage hydrated. A drill press with 11 mm outer diameter drill bit was used to create a cylindrical specimen. During drilling water was supplied continuously as a coolant. Finally, the specimens were collected by cutting again with the band saw. Specimens were cut to a final length of 18mm with a low speed saw (Isomet, Behler, USA). Finally, all specimens were stored at $-20^0$C until next use.

Fiber orientation was determined by using a pin and India ink. The edge of the cartilage was pierced in 14-16 locations and India ink was applied to the surface of the specimens. After a few seconds the India ink fills the space in the cartilage created by the pin. The remaining India ink was removed with dry gauze and the fiber line was somewhat visible to the naked eye. A stereomicroscope (Stemi 508, Zeiss) equipped with a digital camera was used to detect the fiber orientation (Fig: 2.2). Maximum aligned fibers direction was taken as direction of fiber. This fiber direction was marked on the bone part with a carbon pencil (Fig: 2.3). Then the cartilage was returned to PBS solution for rehydration before the experiment.

A pin on plate wear test was carried out by using Brukar Tribolab machine (Brukar UMT Tribolab)(Appendix A). Here the osteochondral specimen was used as a pin and a stainless-steel sheet as a plate (T316, 8# mirror finish) (Fig: 2.4 & 2.5). A hydrating solution of PBS with protease inhibitors (1mM ethylenediaminetetraacetic acid, 5mM

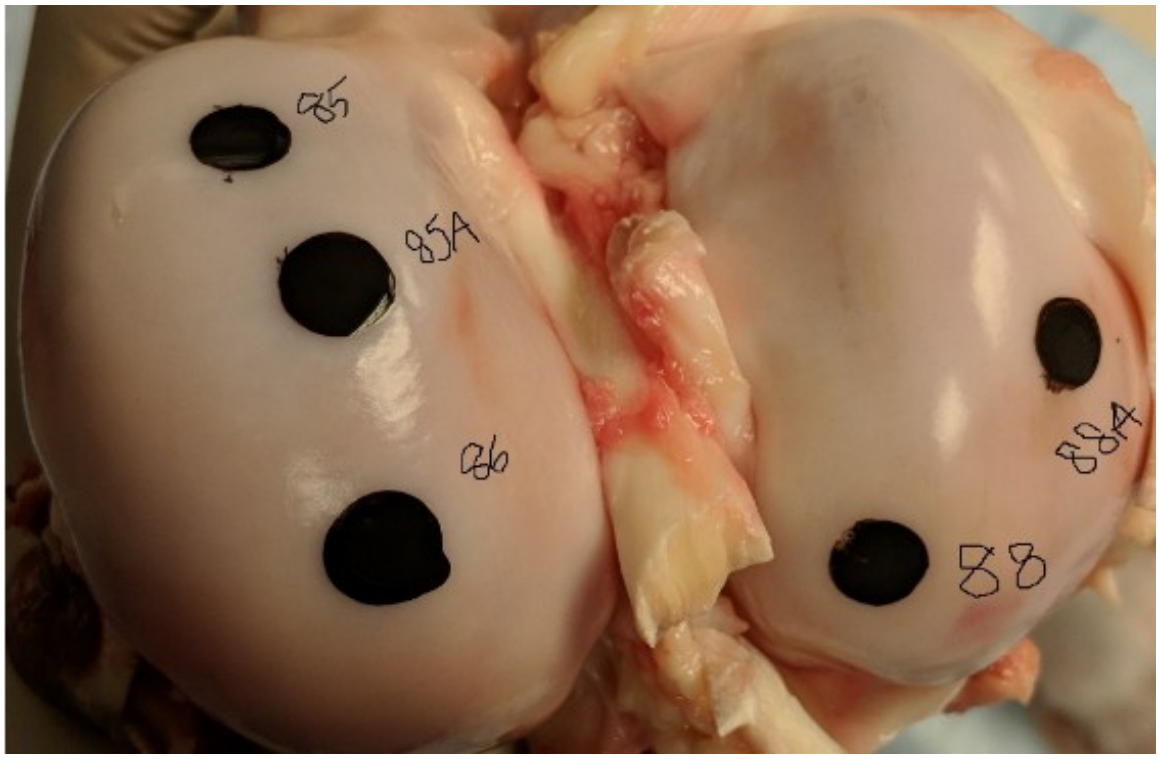


Fig. 2.1. Picture of knee with marking the anatomic location of cartilage specimens.

Fig. 2.2. Split line analysis shows orientation of fiber.

Fig. 2.3. Specimen after complete processing and with marking of fiber direction.

benzamadine and 10mM n-ethylmaleimide) was used as a solution such that the plate and cartilage were submerged in the solution (Fig: 2.4 & 2.5). An average of 22.5ml solution was used in each experiment. A 160N constant load was applied for 14000 cycles of reciprocal sliding motion with 4mm/s velocity and a travel distance of 18mm in each direction; these parameters were determined in preliminary tests and created visible cartilage wear in the specimens. Each wear test took 43hrs and the total wear distance was around 504m. The total contact pressure was 2.21MPa. A total 24 specimens were used for this analysis; 12 specimens were tested in the longitudinal direction or parallel to the fiber direction and 12 specimens were tested in the perpendicular direction or transverse direction to the fiber.

To quantify the angle between the fiber orientation and wear direction after the wear test was complete, the split line analysis was conducted again on the full cartilages surface. Another picture was taken with the microscope equipped with a digital camera, and average fiber angle was determined using Image J software (NIH, USA) (Fig: 2.6). Between each experiment, the steel plate and other equipment were cleaned thoroughly with water and dried with a paper towel and soft tissue. The steel plate was changed when the surface was a little rough. An average of 9-10 experiments were carried out on each steel plate in three consecutive locations.

Friction and normal force ratio was taken to compute COF over the 43hrs duration of the experiment. During analysis, COF data for acceleration and deceleration was discarded, and only the COF data for 4mm/s velocity was considered. COF data were averaged for each reciprocating cycle (both directions) using Matlab Software (Mathworks, USA)(Appendix B). After taking the average COF data for complete experiment, the first data point was considered the initial COF and because the COF reached equilibrium after around 4hrs, the average COF of the last 39hrs was taken as equilibrium COF.

To quantify the amount of wear of the cartilage, the solution bath was collected after the wear test and stored at $-20^0$C before further analysis. Biochemical assays were used to measure the amount of hydroxyproline (HYP) and glycosaminoglycans

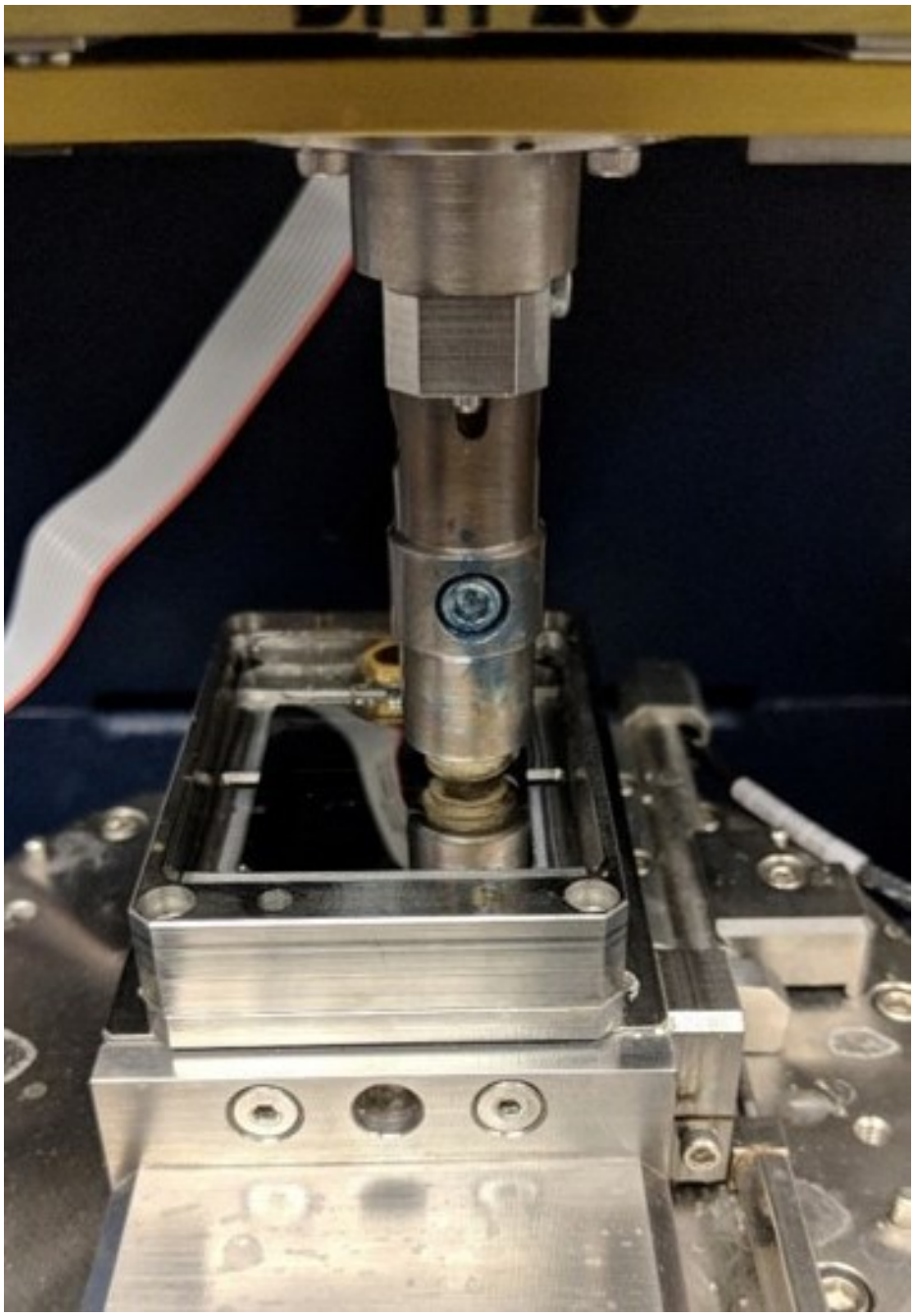

Fig. 2.4. Wear test of cartilage on plate in the solution bath.

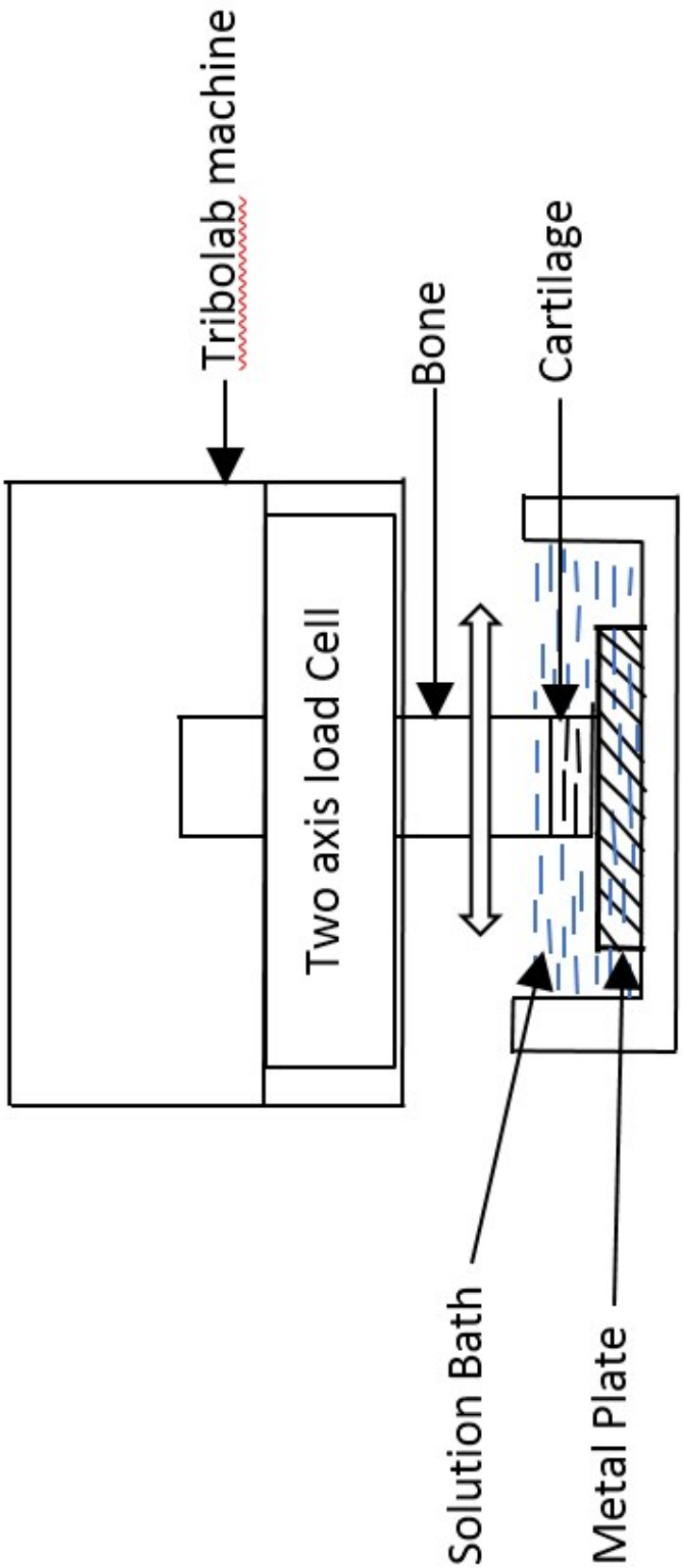


Fig. 2.5. Schematic diagram of experimental set up.

(GAGs) released to the solution bath. HYP is an amino acid constituent and is found almost exclusively in collagen [2].

Samples were freeze-dried (Labconco, USA) at $-48^0$C for 48hrs, collected in 1ml activated papain digestion, and aliquots were hydrolyzed in concentrated HCl. To quantify the amount of HYP, hydrolyzed specimens were assessed with a chloramine-T assay [Appendix C] . To quantify the amount of GAGs, papain digested solutions were assessed with DMMB assay [Appendix D]. Samples were diluted 10:90 ratio with papain buffer to create 100$\mu$l sample.

Friction and wear were analyzed in the following four groups. 1) All longitudinal and transverse samples ($n = 12$). 2) Only cartilage specimens that were collected from medial condyles ($n = 6$). 3) Only cartilage specimens that were collected from lateral condyles ($n = 5\ to\ 6$). 4) Only those in which both longitudinal and transverse cartilage specimens were harvested from the same knee ($n = 7\ to\ 8$). For each group, initial COF, equilibrium COF and amount of wear of the cartilage or HYP and GAGs released in the solution bath were reported. Data was analyzed with a Students t-test, and group 4 was analyzed with a paired t-test. Significance was set at $p < 0.05$.

## 2.3 Results

The average angle between directions of fiber and of wear for longitudinal specimens was 16.1±7.5 and the average angle for the transverse specimens was 69.7±11.4. Figure 2.6 shows the split line directions before and after the wear test.

Initially the COF was low, and with increasing time, the COF increased (Fig:2.7 a & b). After approximately 4hrs it became almost constant for the remaining 39hrs. For the longitudinal case, the average COF increased from an initial value of 0.004± 0.001 to an equilibrium value of 0.265±0.032, and for transverse case the COF rose from 0.00345±0.000388 to 0.248±0.0324. From the statistical analysis, no significant differences were found between longitudinal and transverse direction for all four groups for both the initial and equilibrium COF (Fig: 2.8A & B).

