Biotribology:
Studies of the Effects of Biochemical Environments on the Wear and Damage of Articular Cartilage

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(ABSTRACT)

Tribology is the science of interacting surfaces in relative motion. It is specifically concerned with the friction, wear and lubrication of these surfaces. Although tribology has conventionally been associated with the surface interaction of mechanical systems, concepts of tribology have also been important in the study of biological systems. Biotribology is one of the newest fields to emerge in the discipline of tribology. It can be described as the study of friction, wear and lubrication of biological systems, mainly synovial joints such as the human hip and knee.

Osteoarthritis (OA) is partially characterized by the loss of articular cartilage from the contacting surface of the articulating bones in synovial joints. Although it has been studied extensively, the exact pathways and pathogenesis of OA have yet to be determined. Several factors have been cited as possible contributors to the condition. These factors can primarily be grouped into two categories of mechanical or biochemical abnormalities. Research in biotribology enables the examination of both the mechanical and biochemical factors involved in joint lubrication and OA. This research has focussed on the mechanisms of normal joint lubrication, as well as the possible connections between biotribology and osteoarthritis. Particular emphasis is placed on the effects of biochemical changes and environment on cartilage wear and damage.

Studies were carried out using a test device developed to study the tribological properties of articular cartilage, *in vitro*. A cartilage-on-cartilage test configuration was used with bovine articular cartilage and a cartilage-on-stainless steel configuration used with lapine articular cartilage. Articulating surfaces were put in sliding contact under a normal load. Natural and biochemically modified environments were created to simulate possible normal and pathologic *in vivo* conditions. Wear and friction of the articular cartilage were measured and related to

biochemical environments which are suspected in clinical cases of OA. Quantitative measurement of cartilage wear was achieved through hydroxyproline assay of the post-test lubricants. Surface and subsurface damage were also examined through the use of scanning electron microscopy and histological staining techniques.

The results of four separate studies demonstrated that: (1) exposure of bovine cartilage to collagenase-3, an enzyme suspected in the cartilage degeneration seen in OA, significantly increased cartilage wear ($p = 0.001$); (2) lapine cartilage with surgically induced OA exhibited higher coefficients of friction, but no significant increase in wear over normal cartilage from the same animal; (3) the addition of white blood cell lysate, comparable to what would be seen in mild joint inflammation, to synovial fluid significantly increased cartilage wear over normal synovial fluid ($p = 0.002$); (4) the removal of "boundary lubricating" surface-active phospholipids (SAPLs) from normal synovial fluid had no significant effect on cartilage wear.

These results demonstrate that biochemical changes in the cartilage, as well as the synovial fluid, can lead to increased wear of and damage to the articular cartilage surface. How these changes may occur in living systems remains to be determined. The use of the tribological test device developed and various analytical techniques has made it possible to quantitatively evaluate the effects of biochemical changes and environment on the wear and damage of articular cartilage. These studies have demonstrated that research in biotribology has the potential to make significant contributions to the current knowledge not only of normal joint lubrication but of joint pathology as well.

Dedication

I dedicate this work and the accomplishment it represents to the One who has made it possible. You have shown me that through faith in You, any and all things are possible. I thank You for all the blessings You have bestowed upon me. I will live my life in service to You.

The Lord is my strength and my shield, my heart trusts in him and I am helped.

Psalm 28:7

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TABLE OF CONTENTS

List of Figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

List of Tables

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

1 INTRODUCTION

1.1 Tribology

Tribology was officially defined in 1966 by the U.K. Department of Education and Science Report as the '*science and technology of interacting surfaces in relative motion and the practices related thereto*,' [1]. The purpose of establishing tribology as a scientific discipline was to focus scientific and technological attention on the processes involved when surfaces are rubbed together [2]. The discipline is specifically concerned with the fiction, wear and lubrication of surfaces in relative motion, usually under some form of normal load [2].

Tribology is an interdisciplinary subject involving principles of physics, chemistry, material science, mechanics, engineering, rheology and mathematics, often all at once [3,4]. Tribological behavior is influenced by the physical, mechanical and chemical properties of the surface and subsurface material [5], in addition to the conditions under which the relative motion takes place. Applied load, sliding velocity, temperature, geometry and other physical and environmental conditions all influence the tribological behavior of any given system [3]. This is important to remember when evaluating data from tribological studies. The test conditions and parameters are of crucial importance when comparing tribological systems or predicting behavior.

The economic and technological importance of tribology have been substantially demonstrated [1]. Losses due to energy dissipation and material wear add up to billions of dollars annually in industrialized countries [4]. A proper understanding of tribological processes can provide a basis on which to improve standards of design, and increase engineering efficiency [2]. This has already had a significant impact on energy conservation issues which concern the future of mankind.

Although it has only recently been termed and defined, mankind's interest in the principles of tribology predate recorded history [1,4]. From the function of articular joints to that of the modern combustion engine, in almost every aspect of our daily lives, we are meeting some manifestation of tribology. However, tribology is still an emerging science, as indicated by the

numerous new academic disciplines and research centers emerging in the area. Metals are the most common tribological materials and have been studied in the greatest detail [5]. However, more recently there has been an increase in study of the tribological properties of ceramics, polymers and even biological tissues.

1.2 Biotribology

Tribological studies have been applied to every form of bearing system designed by man [6]. Biotribology is one of the newest fields of study to emerge in the area of tribology. In the manner in which tribology encompasses the study of friction, wear and lubrication of engineering materials, biotribology can be described as the study of these same phenomena in biological systems. Biological systems such as the jaw, spine, eyes and diarthrodial joints are all tribological systems that involve some form of friction, wear or lubrication of biological tissues. Like tribology, biotribology is also interdisciplinary. In addition to those disciplines associated with tribology, biotribology also involves of biomechanics, biochemistry, biology, physiology, clinical medicine and pathology.

Historically, biomechanical studies of joints were based on the need to better understand their structure-function relationship in providing joint motion and the pathomechanical processes involved in joint diseases such as osteoarthritis [6]. The goal of biotribologists is to evaluate biological systems and understand how they function with such tribological efficiency, providing increased understanding of their normal, as well as their pathologic states. In studies of the diarthrodial or synovial joint, the synovial fluid, articular cartilage and supporting bone are viewed as the essential materials forming a bearing system. The performance depends on the mechanical behavior of the materials comprising the joint [6]. Depiction of joint disease as the failure of bearing lubrication processes is an obvious oversimplification; however, the correlation between an engineering bearing and a synovial joint is an appropriate one. Examples of biotribology research include: the study of lubrication by synovial fluid, measurement of friction in synovial joints, the mechanisms of joint lubrication, measurement and analysis of cartilage wear and damage, study of joint mechanics, and the development of artificial joints. Research in

this area has contributed a great deal to the knowledge of normal joint function and the treatment of joint disease through joint replacement technology.

1.3 Possible Connections between Biotribology and Osteoarthritis

Osteoarthritis (OA), which is also known as osteoarthrosis or degenerative joint disease, is the most common form of arthritis and one of the most prevalent medical conditions in the western world. It is defined as *an extremely common, non-inflammatory, progressive disorder of movable joints, particularly weight-bearing joints, characterized pathologically by deterioration and progressive loss of the articular cartilage and by formation of new bone in the subchondral areas and at the margins of the joint* [7]. Despite the prevalence of the disorder, the exact etiology and sequence of events leading to OA have yet to be determined.

Due to the fact that one of the most profound changes seen in OA is the loss of cartilage which acts as the bearing material for articulating bones, there may be a natural inclination for the tribologist to presume that increased wear seen in OA can be studied from a tribological point of view. It is not being stated that OA is a tribological problem, only that biotribology may provide useful methodologies for the study of types of disease that involve changing tribological properties. Whether joint disease originates from changes in joint lubrication by synovial fluid or from changes in the *wear resistance* of the articular cartilage, both have the potential to be studied using the principles of tribology. Although there is a general absence of hypotheses connecting normal synovial joint lubrication and synovial joint degeneration, questions have been raised that suggest the role of biochemical constituents of the synovial fluid in the wear properties of cartilage [3,8]. The wear properties of the articular cartilage itself have also been discussed [9,21,23-26].

Over the past several years, studies have been carried out to examine possible connections between tribology and the mechanisms of normal joint function, as well as joint degeneration such as seen with osteoarthritis [3,8-25]. The majority of papers written in the engineering discipline have focused mainly on friction and lubrication of normal articular joints. There have been well over thirty theories proposed to explain the lubrication mechanism in natural joints that

makes them tremendously effective in minimizing the friction between articular cartilage surfaces. However, none of these proposed theories completely describe normal joint behavior, nor do they provide a convincing model for the breakdown of these mechanisms that would lead to osteoarthritis. In addition, the previous work in this area has not included *in vitro* studies that use the cartilage- sliding-on-cartilage configuration of the natural joint.

Currently, there are several disciplines involved in trying to better understand the progression and causes of osteoarthritis. Papers are published regularly that individually explore the complex biochemistry, rheology, biomechanics, and clinical pathology of the disease. The novelty of biotribology research is that it is an interdisciplinary field that allows the contributions from many of these areas to be equally and simultaneously considered.

1.4 Rationale of Current Research Project

This project is part of ongoing research in the area of biotribology at Virginia Tech [8,9,18,19]. The overall objective of the research, as stated by Dr. Michael Furey, is "*to examine possible connections between tribology – the study of friction, wear and lubrication -- and mechanisms of synovial joint lubrication and articular cartilage degeneration, such as seen with osteoarthritis.*" As part of this objective, the studies done in this project are aimed at examining the effects of biochemical environments on the tribology of articular cartilage, particularly the wear and damage of the tissue.

Wear and damage of the cartilage is the predominant symptom of degenerative joint disease. Despite the fact that the precise etiology is unknown, it is generally accepted that the disease is a cascade of events, involving both mechanical and biochemical changes, leading to a common pathway. Due to the complex nature of the disorder, it is difficult to determine which of these changes contribute to the direct loss of the articular cartilage. The literature reflects an absence of information on how particular biochemical conditions detected in osteoarthritis affect cartilage wear and damage. In addition, there exists a lack of sufficient studies done with cartilaginous surfaces in sliding contact that adequately represent physiologic conditions.

The purpose of this project is to isolate several physical and biochemical conditions suspected in the etiology of osteoarthritis and determine their individual influences on cartilage wear and damage. Tribological wear tests done with a cartilage-on-cartilage testing configuration will be employed to more closely represent synovial joint physiology. This project has been designed to approach the study of joint disease in a novel and interesting manner and the results have the potential to lead to new approaches in the prevention and treatment of osteoarthritis and other joint diseases.

1.5 Objectives of the Project

The overall purpose of this research project is to examine the effects of the biochemistry of the synovial joint on the wear and damage of articular cartilage. The specific objectives are aimed at investigating effects of biochemical environment in relation to some of the current theories of osteoarthritis etiology. The following outlines the broad questions to be addressed by the research project, along with the more specific objectives for the experimental studies to examine:

1. Do certain biochemical changes in the pathologic synovial joint affect the wear and damage of articular cartilage?

- Do biochemical changes in the cartilage matrix lead to increased wear and damage?
- Do pathological environments, such as inflammation, affect cartilage wear and damage?

2. Does osteoarthritic cartilage have different tribological properties than normal cartilage?

- Does initial fibrillation of cartilage lead to accelerated wear and damage?

3. Do lipid constituents of synovial fluid provide boundary lubrication of the cartilage surfaces and protect them from excessive wear and damage?

- Do phospholipids in the synovial fluid protect cartilage surfaces from wear and damage?

2 BACKGROUND AND LITERATURE REVIEW

2.1 Normal Synovial Joints

There are over three hundred joints in the human body [26]. They can be classified into three main groups: synarthroses, amphiarthroses, and diarthroses. Diarthroses are movable joints that are encapsulated by a membrane called the synovial membrane or synovium. The synovium secretes a lubricating fluid, called synovial fluid, into the joint space between articulating bones. For this reason diarthroses are often called synovial joints. Synovial joints are remarkably complex systems that account for most of the body's articulation.

Synovial joints provide humans and animals with the facility of motion while minimizing friction and wear. Their purpose is to facilitate the articulation of one bone segment over another, allowing a wide range of motion and supporting considerable load. In addition, they are expected to function with little to no wear and provide nearly frictionless motion for the lifetime of the user. This is accomplished by their intricate design and complex components.

Synovial joints are generally regarded as consisting of a number of discrete elements, notably the capsule, articular cartilage surfaces, synovial fluid and the synovium (also referred to as the synovial membrane or synovial lining)[27]. **Figure 2.1** is a representation of a typical synovial joint, a simple human diarthrodial joint. The entire joint is enclosed in a strong fibrous capsule, referred to as the joint capsule or capsular ligament. The inside of the joint capsule is lined with the synovium, which is

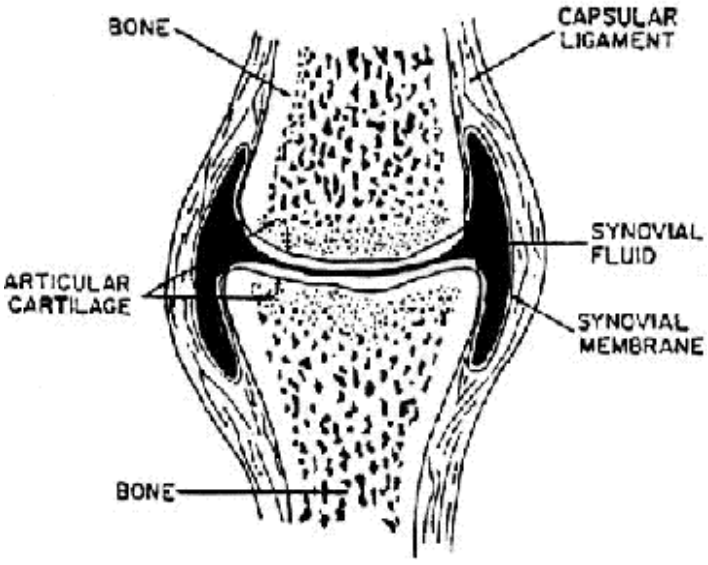

Figure 2. 1 Synovial Joint: a simple diarthrodial joint [14]

comprised of a thin layer of cells called synoviocytes. These cells secrete the synovial fluid into the joint space. In the joint space, the ends of the articulating bones are covered with a thin plate of dense cortical bone. A layer of articular cartilage covers the cortical bone and acts as an excellent bearing surface. Very little synovial fluid is present between the cartilage surfaces. Stability of the entire system is provided by surrounding ligaments and muscles that restrict motion while allowing necessary flexibility.

From an engineering standpoint, the synovial fluid, articular cartilage and supporting bone form a closed bearing system which provides the near frictionless articulation of the skeletal system [6]. The chemical, physical and structural characteristics of each joint element are presumably important to their exceptional function [27].

2.1.1 *Articular Cartilage*

Cartilage is a highly specialized skeletal tissue. It provides shape and form, yet ensures flexibility, durability and deformability. There are several types of cartilage found in the body. Articular cartilage is a form of hyaline cartilage and is found primarily covering the cortical bone in synovial joints. Its basic purpose is to provide a low-friction, low-wear bearing material for the articulating ends of bones in synovial joints. The thickness and make-up of articular cartilage varies from location to location, joint to joint and from species to species; however, its basic structure and function remain the same.

Cartilage is a composite material with both solid and fluid components that vary in concentration and structure through the depth of the material. It consists of both cellular (~5% of cartilage volume) and extracellular components (~95% volume). The cells of the cartilage are called chondrocytes and they secrete the extracellular cartilage matrix components. The extracellular components consist mainly of collagen, water, proteoglycan aggrecan, and glycosaminoglycans (GAGs). These components help cartilage to absorb and distribute the compressive forces experienced by the synovial joint.

The following sections describe the constituents and structure of articular cartilage and how they function together to provide synovial joints with their remarkable tribological properties (i.e., friction, wear and lubrication properties). The morphology, histology and mechanical properties of the cartilage will also be discussed in relation to the studies done as part of this research project.

Constituents and Structure

Most human adult articular cartilage is of the hyaline type [28]. Hyaline cartilage is the precursor of developing bones [29] and is named for its bluish-white, translucent appearance. Mature articular cartilage is avascular and aneural, with very few cells. The cells are called chondrocytes and they are responsible for maintaining the cartilage matrix. The extracellular components consist of interstitial water (65-80%), type II collagen, and ground substance. The ground substances consist of proteoglycan aggrecan, GAGs and other smaller unbound proteins. The structure and constituents of articular cartilage are responsible for its exceptional material and mechanical properties.

Type II collagen accounts for more than half of the dry weight of cartilage and about 15-25% of the wet weight [28,30]. This collagen has three long polypeptide chains that are wound together to form a triple helical structure. This structure gives collagen remarkable stability. Hydroxyproline is one of the amino acids found in the collagen structure and makes up roughly 8-12% of the amino acid sequence. This amino acid is found almost exclusively in type II collagen. The collagen structure is mainly observed in long fibers that group together in bundles of varying orientation in the articular cartilage.

Proteoglycans are the major family of macromolecules of the ground substance, comprising 22-38% of the dry weight of adult articular cartilage [31]. The structure model of these molecules is similar to that of a bottle brush. Hyaluronic acid, a non-sulfated glycosaminoglycan, is the root of the structure. Proteins link hyaluronic acid to collagen in a “bottle brush” configuration. Core proteins attach to the linking proteins and provide a place for proteoglycans to bind. The

proteoglycans continue to bind and form proteoglycan aggregates, also called aggrecan aggregates [28,31,32].

The individual proteoglycans consist of polysaccharide chains called glycosaminoglycans, or GAGs. The GAGs of the proteoglycan include chondroitin sulfate and keratan sulfate. These form long negatively charged chains which make the proteoglycans highly negatively charged molecules that can bind up to 50 times their weight in water. These aggregates stabilize the cartilage matrix and provide its compressive properties. The proteoglycan structure is shown in **Figure 2.2**.

The distribution of the chondrocytes and of matrix constituents varies throughout the thickness of the cartilage. The cartilage is composed of four different zones or layers: superficial, intermediate (or middle), deep and calcified layers. These layers are situated parallel to one another and anchored to the subchondral bone. The chondrocyte and collagen alignment, as well as the proteoglycan distribution, vary throughout these layers in a fashion that reflects the tensile and compressive forces and shear stresses acting on the tissue.

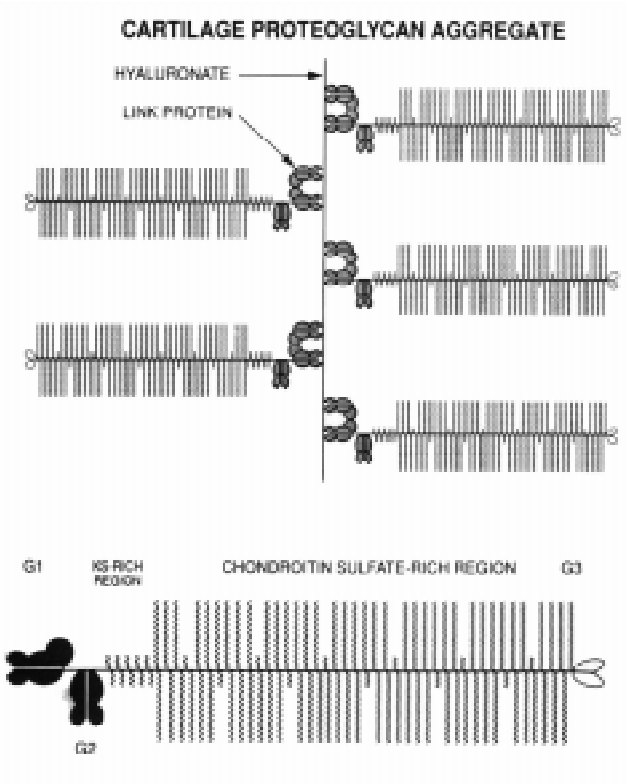

Figure 2. 2 Proteoglycan aggrecan and individual proteoglycan structures. [32]

The superficial layer is the uppermost layer of the cartilage and is tangent to the joint surface. It is the thinnest of the cartilage layers and is maximally exposed to the shearing, compressive and tensile forces seen in the synovial joint. In this layer the chondrocytes are most dense and are

aligned with their long axis parallel to the surface. They are long and flat in shape and relatively inactive. The collagen fibers are smallest in diameter in the superficial layer and are tightly packed and aligned parallel to the joint surface. The proteoglycan aggrecan is in its lowest concentration in the superficial layer.

The middle or intermediate layer lies directly below the superficial layer. This layer is several times the volume of the preceding superficial layer [32]. Here, the collagen fibers are larger in diameter and are oriented in a random meshwork. The proteoglycans are more abundant in this layer and fairly evenly distributed throughout the meshwork of collagen fibers. The chondrocytes are more spherical or elliptical in shape and randomly oriented through the matrix.

The deep layer of cartilage is located between the middle and calcified layers. This layer is also referred to as the radial zone because of the radial orientation of the collagen fibers. The fibers are still large, as in the middle zone, but are arranged in a tighter mesh and run radially into the calcified layer and subchondral bone. Chondrocyte volume and density are at their lowest here and the cells are generally found in clusters that line up in a columnar fashion perpendicular to the joint surface. Proteoglycans are found in their highest content in this layer, while water content is at its lowest. In general, for the uncalcified cartilage layers, water content decreases with depth, while proteoglycan content and collagen fiber diameter increases [30].

The calcified layer of cartilage is located adjacent to the subchondral bone. The junction between the deep layer and the calcified layer is denoted by a tidemark that can be seen when cartilage is stained with hematoxylin and eosin. The calcified layer has a few small cells and acts as a "buffer zone" between the uncalcified cartilage and the subchondral bone [30]. Collagen fibers penetrate from the deep layer directly into the calcified cartilage, anchoring it to the underlying bone. Thus, its purpose mainly appears to be to provide the attachment of the uncalcified cartilage to the subchondral bone. **Figure 2.3** illustrates the cartilage layers and structure.

The cellular density, collagen fiber alignment and proteoglycan distribution of articular cartilage are specifically arranged to give the cartilage the necessary material and mechanical properties to withstand and distribute the tensile, compressive and shear stresses experienced during normal motion. These properties, explained in the following section, make articular cartilage an ultra-efficient bearing material for one of the most sophisticated tribological systems found in nature.

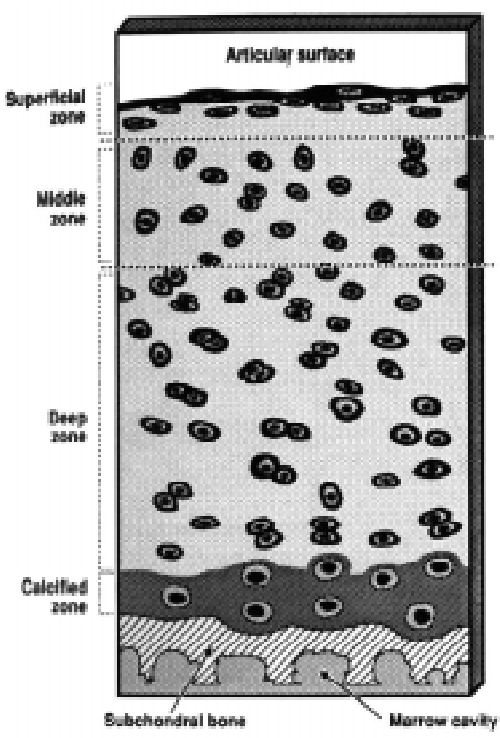

Figure 2. 3 Articular cartilage structure [30]

Material and Mechanical Properties

Human joints are specifically designed for transmitting large normal loads from one bone to another while allowing an efficient relative motion in a direction tangential to the surfaces [26]. These loads are often dynamic and vary from joint to joint. The structural organization of the articular cartilage matrix endows this tissue with special physical properties that enable it to absorb and dissipate loads and to provide a nearly frictionless articulating surface [26,30,31]. The matrix constituents have properties that are specially designed to provide these needed attributes.