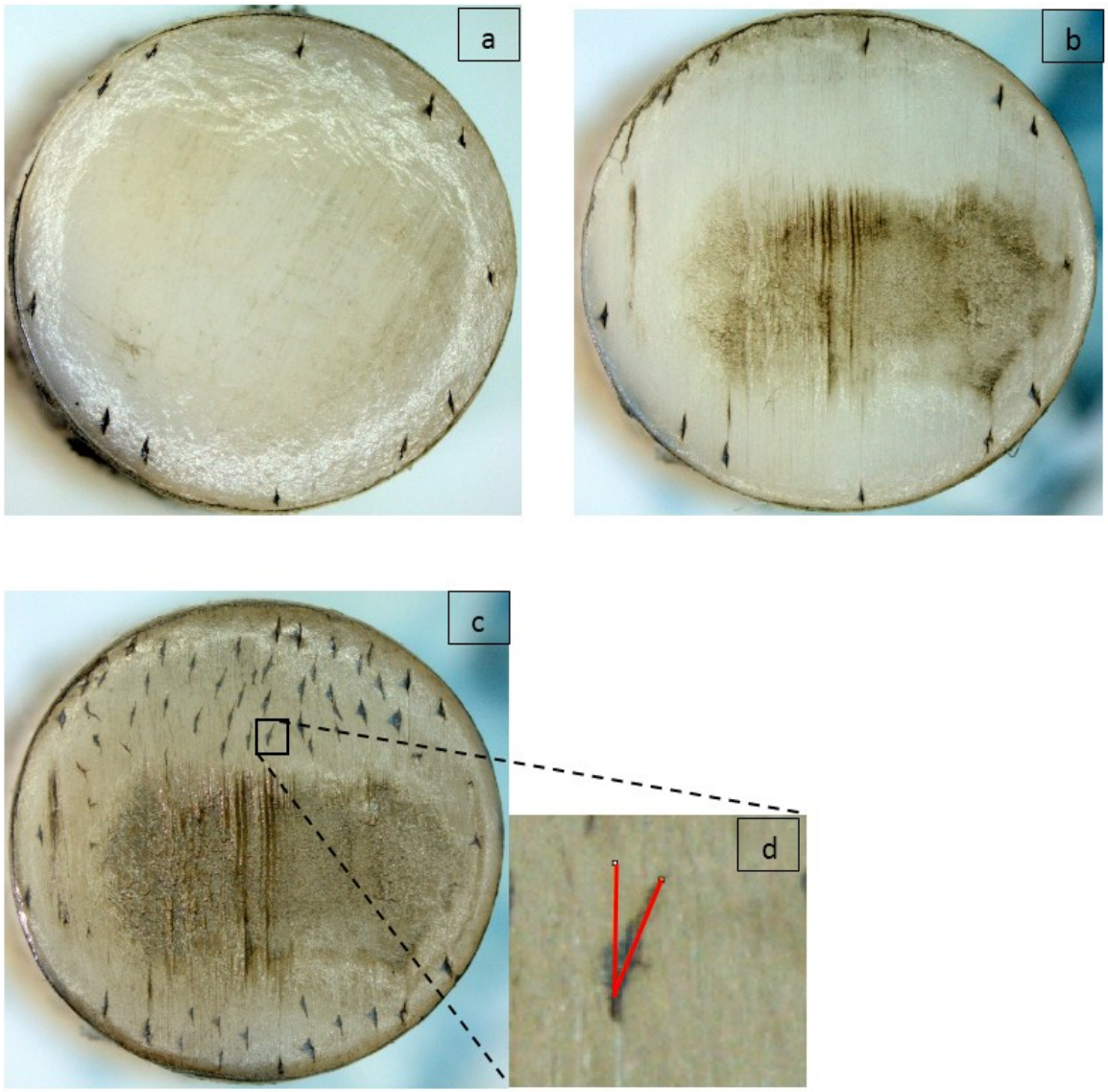


Fig. 2.6. a) Split line of specimen or cartilage around the edge, b) cartilage after wear test, c) split line or fiber direction of cartilage in the surface, d) determining the angle of the split lines or fibers.

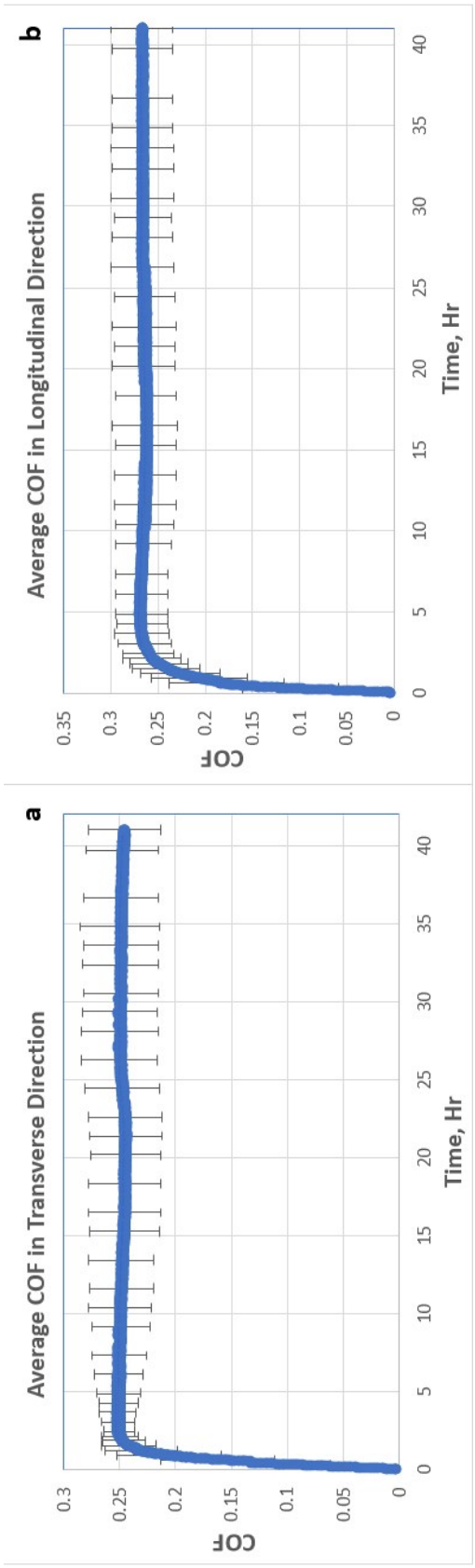


Fig. 2.7. Average COF vs Time, a) transverse direction of fiber, b) longitudinal direction of fiber.

The amount of HYP released to the solution bath during the wear test did not show any significant difference between the longitudinal and transverse direction of wear of the cartilage for any of the four groups (Fig: 2.8C).

A statistically significant differences between the longitudinal and transverse direction of GAGs released in the solution bath during the wear test was seen. The differences were significant in the cases where all samples were considered, when just the medial side was considered and when specimens from the same knees were considered. There was no significant different between longitudinal and transverse GAG wear when specimens from just the lateral side were analyzed (Fig: 2.8D).

## 2.4 Discussion

Based on the amount of GAGs released in the solution bath, our hypothesis regarding the anisotropic nature of the cartilage tissue was supported by the accelerated wear test. Though the specimens from the lateral condyles did not show a significant difference, the results from the other three groups have supported our hypothesis. We also hypothesized that the COF will be different between the longitudinal and transverse direction, but no significant difference was found between the two directions of the cartilage in the cases of initial and equilibrium COF. Results from the accelerated wear test are consistent with reports of cartilage anisotropy in a tension test [40].

When no significant differences were seen in the HYP wear, it was assumed that there might be a difference between the AC collected from the lateral side and the medial side. Therefore, AC collected from the medial and later side were analyzed separately. Moreover, different donor ages, origins or health conditions might cause different AC structure. Therefore, the wear test for the same knee was analyzed, where both samples were harvested from the same donor. However, none of these analyses have shown difference in HYP wear in the longitudinal or transverse directions. While the amount of GAGs released to the hydrating bath due to wear was different between the longitudinal and transverse specimens, differences in the HYP wear were not

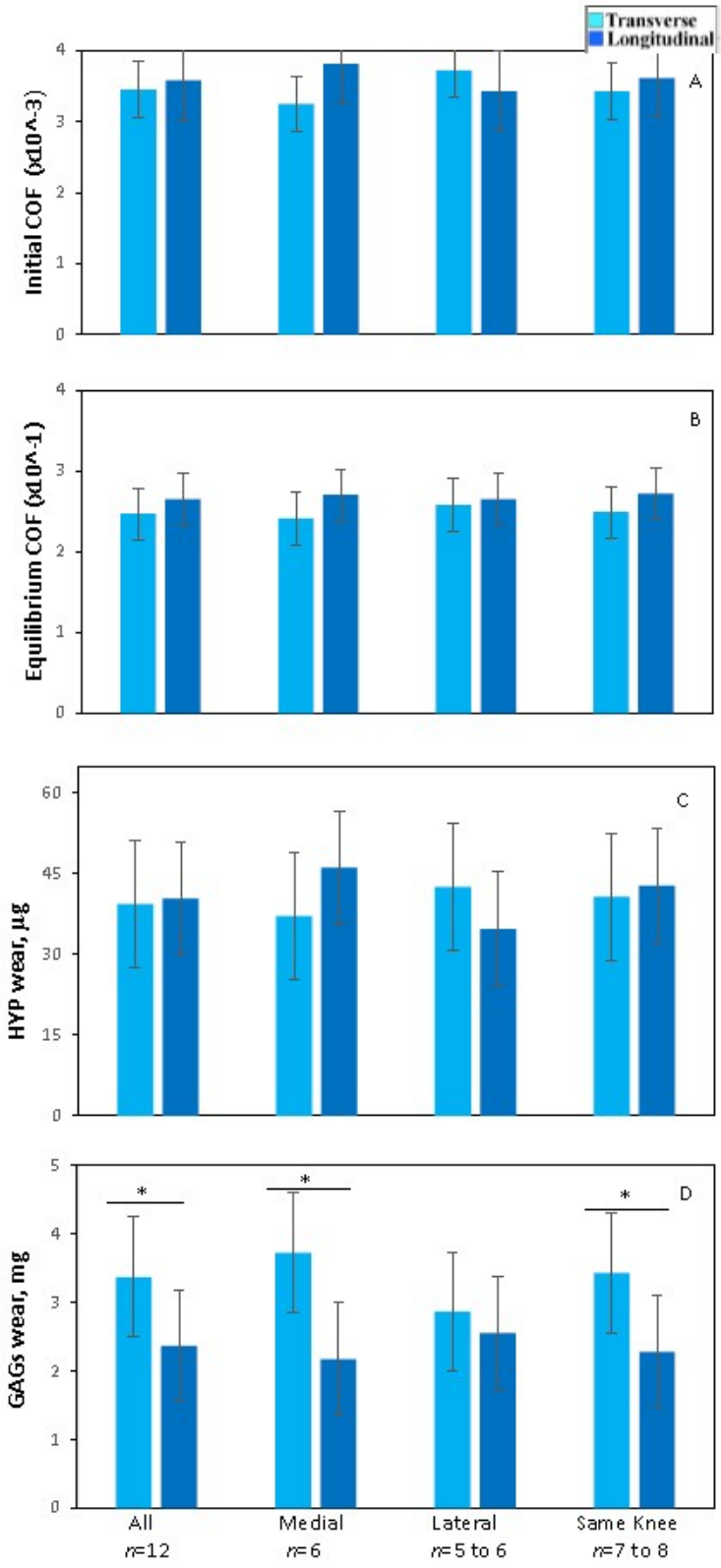


Fig. 2.8. A) Initial COF measurement taken immediately upon onset of the testing, B) Equilibrium COF measured at the end 43hrs testing, C) HYP that was removed from the tissue during wear, D) GAGs that were removed from the tissue during wear.

detected. It is possible that the collagen network was more damaged in transverse wear than longitudinal wear but was not released to the solution bath completely. Damaged collagen could stay attached to the cartilage surface, while the GAGs release to the solution bath. In the future, differences in collagen fiber damage in longitudinal and transverse wear specimens may be detected by using collagen hybridizing peptide [41].

The average fiber angle for longitudinal and transverse direction was not exactly $0^0$ or $90^0$. The specimens were aligned using the fiber angles that were determined at the edges, but after the wear test more data on the fiber angles was collected across the cartilage surface. Because the fiber angle varies across the surface, the preferential direction that was determined after the wear test was not the same as the one that determined before the test started and was used to align the test specimen. This lead to a deviation in the angle between wear and fiber lines from $0^0$ or $90^0$ (Fig:2.6C). Besides that, the specimens were aligned in the wear test manually, which lead to some experimental positioning errors. In spite of this, the angle was close to $0^0$ or $90^0$ with an average deviation within $20^0$.

Initially the COF increased rapidly, but after time it became almost constant. During the wear test, the load is initially carried by the fluid pressure. With increasing time, the fluid leaves the AC, the load transfers from the fluid to the solid matrix and the COF increases. In the equilibrium condition, fluid flow stops and the solid matrix carries its maximum load. The fluid does not create frictional forces, so initially the frictional force is low because the load is mainly carried by the fluid. By the time, the COF increases as the load is transferred from the fluid to the solid and is a maximum at equilibrium. This COF behavior was seen by many other groups and our result is consistent with their reports [20, 42–44].

Significant differences in GAGs released to the hydrating bath during the wear test may be helpful in improving grafting treatments. The results suggest that the orientation of full-thickness tissue grafts will affect wear, and if oriented such that the fibers are transverse to joint articulation they will wear more. Surgeons may need to

pay attention to the fiber orientation of the grafts. This result is also important for the biomechanical engineers that are performing an in vitro wear test. They should be careful about the orientation of the cartilage to conduct a consistent wear analysis.

# 3. EFFECT OF ANATOMIC LOCATION ON CARTILAGE ON WEAR RESISTANCE

## 3.1 Introduction

From different studies, it has been found that when people exercise regularly their cartilage becomes stronger than the cartilage of peoples who do not. Regular exercise and walking will increase the proteoglycan content and compressive stiffness, decrease the rate of fluid flux during loading and reduce the probability of OA [6, 45, 46]. This may be due to the increased mechanical load to the cartilage, as mechanical loading enhances cartilage matrix production. Generally, the medial side of the knee joint carries more of the body mass than the lateral side [47]. The knee joint AC doesnt come in contact uniformly during movement. Due to knee anatomy, the middle portion of the knee takes the maximum load or contact pressure during movement, whereas other areas take much less. We have hypothesized that the cartilage in the middle of the medial condyle or load bearing zone takes maximum pressure during movement and as a result, the AC in that zone is stronger than the other zones and will have better wear resistance. So, the cartilage from the condyles in the bovine knee were divided in four zones, 1) distal from middle of medial, 2) middle of medial, 3) middle in lateral, and 4) distal from middle of lateral. In this study, an accelerated wear test of cartilage was carried out on specimens from those four zones and the amount of wear was measured. The amount of hydroxyproline (HYP), glycosaminoglycan (GAGs), DNA (cellular content) and water content of adjacent cartilage samples were quantified to compare the composition of the cartilage in those four locations. This study would be beneficial to medical science if it could show which anatomic locations were the best sources of tissue for transplants.

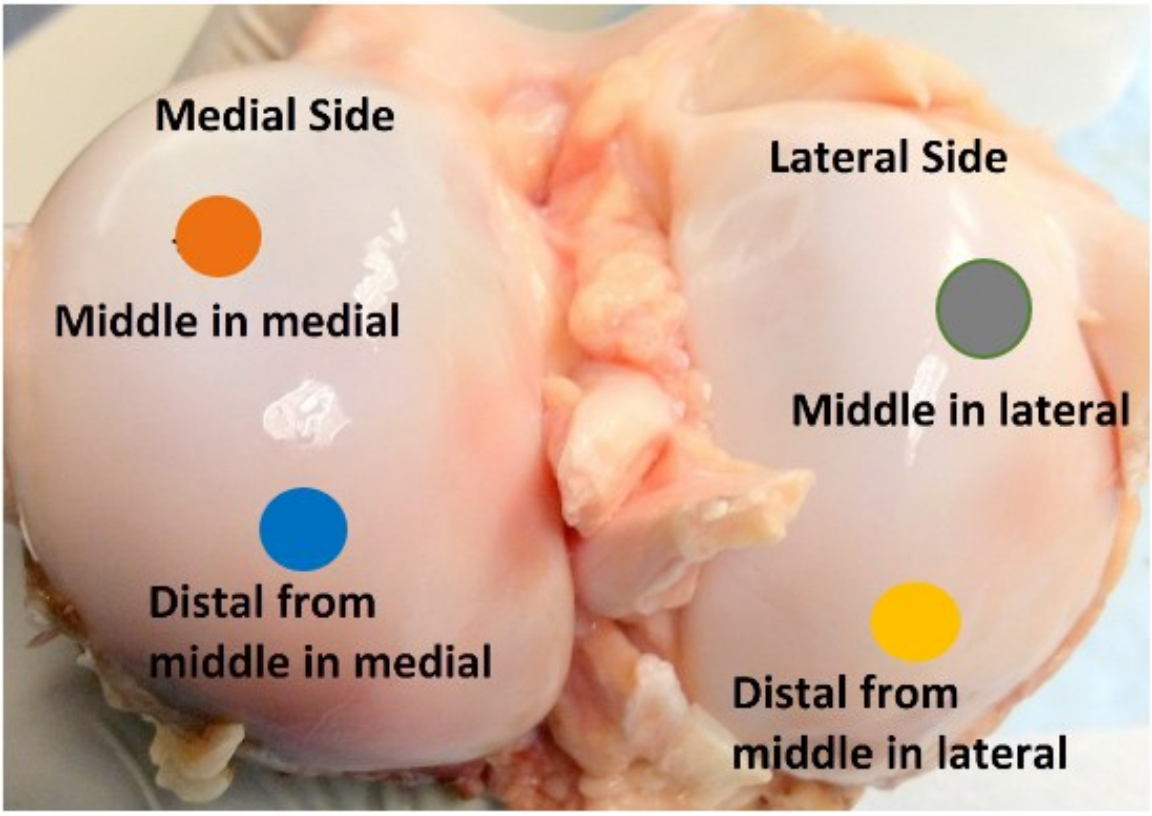


Fig. 3.1. Bovine knee with four locations identified.