Collagen is a tough fibrous protein that provides high tensile strength to biological tissues. The protein consists of three identical α-chains whose amino acid sequence consists mainly of glycine, proline and hydroxyproline, with glycine as every third amino acid [33]. This sequence allows the polypeptide to assume a triple helical structure. The triple helix is a very stable structure that resists tension and thereby confers a high tensile strength to the resulting collagen fibers. In articular cartilage, the tensile strength is primarily determined by the collagen fiber network, whose backbone is type II collagen.

Cartilage can experience compressive stresses up to 18 MPa [6]. The ability of cartilage to resist these loads is determined by the proteoglycan content. The proteoglycan aggregate is a group of large elastic macromolecules that are highly negatively charged and bind water. They expand in solution and resist compression into a smaller volume. The molecules swell, creating an internal pressure that gives articular cartilage great compressive strength and the ability to resist deformation and dissipate loads [29-31].

The distribution and structure of the collagen fibrils and proteoglycans come together in cartilage to provide optimal mechanical properties for normal joint function. In the superficial layer, the collagen fibers are tightly packed and aligned tangentially to the joint surface. The tensile strength of the collagen fibers allows the cartilage to resist the large tensile stresses that are experienced at the joint surface during articulation. The correlation between collagen content and tensile strength lessens as cartilage depth increases. This is due to the fact that less tensile stress is experienced in the deep layer of cartilage. However, the collagen network is maintained in the intermediate and deep layers in order to provide structure and stiffness, in conjunction with the proteoglycans.

The proteoglycans provide high compressive strength by binding water and keeping it in the matrix. The swelling of the proteoglycans is restricted by the collagen network. The proteoglycans, in turn, retard the rate of stretch and alignment of collagen when tensile load is suddenly applied [34]. Together, the collagen-proteoglycan matrix present in the middle and deep layers give cartilage elasticity, compressive stiffness and high energy- dissipation properties when subjected to shear stress.

With the combination of the above properties and the aforementioned structure, cartilage has been described as a viscoelastic, fluid-filled, fiber-reinforced, porous-permeable, composite material [35]. The viscoelasticity is determined by the cartilage response to loading, which combines viscosity and elasticity. Under a constant load or deformation, the cartilage response varies with time. The fact that articular cartilage can be 65-80% water makes it a fluid filled material that is reinforced by collagen fibers. The matrix structure makes the material porous and

permeable to certain solutes and nutrients that diffuse through the matrix. Varying content and alignment of the constituents throughout the depth of the cartilage make it comparable to a composite material. All these factors give normal articular cartilage mechanical properties that allow it to distribute stress applied to the subchondral bones with very low fiction and little to no wear.

Morphology

Electron microscopy is often used to look at the surface characteristics of biological tissues. Transmission Electron Microscopy (TEM), Environmental Scanning Electron Microscopy (ESEM) and Scanning Electron Microscopy (SEM) are the most frequently used techniques in examining cartilage morphology and topography. There has been much debate over the years about the true morphology of articular cartilage. Early morphological studies showed that contrary to information obtained from macroscopic examination, cartilage does not have an ultra smooth surface, but has an undulating surface with pits and bumps that correspond to underlying chondrocytes and proteoglycan molecules [36,37]. Many have contributed these surface features to the microscopy preparation techniques which cause dehydration and shrinkage of the tissue and create undulations and pitting of the surface [38,39].

Current improved preparation techniques reduce the dehydration of tissues prepared for viewing with electron microscopy. They have resulted in information supporting the idea that the cartilage surface in articular joints in indeed smooth, with very few surface features at all [38-41]. The protective superficial layer of the cartilage provides a very smooth articulating surface when observed with scanning electron microscopy. SEM shows very few surface asperities or undulations. In deeper layers, collagen fibril bundles can be observed and some large protein macromolecules are visible as well. The directionality of collagen fibers in the different layers of the cartilage can be detected if sectioning is done properly [42]. Clarke [43] concluded from high magnification SEM that dead cartilage tissue showed no visible difference from living tissue. Chai [44] and Pelligrini [37] also used SEM to classify various stage of cartilage degeneration.

Histology

Histochemical techniques are used to help evaluate the histological changes in biological tissues. For cartilage histology, thin sections are cut perpendicular to the joint surface and the layers of cartilage can be seen. Most biological tissues are colorless and need to be stained to make certain tissue components conspicuous and distinct. Stains or dyes are used to elucidate certain aspects of the tissue. Dyes used to stain tissues act like acid or basic compounds and form electrostatic (salt) bonds with ionizable groups of the tissue [45]. Tissue components that readily stain with basic dyes are termed *basophilic* and those with an affinity for acid dyes are called *acidophilic*. The most common basic dyes are: toluidine blue, methylene blue, hematoxylin and alcian blue. Common acid dyes are: orange G, eosin, and acid fuchsin. The acid components of the tissue bind to the basic dyes and basic components attract the acid dyes, hence the suffix *philic* at the end. In some histochemical techniques, the intensity of the color produced is directly proportional to the concentration of the substance being analyzed [46].

Collagen is the primary basic element of cartilage and proteoglycans and proteins, are the acidic elements. With most stains the basic elements appear light to dark pink to and the acidic elements appear blue to purple.

Some acid and basic dyes are used together to highlight different tissue components on the same sample. The most common of these is the hematoxylin and eosin stain (H&E). H&E is commonly used to examine the structure and distribution of cartilage constituents. In the superficial layer where the collagen is tightly packed and there are few cells and little to no proteoglycans, the thin layer appears as a dense dark pink strip at the top of the cartilage. The intensity of the pink stain lessens with the depth of cartilage because of the more random and spaced out organization of the collagen in the deeper layers. The pericellular matrix surrounding the chondrocytes are stained a more intense pink due to the increased collagen content around the cells. The cell nuclei appear blue due to the acidic nature of the cell cytoplasm.

Alcian blue is used as a basic stain that adheres to the proteoglycan and protein elements of the tissue. Normal tissue stained with alcian blue shows little affinity for the stain in the superficial layer where there are no proteoglycans and increasing affinity is seen in the deeper layers where the proteoglycan concentration increases. The chondrocytes stain an intense blue because of the basophilic nature of the cell nuclei.

2.1.2 Synovial Fluid

Synovial fluid is the lubricating fluid of the synovial joint. It is secreted into the joint space by the cells of the joint lining. The lining of the synovial joint is called the synovium and the cells that comprise the synovium and secrete the fluid are called synoviocytes. Normal synovial fluid is a dialysate of blood plasma that is comprised mainly of water (85%), hyaluronic acid and protein [27]. Most of the protein in synovial fluid is albumin. Other larger proteins derived from blood are present in small quantities. Phospholipids and cholesterol are also present in small quantities.

The synovial fluid appears to have three main purposes. The first is to carry nutrients to the articular cartilage, and the second to remove the waste products. The third purpose of the fluid is to provide lubrication for the articulating cartilage surfaces. The complete constituency of synovial fluid has yet to be determined. However, it has long been speculated that certain constituents are responsible for the extraordinary lubrication properties of the synovial joint. What these constituents are and by what mechanisms they provide joint lubrication are still being investigated. The numerous forms of joint lubrication theories that have been put forth thus far will be discussed in more depth in **Section 2.2**.

2.2 Tribology of Normal Joints

2.2.1 *Mechanical Function*

Human joints are specifically designed for transmitting large normal loads from one bone segment to another while allowing efficient relative motion in a direction tangential to the surfaces [47]. Due to the large mechanical disadvantage in the body, compressive stresses in diarthrodial joints can be much larger than that of body weight. Joints of the lower extremities

can experience loads of two and a half to ten times the actual body weight. Joint motion is characterized by frequent rapid starts and stops, both associated with high compressive loads. As a result, most physiologic loads are transient and result in high peak dynamic stresses.

During the walking cycle, high loads occur for a short period of time, while low loads exist for a longer period. To complicate things further, when the load is high, the sliding velocity is low (reaching zero at reversal of motion) and when the load is low, the velocity is high [47]. The specific joint configurations are designed to allow motion and create contact areas that are able to carry these complex dynamic loads. However, from a tribological viewpoint, these performance characteristics are severe and demand efficient lubrication processes to minimize friction and wear. The properties of the articular cartilage and lubrication by synovial fluid work in concert to achieve this. A brief review of some of the postulated mechanisms of joint lubrication are presented below.

2.2.2 *Joint Lubrication*

Lubrication is defined as the process of adding a solid, liquid, or gas to reduce friction and/or wear at the interface between two surfaces in relative motion [3]. In the synovial joint, lubrication is provided by the synovial fluid, which has been previously described. Joint lubrication has been studied extensively, with over two dozen proposed theories. There are several excellent reviews of joint lubrication theories located in literature [3,22,47-50]. Most of these theories are based on friction measurements and do not address wear. The following section is not a extensive review of the proposed theories, but rather a brief overview of the progression of joint lubrication research.

Early theories of joint lubrication were mainly variations of the hydrodynamic theory of lubrication first proposed by MacConaill [51]. Friction studies later carried out by Jones [52] with horse stifle joints led him to conclude that fluid film lubrication was present. Charnley [53] repeated these experiments and found conflicting results that led to the conclusion that boundary lubrication was the active mechanism in joints. The low coefficients of friction obtained in Charnely's work led to the proposed 'weeping' lubrication theory by McCutchen [54].

Dintenfass [55] was the first of several researchers to look at elastrohydrodynamic lubrication in synovial joints. Out of this work, Dowson [56] and Fein [57] added the importance of 'squeeze-film' lubrication. Mixed lubrication theories, by Linn [58], concluded that both hydrodynamic and boundary lubrication were acting concurrently in the joint. Investigation of the boundary lubrication of joints led to identification of a protein component of the synovial fluid called the lubricating glycoprotein [59]. These proteins are regarded as the boundary lubricating element of the synovial fluid. Other theories such as 'boosted' lubrication [60] and multimode lubrication [6,61] have also been proposed.

There continues to be much debate about the modes of joint lubrication operating in human synovial joints. The dynamic forces, velocities and nature of loading experienced by the joint require that different modes of lubrication act at different intervals of joint motion. This supports the more recent theories of mixed or multimode lubrication. A logical conclusion from the experiments carried out is that during periods of high velocity, fluid film lubrication exists while, during intervals of low to zero velocity, mixed lubrication is present with boundary friction dominating [22]. During periods of boundary lubrication, wear and damage of cartilage are more likely. This is the regime studied in this research project and the experimental set-up is such that it lends itself to the study of boundary lubrication of cartilage surfaces.

2.2.3 *Friction*

Synovial joints enjoy very low coefficient of friction when compared to engineering bearings. Many of the early friction studies have been cited above. In the numerous studies done to measure the friction of diarthrodial joints, coefficients of friction ranging from 0.002 to 0.02 have been reported for hydrated cartilage loaded under dynamic conditions and lubricated with synovial fluid. In many cases, however, the friction studies have oversimplified the cartilage contact, and many do not use the cartilage-on-cartilage configuration lubricated with synovial fluid. Many of the literature studies isolate the synovial fluid and test its 'lubricity' with non-cartilaginous materials. Cartilage sliding against a metal or glass have been used with non-physiological loading conditions.

Although an accurate *in vitro* test to simulate *in vivo* joint function would be very difficult to achieve, it is likely essential to obtaining accurate information about actual joint function and lubrication. Examining the interaction of both cartilage and synovial fluid is crucial to understanding how the efficient joint operates because biological materials react uniquely to load, stress and shear.