## 3.2 Methods

Using the same technique as described in Chapter 2, 28-osteochondral cylindrical specimens were collected from seven knees, with four specimens from each knee (Fig: 3.1). To maintain consistency, the split line on the edge of the cartilage specimens was found in 14-16 different locations and the fiber direction was determined. An accelerated, in vitro, reciprocal wear test was carried out for all the samples using the same protocol as described in chapter 2 (14000 cycles, 160N load, 4mm/s velocity, 18mm travel distance in each direction) with the direction of travel oriented in the direction longitudinal to the preferred fiber orientation.

To quantify the amount of wear of the cartilage, the solution bath was collected after the wear test and stored at $-20^0$C before further analysis. Chloramine-T and DMMB assays were used to measure the amount of HYP and GAG content in the solution bath, respectively [Appendix C & Appendix D].

Cartilage that had been adjacent to the wear test specimens were collected from three of the knees or 12 of the specimens. At each site, three to four full thickness cartilage samples were collected using a surgical blade and stored at $-20^0$C before further analysis.

In each location, adjacent cartilage samples were divided in two groups, full thickness cartilage and superficial zone cartilage. For the superficial zone, 250 $\mu$m thickness of the cartilage was taken off from the articular surface using a sledge microtome (Microm GmbH HM430, Germany) of one to two cartilage samples from each location and the remaining one to two cartilage samples were used in full thickness.

The mass of the samples were taken before and after freeze-drying. HYP (collagen) content was assessed through a Chloromine-T analysis [Appendix C], GAG content was quantified through a DMMB analysis [Appendix D], and cellular content was evaluated by quantifying the amount of DNA through the PicoGreen kit (Appendix E; Invitrogen, CA, USA) following the manufacturers protocol.

For HYP analysis in full thickness and superficial zone, 10$\mu$l and 50$\mu$l papain digested solution, respectively, were used. This was less than the normal protocol (Appendix C) because the amount of HYP in full thickness or superficial zone was otherwise beyond the assay limits. For GAGs analysis in full thickness and superficial zone, 2.5$\mu$l and 5$\mu$l papain digested solution or stock solution, respectively, were used, and diluted to a final volume of 100$\mu$l.

For measuring water content as a percentage the following formula was used (3.1),

$$\%Water\ content = \frac{Wet\ cartilage\ weight - Dry\ cartilage\ weight}{Wet\ cartilage\ weight} \times 100\% \quad (3.1)$$

Total HYP, GAGs, and DNA content were reported normalized to wet weight. The

following comparison among the four locations were analyzed: a) HYP released to the solution bath during wear test, $n$= 7 per location, b) GAGs released to the solution bath during wear test, $n$= 7 per location, c) HYP normalized to wet weight in the full thickness and superficial zone of the cartilages, $n$= 3 per location, d) GAGs normalized to wet weight in the full thickness and superficial zone of the cartilages, $n$= 3 per location, e) DNA normalized to wet weight in the full thickness and superficial zone of the cartilages, $n$= 3 per location, f) % water content in the full thickness of the cartilage, $n$= 3 per location. One-way ANOVA with repeated-measures with Tukeys post-hoc analysis was performed with GraphPad Prism software.

## 3.3 Results

### 3.3.1 Comparison of Wear Test Results

Quantification of HYP and GAGs released to the solution bath during the wear test have not shown any significant difference among the four locations (Fig: 3.2A & C). When looking at the HYP and GAGs released in the solution for each individual donor, no specific pattern is evident (Fig: 3.2B& D). It was expected that average wear might lowest in middle position of the medial side, but in some donors the wear at this location is the highest.

### 3.3.2 Comparison of Compositions in Four Locations

Comparison of HYP, GAGs, DNA content & % of water content among the four locations were analyzed for full thickness and superficial zone of the cartilage. No significant differences were seen between the different locations for any of the constituents of cartilage that we measured (Fig: 3.3).

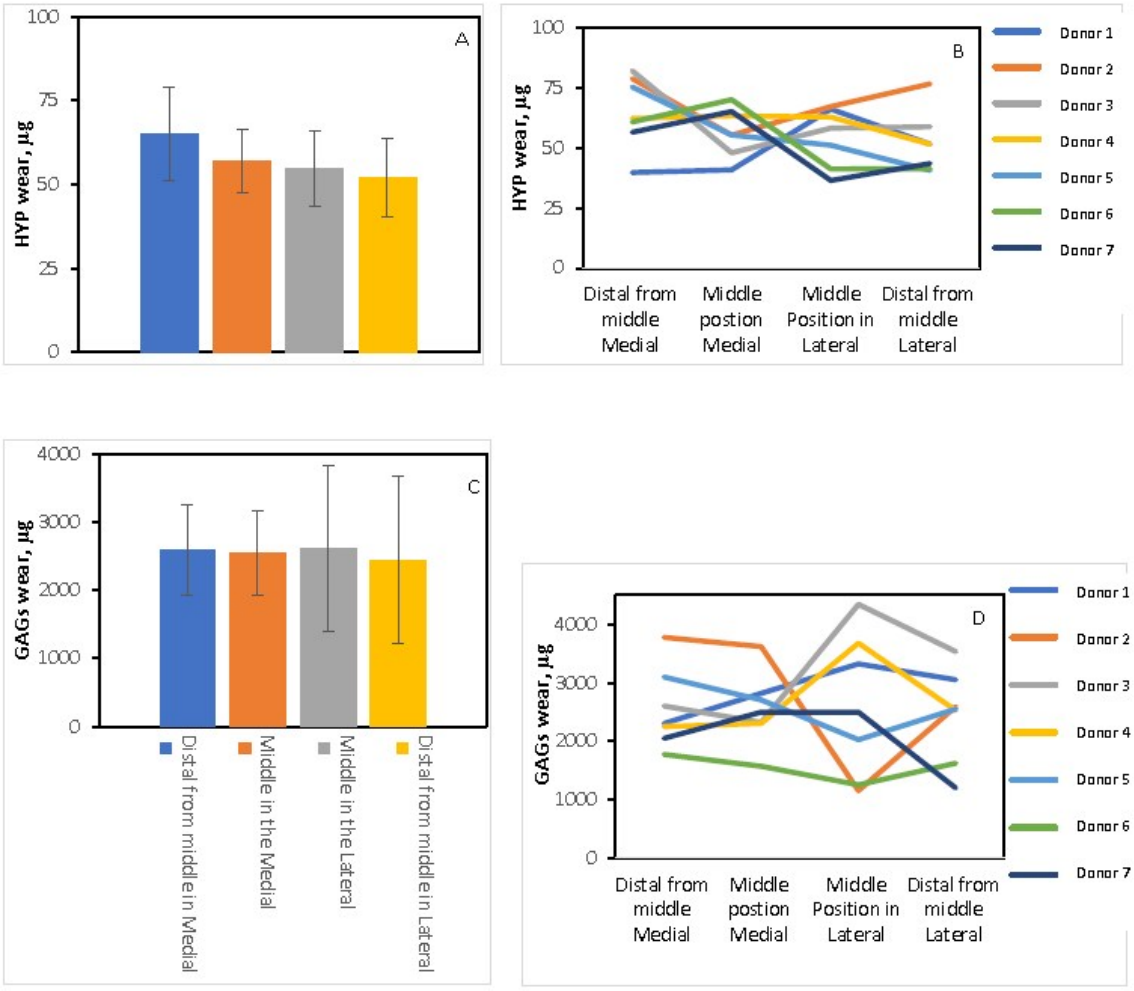


Fig. 3.2. A& C) Average amount of wear in terms of HYP & GAGs released in the solution bath for the four locations, B &D) Amount of wear for four locations samples for each donor in terms of HYP and GAGs released to the solution bath.

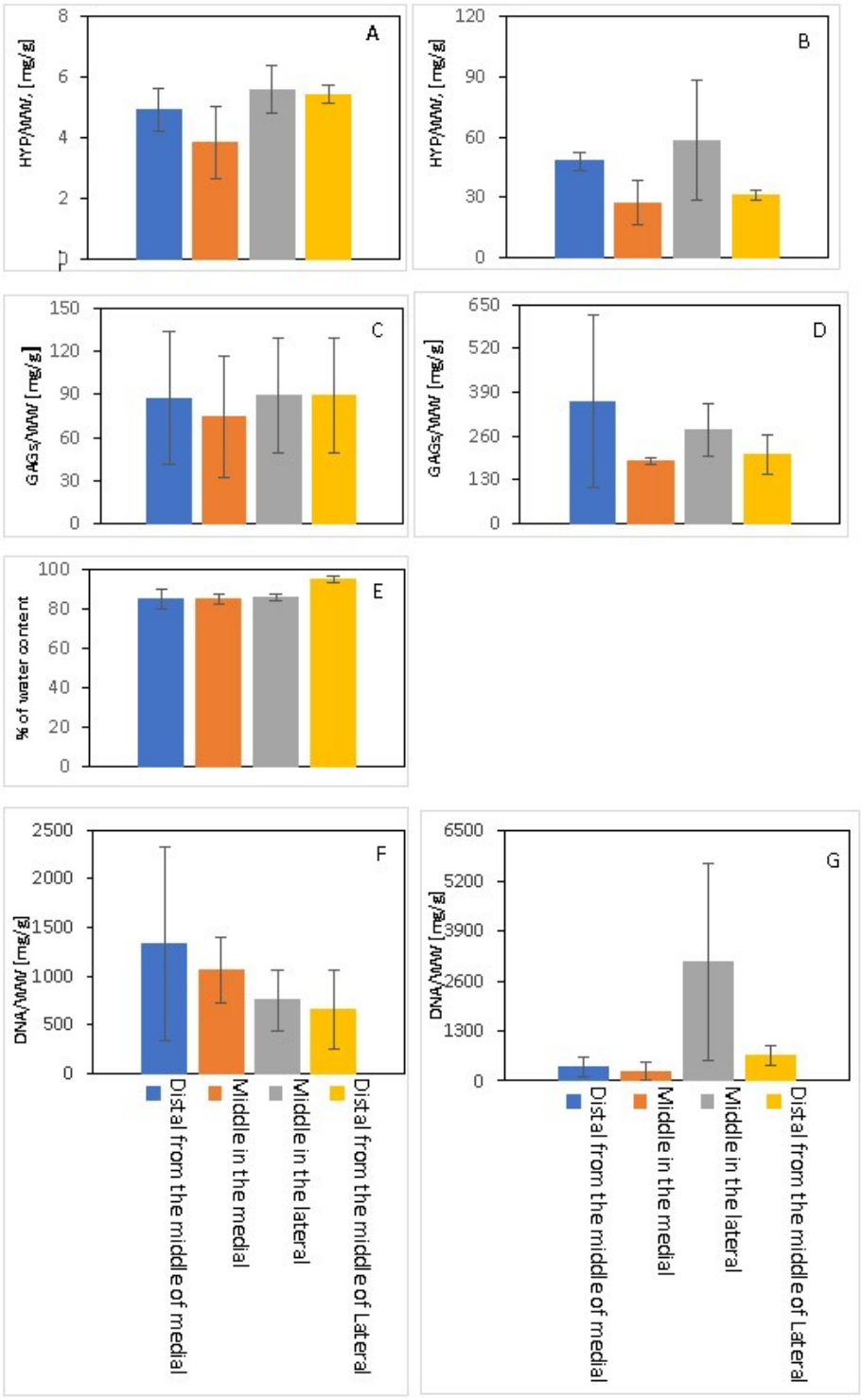


Fig. 3.3. HYP content (A-B), GAG content (C-D), DNA content (F-G) and water content (E) in the full thickness (A, C, E, F) or superficial zone (B, C, G) of the cartilage in four specific locations.

### 3.4 Discussion

It was hypothesized that cartilage from different anatomic locations would give different wear and composition based on different loading. Our hypothesis is not supported by the experimental results. No significant differences were found among the four locations in terms of amount of wear or cartilage compositions. There may not be a large difference in the loading at the different sites that we tested. The congruent shapes of the articular cartilage may distribute the load relatively evenly to different sites, leading to consistent composition throughout the articular joint. Another reason could be that samples were harvested from the flattest locations of the femoral condyle. This flat surface maintains a consistent contact pressure between cartilage and the steel plate for all specimens. However, the flat surfaces may take a similar load to one another. For that reason, any significant differences might not be acquired in the wear and the composition of cartilage among the four locations. Our results are consistent with measurements of the shear and aggregate moduli of the cartilage from the medial and lateral condyles of the human knee [48], which also did not differ with anatomic location.

S. Akizuki et al. had shown that cartilage from the lower weight bearing area had a higher tensile modulus than the cartilage from the higher weight bearing area of the human knee joint [39]. They have also shown that cartilages from lower weight bearing site had a higher collagen/proteoglycan ratio than the higher weight bearing site. Poissons ratio and stiffness were also reported to be higher in the cartilage from femoral medial condyle than the femoral lateral condyle for the canine and human knee [48,49]. These properties, such as tensile modulus and Poissons ratio, may not be related to wear resistance. Moreover, in S. Akizukis studies, higher and lower weight bearing sites had large distances between the two locations [39]. In our studies, all four harvest sites were on the condyles (Fig:3.1). This may also explain why we did not observe any significant differences in the wear or biochemical composition in the

four locations. Due to different testing procedures, different species, and different harvest sites, our results are not similar to these previous studies.

# 4. ANALYSIS OF CARTILAGE COMPOSITION IN RELATION TO WEAR AND FRICTION

## 4.1 Introduction

Different cartilage specimens showed different amounts of wear in the accelerated wear test. For example, in Chapter 3, the standard deviation of HYP wear was more than 25% of the average value (Fig: 3.2A). To better understand cartilage wear, it would be helpful to understand whether variations in the composition of the cartilage can explain the variations in wear. The main components of cartilage are collagens, proteoglycans, cells and water. Previous studies have determined that mechanical properties vary with the cartilage composition. For example, tensile strength is correlated with the water, collagen, and pyridinoline content [29], tensile modulus is correlated with collagen/proteoglycan ratio [39], and Poissons ratio is correlated with hydroxyproline/uronic acid ratio [49]. However, it has not been determined, whether the friction and wear is also correlated with cartilage composition.

The correlations between friction and wear quantities and cartilage composition were analyzed. The friction and wear quantities that we assessed were initial COF, equilibrium COF, friction slope and time constant, HYP wear, and GAG wear. Since wear occurs at the articular surface, the composition of the superficial zone may play an important role in the wear. Therefore, the composition of both the superficial zone and full thickness of cartilage samples that were adjacent to the wear test specimen were evaluated. Correlations between the friction/wear parameters and cartilage composition were determined. This information could help predict the wear of allograft and autograft tissues based on their composition.

## 4.2 Methods

For this analysis, 14 osteochondral cylindrical specimens were collected with 9.52mm diameter from three knees for accelerated wear testing. Moreover, we also collected three to four samples of cartilage that were adjacent to the wear test specimens. All wear test specimens and adjacent cartilage samples were stored in the freezer at $-20^0$C before further analysis.

To maintain consistency, the split line analysis was performed at the edge of the cartilage in 14-16 different locations and the fiber direction was determined. The wear test was carried out for all of the samples($n$=14) using same protocol as described in Chapter 2 (14000 cycles, 160N load, 4mm/s velocity, 18mm travel distance on each side) with specimens oriented in the longitudinal direction.

The amounts of HYP and GAGs released in the solution bath due to the wear test were quantified as described in Chapter 2. Friction and normal force was taken to calculate the COF, also as described in Chapter 2.

The COF rises initially and reaches equilibrium at about 4.5 hours. To characterize this transient behavior, coefficients A and B of an exponential curve were fit to the COF data for 4.5hrs, using Matlab (Mathworks, USA), with initial coefficient values of $A_0 = 0.5$ and $B_0 = 0.5$.

$$COF = A \times (1 - exp^{Bt}) \tag{4.1}$$

where $t$ is time. The initial slope of COF with respect to time was found by taking the derivative of the exponential equation and setting the time to zero.

$$Slope = A \times B \tag{4.2}$$

The time constant ($\tau$) of equation (4.1) is

$$\tau = 1/B \tag{4.3}$$

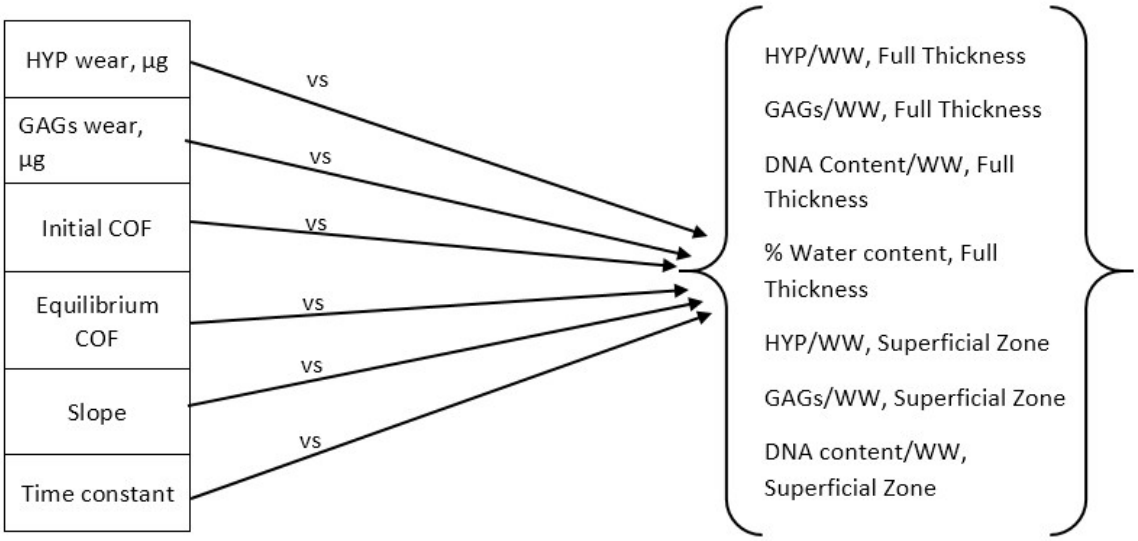


Fig. 4.1. Diagram of correlations among different wear and friction properties of cartilage and cartilage composition.

Cartilage compositions of the adjacent samples were analyzed with the same protocol as described in Chapter 3. A total of 28 samples for 14 locations were examined; 14 cartilage samples for the full thickness analysis and 14 cartilage samples for the superficial zone analysis. To quantify the content of HYP, GAGs, DNA and water, the same protocols were used as described in Chapter 3 and HYP, GAGs, and DNA data were reported as normalized to wet weight.

Correlation analysis were conducted between different components of cartilage composition and friction or wear (Fig: 4.1) using GraphPad Prism Software with $p < 0.05$ considered as significant.

The results were reported with graphs showing the amounts of particular components of cartilage composition on the y-axis and the values of the friction or wear properties on the x-axis for each specimen(Fig: 4.2).


Fig. 4.2. A schematic of correlation analysis.

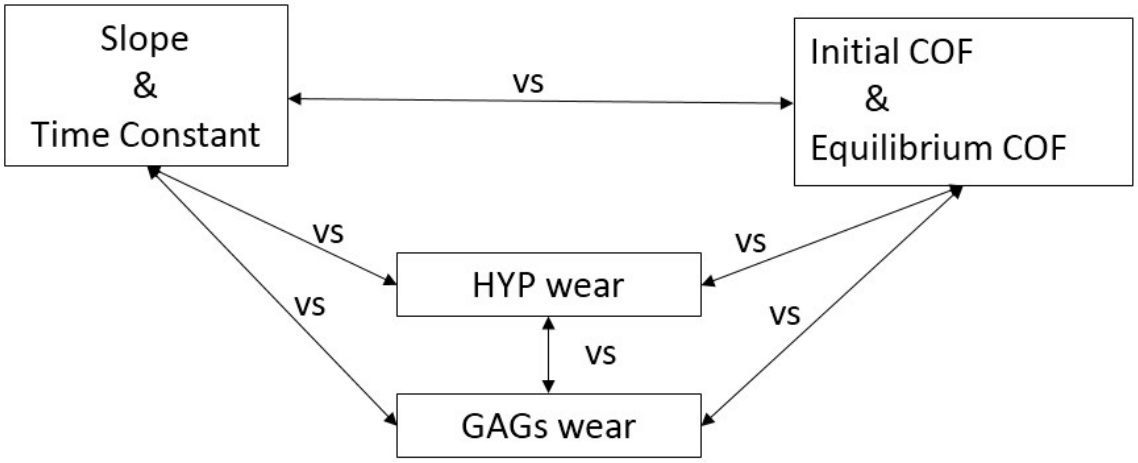


Fig. 4.3. Diagram of correlation among different friction and wear properties of cartilage.

Alternately, correlations were conducted between different measures of friction and wear (Fig:4.3), also using GraphPad Prism Software with $p < 0.05$ considered as significant.

## 4.3 Results

### 4.3.1 Best Fit Curve Between COF and Time

The exponential equation fit the COF vs. time data well, with $R^2$ values all above 0.94 (Fig:4.4). Average values for A and B were 0.2943 and 0.0005$s^{-1}$ respectively.

### 4.3.2 Correlation Between Measures of Friction and Wear vs. Cartilage Composition

From the linear regression analysis, there was a significant correlation between the wear and the full-thickness collagen content. The HYP released to the fluid during the wear test and HYP content of the full thickness of the cartilage were a negatively

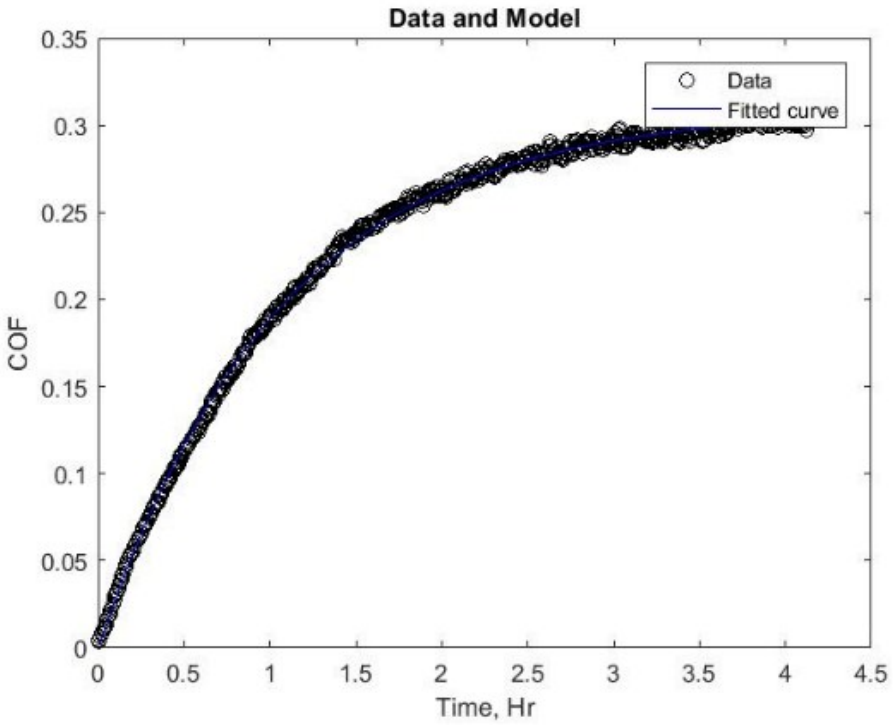


Fig. 4.4. Typical best fit curve for COF vs Time data.

correlated (Fig: 4.5a). Similarly, the GAGs released to the hydrating fluid during the wear test was also negatively correlated with the collagen content in the full thickness of the cartilage (Fig: 4.5f).

There was also another trend between HYP wear and % of water content in the full thickness of the cartilage ($p = 0.0599$). The slope was negative, and the line was nearly flat. (Fig: 4.5e)

The initial COF tends to correlate with HYP content in the superficial zone of the cartilage (Fig: 4.5i). So, lower amount of HYP in the superficial zone increase the initial COF ($p$= 0.0034). We have not found any other correlation between the friction and wear parameters with the cartilage composition.

DNA content was not correlated to anything, so it is not shown in the figure.

### 4.3.3 Correlation Graphs Between Measures of Friction and Wear

A positive tendency was found between the friction time constant and HYP wear ($p = 0.0654$; Fig: 4.6a). A significant negative correlation was found between slope of COF and HYP removed from the tissue in the wear test (Fig: 4.6e). Time constant and equilibrium COF also correlated significantly; they are correlated positively (Fig: 4.6d). The HYP and GAG wear are positively correlated with each other (Fig: 4.6m). We have not found any other significant correlation among the other groups.

## 4.4 Discussion

Previous studies have determined a relationship between composition and mechanical properties of cartilage. T. Lyrra et al., A.K. Williamson et al., and J.S.Jurvelin et al. had studied the relationship between the cartilage composition and equilibrium modulus, tensile strength, and Poissons ratios [29,48,49]. Significant correlations have also been found in the current study between biochemical composition, friction and wear in a standardized accelerated wear test. For example, wear and collagen content in the full thickness of the cartilage was negatively correlated; lower HYP content

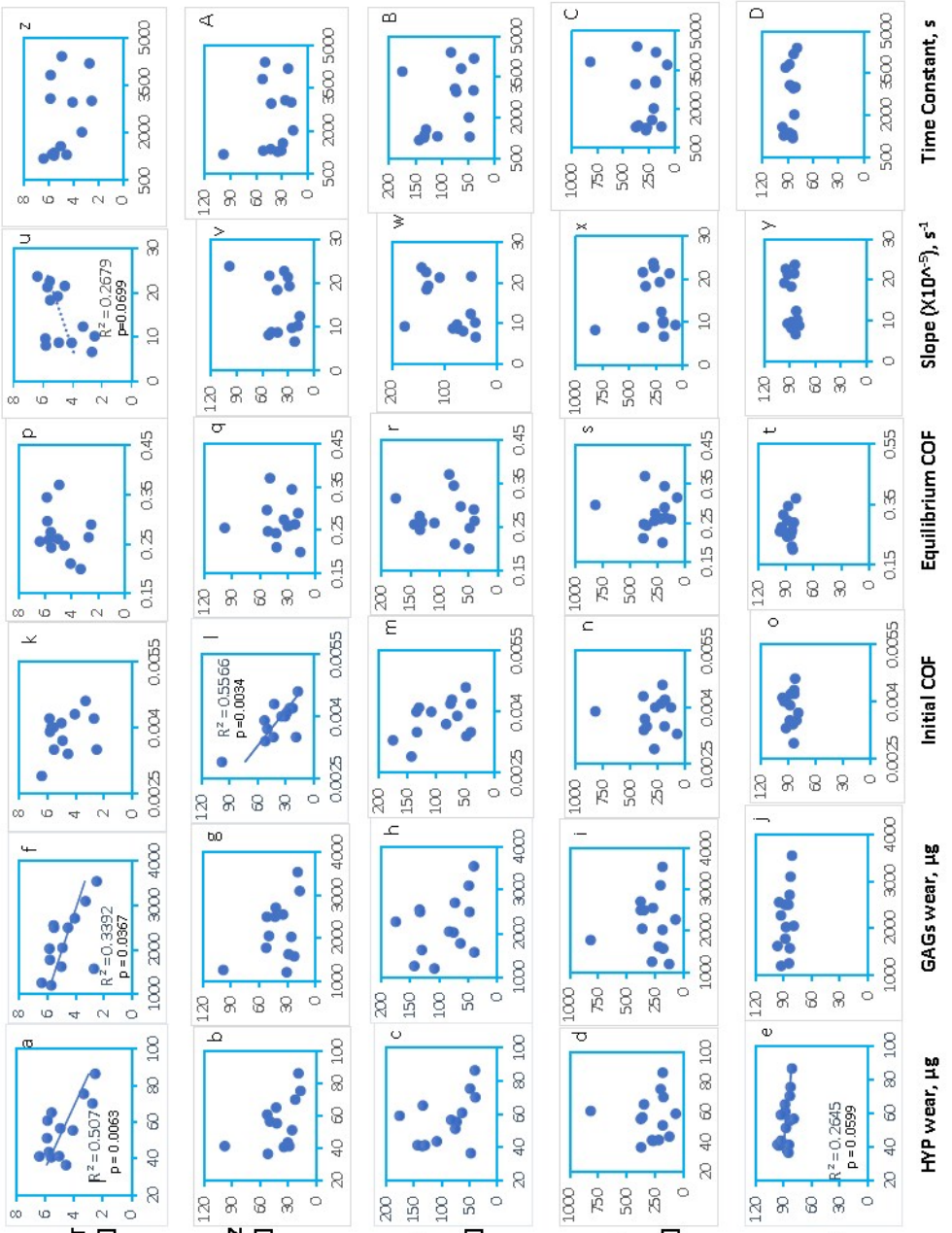


Fig. 4.5. Relationships between cartilage constituent concentrations (y-axis) and friction/wear properties (x-axis). Regression lines and corresponding $R^2$ values are shown where $p < 0.1$. [HYP= Hydroxyproline, GAGs= glycosaminoglycans, FT= Full thickness of the Cartilage, SZ= Superficial zone of the cartilage]

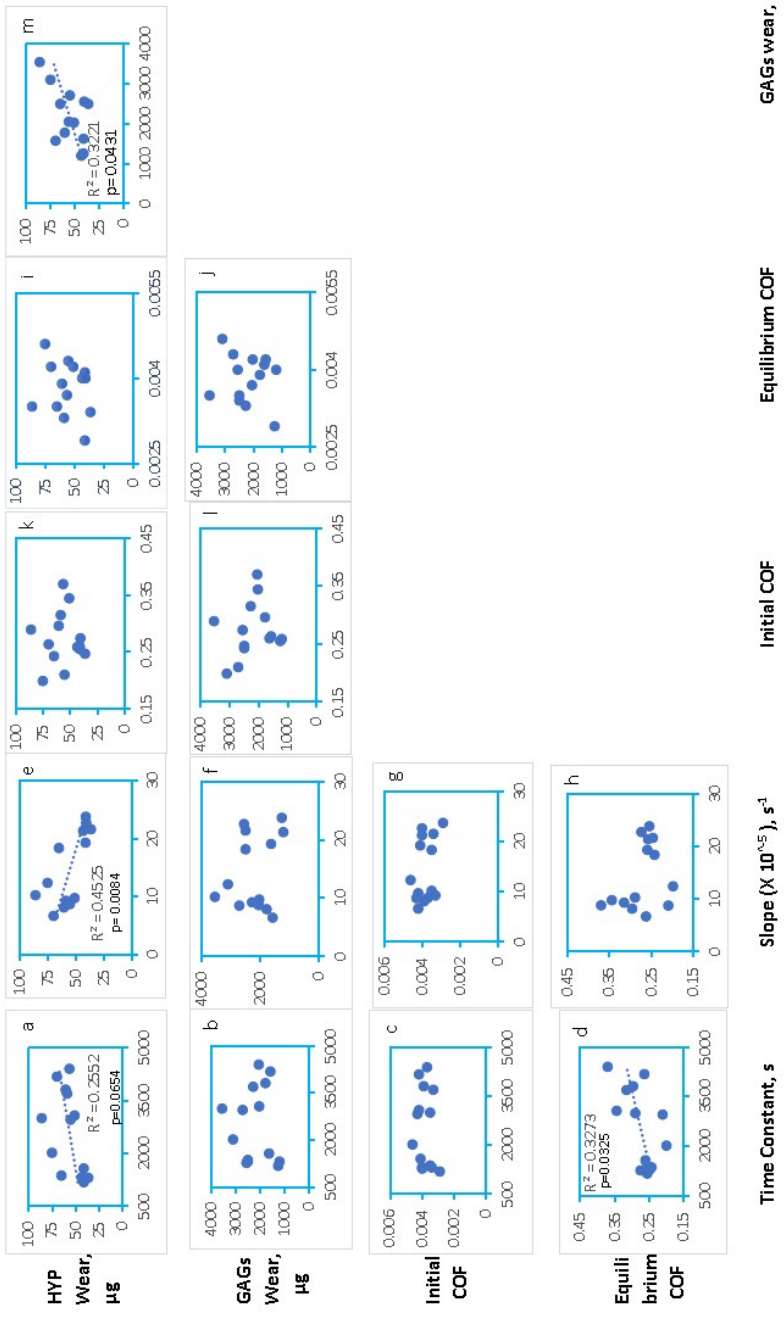


Fig. 4.6. Correlation graphs between different measures of friction and wear. Regression lines and corresponding $R^2$ values are shown where $p < 0.1$. [HYP= Hydroxyproline, GAGs= Glycosaminoglycans].