The value of the numerous studies that measure friction of non-cartilaginous materials lubricated with synovial fluid is that they may give insight to properties of synovial fluid and how they may contribute to the low friction coefficients seen in synovial joints. In the same way, i*n vitro* studies of cartilage-on-cartilage contacts lubricated with synovial fluid give insight to factors that effect the tribological behavior of the system.

2.2.4 *Wear*

The study of cartilage wear is imperative to understanding normal and abnormal joint function. Excessive joint wear is the major symptom of noninflammatory osteoarthritis. Cartilage wear and damage have been measured *in vivo* and *in vitro* by methods such as biochemical markers, physical measurements, and microscopy [16].

There are a several different techniques available for measurement of *in vivo* cartilage wear. X-ray techniques show the deterioration of the joint over time. The articulating ends of bones become closer to one another as the cartilage is worn and this can be used as a measure of how much wear the joint has experienced [18]. Magnetic resonance imaging (MRI) has also been used to measure cartilage wear [62]. MRI scans are very detailed and allow the view of the tissue health and damage. Biochemical markers are also used to measure the presence of cartilage degradation products in the joint fluids. This gives an indication of the rate and volume of cartilage wear occurring during a pathological process [63-66]. The extent of cartilage wear in the joint can also be determined by post-mortem examination. Unfortunately, these methods have drawbacks and limitations and cannot guarantee an accurate measurement of wear. In addition, these types of measurement techniques do not give much insight as to which factors are influencing the wear behavior of the cartilage.

Cartilage wear has been extensively studied *in vitro* under certain non-physiological conditions. Whether the altered condition be the load, speed, material, or lubricant, these conditions differ from the physiologic environment and the results cannot be directly related to *in vivo* cartilage wear. Nonetheless, *in vitro* studies are useful in evaluating cartilage wear behavior and the parameters significantly affecting it. Several techniques have been employed to measure *in vitro* cartilage wear. Techniques such as wear transducers [25], liquid chromatography [67-69], assays for cartilage constituents [24,70,71] and ferrography [72,73] have all been used to measure cartilage wear in *in vitro* studies. In studies such as the work of Dr. M.J. Furey at the Children's Hospital [3], the wear of cartilage sliding against stainless steel was measured using hydroxyproline analysis of the test lubricants. Additional studies have been done that measure the wear of cartilage against steel, cartilage, and other materials [3,24,18,19,74,75]. In these studies, factors such as load and lubricating fluid were found to significantly effect cartilage wear. How these wear studies relate to wear seen *in vivo,* as in the case of OA, has not been examined.

2.3 Osteoarthritis

The term osteoarthritis (OA) is a misnomer, implying that the disease is primarily an inflammatory process. The pathologically accurate term is degenerative joint disease or osteoarthrosis. There is still a lack of consensus about the true definition of OA, but it is generally accepted that the disease involves both mechanical and biological factors. OA is an end response to numerous possible injuries to the joint. Changes seen in OA include the removal of bone in some sites and new formation in others, in addition to the fibrillation and eventual loss of the articular cartilage from the joint surface. Overall, it is the degeneration of the articular cartilage progressively leading to the denudation of the joint surface. There are secondary, often mild inflammatory changes usually within the joint capsule or synovial fluid [31,105]. There are also some commonly seen changes in osteoarthritic cartilage. Some of the most common morphological and histological changes seen in OA cartilage over the progression of the disease will be discussed.

2.3.1 *Morphology of OA Cartilage*

Ultrastructure surface topography of OA cartilage has been previously studied [37,44,76]. In early stages of OA, fragmented collagen fibers with large diameters, along with radial reorientation have been seen with electron microscopy and x-ray diffraction. Ridges, grooves, undulations, and cavities have all been described as being integral to the surface topography of diseased articular cartilage [77,78]. Loss of superficial tangential layer of OA joint surfaces has also been demonstrated with SEM [76]. Disruption confined to the superficial layer is called flaking and is often hard to view with electron microscopy. As degeneration progresses to fibrillation, it is more detectable as surface disruption under microscopic examination. In intermediate stages of the disease, prominent ridge and crater patterns can be found [37]. At high magnifications, no fibrillar network has been identifiable, and a grossly disorganized wave and ridge surface topography devoid of a tangential layer are observed. At the end-stage disease, full thickness wear of cartilage occurs (ulcers and erosions) and highly polished eburnated surfaces of bone are observed with adjacent areas of residual articular cartilage. At high magnifications, fissuring and cracks can be seen at the base of the craters and a complete absence of the organized collagen fiber matrix seen [44].

There are also changes seen in OA synovial fluid. The total number of cells, including white blood cells, increases above normal. The cellularity and nature of the matrix change, influencing the physical and chemical behavior of the fluid [27].

2.3.2 *Histology of OA Cartilage*

The onset of osteoarthritis is a difficult event to determine and there are many theories about the initial causes and pathway of the disease. Despite the nature of the predisposing cause, certain lesion characteristics are common at certain stages of the disorder. In the early stages of OA, damage to the surface is not always visible to the naked eye. The first detectable changes are a softening and swelling of the articular surface. This is thought to happen due to increased enzymatic activity early in the progress of the disease [31,95]. There is a loss of affinity for the hematoxylin stain in the deep radial layer of the cartilage. This corresponds to evidence that hyaluronic acid and proteoglycan contents are reduced early in OA [31]. The initial changes in

the integrity of the collagen network can lead to surface damage and more profound lesions. Disruption of the collagen fibers in the superficial zone leads to splitting and tearing in vertical lines through the cartilage; this is termed fibrillation. These lesions that are initially confined to the superficial layer of the articular surface are classified as *flaking* or *minimal fibrillation*. Chondrocytes have been observed in clusters around these areas of fibrillation and damage.

As OA progresses, more severe and destructive fibrillation occurs, involving splitting that can penetrate the deeper zones of cartilage and affect the calcified cartilage layer as well. The splitting follows the alignment of the collagen fibers and has been associated with theories of a mechanical fatigue failure within the articular cartilage [79]. In response to these changes, the collagen content increases during this intermediate stage, along with a continual degradation of GAGs and proteoglycans. Loss or necrosis of chondrocytes can be observed in all layers of the cartilage. However, proliferation of cells into clusters, called *chondrones*, may also be present.

In the late stages of OA, the histological changes seen are similar to those of the previous stages, but more severe in nature. In addition to continued fibrillation and loss of ground substance, full to nearly full thickness loss of cartilage can occur with subchondral cyst formation, growth of new fibrous cartilage, and bone remodeling.

Histotechnology has been useful in evaluating the progression and severity of OA. Methods such as the Mankin Scoring System for osteoarthritis have aided in standardizing the methods of evaluating histological changes in pathological cartilage and bone [80].

2.3.3 *Theories of Etiology*

There are numerous theories about the etiology of osteoarthritis. Most accounts of early OA show that fibrillation of the cartilage usually precedes other lesions and is the earliest gross change seen [81]. This had led some researchers to believe that the bone remodeling commonly observed in OA is due to normal age-related changes. Other theories propose that the fibrillation of cartilage triggers other changes in the joint which include bone remodeling. The relationship between cartilage lesions and bone remodeling has been difficult to establish.

It has been suggested [36] that once osteoarthritis progresses to the point were fibrillation is apparent, the disruption of the integrity of the collagen network may lead to increased susceptibility to wear and damage. The tribological properties of osteoarthritic cartilage have not been examined in an effort to test the validity of this hypothesis.

Another hypothesis put forth is that stiffening of the subchondral bone is the initial cause of OA [82,83]. It has been suggested that fractures in the subchondral bone precede cartilage fibrillation because the bone is the weight bearing element of the joint. Once damage to the bone occurs, the cartilage then has to absorb more of the shock and stress and this results in increased damage and fibrillation.

Still other theories included biochemical changes in the cartilage that reduce proteoglycan and GAG content or lead to collagen degradation. This group of theories is centered around the activity of enzymes, phagocytes, neutrophils, or other bioactive substances that have the ability to lyse, phagocytize, or degrade the constituents of the cartilage matrix. These theories can be divided into two categories: 1) those which propose a direct activity of proteolytic enzymes within the cartilage, and 2) those that suggest enzymatic activity present in the synovial fluid that alter its constituents and change its lubrication properties. In addition, there are still other theories that point to genetic factors involved in OA that can produce any one or more of the aforementioned conditions.

The degenerative changes seen in OA overlap and it has not been possible yet to determine exactly which precedes another or triggers another in their progression. Research in this area continues in an effort to gather more information about the initial events that occur early on in OA and the chain of events that occur during the progression of the disease.

2.4 Biotribology Research at Virginia Tech

We are still a long way from understanding the factors involved in the degeneration and breakdown of the synovial joint. In a continuing study of biotribology at Virginia Tech, research

has been concerned not only with the lubrication and wear of normal, healthy joints, but also with tribological phenomenon which could lead to joint degeneration seen in osteoarthritis. The research is designed to study biological systems using an engineering methodology coupled with biochemical, physiological and pathological conditions and effects.

A collaborative research effort in biotribology, headed by Dr. Michael Furey in the Department of Mechanical Engineering, has employed a unique approach involving *in vitro* experiments with bovine articular cartilage and emphasizing the effects of fluid biochemistry on cartilage wear and damage [8-13, 18-20]. The foundation for this research is built upon the sabbatical work of Dr. Furey at the Children's Hospital Medical Center at the Harvard Medical School in 1983-84. This work examined the hypothesis that the detailed biochemistry of the fluid/cartilage system has a pronounced and possibly controlling influence on cartilage wear [3, 14-17]. In these experiments, a cartilage sliding against stainless steel configuration was used. The effects of various lubricating test fluids on cartilage wear were determined by biochemical analysis of wear debris. The test fluids included a buffered saline reference fluid, distilled water, synovial fluid, and several constituents added to the reference fluid: hyaluronic acid, a lubricating glycoprotein isolated by Swan (LGP-1), and a protein complex from the synovial fluid. Analysis of the post-test fluids by hydroxyproline assay showed that distilled water and the saline reference fluid produced similarly high levels of cartilage wear. The synovial fluid produced significantly less (e.g. 90%) wear than the reference fluid. The protein complex isolated from synovial fluid was quite effective in reducing wear when added to the reference fluid, producing similar results as the synovial fluid. Hyaluronic acid was effective in reducing wear of cartilage when added to the reference fluid, but was not as effective as the protein complex or the synovial fluid. Swann's LGP-1, although found to be effective in reducing friction, did not reduce cartilage wear when added to the reference fluid. The cartilage specimens were also examined with scanning electron microscopy.

The most significant aspects of these tests are that: 1) normal synovial fluid was found to produce very low cartilage wear and damage, and 2) the biochemical properties of the lubricants showed significant effects on the cartilage wear and damage observed. A consequence of these

results is that a lack or deficiency of certain biochemical constituents in the synovial fluid may be one factor contributing to the initiation and progression of cartilage wear, damage and possibly osteoarthritis.

More recently at Virginia Tech, Dr. Furey has organized a collaborative effort to continue the biotribology research started in Boston. Faculty, graduate and undergraduate students from the Colleges of Engineering and Veterinary Medicine, and the Departments of Biochemistry and Animal Science have worked together in recent years to continue this interesting work and gain a better understanding of how synovial joints function from a tribological viewpoint.

The graduate work of several Masters and undergraduate research students has been instrumental in the advances made in this area. The work of Ms. Bettina Burkhardt led to the design and development of an experimental device for wear testing of cartilage-on-cartilage and cartilage-on-steel systems [2,5,48]. This device underwent several redesigns, with the final improvements done by Mr. Matthew Schroeder.

The graduate work of Mr. Schroeder led to the first cartilage-on-cartilage wear tests. This study developed, explored, and refined techniques for the *in vitro* study of cartilage-on-cartilage wear, friction and deformation [18]. Load, sliding speed and sliding distance were kept constant, while lubricating fluid and test length were varied. Lubricants used consisted of phosphate buffered saline, bovine serum, and bovine synovial fluid. The results indicated that synovial fluid lubricant produced the least amount of wear followed by the bovine serum. The serum produced more wear and damage to the cartilage than synovial fluid, but less than the buffered saline. They also showed that the three hour tests produced more wear and damage than the one hour tests.

The significance of this work is that it established the use of several techniques and procedures employed by Furey in Boston, at Virginia Tech facilities. It also confirmed that the effects of the biochemical constituents of the lubricating fluid on cartilage wear seen by Furey were also observed in a cartilage-on-cartilage system.

This study was followed by the graduate work of Mr. Michael Owellen. In these studies, cartilage wear, damage and friction were examined in a cartilage-on-stainless steel system [19]. Two loads of 20 and 65 N were used along with three lubricants: buffered saline reference fluid, synovial fluid, and the reference fluid with hyaluronic acid added. Analysis of wear debris with hydroxyproline assay showed that under high load, saline lubricant produced the most wear. Hyaluronic acid produced less wear than the saline and synovial fluid produced the least wear of the three lubricants. Wear and damage was sensitive to load with all three lubricants, but was not significantly affected by the lubricant under low loads. Friction of the system was found to be affected by load, but not significantly by lubricant. This work reestablished the results obtained by Furey in Boston with the equipment and procedures available at Virginia Tech.

The biotribology research done at Virginia Tech thus far has focussed on determining the beneficial constituents of synovial fluid that assist in the prevention of wear and damage of articular cartilage during sliding motion. There is also a separate, but related area of interest that concerns synovial fluid constituents which may act to increase wear and further damage of articular cartilage under tribological contact. They could include enzymes and other bioactive components of the joint that are present during both normal and pathological processes. This is the focus of the present research which looks specifically at the effects of biochemical changes in the synovial joint on cartilage wear and damage. Collaboration with other University departments will likely continue through the new Center for Biomedical Sciences.

3 EXPERIMENTAL

3.1 Material Preparation

3.1.1 *Bovine Specimens*

Bovine articular cartilage was obtained from the Virginia/Maryland College of Veterinary Medicine with the assistance of Dr. Hugo Veit. The shoulder and stifle joints of 24 mature steer, sacrificed in an unrelated study, were dissected, harvested and stored at -25 ^{o}C. The joints were stored with saline saturated gauze pads to prevent cartilage dehydration. Each joint was labeled to identify the animal source and type of joint.

Specimens of cartilage and underlying bone were prepared for *in vitro* tribological testing. Cylindrical plugs of 6.35 mm (¼-*inch*) and 25.4 mm (1-*inch*) diameters were cut on a *Tradesman*® variable speed torque drill press and are shown in **Figure 3.1**. The plugs were obtained by scoring the articular cartilage of the joint surface with a cork bore and then using a custom-made hollow cutter to drill plugs perpendicular to the cartilage surface. The resulting plugs consisted of a top layer of cartilage followed by a larger layer of underlying bone. The cartilage was kept hydrated and cool by lubricating with deionized water during the cutting process. The 6.35 mm plugs were obtained from the femoral condyles, and the 25.4 mm plugs were cut from the tibial surfaces. This allowed for the *in vitro* test configuration to model that of the natural joint (see **Section 3.2.1**).The resulting cylindrical plugs were removed from the joint, labeled and stored in deionized water at -25 ^{o}C until testing.

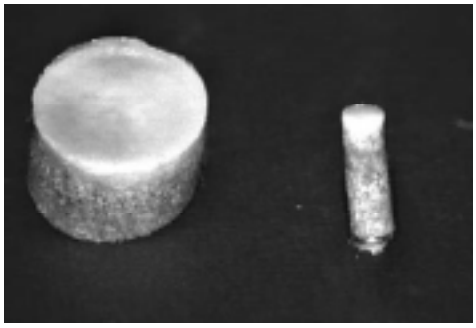

Figure 3. 1 Bovine cartilage plugs

3.1.2 *Bovine Synovial Fluid*

Synovial fluid was collected from the shoulder and knee joint spaces of each of the bovine steer used. The fluid volumes varied, but an average of 4 ml of synovial fluid was collected from each joint. The fluids were separated into left and right joint fluids, meaning left stifle and shoulder fluids were combined and separated from the mixture of right stifle and shoulder fluids. Most

fluids collected appeared normal, with a transparent yellow color and high viscosity. However, some fluids contained blood and were less viscous. After collection, the fluids were labeled and frozen along with the joints at -25 ^{o}C until their use in specified tests.

3.1.3 *Lapine Specimens*

Lapine articular cartilage was obtained with the help of Dr. Hugo Veit and Dr. Anthony Pease of the Virginia/Maryland College of Veterinary Medicine. Osteoarthritis was surgically induced in New Zealand White rabbits by meniscectomy of the lateral meniscus and transection of the lateral collateral ligament of one knee. The remaining knee was left unaltered for use as a non-surgical control. The rabbits were maintained for six weeks with no additional procedures other than a sampling of synovial fluid. At the end of the six week period, the rabbits were euthanatized. Both the surgical and non-surgical knees were removed, post-mortem, and frozen at -70 ^{o}C. Three sets of normal and OA joints were submitted to the tribology laboratory for use in tribological wear and friction studies.

After receipt of several osteoarthritic and normal rabbit knee joints, the specimens were thawed, trimmed of muscle and ligaments, and refrozen at -25 ^{o}C. On the day of testing, the knees were thawed and further prepared for accommodation into the test apparatus. The femoral condyles of selected knee joints were separated on a band saw and the surrounding bone trimmed so that the articulating surface was perpendicular to the underlying femur. The bone shaft was then coated with an epoxy and covered with a sheath of heat shrinkable tubing. The tubing was heated and formed into a cylindrical plug before the epoxy could dry. When the epoxy hardened, the result was a solid cylindrical plug with the articular cartilage of the lapine femoral condyle exposed at the top. This procedure made it possible to use these smaller, more fragile rabbit condyles in the test apparatus. The specimens were tested on same day as their preparation.

3.1.4 *Phosphate Buffered Saline*

Phosphate buffered saline (PBS) with a pH of 7.4 was purchased from Sigma Chemical (lot #047H8933). The PBS solution was prepared with deionized water and stored at room temperature in a glass bottle. Aliquots were removed and used as lubricants in specified tests.

3.2 Experimental Apparatus and Data Acquisition

3.2.1 *Test Apparatus*

The tribological test apparatus, previously designed by Burkhardt [8,18,48], is shown schematically and photographically in **Figures 3.2** and **3.3**, respectively. The device consists of a shaft and bearing assembly for vertical translation of the upper specimen holder which is connected to the shaft via an octagonal strain ring. The attached strain gages measure normal and tangential load on the upper specimen. Normal load is applied via dead weights at the top of the vertical shaft. If high loads are required, a pneumatic load cylinder is also available. The upper specimen is placed in contact with the lower specimen which is mounted in an additional specimen holder on an X-Y translation table. The lower specimen translates with a linear reciprocating velocity of 8 mm/sec. The total traverse in the positive direction is 6.5 mm. Accompanying data acquisition equipment allows for the measurement of friction, normal load and deformation under conditions of variable load and reciprocating velocity. The system currently operates at room temperature (23 ± 2 °C) with no environmental controls.

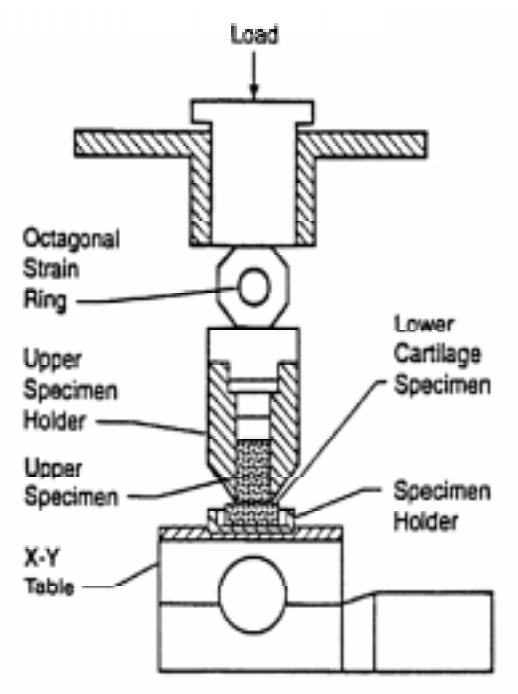

Figure 3. 3 Schematic of tribological test device

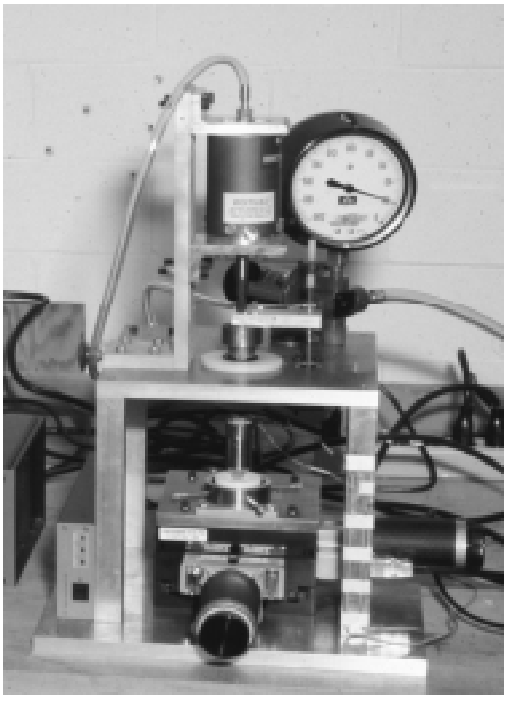

Figure 3. 2 Tribological test device

3.2.1 *Data Acquisition Equipment*

The upper and lower specimen holders are designed to hold samples of 6.35 mm and 25.4 mm diameter, respectively. The upper specimen holder is attached to the octagonal strain gage unit whose signal is transmitted to a multi-meter and IBM 286 desktop computer. Data are obtained with Global Lab Data Acquisition Software. This software measures voltage changes over a 3 second period with a frequency of 250 Hz.

The lower specimen is mounted on a linear displacement table programmed by a NEAT-310 motion controller. The table is powered by stepper motors and programmed for linear reciprocating motion along one axis.

For the studies in this project, one of two test configurations was used. In each configuration, the 6.35 mm plug of articular cartilage, from the femoral condyle of an articular joint, was placed in sliding contact with the 25.4 mm diameter cartilage plug from the tibial surface of the same joint or a disk of highly polished 303 stainless steel. This simulates the sliding motion and configuration of articulating cartilage seen in natural and some partial artificial joints, respectively. **Table 3.1** shows the range of parameters of the tribological test device used for the included studies.

Table 3. 1 Device specifications

Test Configuration	cartilage-on-cartilage cartilage-on-stainless steel
Contact Geometry	flat-on-flat convex-on-flat irregular-on-irregular
Cartilage Type	bovine articular lapine articular
Applied Load	39 - 79 N
Average Pressure	1.2 - 2.4 MPa
Type of Motion	linear, reciprocating
Sliding Velocity	8 mm/sec

3.3 Analysis Methods and Techniques

3.3.1 *Hydroxyproline Analysis*

Hydroxyproline analysis was used for the determination of cartilage wear in post-test lubricants. Colorimetric assay of test fluids produces a color yield that is accurately proportional to the hydroxyproline concentration over a wide range of concentrations.

A procedure, developed by Neuman and Logan [70] and modified by Mr. Delbert Jones and the author, has been employed for hydroxyproline analysis. After wear testing was performed, wear debris, suspended in the lubricant, was collected along with washings of the articular surfaces. The fluids were prepared for analysis of hydroxyproline content according to the following procedure:

REAGENTS:

1. 0.01 M copper sulfate solution (anhydrous or nH_20)
2. 2.5 & 3 N sodium hydroxide
3. 6% hydrogen peroxide
4. 3 N sulfuric acid
5. α- dimethylaminobenzaldehyde in c.p. n-propanol

PROCEDURE:

1. Put liquid samples in glass screw top vials, label and freeze at -70 ^{o}C.
2. Completely lyophilize frozen samples (approx. 15-18 hours).
3. Hydrolyze dry samples with 2 ml of 6 M HCl for 24 hours at 100 ^{o}C.
4. Cool samples and filter through 0.2 μm pore size syringe filter.
5. Take 1 ml aliquot, add 2 ml of 3 N NaOH and shake.
6. Make 4-trans-hydroxy-L-proline standards with total volume of 1 ml. Assay along with the neutralized samples.