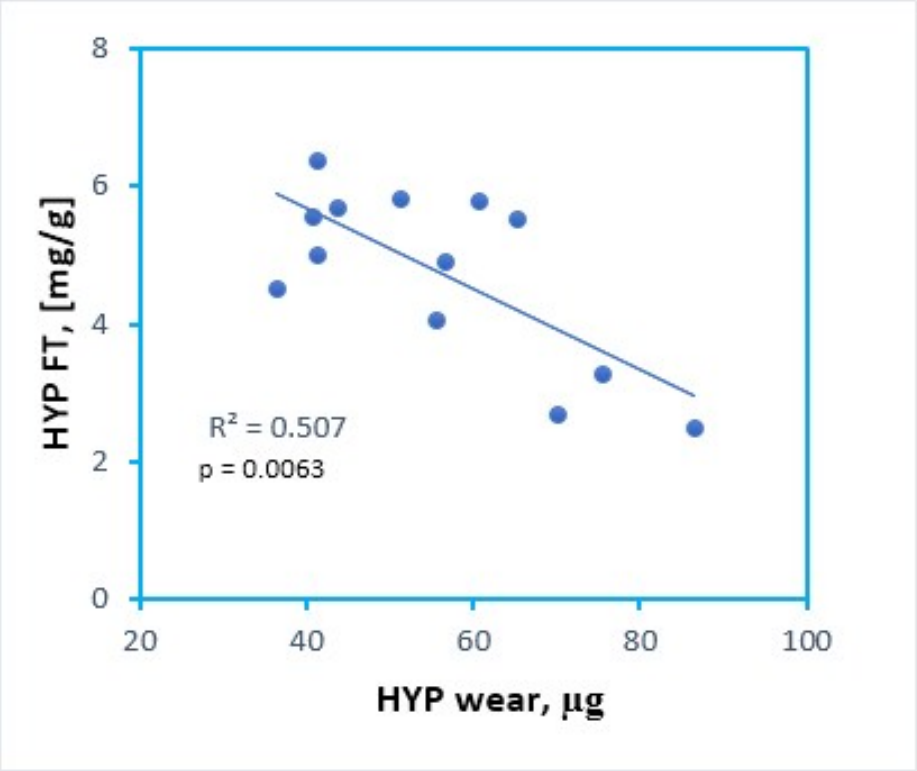


Fig. 4.7. Correlation between HYP content in the full thickness(FT) of the cartilage and HYP removed from the tissue during the wear test.

leads to a higher amount of wear (Fig: 4.7). As HYP is an amino acid component of type II collagen network, less of the type II collagen network leads to a higher amount of wear. A reduced collagen network has less strength, and as a result, it is more easily broken down in the accelerated wear test. In contrast, a higher amount of HYP or collagen network does not break down easily, and as a result, it wears less. The additional significant correlation between GAG wear and collagen content of the full thickness of the cartilage confirms the idea that a lower amount of type II collagen network leads to a higher amount of wear in accelerated wear test. Both results indicate that a higher amount of wear of the cartilage may be due to the lower amount of HYP or collagen in the full thickness of the cartilage.

Another correlation has also been observed between HYP removed from the tissue during the wear test and water content in the full thickness of the cartilage. The water content among all specimens is almost constant, so the correlation may not be meaningful.

Another significant negative correlation has been found between initial COF vs HYP content in the superficial zone, meaning that higher HYP content in the superficial zone creates lower initial COF whereas lower HYP content in the superficial zone creates higher initial COF. Higher HYP/collagen content in the superficial zone might be able to constrain higher amounts of fluid pressure than the cartilage with lower HYP, and more fluid pressure leads to lower initial COF.

Moreover, GAG and HYP wear are also directly correlated (Fig: 4.6m). The type II collagen network holds the GAGs in the cartilage tissue. So, if this collagen network is damaged, then GAGs release from the cartilage.

A significant positive correlation has been found between friction slope and HYP wear. This seems counterintuitive, because higher slope means the COF rises faster, and as a result, it should give more wear than the cartilage with lower slope. However, intuition would also suggest that higher friction leads to more wear, but there was no correlation that would support this. This suggests that the relationship between friction and wear in cartilage is complicated and may not follow intuition. There are also other properties, like permeability, that may be responsible for the transient frictional behavior.

This analysis may be helpful in improving the surgical grafting procedure. Before allograph or autograph of cartilage tissue is transplanted, the HYP content in the cartilage should be analyzed. Cartilage with lower HYP content in the full thickness and superficial zone of the cartilage should not be used as this will be associated with higher wear and higher friction.

# 5. CROSSLINKING OF CARTILAGE TISSUE TO IMPROVE RESISTANCE DUE TO WEAR

## 5.1 Introduction

High trauma to the cartilage surface due to accident causes cartilage fissures and micro damage [50, 51]. A high energy trauma to the AC surfaces causes a mechanical, biochemical and cellular response that can lead to post traumatic osteoarthritis [PTOA]. A single traumatic event can generate pro-inflammatory cytokines that cause an upregulation of degenerative enzymes that leads to reduce mechanical properties and ultimately wear resistance [52–55]. An AC injury can also cause microstructural damage with or without creating fissures [56]. PTOA often leads to joint replacement. But for younger individuals, arthroplasty is not an effective option, due to limited life span of the implants [57–59]. Present surgical treatment for joint injury, such as anatomic reduction of intra-articular fractures, ligament repair, and joint stabilization can improve joint instability or incongruity, but do not repair the mechanical damage of the AC surfaces [60–66]. Recent studies have shown that chondrocyte damage due to mechanical overload can be limited by biological therapies, they also prevent the consequent activation of catabolic pathways. For example, D′Lima et al. showed that a caspase inhibitor reduces degradative chondrocytes pathways [67] while Haut et al. showed that cellular necrosis due to mechanical insult to cartilage tissue is reduced by surfactant [68]. That antioxidants are effective for reducing chondrocyte death was reported by Martin et al. [69, 70]. However, while these analyses are promising biological treatments, they do not repair the mechanical responses or mechanical integrity.

In previous studies, collagen crosslinking using genipin, a natural plant extract, improved mechanical properties like stiffness and wear resistance of cartilage [27]. So,

our long-term goal is to induce exogenous crosslinks to the collagen network to prevent wear and degradation in damage articular cartilage. But, higher concentrations of genipin causes chondrocytes death [21]. Besides that, genipin treatment is difficult to apply in specific locations because the genipin solution will crosslink all the tissues that it contacts in the joint. Photo-initiated crosslink protocols have been developed by using chloro-aluminum phthalocyanine tetrasulfonic acid (CASPc) with 670nm laser light [71,72]. This laser light interacts with CASPc to cause an oxidation reaction that crosslinks the collagen network. Due to this crosslink the bond among the collagen fibers increases. This treatment can be used in a specific location of joints, because in this process only the location that is exposed to light will be crosslinked. In this analysis, two protocols were used. The first protocol was first reported by another group; a crosslinked bond was created at the interface between two cartilage tissues for the purpose of adhering a graft to the cartilage surrounding a defect site [73]. Whether this protocol can be used to improve the mechanical wear response of cartilage is currently unknown. Moreover, in their protocol, the laser exposure time was larger than the time that the tissue is submerged in the CASPc solution. But, as a treatment for PTOA, less laser time will be more convenient to the doctor and the patient. Therefore, a second protocol was developed that reduced laser exposure time and increased CASPc submersion time. The second protocol was optimized previously by indentation test in our lab. The objective of this study was to test both the photochemical crosslinking protocols in an accelerated wear test of the cartilage.

## 5.2 Methods

For this analysis, a total 27-osteochondral cylindrical specimens with 9.52mm diameter were collected from four knees using same protocol as describe in chapter 2. Those samples were stored in the freezer at $-20^0$C before further analysis.

The wear test was carried out in three different groups, two treated groups and one untreated group. Additionally, in a control group, zero load was applied, and

other protocols remained same as in Chapter 2 (n=3). During processing, cartilage specimens were cut using different tools to get the cylindrical specimens. As a result, during the wear test some collagen was released in the solution bath through the cut surfaces. So, this amount of collagen released in the solution bath in the control group is consistent for all groups but does not come from cartilage wear.

For treated groups, a total ten specimens were used, five specimens for each group. First, the specimen was removed from the freezer and rehydrated in PBS solution for 15min before the crosslink treatment. For crosslinking, 2ml 0.5mM CASPc was prepared. For getting stable laser power, the laser power (current 0.82A) was kept on for 30min before laser exposure.

In the first protocol, the specimen was submerged in the 0.5mM CASPc solution for 20s. After that, the articular surface immediately was exposed to laser light covered with plastic wrap for 10 minutes. The laser power was 265mW and the spot size diameter was around 5.5mm (Fig: 5.1). The plastic wrap was used on the top of the specimen to keep the cartilage hydrated. After the laser treatment, a photo was taken of the crosslinked cartilage using a stereomicroscop equipped with a digital camera, then the cartilage was submerged in PBS for rehydration and equilibration at the room temperature. The wear test was carried out by using same protocol as described in the chapter 2.

In the second protocol, the specimen was submerged in the 0.5mM CASPc for 20min and the laser treatment was 2min. Other procedures were the same as in the first protocol.

In the untreated group, a total of fourteen specimens were used, and wear test was carried out by using same wear test protocol as in Chapter 2, without laser and without CASPc.

After the wear test the solution bath was collected in the freezer at $-20^0$C before further analysis. To quantify the amount of HYP wear, the same steps were taken as in Chapter 2 and Appendix C.

Initial COF and equilibrium COF were reported as described in Chapter 2.

The average HYP released to the water bath from the control group was subtracted from the other groups to give the HYP in the bath that was only due to the wear.

One way ANOVA followed by LSD post hoc test was carried out with SPSS software to evaluate statistical differences in the mean Initial COF, Equilibrium COF and HYP wear of the three groups (Untreated, Crosslink protocol-1, Crosslink Protocol-2).

## 5.3 Results

Specimens with both crosslink treatments showed significantly lower wear than the untreated specimens (Fig:5.2). In the post hoc test, statistically significant differences were found between untreated and crosslink protocol-1, and untreated and crosslink protocol-2. However, no differences were found between crosslink protocol-1 & crosslink protocol-2 (Fig: 5.3C). Initial and equilibrium COF were not significantly different among the 3 groups in the one-way ANOVA analysis (Fig: 5.3A & B).

## 5.4 Discussion

The wear resistance of the cartilage increased due both crosslinking treatments. The first crosslinking protocol was used by Arvayo et al., where strength of a crosslinked bond was analyzed between graft and host cartilage [73], and was used in the accelerated wear test.

In our lab, different combinations of laser timing and submerging time of cartilage in CASPc was first analyzed with an indentation test. The indentation test characterizes the mechanical and viscoelastic properties of the cartilage tissue [74, 75]. A constant indentation depth or stress relaxation test can be useful for AC because its mechanical response changes as a function of time [72]. In this technique, maximum load will be achieved just as the maximum indentation depth is reached, after that load will reduce and will eventually become equilibrium [76].

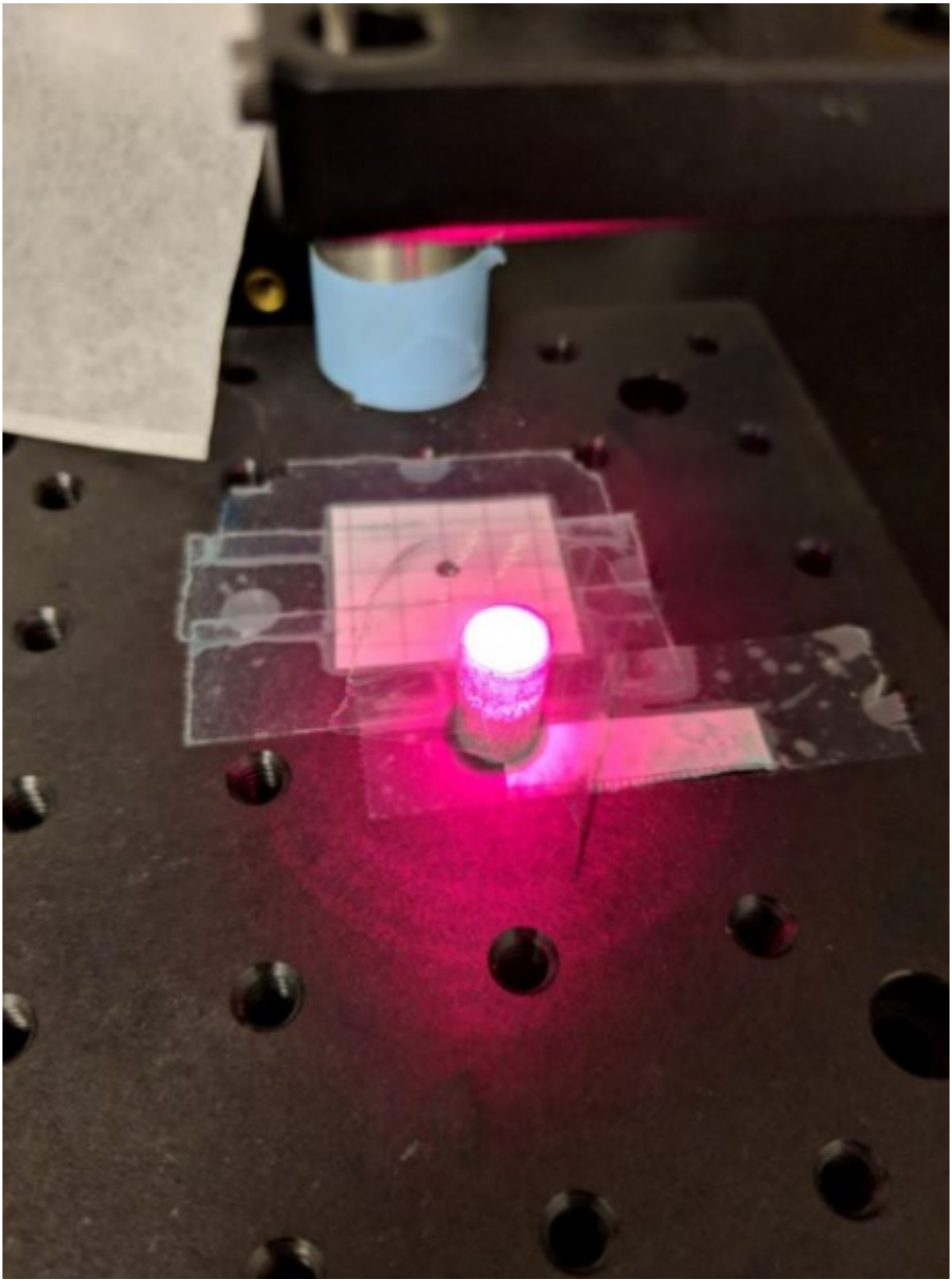

Fig. 5.1. Laser treatment of the cartilage.