ASSAY:

1. Take 1 ml of neutralized sample.
2. Add 0.5 ml of each of the following in succession: 0.01 M copper sulfate, 2.5 N sodium hydroxide, 6% hydrogen peroxide.
3. Vortex for 5 minutes.
4. Place in water bath at 80 ^{o}C for 5 minutes with frequent vigorous shaking.
5. Chill in ice and water bath.
6. Add 2 ml of 3 N sulfuric acid.
7. Add 1 ml of 5% α- dimethylaminobenzaldehyde.
8. Vortex for 1 minute.
9. Place in water bath at 70 ^{o}C for 16 minutes.
10. Cool in tap water.
11. Transfer contents of each tube to selected absorption tubes.
12. Read at 540 nm on spectrophotometer.

The amount of hydroxyproline in each test fluid was determined by spectrophotometry and converted to the amount of hydrated cartilage with a conversion factor of 125. This factor corresponds to an 8% hydroxyproline content in dehydrated collagen and a 10% collagen content in hydrated cartilage.

3.3.2 *Scanning Electron Microscopy*

After the completion of the wear tests, selected plugs were prepared for scanning electron microscopy. The samples were rinsed with deionized water to remove any loose wear debris. The plugs were then cut parallel to the sliding direction, leaving the articular surface and a small layer of underlying bone. The remaining sample was fixed in a solution of 5% glutaraldehyde, 3% formaldehyde, and 2.75% picric acid in 0.1M Na cacodylate buffer at pH 7.3 to 7.4 and refrigerated at 5 ^{o}C for at least 24 hrs. Samples were prepared according to procedures used by Boyde [40] and Bozzola [41]. The samples were processed at the Virginia-Maryland College of Veterinary Medicine. The following is the exact 2-day protocol used at the Morphology Laboratory in the Veterinary College:

Day 1:

1. Rinse sample twice in 0.1 M Na cacodylate buffer for 15 minutes each
2. Post fix the sample with 1% OsO4 in 0.1 M cacodylate buffer for 1 hour
3. Rinse sample twice in 0.1 M cacodylate buffer for 10 minutes
4. Dehydration: using 15%, 50 %, 70%, 95% EtOH for 15 minutes
5. Leave in 100% EtOH overnight

Day 2:

1. Place sample in fresh EtOH for 15 minutes
2. Place sample in a critical point dryer at 9 ^{o}C for 5 minutes (repeat 8 times)
3. Place sample on a stub of appropriate size using double sided tape and label
4. Put silver paint on the periphery of the sample for conduction to the stub
5. Allow the paint to dry thoroughly
6. Place sample in the sputtering machine and gold coat for 120 seconds (the coat should be 18 mA and pressure should be between 2-4 atm)
7. After coating, sample is ready for viewing in the JEOL JSM-35C Scanning Electron Microscope.

After viewing with the electron microscope, photographs were taken of the dominant and reoccurring features on each cartilage surface. These photographs were then used to qualitatively determine damage to the cartilage surface resulting from the test treatments.

3.3.3 *Histology*

After the completion of the wear tests, selected samples were prepared for histological examination. The plugs were rinsed with deionized water to remove any loose wear debris. The sample plugs were then cut in half perpendicular to the sliding direction. The cross section of cartilage and bone was then fixed in 10% neutral buffered formalin for minimum of 24 hours.

Histology slides were then prepared, according to standard histotechnological procedures [84], at the Histopathology Laboratory in the Veterinary College. The procedure consists of the following steps:

1. Decalcification
2. Dehydration
3. Embed in paraffin wax
4. Cut 3 μm section with microtome
5. Set in gelatin and water bath at 40 ^{o}C
6. Attach samples to slide and remove wax
7. Heat slides and stain with hematoxylin & eosin (H&E) or alcian blue (pH = 2.5) for 20 minutes

The resulting slides were viewed with a high magnification microscope and the dominant and reoccurring features photographed. These photographs were used to qualitatively determine damage to the cartilage surface and subsurface, as well as changes in cartilage biochemistry resulting from the test treatments.

3.3.4 *Friction Measurement*

Friction was determined from the output of the strain gage unit. The gage was calibrated prior to the beginning of each new experimental phase and new calibration curves were generated. The voltage acquired by the Global Lab software was then translated into tangential load via these calibration curves. The coefficient of friction was calculated as the ratio of tangential force (friction) to normal load. The normal load and applied load were assumed to be equivalent due to the negligible variation in normal load observed over the test duration. Friction data were available for Experimental Phases II and III only.

3.4 Experimental Phases

The following section provides a brief description of the experimental phases completed in this research project. The phases examine three different possible modes of cartilage degradation seen in osteoarthritis. The following chapters discuss each experimental phase, their results and conclusions.

Phase I: The Effect of Exposure to Collagense-3 on the *In Vitro* Wear and Damage of Bovine Articular Cartilage

The Phase I study was designed to examine the effect of an adverse biochemical environment on the wear and damage of bovine articular cartilage. An adverse biochemical environment, in the form of exposure to the collagenase-3 enzyme was created and the subsequent effect on wear and damage of the cartilage evaluated. Collagenase-3 is an enzyme recently suspected in the degeneration of osteoarthritic cartilage. Through the experimental design, examination of the effects of the enzyme, test lubricant, type of joint and all interactions were possible.

Phase II: Wear of Osteoarthritic Lapine Articular Cartilage

The Phase II study investigates the tribological properties of osteoarthritic cartilage. Articular cartilage from rabbits with surgically induced osteoarthritis was tested and the wear and friction properties were evaluated and compared to those of normal cartilage from the same animal. The study examines the effect of fibrillation and disruption of the cartilage matrix, caused by osteoarthritis, on the subsequent wear of the cartilage. This study investigates the theory that osteoarthritis has a progressive pathogenesis in which the effects of the onset of the disease accelerate its further progression, particularly cartilage loss.

Phase III: Effect of Exposure to White Blood Cell Lysate on the *In Vitro* Wear of Articular Cartilage

The Phase III study examines the effects of the changing biochemistry of the synovial fluid during inflammation on wear, damage and friction of articular cartilage. High numbers of white blood cells are often found in synovial fluid with low-grade synovitis which is commonly seen in more than 50% of OA patients. Pathological fluid was created artificially by combining a white blood cell lysate with normal bovine synovial fluid. The wear and friction of the cartilage surfaces were compared with normal synovial fluid and the pathological fluid. Through this experiment, we examine whether the byproducts of joint inflammation adversely effect the ability of synovial fluid to act as an optimal lubricant for articulating surfaces.

Phase IV: The Effect of Digestion of Synovial Fluid Phospholipids, by Phospholipase A_2, on the Lubricating Properties of Synovial Fluid

In Phase IV, we examined the effect of additional biochemical changes in the synovial fluid on cartilage wear. In this set of experiments, a suspected beneficial constituent of synovial fluid was removed. Surface-active phospholipids (SAPLs) have been recently cited as the effective boundary lubricating component of synovial fluid. A pathological fluid was created artificially by destroying the SAPLs in the synovial fluid with Phospholipase A_2. The wear and friction of the cartilage surfaces were compared with normal synovial fluid and the pathological fluid. This experiment examines whether the removal of SAPLs from the synovial fluid adversely affects its ability to act as a highly efficient lubricant for articulating surfaces.

4 PHASE I: The Effect of Exposure to Collagenase-3 on the *In Vitro* Wear of Bovine Articular Cartilage

4.1 Theory and Background

During normal growth processes and during traumatic experiences, changes in the biochemistry of the lubricating fluid and cartilage in the synovial joint can occur. Inflammation and swelling can occur, bringing harmful agents in contact with the joint capsule, synovial fluid and cartilage. Repeated exposure to these agents may result in biochemical changes of the synovial fluid and articular cartilage, which may alter the tribological properties of the joint and possibly lead to increased wear and damage. Numerous phagocytic cells and infectious agents gain access to the joint space during the life of the joint and there has been continued research to identify those with the ability to destroy or degrade cartilage.

Enzymes are capable of removing connective tissues in the body during normal turnover, as well as during pathological processes [31,85]. As a result, enzymatic degradation has long been suspected as a mechanism of cartilage degeneration. Several studies have shown that degradative enzymes are present in increased quantities in osteoarthritic joints [86-90]. Hence, the identification of specific enzymes involved in the destruction of articular cartilage has been a frequent topic of interest to pathobiologists.

Collagenases are a family of proteolytic enzymes that are capable of cleaving type II collagen in its triple helical form found in articular cartilage [85]. The detection of increased levels of active collagenases in the synovial fluid and cartilage of arthritic joints has implicated them in mechanisms of cartilage degradation in joint diseases [64,77,81,85,87,88,91-97].

Recent studies have shown that collagenase-3 (MMP-13) plays a significant role in the cleavage of type II collagen in osteoarthritic articular cartilage [98,99]. Collagenase-3 has demonstrated a higher cleavage activity than collagenase-1, which was previously thought to be the main collagenase involved in the destruction of type II collagen [99]. Collagenase-3 has also been

identified in the chondrocytes of pathologic cartilage [99]. It is therefore of interest to examine the effect of exposure to collagenase-3 on the biomechanical wear of articular cartilage.

4.2 Objective

The primary objective of the Phase I study in this research project was to determine if, and to what extent, collagenase-3 affects cartilage wear and damage *in vitro*. Although numerous *in vitro* experiments have shown that collagenase-3 has the ability to degrade cartilage in a biological tissue culture, none have measured its effect on the biomechanical wear of cartilage after exposure to the enzyme. The extent of biomechanical wear is relevant when studying the role of the enzyme in the cartilage loss associated with osteoarthritis. As secondary objectives, the effect of the lubricating fluid was examined, in addition to the differences in wear of knee and shoulder joints.

In this phase of study, physiologic and histologic changes produced in the cartilage, due to exposure to collagenase-3, were examined. These changes represent an alteration in the biochemical make-up of the articular cartilage which may be related to changes seen in osteoarthritic joints. The effect these changes have on the wear, or loss, of cartilage is the primary focus of the Phase I study.

4.3 Materials and Methods

4.3.1 *Cartilage Preparation*

Prior to testing, bovine cartilage plugs were thawed overnight at -2 to -4 ^{o}C. The plugs were previously frozen in vials of deionized water to maintain their moisture (see **Section 3.1.1** for complete description of bovine cartilage preparation). When thoroughly defrosted, the plugs were removed from refrigeration and prepared for enzyme treatment. All bovine cartilage plugs from the same animal were prepared simultaneously. Plugs from different animals were treated separately in individual batches.

4.3.2 *Collagenase-3 Treatment*

The bovine cartilage plugs were taken to a sterile hood and transferred into tissue culture wells. The ¼-*inch* plugs were put in 3 ml volume wells and the 1-*inch* plugs were put in 14 ml volume wells. A solution of biologically compatible media was prepared by combining RPMI (49 ml) and penicillin streptomycin (1 ml), both purchased from Sigma Chemical. The collagenase-3 solution was prepared by combining 50 mg of lyophilized collagenase-3 powder (Worthington Biochemical Corp; Lot #S6K495; 140 u/mg) with 5 ml of the RPMI media solution, making a 10 mg/ml solution. The solution was then filtered through a sterile 0.2 μm pore size syringe filter. A range of collagenase-3 activities in OA joints was determined from current literature [85,98]. Activities of 50 and 100 u/ml were chosen for use in this study. These represent median and high-end values of those found in the literature. For the collagenase-3 used, **Table 4.1** shows the volumes of collagenase-3 and media added to the bovine cartilage in each of the tissue culture wells for the desired activity.