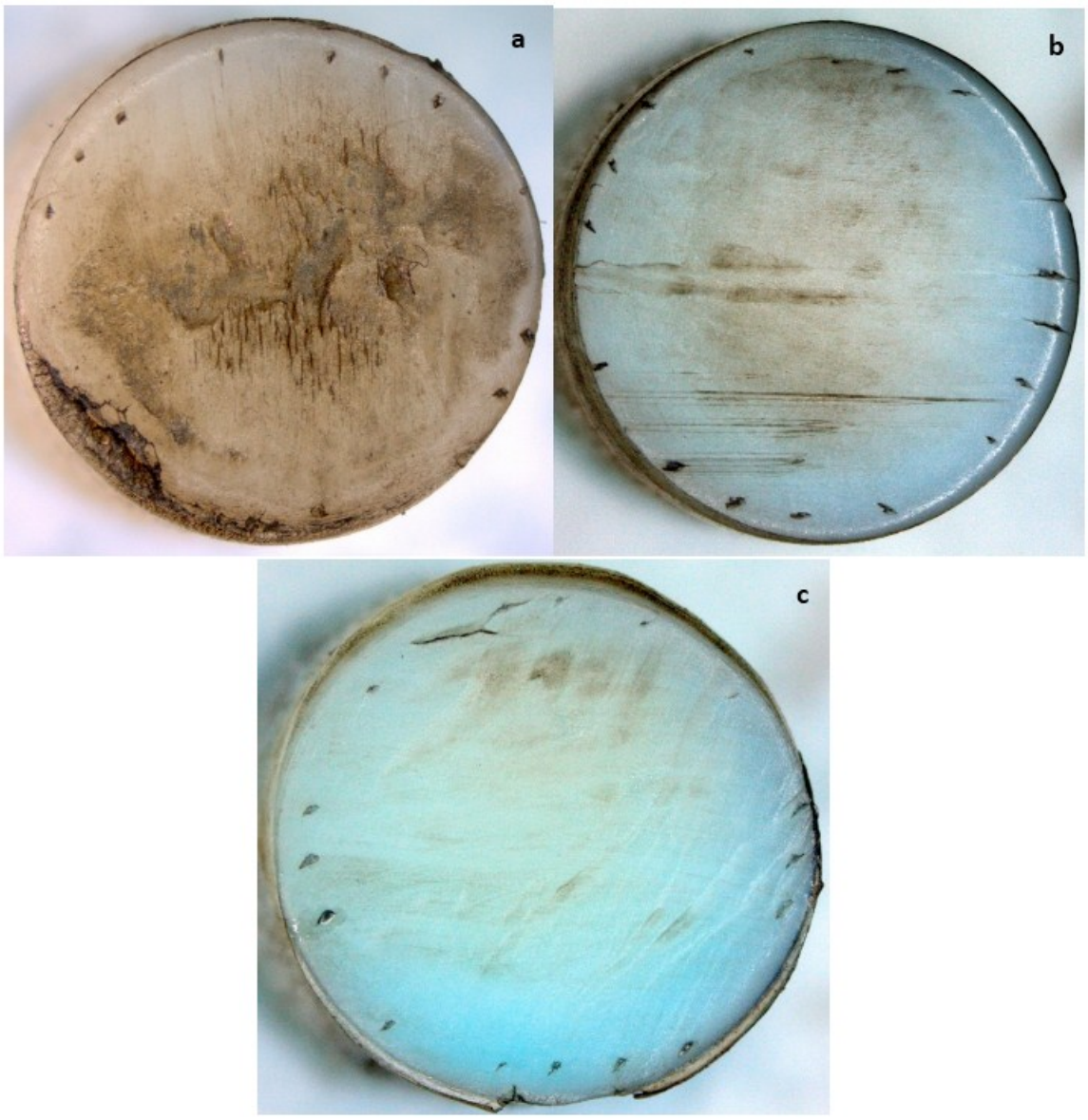


Fig. 5.2. Cartilage after the wear test a) untreated, b) treated with Crosslinking protocol-1 b) treated with Crosslinking protocol-2.

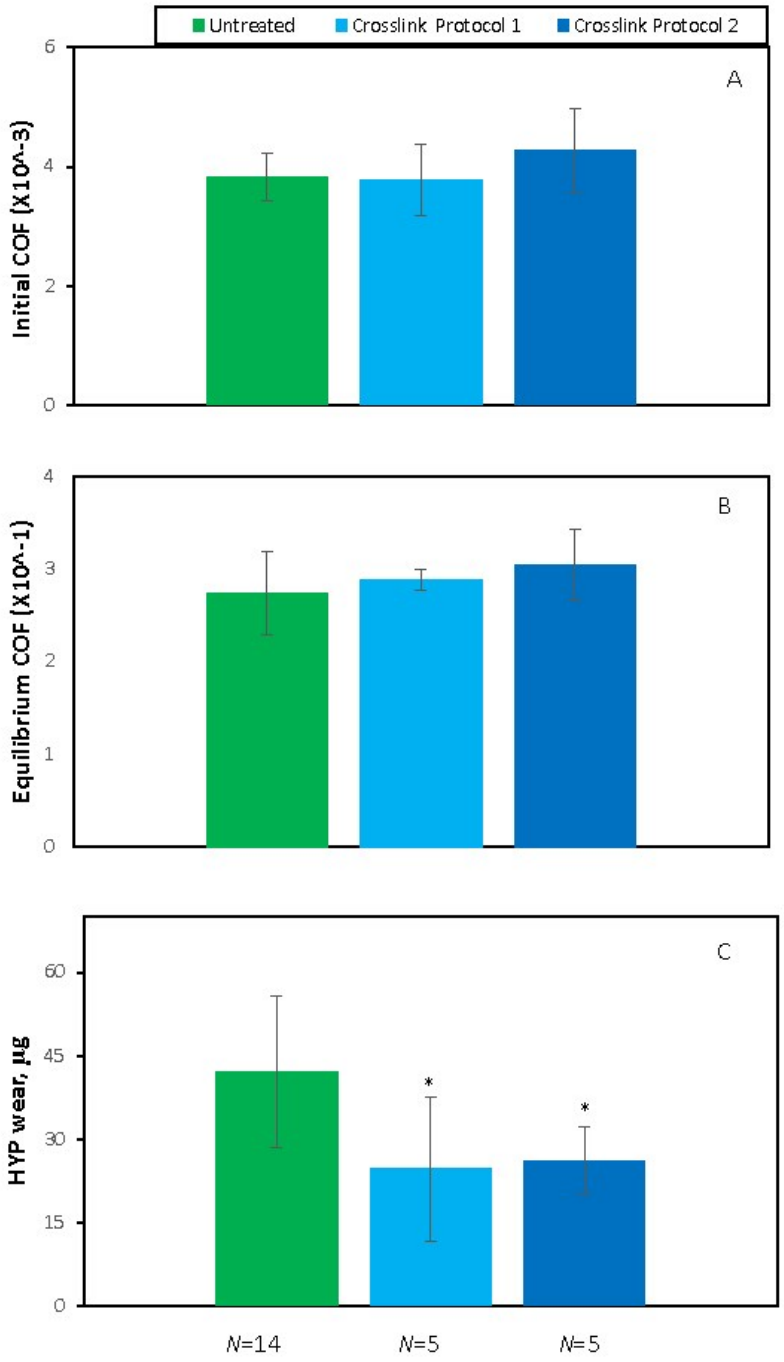


Fig. 5.3. A) Initial COF, B) Equilibrium COF, C) HYP due to wear of untreated and, two treatment groups (Crosslink protocol-1 & 2). *Indicates significant difference from untreated.

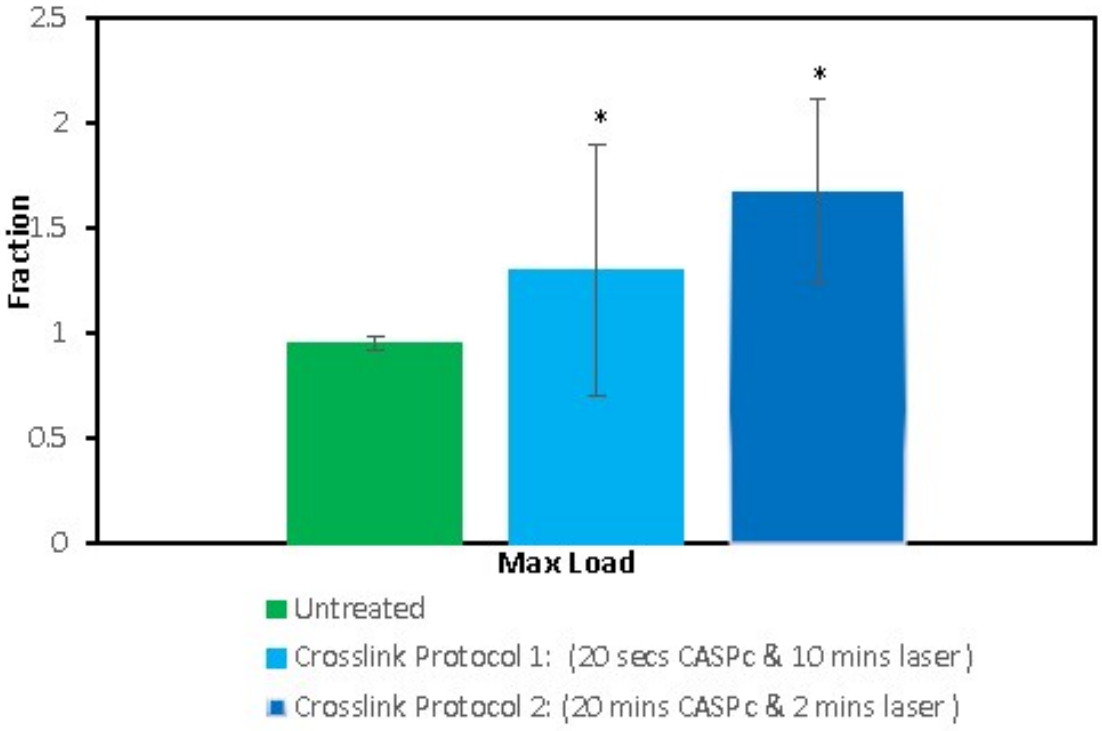


Fig. 5.4. Indentation test result for untreated, crosslink protocol-1 & 2. Results are shown as max load after crosslinking divided by max load before crosslinking. [Wagner Lab]. *Indicates significant difference from untreated.

From the indentation test result, no significant difference was found between the crosslink protocol 1 and 2, and both protocols were stiffer after crosslinking, compare to the untreated specimens (Fig: 5.4). From this analysis, crosslink protocol-2 was chosen with minimum laser time. In the accelerated wear test, the crosslink protocol-2 has also given higher wear resistance of cartilage than the untreated group. No significant difference was found between crosslinking protocol 1 & 2, which is consistent with the indentation test result.

No significant differences have been observed between untreated and treated group in frictional analysis. So, these crosslink protocols have not changed any frictional properties of the cartilage tissue. Bonitsky et al. had not found any significant differ-

ences in friction of cartilage tissue with genipin crosslinking treatment [27]. Moreover, Sitterle et al. had reported that photochemical treatment with CASPc does not cause cell death [35].

One limitation of our analysis that the diameter of the laser light was 5.5mm, whereas the diameter of the specimen or cartilage surfaces was 9.52mm. For that reason, the crosslinking treatment was carried out in a smaller area or in the middle of the cartilage instead of the full surfaces and during wear test some wear was observed in the untreated area surrounding the location of laser light (Fig: 5.2b & c). If a larger laser spot size could be applied, it might give a better result.

This finding may be important in the treatment of impacted AC. Doctor may be able to use CASPc crosslinking treatment to improve the wear resistance properties of the AC to prevent or slow down the progression of cartilage damage. In addition, crosslink protocol-2 is more convenient to apply during treatment than the crosslinking protocol-1 because the doctor would not need to hold the laser light for as long of time.

# 6. CONCLUSION AND FUTURE DIRECTIONS

## 6.1 Conclusions

One limitation in our analysis is that the wear test is an accelerated wear test and is not physiologic. In real life, cartilage does not experience a large, constant load for a long period of time, nor does it experience significant wear during a 43 hours time period. During walking or running, the maximum load occurs for short period of time and then the load releases; water exudation and re-absorption occur cyclically. Despite this limitation, the studies in this thesis describe various factors that affect the wear of bovine articular cartilage in accelerated testing in vitro. In Chapter 2, the hypothesis regarding the anisotropic nature of cartilage wear was proven by quantifying the GAGs released to the hydrating fluid. The COF of the cartilage tissue did not show any anisotropic behavior in the accelerated wear test. In Chapter 3, no significant differences in wear were seen between the anatomic locations that were tested. In Chapter 4, a significant negative correlation has been found between HYP wear and HYP content in the full thickness of the cartilage, and between GAG wear and HYP content in the full thickness of the cartilage. Another significant correlation had been observed between initial COF and HYP content in the superficial zone of the cartilage. No other interesting correlation has been observed between friction properties and wear quantities and composition of the cartilage. In Chapter 5, the two photo-initiated crosslinking protocols have given better wear resistance in cartilage tissue without changing the frictional behavior of the cartilage.

## 6.2 Future Directions

While these studies have uncovered some important relationships between the orientation, composition, and degree of crosslinking with the wear and friction of cartilage tissue, open questions remain. For example, a significant difference has been found between the longitudinal and transverse directions in GAG wear but not in HYP wear (Chapter 2), even though HYP and GAG wear are positively correlated (Chapter 4). So, to quantify wear, it may be important to analyze both the HYP and GAGs release during the wear test. Furthermore, the use of collagen hybridizing peptides should be considered to quantify the amount of damaged collagen remaining on the cartilage specimen.

Although some significant correlations were found between cartilage composition and measures of friction and wear, other material properties of cartilage may also determine the friction and wear characteristics. For example, permeability and native collagen crosslinks may partially determine the friction and wear properties.

In the future, after damaging the cartilage with an impact load, the photochemical crosslinking treatment will be applied to the damaged cartilage. The wear test will be carried out with the impacted and treated cartilage. This will help us to understand the effect of photochemical crosslinking treatment for the post traumatic osteoarthritis(PTOA) patients.

# REFERENCES

# REFERENCES


[1] J. M. Mansour and J. F. Welter, "Multimodal evaluation of tissue-engineered cartilage," *Journal of medical and biological engineering*, vol. 33, no. 1, p. 1, 2013.

[2] S. R. Simon, *Orthopaedic basic science*. Amer Academy of Orthopaedic, 1994.

[3] A. Joukar, H. Niroomand-Oscuii, and F. Ghalichi, "Numerical simulation of osteocyte cell in response to directional mechanical loadings and mechanotransduction analysis: Considering lacunar–canalicular interstitial fluid flow," *Computer methods and programs in biomedicine*, vol. 133, pp. 133–141, 2016.

[4] S. Below, S. P. Arnoczky, J. Dodds, C. Kooima, and N. Walter, "The split-line pattern of the distal femur: a consideration in the orientation of autologous cartilage grafts," *Arthroscopy*, vol. 18, no. 6, pp. 613–617, 2002.

[5] N. S. Landínez-Parra, D. A. Garzón-Alvarado, and J. C. Vanegas-Acosta, "Mechanical behavior of articular cartilage," in *Injury and Skeletal Biomechanics*. InTech, 2012.

[6] C.-B. James and T. L. Uhl, "A review of articular cartilage pathology and the use of glucosamine sulfate," *Journal of athletic training*, vol. 36, no. 4, p. 413, 2001.

[7] R. C. Lawrence, D. T. Felson, C. G. Helmick, L. M. Arnold, H. Choi, R. A. Deyo, S. Gabriel, R. Hirsch, M. C. Hochberg, G. G. Hunder *et al.*, "Estimates of the prevalence of arthritis and other rheumatic conditions in the united states: Part ii," *Arthritis & Rheumatism*, vol. 58, no. 1, pp. 26–35, 2008.

[8] J. A. Buckwalter and J. A. Martin, "Sports and osteoarthritis," *Current opinion in rheumatology*, vol. 16, no. 5, pp. 634–639, 2004.

[9] J. A. Buckwalter and T. D. Brown, "Joint injury, repair, and remodeling: roles in post-traumatic osteoarthritis," *Clinical Orthopaedics and Related Research®*, vol. 423, pp. 7–16, 2004.

[10] A. K. Gupta, A. Joukar, R. OConnell, and V. K. Goel, "The evaluation of shoulder abduction with and without rotation on the supraspinatus tendon and labrum: A finite element study," *Orthopaedic journal of sports medicine*, vol. 5, no. 7_suppl6, p. 2325967117S00380, 2017.

[11] J. Martel-Pelletier, C. Boileau, J.-P. Pelletier, and P. J. Roughley, "Cartilage in normal and osteoarthritis conditions," *Best Practice & Research Clinical Rheumatology*, vol. 22, no. 2, pp. 351–384, 2008.

[12] P. Cram, X. Lu, S. L. Kates, J. A. Singh, Y. Li, and B. R. Wolf, "Total knee arthroplasty volume, utilization, and outcomes among medicare beneficiaries, 1991-2010," *Jama*, vol. 308, no. 12, pp. 1227–1236, 2012.

[13] D. A. Grande, J. A. Schwartz, E. Brandel, N. O. Chahine, and N. Sgaglione, "Articular cartilage repair: where we have been, where we are now, and where we are headed," *Cartilage*, vol. 4, no. 4, pp. 281–285, 2013.

[14] D. A. Grande, M. I. Pitman, L. Peterson, D. Menche, and M. Klein, "The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation," *Journal of Orthopaedic Research*, vol. 7, no. 2, pp. 208–218, 1989.

[15] D. B. Saris, J. Vanlauwe, J. Victor, M. Haspl, M. Bohnsack, Y. Fortems, B. Vandekerckhove, K. F. Almqvist, T. Claes, F. Handelberg *et al.*, "Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture," *The American journal of sports medicine*, vol. 36, no. 2, pp. 235–246, 2008.

[16] C. G. Jeong and S. J. Hollister, "A comparison of the influence of material on in vitro cartilage tissue engineering with pcl, pgs, and poc 3d scaffold architecture seeded with chondrocytes," *Biomaterials*, vol. 31, no. 15, pp. 4304–4312, 2010.

[17] T. Hraha, K. Doremus, C. McIlwraith, and D. Frisbie, "Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (irap and irap ii) using equine blood," *Equine veterinary journal*, vol. 43, no. 5, pp. 516–521, 2011.

[18] L. S. J. Berrien, "Biotribology: studies of the effects of biochemical environments on the wear and damage of articular cartilage," Ph.D. dissertation, Virginia Tech, 1999.

[19] J. H. Dumbleton, *Tribology of natural and artificial joints*. Elsevier, 1981, vol. 3.

[20] E. Northwood and J. Fisher, "A multi-directional in vitro investigation into friction, damage and wear of innovative chondroplasty materials against articular cartilage," *Clinical Biomechanics*, vol. 22, no. 7, pp. 834–842, 2007.

[21] R. Krishnan, M. Caligaris, R. L. Mauck, C. T. Hung, K. Costa, and G. A. Ateshian, "Removal of the superficial zone of bovine articular cartilage does not increase its frictional coefficient," *Osteoarthritis and cartilage*, vol. 12, no. 12, pp. 947–955, 2004.

[22] M. Furey, "The influence of synovial fluid constituents on cartilage wear: A scanning electron microscope study," in *Conference on Joint Destruction, XVth Symposium on the European Society of Osteoarthrology*, 1987.

[23] J. Beltran, A. M. Noto, L. Herman, J. Mosure, J. Burk, and A. Christoforidis, "Joint effusions: Mr imaging." *Radiology*, vol. 158, no. 1, pp. 133–137, 1986.

[24] R. H. Shmerling, "Synovial fluid analysis. a critical reappraisal." *Rheumatic diseases clinics of North America*, vol. 20, no. 2, pp. 503–512, 1994.

[25] A. Rorvik and A. Grondahl, "Markers of osteoarthritis: a review of the literature," *Veterinary Surgery*, vol. 24, no. 3, pp. 255–262, 1995.

[26] C. J. Malemud, "Markers of osteoarthritis and cartilage research in animal models." *Current opinion in rheumatology*, vol. 5, no. 4, pp. 494–502, 1993.

[27] M. E. McGann, C. M. Bonitsky, M. L. Jackson, T. C. Ovaert, S. B. Trippel, and D. R. Wagner, "Genipin crosslinking of cartilage enhances resistance to biochemical degradation and mechanical wear," *Journal of Orthopaedic Research*, vol. 33, no. 11, pp. 1571–1579, 2015.

[28] T. Ahsan, F. Harwood, K. McGowan, D. Amiel, and R. Sah, "Kinetics of collagen crosslinking in adult bovine articular cartilage," *Osteoarthritis and cartilage*, vol. 13, no. 8, pp. 709–715, 2005.

[29] A. K. Williamson, A. C. Chen, K. Masuda, J.-M. T. Eugene, and R. L. Sah, "Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components," *Journal of Orthopaedic Research*, vol. 21, no. 5, pp. 872–880, 2003.

[30] H. Lipshitz, R. Etheredge, and M. J. Glimcher, "In vitro studies of the wear of articular cartilageiii. the wear characteristics of chemically modified articular cartilage when worn against a highly polished characterized stainless steel surface," *Journal of biomechanics*, vol. 13, no. 5, pp. 423–436, 1980.

[31] E. L. Radin, D. A. Swann, I. L. Paul, and P. J. Mcgrath, "Factors influencing articular cartilage wear in vitro," *Arthritis & Rheumatology*, vol. 25, no. 8, pp. 974–980, 1982.

[32] S. R. Oungoulian, K. E. Hehir, K. Zhu, C. E. Willis, A. G. Marinescu, N. Merali, C. S. Ahmad, C. T. Hung, and G. A. Ateshian, "Effect of glutaraldehyde fixation on the frictional response of immature bovine articular cartilage explants," *Journal of biomechanics*, vol. 47, no. 3, pp. 694–701, 2014.

[33] J. DeGroot, N. Verzijl, W.-V. Wijk, J. Marion, R. A. Bank, F. P. Lafeber, J. W. Bijlsma, and J. M. TeKoppele, "Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase–mediated degradation: The role of advanced glycation end products," *Arthritis & Rheumatology*, vol. 44, no. 11, pp. 2562–2571, 2001.

[34] S. Elder, A. Pinheiro, C. Young, P. Smith, and E. Wright, "Evaluation of genipin for stabilization of decellularized porcine cartilage," *Journal of Orthopaedic Research*, vol. 35, no. 9, pp. 1949–1957, 2017.

[35] V. Sitterle, J. Nishimuta, and M. Levenston, "Photochemical approaches for bonding of cartilage tissues," *Osteoarthritis and cartilage*, vol. 17, no. 12, pp. 1649–1656, 2009.

[36] Q. Meng, S. An, R. A. Damion, Z. Jin, R. Wilcox, J. Fisher, and A. Jones, "The effect of collagen fibril orientation on the biphasic mechanics of articular cartilage," *Journal of the mechanical behavior of biomedical materials*, vol. 65, pp. 439–453, 2017.

[37] V. Roth and V. Mow, "The intrinsic tensile behavior of the matrix of bovine articular cartilage and its variation with age." *The Journal of bone and joint surgery. American volume*, vol. 62, no. 7, pp. 1102–1117, 1980.

[38] S.-Y. Woo, W. Akeson, and G. Jemmott, "Measurements of nonhomogeneous, directional mechanical properties of articular cartilage in tension," *Journal of biomechanics*, vol. 9, no. 12, pp. 785–791, 1976.

[39] S. Akizuki, V. C. Mow, F. Müller, J. C. Pita, D. S. Howell, and D. H. Manicourt, "Tensile properties of human knee joint cartilage: I. influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus," *Journal of Orthopaedic Research*, vol. 4, no. 4, pp. 379–392, 1986.

[40] C.-Y. Huang, A. Stankiewicz, G. A. Ateshian, and V. C. Mow, "Anisotropy, inhomogeneity, and tension–compression nonlinearity of human glenohumeral cartilage in finite deformation," *Journal of biomechanics*, vol. 38, no. 4, pp. 799–809, 2005.

[41] J. L. Zitnay, Y. Li, Z. Qin, B. H. San, B. Depalle, S. P. Reese, M. J. Buehler, S. M. Yu, and J. A. Weiss, "Molecular level detection and localization of mechanical damage in collagen enabled by collagen hybridizing peptides," *Nature communications*, vol. 8, p. 14913, 2017.

[42] P. Lewis and C. McCutchen, "Mechanism of animal joints: experimental evidence for weeping lubrication in mammalian joints," *Nature*, vol. 184, p. 1285, 1959.

[43] R. Krishnan, M. Kopacz, and G. A. Ateshian, "Experimental verification of the role of interstitial fluid pressurization in cartilage lubrication," *Journal of Orthopaedic Research*, vol. 22, no. 3, pp. 565–570, 2004.

[44] H. Forster and J. Fisher, "The influence of continuous sliding and subsequent surface wear on the friction of articular cartilage," *Proceedings of the Institution of Mechanical Engineers, Part H: Journal of Engineering in Medicine*, vol. 213, no. 4, pp. 329–345, 1999.

[45] H. J. Helminen, M. M. Hyttinen, M. J. Lammi, J. P. Arokoski, T. Lapveteläinen, J. Jurvelin, I. Kiviranta, and M. I. Tammi, "Regular joint loading in youth assists in the establishment and strengthening of the collagen network of articular cartilage and contributes to the prevention of osteoarthrosis later in life: a hypothesis," *Journal of Bone and Mineral Metabolism*, vol. 18, no. 5, pp. 245–257, 2000.

[46] K. Puustjärvi, J. Nieminen, T. Räsänen, M. Hyttinen, H. J. Helminen, H. Kröger, J. Huuskonen, E. Alhava, and V. Kovanen, "Do more highly organized collagen fibrils increase bone mechanical strength in loss of mineral density after one-year running training?" *Journal of bone and mineral research*, vol. 14, no. 3, pp. 321–329, 1999.

[47] D. L. Bartel, D. T. Davy, and T. M. Keaveny, *Orthopaedic biomechanics: mechanics and design in musculoskeletal systems*. Upper Saddle River, 2006.

[48] T. Lyyra, I. Kiviranta, U. Väätäinen, H. J. Helminen, and J. S. Jurvelin, "In vivo characterization of indentation stiffness of articular cartilage in the normal human knee," *Journal of biomedical materials research*, vol. 48, no. 4, pp. 482–487, 1999.

[49] J. S. Jurvelin, J. P. Arokoski, E. B. Hunziker, and H. J. Helminen, "Topographical variation of the elastic properties of articular cartilage in the canine knee," *Journal of biomechanics*, vol. 33, no. 6, pp. 669–675, 2000.

[50] J. M. Donohue, D. Buss, T. R. Oegema Jr, and R. C. Thompson Jr, "The effects of indirect blunt trauma on adult canine articular cartilage." *JBJS*, vol. 65, no. 7, pp. 948–957, 1983.

[51] D. R. Osmon, E. F. Berbari, A. R. Berendt, D. Lew, W. Zimmerli, J. M. Steckelberg, N. Rao, A. Hanssen, and W. R. Wilson, "Diagnosis and management of prosthetic joint infection: clinical practice guidelines by the infectious diseases society of america," *Clinical Infectious Diseases*, vol. 56, no. 1, pp. e1–e25, 2012.

[52] R. Haut, T. Ide, and C. De Camp, "Mechanical responses of the rabbit patello-femoral joint to blunt impact," *Journal of biomechanical engineering*, vol. 117, no. 4, pp. 402–408, 1995.

[53] J. D. Backus, B. D. Furman, T. Swimmer, C. L. Kent, A. L. McNulty, L. E. DeFrate, F. Guilak, and S. A. Olson, "Cartilage viability and catabolism in the intact porcine knee following transarticular impact loading with and without articular fracture," *Journal of Orthopaedic Research*, vol. 29, no. 4, pp. 501–510, 2011.

[54] W. N. Newberry, C. D. Mackenzie, and R. C. Haut, "Blunt impact causes changes in bone and cartilage in a regularly exercised animal model," *Journal of orthopaedic research*, vol. 16, no. 3, pp. 348–354, 1998.

[55] M. Lotz, "Cytokines in cartilage injury and repair." *Clinical orthopaedics and related research*, vol. 391, pp. S108–S115, 2001.

[56] W. Wilson, C. van Burken, C. van Donkelaar, P. Buma, B. van Rietbergen, and R. Huiskes, "Causes of mechanically induced collagen damage in articular cartilage," *Journal of Orthopaedic Research*, vol. 24, no. 2, pp. 220–228, 2006.

[57] A. C. Gelber, M. C. Hochberg, L. A. Mead, N.-Y. Wang, F. M. Wigley, and M. J. Klag, "Joint injury in young adults and risk for subsequent knee and hip osteoarthritis," *Annals of internal medicine*, vol. 133, no. 5, pp. 321–328, 2000.

[58] E. M. Roos, "Joint injury causes knee osteoarthritis in young adults," *Current opinion in rheumatology*, vol. 17, no. 2, pp. 195–200, 2005.

[59] M. K. Lotz, "New developments in osteoarthritis: posttraumatic osteoarthritis: pathogenesis and pharmacological treatment options," *Arthritis research & therapy*, vol. 12, no. 3, p. 211, 2010.

[60] W. C. Kramer, K. J. Hendricks, and J. Wang, "Pathogenetic mechanisms of posttraumatic osteoarthritis: opportunities for early intervention," *International journal of clinical and experimental medicine*, vol. 4, no. 4, p. 285, 2011.

[61] F. Nelson, R. Billinghurst, I. Pidoux, A. Reiner, M. Langworthy, M. McDermott, T. Malogne, D. Sitler, N. Kilambi, E. Lenczner *et al.*, "Early post-traumatic osteoarthritis-like changes in human articular cartilage following rupture of the anterior cruciate ligament," *Osteoarthritis and cartilage*, vol. 14, no. 2, pp. 114–119, 2006.

[62] J. A. Buckwalter, "Articular cartilage: injuries and potential for healing," *Journal of Orthopaedic & Sports Physical Therapy*, vol. 28, no. 4, pp. 192–202, 1998.

[63] G. Schulze-Tanzil, "Activation and dedifferentiation of chondrocytes: implications in cartilage injury and repair," *Annals of Anatomy-Anatomischer Anzeiger*, vol. 191, no. 4, pp. 325–338, 2009.

[64] A. Joukar, A. Shah, A. Kiapour, A. S. Vosoughi, B. Duhon, A. K. Agarwal, H. Elgafy, N. Ebraheim, and V. K. Goel, "Gender specific sacroiliac joint biomechanics during standing upright: A finite element study." *Spine*, 2018.

[65] A. Joukar, J. Mehta, D. Marks, and V. K. Goel, "Lumbar-sacral destruction fixation biomechanics: A finite element study," *The Spine Journal*, vol. 17, no. 11, p. S335, 2017.

[66] A. Joukar, "Gender specific sacroiliac joint biomechanics: A finite element study," Ph.D. dissertation, University of Toledo, 2017.

[67] D. D'lima, S. Hashimoto, P. Chen, C. Colwell, and M. Lotz, "Human chondrocyte apoptosis in response to mechanical injury," *Osteoarthritis and Cartilage*, vol. 9, no. 8, pp. 712–719, 2001.

[68] D. M. Phillips and R. C. Haut, "The use of a non-ionic surfactant (p188) to save chondrocytes from necrosis following impact loading of chondral explants," *Journal of orthopaedic research*, vol. 22, no. 5, pp. 1135–1142, 2004.

[69] J. Martin and J. Buckwalter, "Post-traumatic osteoarthritis: the role of stress induced chondrocyte damage," *Biorheology*, vol. 43, no. 3, 4, pp. 517–521, 2006.

[70] J. A. Martin, D. McCabe, M. Walter, J. A. Buckwalter, and T. O. McKinley, "N-acetylcysteine inhibits post-impact chondrocyte death in osteochondral explants," *The Journal of Bone and Joint Surgery. American volume.*, vol. 91, no. 8, p. 1890, 2009.

[71] A. Joukar, E. Nammakie, and H. Niroomand-Oscuii, "A comparative study of thermal effects of 3 types of laser in eye: 3d simulation with bioheat equation," *Journal of thermal biology*, vol. 49, pp. 74–81, 2015.

[72] C. M. Bonitsky, *Wear and friction characteristics and crosslinked properties of genipin and photochemical crosslinked articular cartilage.* University of Notre Dame, 2015.

[73] A. L. Arvayo, I. J. Wong, J. L. Dragoo, and M. E. Levenston, "Enhancing integration of articular cartilage grafts via photochemical bonding," *Journal of Orthopaedic Research®*, 2018.

[74] Y.-R. Jeng, C.-P. Mao, and K.-T. Wu, "Instrumented indentation investigation on the viscoelastic properties of porcine cartilage," *Journal of Bionic Engineering*, vol. 10, no. 4, pp. 522–531, 2013.