Table 4. 1 Volumes of collagenase-3/media solution added to tissue culture wells

Cartilage Plug Size	Media Solution	Collagenase-3 Solution for Desired Activity	
		50 u/ml	100 u/ml
6.25 mm dia.	1 ml	0.03 ml	0.07 ml
25.4 mm dia.	3 ml	0.10 ml	0.21 ml

Controls were also prepared with cartilage in media solution containing no collagenase-3 solution. When the concentrations of collagenase-3 were added, the wells were covered and put in an incubator at 37 ^{o}C for 24 hours. The cartilage plugs were then removed from the wells, rinsed to remove residual enzyme and refrigerated with the cartilage surface submerged in the selected test fluid until start of test.

4.3.3 *Lubricant Preparation*

Synovial fluids from joints corresponding to the test plugs were also thawed in the refrigerator overnight before testing. The fluids were removed in 1 ml aliquots and put between the cartilage surfaces during the test.

Phosphate buffered saline was removed in 1 ml aliquots from a bottle stored at room temperature (23 ± 2°C) and put between the cartilage surfaces during testing.

4.4 Design

Phase I is the first set of statistically designed experiments in this research project. Six sets of femoral (upper) and tibial (lower) cartilage plugs from four steer were used for a total of twenty-four tests. From each steer, two sets of cartilage plugs came from each knee and one set from each shoulder for the total of six. Both synovial fluid and phosphate buffered saline were used as lubricants. In addition, the two levels of collagenase-3 concentration were used along with several controls. The complete study allowed for the determination of effects due to collagenase treatment and concentration, type of joint, lubricant, and all interactions. **Table 4.2** is a statistical design that was constructed with advice from Statistical Consulting Services on campus:

Table 4. 2 Statistical design: variation of the Latin Rectangle

	rk1	rk2	lk1	lk2	rs	ls
steer 1	1	2	3	4	5	6
steer 2	3	4	5	6	1	2
steer 3	1	2	5	6	3	4
steer 4	1	4	5	6	2	3

The following are the definitions for the components of the rectangle:

rk = right knee rs = right shoulder
lk = left knee ls = left shoulder

Table 4. 3 Definition of treatments

Treatment	**Lubricant**	**Enzyme Concentration (u/ml)**
1	Saline	0
2	Saline	50
3	Saline	100
4	Synovial Fluid	0
5	Synovial Fluid	50
6	Synovial Fluid	100

The numbers 1-6 represent one of the 6 combinations of treatment for the Phase I experiment shown in **Table 4.3**. The steers were randomly assigned and the treatments within each block of steers were randomly assigned, first the knees and then the shoulders. This design blocks out any variation based on the animal, but allows for evaluations of variation based on joint type and treatment. Type III Sums of Squares was used to analyze significance of all factors and any linear or quadratic effect of the enzyme.

4.5 Wear Tests

After enzyme treatment and incubation, the cartilage sets were allowed to sit in their respective lubricants for 2-3 hours before testing in order for the cartilage to equilibrate with the fluid. The upper and lower specimens were then put in their respective specimen holders and mounted on the test apparatus. Lubricating fluid was added between the two specimens and the test begun. Tests were performed as described in **Section 3.2**. The specific test parameters for this phase of study are shown below in **Table 4.4**.

Table 4. 4 Test parameters

Phase I Test Parameters	
Configuration	cartilage-on-cartilage
Load	78.4 N dead weight
Duration	60 minutes
Enzyme Concentration (Collagenase-3)	0, 50, 100 u/ml in tissue culture media
Lubricants	synovial fluid, phosphate buffered saline
Post-test Analysis	SEM, histology, hydroxyproline assay

Upon termination of the test, the cartilage specimens were immediately removed, rinsed, trimmed and fixed for further analysis. The rinses and lubricating fluid were collected, combined and frozen until analysis by hydroxyproline assay.

The procedures shown in **Table 4.5** were used to analyze the surface and subsurface damage, and wear of the articular cartilage during the *in vitro* test. The information gathered from the analysis of each set of cartilage plugs within a treatment group was combined and compared to those from different treatment groups. The results of the analysis are described in the following section.

Table 4. 5 Analysis techniques used for evaluation of wear and damage

Analysis	**Procedure**
Surface damage	SEM, histological sectioning
Subsurface damage	histologic evaluation
Wear	hydroxyproline assay

4.6 Results

After all tests in the selected test design were completed, analysis was performed according to the protocols outlined in **Section 3.3**. Wear and damage of the cartilage specimens were measured and related to the test lubricants and treatments. Hydroxyproline assay of test lubricants was used to quantitatively determine wear of the cartilage surface. Information obtained from scanning electron microscopy and histological sectioning and staining was combined and used to qualitatively determine surface damage and biochemical changes in the cartilage.

4.6.1 *Wear Results*

The test lubricants collected after wear testing were analyzed for hydroxyproline content. In cases where synovial fluid was used as the test lubricant, a sample of the fluid was assayed before testing to determine the amount of hydroxyproline content and that amount was subtracted from the post- test measurements.

Using the assay procedure, cartilage wear was determined for 23 wear tests. The original test design called for 24 tests, but the final test failed and the test fluid was not recoverable. The resulting design consisted of twelve tests done using synovial fluid as the lubricant and eleven tests with phosphate buffered saline as the lubricant. In both of these cases, four wear tests were

performed using normal cartilage which served as controls. The remaining tests involved cartilage treated with collagenase-3 at the 50 or 100 u/ml concentration level. In each of the twenty-three tests, the fluids were assayed in triplicate and their average used as the hydroxyproline content.

The wear results obtained from the twenty-three tribological tests are shown in **Figure 4.1**. The average wear values and standard deviations for each test treatment are shown in **Table 4.6** and graphically represented in **Figure 4.2**. Overall, there were three variables within the test data: lubricant used, type of joint, and level of collagenase-3 concentration. (See **Appendix A** for data tables.)

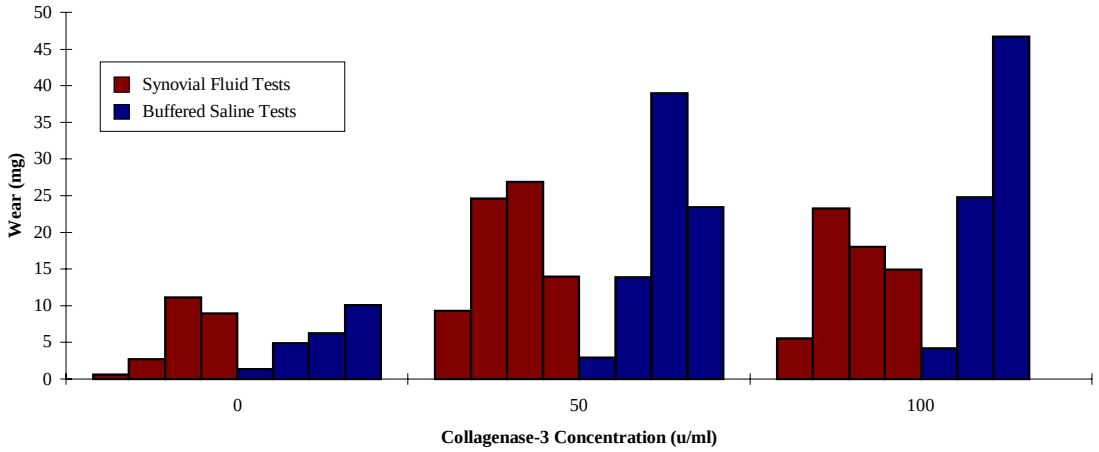

Figure 4. 1 Wear values for Phase I study

Table 4. 6 Average wear values (mg) for Phase I study

	Collagenase-3 Concentration		
Lubricant	**0**	**50**	**100**
Synovial Fluid	5.8 ± 5.0	18.7 ± 8.4	15.4 ± 7.4
Buffered Saline	5.6 ± 3.6	19.6 ± 15.5	25.2 ± 21.2

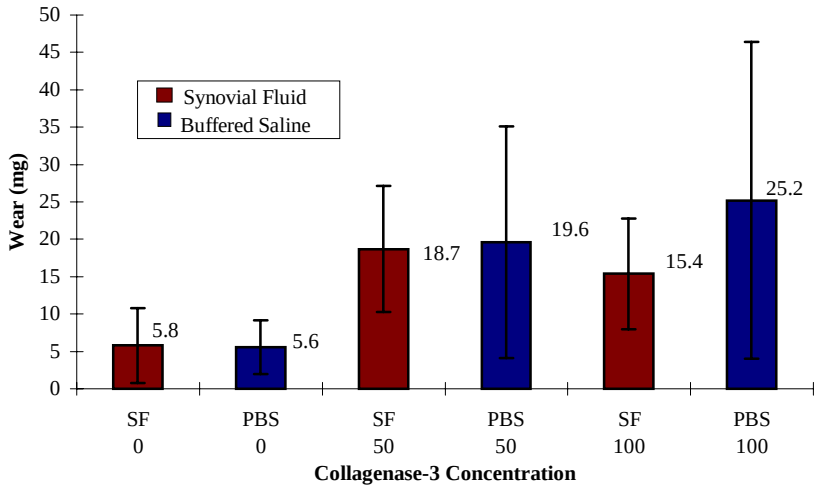

Figure 4. 2 Average wear values for Phase I study

There is a definite increase in the biomechanical wear of bovine articular cartilage after exposure to the collagenase-3 enzyme. For the twelve tests done with synovial fluid, the increase in wear of the enzyme treated cartilage is 3 times that of the untreated cartilage, as seen in **Figure 4.3**. Tests done with buffered saline produced a 4-fold increase in wear of the enzyme treated cartilage over the untreated controls. This is illustrated in **Figure 4. 4**.

The average wear of all tests done with synovial fluid was 13.3 mg, as compared to 16.1 mg of wear from all buffered saline tests. Average wear from tests done on knee joints was 13.9 mg, compared to 16.4 mg from tests with shoulder joints. Overall average wear from the enzyme treatment was 5.7, 19.2 and 19.6 mg for the 0, 50 and 100 u/ml collagenase-3 concentrations, respectively.

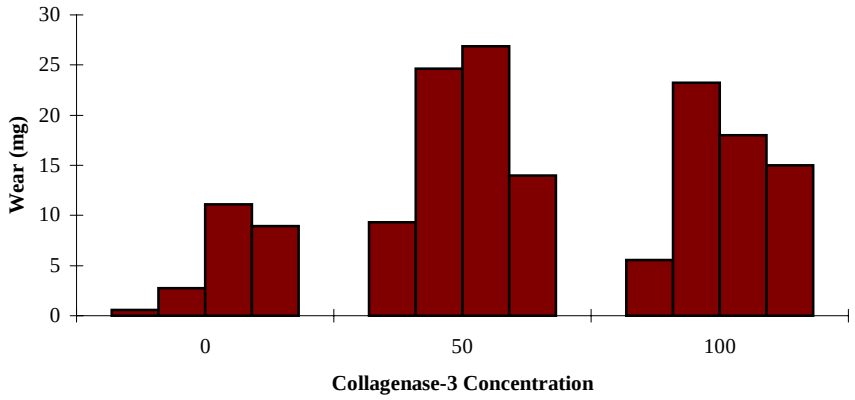

Figure 4. 3 Wear in synovial fluid tests

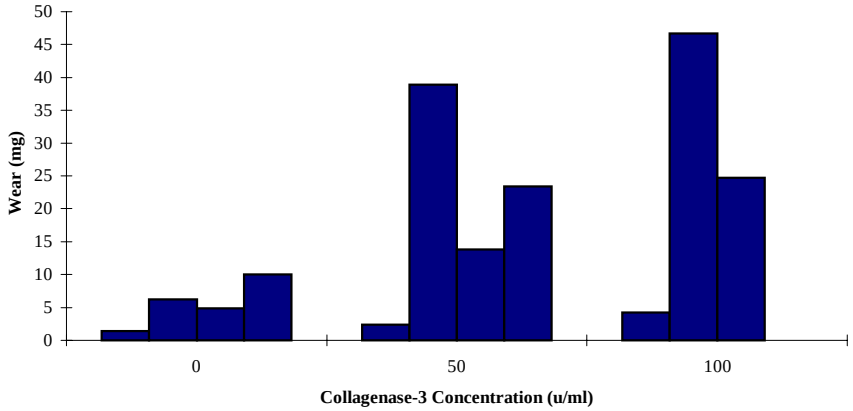

Figure 4. 4 Wear in buffered saline tests

4.6.2 *Statistical Analysis*

The testing in Phase I was done in a partially balanced incomplete block design. Each animal was used as a block because there is an expected variation between cartilage from different animals. The combinations of lubricant, joint type and enzyme treatment constituted the tests treatments. Statistical analysis of the wear data was done using the SAS System software. Analysis of variance was performed and the following data obtained at the $\alpha = 0.05$ confidence interval:

1. There was no significant difference between the synovial fluid and saline lubricants, p= 0.3101 (*p* is the level of significance of the statistical test).