[75] F. Richard, M. Villars, and S. Thibaud, "Viscoelastic modeling and quantitative experimental characterization of normal and osteoarthritic human articular cartilage using indentation," *Journal of the mechanical behavior of biomedical materials*, vol. 24, pp. 41–52, 2013.

[76] J. D. Kaufman and C. M. Klapperich, "Surface detection errors cause overestimation of the modulus in nanoindentation on soft materials," *Journal of the mechanical behavior of biomedical materials*, vol. 2, no. 4, pp. 312–317, 2009.

# APPENDIX

# A. TRIBOLAB WEAR TEST CODE

```
<?xml version="1.0" encoding="UTF-8"?>
<UMT>
    <!--Script-->
    <ScriptVersion CtsxVersion="1"/>
    <ScriptBinder AltUnit="" ChannelsToSave="Fx;Fz;Z3;V3"/>
    <Sequences index="0" IsSelected="1">
        <TakeoffItem>
            <TouchForce Value="0.200000"/>
            <PretouchSpeed Value="1.000000"/>
            <TouchSpeed Value="0.100000"/>
        </TakeoffItem>
        <AutopilotItem>
            <TolerancePlus Value="0.020000"/>
            <LoopExternal>
                <ParamDefault Value="1"/>
            </LoopExternal>
        </AutopilotItem>
        <LandingItem/>
        <BlackboxItem>
            <DataFileName Value="C:\file location\file name.tst"/>
            <FilterSize Value="15"/>
            <ChannelNames>
                <Value index="0" value="Fx"/>
                <Value index="1" value="Fz"/>
                <Value index="2" value="Z1"/>
```

```
            <Value index="3" value="Z2"/>
            <Value index="4" value="Z3"/>
            <Value index="5" value="V1"/>
            <Value index="6" value="V2"/>
            <Value index="7" value="V3"/>
        </ChannelNames>
        <ChannelUnits>
            <Value index="0" value="N"/>
            <Value index="1" value="N"/>
        </ChannelUnits>
    </BlackboxItem>
    <NameItem>
        <SequenceName Value="Apply Load"/>
    </NameItem>
    <Steps index="0">
        <CarriageItem>
            <Force1 Value="160.000000"/>
            <Duration Value="5.000000"/>
            <ParameterDur>
                <ParamDefault Value="5.000000"/>
            </ParameterDur>
            <ForceProfile/>
            <FocusingDist Value="2.000000"/>
            <TargetLocation Value="1"/>
            <OscPID_I Value="0.000100"/>
        </CarriageItem>
        <Spindle1Item/>
        <Spindle2Item/>
        <SliderItem/>
```

```
            <NameItem/>
            <StrainItem/>
            <HeaterItem/>
            <PumpsItem/>
            <CP4Item/>
            <PowerSupplyItem/>
            <PiezoItem/>
            <XYSynchItem/>
        </Steps>
    </Sequences>
    <Sequences index="1" IsSelected="1">
        <TakeoffItem>
            <TouchForce Value="0.200000"/>
            <PretouchSpeed Value="2.000000"/>
            <TouchSpeed Value="1.000000"/>
        </TakeoffItem>
        <AutopilotItem>
            <TolerancePlus Value="0.020000"/>
            <LoopExternal>
                <ParamDefault Value="1"/>
            </LoopExternal>
        </AutopilotItem>
        <LandingItem/>
        <BlackboxItem>
            <CreateDataFile Value="1"/>
            <DataFileName Value="C:\file location\file name.tst"/>
            <FilterSize Value="15"/>
            <ChannelNames>
                <Value index="0" value="Fx"/>
```

```
            <Value index="1" value="Fz"/>
            <Value index="2" value="Z3"/>
            <Value index="3" value="V3"/>
        </ChannelNames>
        <ChannelUnits>
            <Value index="0" value="N"/>
            <Value index="1" value="N"/>
        </ChannelUnits>
    </BlackboxItem>
    <NameItem>
        <SequenceName Value="Reciprocating Test"/>
    </NameItem>
    <Steps index="0">
        <CarriageItem>
            <Force1 Value="160.000000"/>
            <Duration Value="43.500000"/>
            <ParameterDur>
                <ParamDefault Value="156600.000000"/>
            </ParameterDur>
            <TimeUnit Value="2"/>
            <MoveUpAtEnd Value="1"/>
            <ForceProfile/>
            <FocusingSpeed Value="0.200000"/>
            <FocusingDist Value="2.000000"/>
            <TargetLocation Value="1"/>
            <OscPID_I Value="0.000100"/>
            <AbortSpeed Value="1.000000"/>
        </CarriageItem>
        <Spindle1Item/>
```

```
            <Spindle2Item/>
            <SliderItem>
                <Distance Value="18.000000"/>
                <ParameterOffset>
                    <ParamDefault Value="18.000000"/>
                </ParameterOffset>
                <MotionType Value="4"/>
                <CycleDelay2 Value="1.000000"/>
                <Speed Value="4.000000"/>
                <ParameterSpeed>
                    <ParamDefault Value="4.000000"/>
                </ParameterSpeed>
                <CyclesCount Value="14000"/>
                <CycleDelay1 Value="1.000000"/>
            </SliderItem>
            <NameItem/>
            <StrainItem/>
            <HeaterItem/>
            <PumpsItem/>
            <CP4Item/>
            <PowerSupplyItem/>
            <PiezoItem/>
            <XYSynchItem/>
        </Steps>
    </Sequences>
    <SGUIParameters/>
</UMT>
```

# B. MATLAB CODE FOR AVERAGE COF CALCULATION

```
clear all
clc
data = xlsread('F:file directory.xlsx');
a = data(:,5);
b=zeros(596,1);
j=1;
sum=0;
for i = 1:596670;
    sum=sum+a(i);
    if i==1000*j
        b(j) = sum/1000;
        sum=0;
        j=j+1;
    end

end
disp(b);
```

# C. PAPAIN DIGESTION

## C.1 Papain Buffer (PB, can be stored at RT)

To make 10 ml of PB:

1. 10 ml DI H20
2. 100 l EDTA
3. 156 mg Sodium phosphate, monobasic dihydrate
4. pH to 6.5

## C.2 Activated Papain Solution (make fresh)

To make 10 ml Activated Papain Solution

1. Add 8.8 mg L-Cysteine to 10 mL PB
2. Calculate volume of papain needed:Requires 125 $\mu$g/ml final papain concentration. *Note that the stock papain concentration changes from batch to batch. Update any calculations based on the current lot of papain. Total= 125$\mu$g/ml * 10ml = 1250$\mu$g = 1.25mg Stock Papain = 21 mg/ml Therefore (1.25/21) * 1000 = 59.52 $\mu$l
3. Remove papain from bottle using sterile syringe in hood and place in a 500 $\mu$l tube
4. Remove from hood and add calculated volume of papain to Activated Papain Solution

## C.3 Papain Digestion

1. Add Activated Papain Solution to each sample in a 1 ml tube
2. 1 ml per cartilage explant
3. Place tubes in a $60^0$C water bath overnight
4. The next morning, place tubes in oven at $80^0$C for 15 minutes
5. Samples can be stored at $-80^0$C until use for future biochemical assays

## C.4 Hydroxyprolin Assembly

Hydrolyzation & Neutralization procedure for media:

1. Prepare samples/PB (100 l) in 0.5 ml O-ringed screw capped micro-centrifuge tubes.
2. Add 100 $\mu$L of concentrated HCL (38%) to each tube.
3. Seal the micro-tube, vortex and spin briefly using the bench-top centrifuge.
4. Incubate at $110^0$C for 18 hours.
5. After cooling, centrifuge the micro-tubes at 10000g for 5 mins.
6. Dry the samples in a heating block at $60^0$C in a fume hood with the lids off. Usually takes 48-72 hours.
7. Dissolve in 200 $\mu$L ultra-pure $H_2O$.
8. Vortex until re-suspended, and spin the micro-tubes using the small bench-top centrifuge.(For the lower concentration samples, e.g. day 0, there may be some sediment which wont re-suspend, in this case, centrifuge this sediment to the bottom of the tube with bench-top centrifuge and take sample from top of tube, taking care to avoid the sediment.)
9. Samples can be analysed immediately or stored at $4^0$C for up to 1 week.

## C.5 Hydroxyprolin Stock Solution

1. Add 40 mg of trans-4-Hydroxy-L-proline (56250, Fluka) to 40 ml of ultra pure H20 to create 1 mg/ml stock solution.
2. Label and store at $4^0$C.
3. Solution expires after 3 months.

## C.6 Citrate Stock Buffer

1. Prepare 100 ml of solution: Use fresh batch for each experiment.
   - 80 ml Ultra pure $H_20$
   - 5.04 g Citric acid monohydrate (C1909, Sigma, MW 210.14)
   - 11.98 g Sodium acetate trihydrate (S7670, Sigma, MW 136.08)
   - 7.22 g Anyhdrous sodium acetate (71183, Sigma, MW 82.03)
   - 3.4 g Sodium hydroxide (M137, Amresco, MW 40)
   - 1.26 ml Glacial acetic acid (42322-5000, Acros)
2. Adjust pH to 6.1.
3. Add ultra pure H20 to bring total volume to 100 ml.
4. Filter with filter paper (09-790-4B, Fisher).
5. Label and store at $4^0$C.

## C.7 Assay Buffer (per 2 plates)

Prepare fresh in a 15 ml Falcon tube before assay by dissolving:

- 1.5 ml 1-propanol (43848, Alfa-Aesar)
- 1 ml ultra pure $H_20$
- 5 ml citrate stock buffer

### C.8 Choloramin T Reagent (per plate):

1. Prepare fresh in a 15 ml Falcon tube before assay by dissolving:
   - 141 mg Chloramine T (857319-100g, Sigma, MW 227.6)
   - 0.5 ml ultra pure $H^20$
2. Place in oven at $60^0$C for 10 min to dissolve. Then add:
   - 0.5 ml 1-propanol (43848, Alfa-Aesar)
   - 4 ml citrate stock buffer
3. Wrap the tube in tinfoil until use

### C.9 DMBA Reagent (per plate)

1. Prepare fresh in a 15 ml Falcon tube before assay by mixing:
   - 6 ml 1-propanol (43848, Alfa-Aesar)
   - 3 ml 70% perchloric acid (2766-14, Macron)

### C.10 Hydroxyproline Standards for hydrogels (per 5 plates)

1. Create a 50 $\mu$g/ml hydroxyproline solution from the 1 mg/mL stock solution:
   - Take 50 $\mu$l of hydroxyproline stock solution (1mg/ml)
   - Add 950 $\mu$l PB
2. Vortex and briefly spin hydroxyproline solution (50 $\mu$g/ml) in benchtop centrifuge.
3. Prepare standards in 1.5 ml microtubes as detailed in table below
4. Vortex standards and spin briefly in benchtop centrifuge

Table C.1.
Dilution of standard HYP working solution with PB as table below:

| Standard ID | Vol 50g/ml HYP ($\mu$l) | Vol PB to 1000l | Add 60 $\mu$l to each well (ng/well) |
|---|---|---|---|
| 1 | 0 | 1000 | 0 |
| 2 | 5 | 995 | 15 |
| 3 | 20 | 980 | 60 |
| 4 | 40 | 960 | 120 |
| 5 | 70 | 930 | 210 |
| 6 | 100 | 900 | 300 |
| 7 | 125 | 875 | 325 |
| 8 | 150 | 850 | 450 |

### C.11 Assay Procedure

1. Add 60 $\mu$l (in triplicate) of standards(Fluka) and samples to wells of a clear round-bottomed 96 well plate.
2. Using the multichannel pipet, add to each well:
   - 20 $\mu$l of Assay Buffer
   - 40 $\mu$l of Chloramine-T Reagent
3. Cover plate with tinfoil and incubate for 20min at room temperature to allow hydroxyproline oxidation to complete
4. Add 80 $\mu$l of DMBA reagent using the multichannel pipet. Solution will become cloudy. It is essential to mix the contents using the pipet until the solution becomes clear.
5. Cover with SealPlate and incubate in an oven at $60^0$C for 20 mins.
6. Cover plate with tinfoil and place on baking tray and allow to cool for 25 mins.
7. Remove sealplate carefully after cooling.
8. Read in plate reader at 570 nm. Hydroxyproline levels in samples can be estimated from the standard curve.

# D. DMMB ASSAY FOR CARTILAGE TISSUE

## D.1 Materials

- Chondroitin Sulfate (Sigma, C4384)
- 1,9-Dimethyl-Methylene Blue (Sigma, 341088-1G)
- Formic Acid (EMD, FX0440)
- 1 M NaOH
- Papain Buffer (PB)
- Clear, round bottomed 96-well plate

## D.2 Protocol

1. Prepare standards:
   - Dissolve 1mg chondroitin sulfate into 1 ml PB (1 mg/ml, stock solution)
   - Dilute stock solution 1:10 in PB (100 $\mu$g/ml, standard working solution

Table D.1.
Dilution of standard DMMB working solution with PB as table below:

| $\mu$g GAGs | $\mu$L working solution | $\mu$L PB |
|---|---|---|
| 0 | 0 | 100 |
| 1.25 | 12.5 | 87.5 |
| 2.5 | 25 | 75 |
| 5 | 75 | 25 |
| 10 | 100 | 100 |

2. Prepare DMMB Stock Solution (wrap in foil and store at RT):
    - 16 mg DMMB
    - 2 ml Format Buffer (15.4 $\mu$l Formic Acid, 29.4 $\mu$l 1M NaOH, 5 ml DI $H_20$, pH=3.5)
    - 5 ml EtOH
    - 1.91 g GuHCl
    - 93 ml DI $H_20$
3. Prepare DMMB Working Solution (make fresh):
    - Dilute Stock Solution in DI H20 1:4.75
4. Prepare Dissociation Reagent:
    - 90 ml PB
    - 10 ml 1-propanol
    - 38.2 g GuHCl
5. For gels, place a total of 100 $\mu$l (sample+PB) into new 1.5 ml micro-centrifuge tubes. For media samples, place a total of 400 $\mu$l (sample+DMEM) into new 1.5 ml micro-centrifuge tubes. Add 1 ml of DMMB Working Solution to each sample, and rotate/mix well for 30 minutes.
6. Centrifuge at 15,000xg for 10 minutes. All dye bound to sGAG will pellet at the bottom of the tube.
7. Remove solution from the above the and leave pallet at the bottom without disturbance.
8. Add 1 ml Dissociation Reagent to each tube, and vortex/mix until pellet is fully dissolved.
9. Pipet 200 $\mu$l of standards in triplicate into a 96 well plate
10. Pipet 200 $\mu$l of samples in triplicate into plate.
11. Analyze using DMMB Assay protocol on plate reader.

12. Calculate $\mu$g of sGAG by fitting data to linear standard curve.

# E. PICO GREEN ASSAY

## E.1 Solutions

Add 2 ml 20x TE to 38 ml nuclease free $H_2O$. Make up in 50 ml centrifuge tube. Save excess for future use.

### DNA Standards

1. Prepare standard curve with DNA provided in PicoGreen kit.
2. Store at $4^0$C for future use.

### PicoGreen Working Solution

1. Dilute PicoGreen stock solution 1:200 in TE buffer.
2. only what you need. Protect from light.

Table E.1.
Dilution of standard DNA working solution with TE as table below:

| | Final Conc. in well (ng) | Volume DNA ($\mu$l) | Volume TE (ml) |
|---|---|---|---|
| A | 0 | 0 | 1.2 |
| B | 1 | 120 of C | 1.08 |
| C | 10 | 120 of D | 1.08 |
| D | 100 | 120 of E | 1.08 |
| E | 1000 | 26 $\mu$l(100$\mu$g/ml) | 1.17 |

## E.2 Protocol

1. Pipet 100 $\mu$l standards in triplicate into black microplate.
2. Diluted the stock solution 2:1000 $\mu$l with 1X TE Buffer.
3. Add 3 $\mu$l and 20 $\mu$l of diluted solution from the full thickness and the superficial zone. For making 100 $\mu$l of sample 97 $\mu$l and 80 $\mu$l of 1X TE buffer have added in the black plate reader. Note: Those ratios can change based on amount of DNA present in sample, but the total MUST be 100 $\mu$l to match standard.
4. Add 100 $\mu$l PicoGreen working solution to each well using the multipipette.
5. Protect from light. Incubate 5 minutes at room temperature.
6. Read plate using the fluorescent PicoGreen protocol on plate reader.
7. Calculate ng of DNA by fitting data to linear standard curve.