2. There was a significant difference between the three levels of enzyme exposure, p= 0.0049.
3. There was a significant difference between the controls and the enzyme treated cases, p= 0.0014.
4. There was no significant difference between the 50 and 100 u/ml enzyme concentrations, p= 0.7275.
5. There was no significant difference between wear of knee and shoulder cartilage, p= 0.7489
6. There was no significant interaction of lubricant and enzyme treatment, p= 0.4143.

(See **Appendix B** for SAS code and output)

4.6.3 *Scanning Electron Microscopy*

After testing, selected cartilage plugs were prepared and viewed with scanning electron microscopy (SEM) to evaluate surface damage. Both top and bottom plugs from selected tests were examined with SEM. Damage for both plugs were evaluated and compared according to lubricant and enzyme treatment. It is clear that in all cases, the 1-*inch* bottom cartilage plugs sustained greater damage than the ¼-*inch* top plugs.

For control cases (0 u/ml of collagenase-3), the ¼-*inch* top plugs showed no visible signs of damage. Tests done with both synovial fluid and saline produced cartilage surfaces that were virtually unchanged from a normal, untested cartilage sample. **Figures 4.5 - 4.7** are electron micrographs of normal untested cartilage, cartilage tested with synovial fluid and cartilage tested with saline. All variations between the three ¼-*inch* cartilage specimens are within the normal expected variations between cartilage from different animals.

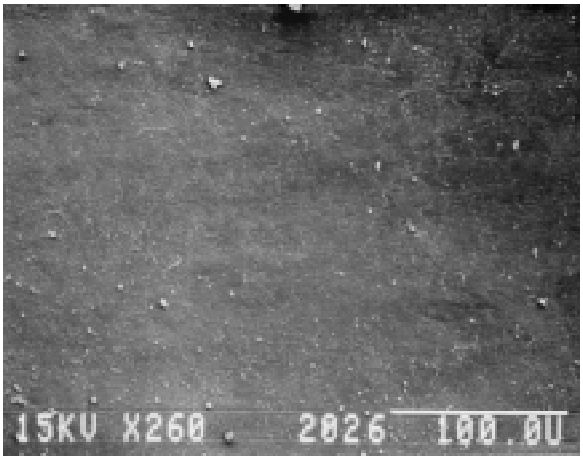

Figure 4. 5 SEM of normal untested cartilage

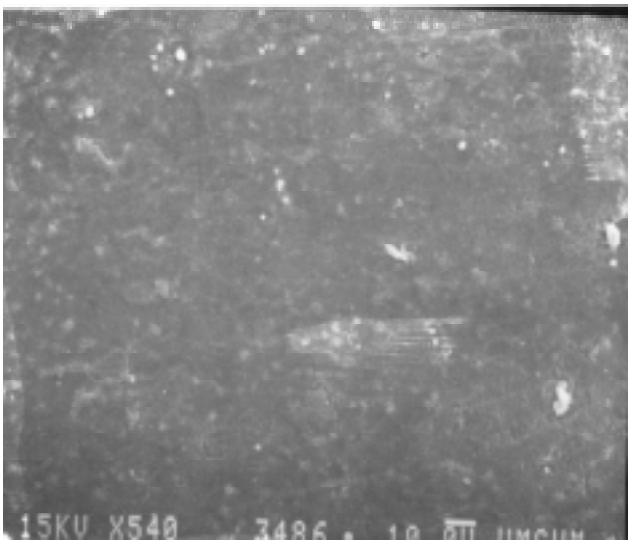

Figure 4. 6 SEM of ¼-*inch* cartilage plug tested with synovial fluid

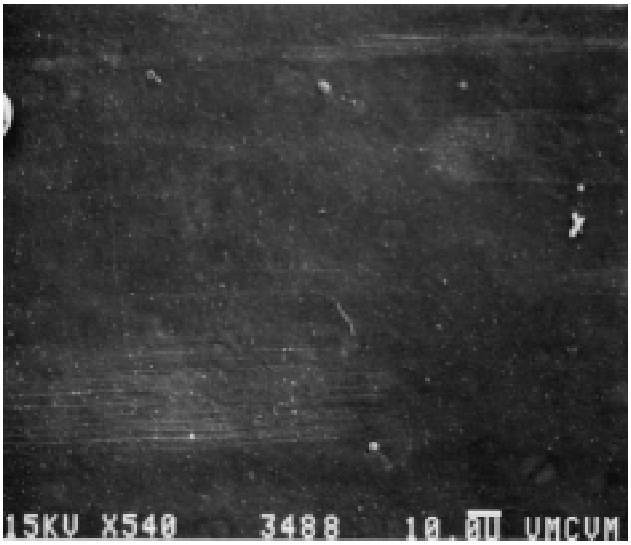

Figure 4. 7 SEM of ¼-*inch* cartilage plug tested with buffered saline

The 1-*inch* bottom plug of both the synovial fluid and saline tested cartilage sustained more damage due to the contact mechanics and loading method. There is a visible difference between the synovial fluid and saline tested cartilage. Both exhibited wear tracks in the direction of sliding, but with different characteristics. The synovial fluid tests produced a wavy, undulating surface with smooth ridges in the direction of sliding. The saline tests produced a dry, cracked surface with splitting and cracking along the direction of sliding. These surfaces are shown in **Figures 4.8** and **4.9** (arrows denote sliding direction).

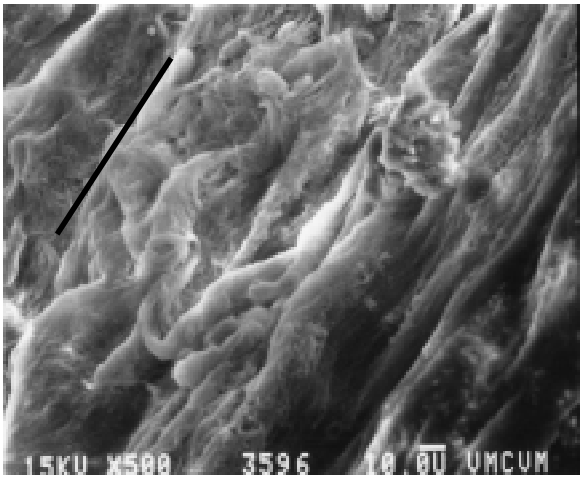

Figure 4.8 SEM of 1-*inch* cartilage plug tested with synovial fluid

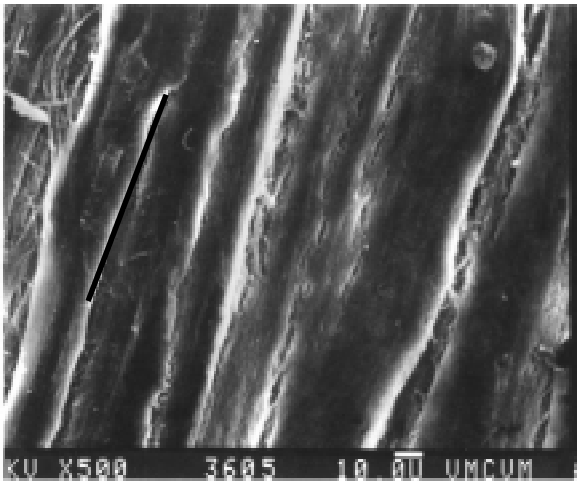

Figure 4.9 SEM of 1-*inch* cartilage plug tested with buffered saline

After exposure to collagenase-3 at the 50 u/ml concentration, tribological testing produced significant damage to both the ¼-*inch* and 1-*inch* cartilage specimens in both the synovial fluid and saline tests. **Figures 4.10-4.12** are micrographs of ¼-*inch* cartilage plugs treated with 50 u/ml of collagenase-3 before testing, after testing with synovial fluid and after testing with saline, respectively. The ¼-*inch* plugs tested with synovial fluid showed a disruption of collagen fibers in the surface layer. The fibers were visible in thick bundles of about 10- 20 microns in diameter and were randomly oriented at high magnifications. At lower magnifications, some directionality was seen in the fiber alignment. In the saline tests, wear tracks were evident at high magnification. The tracks were in the form of smooth undulations, about 15 microns in diameter which were separated by grooves aligned in the direction of sliding on the surface of the cartilage.

Figure 4.10 SEM of cartilage treated with 50 u/ml of collagenase-3 before wear testing

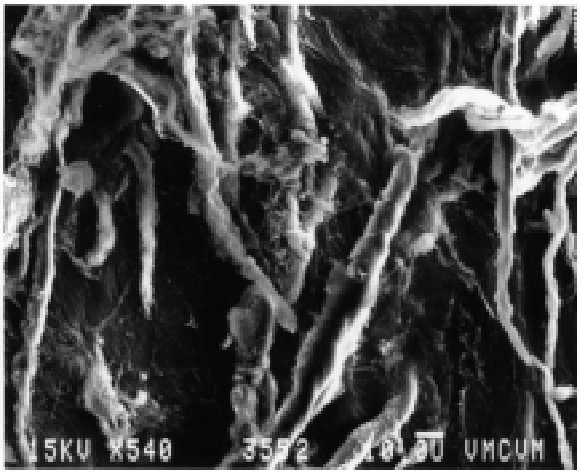

Figure 4.11 SEM of ¼-*inch* cartilage plug treated with 50 u/ml collagenase-3, tested with synovial fluid

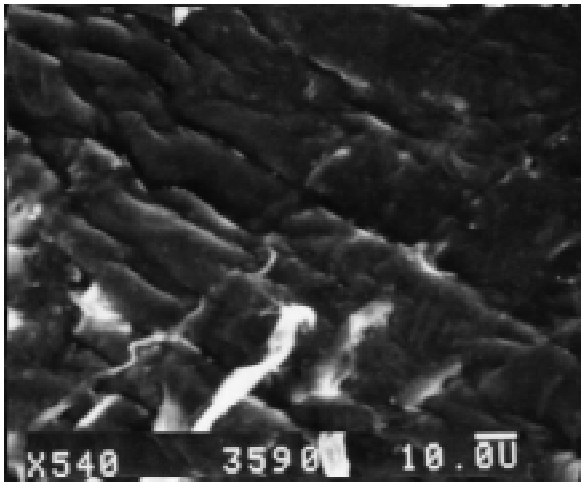

Figure 4.12 SEM of ¼-*inch* cartilage plug treated with 50 u/ml collagenase-3, tested with buffered saline

With the 1-*inch* plugs treated with 50 u/ml, the synovial fluid and saline tested cartilage exhibited similar features. Both tests produced disruption and separation of collagen fibers in the articular surface. The fibers were thinner in diameter than in the ¼-*inch* plugs, averaging about 5 microns for the synovial fluid tests and 2 microns for the saline tests. The alignment of the fibers was random in the synovial fluid tests and along the sliding direction for the saline tests. **Figures 4.13** and **4.14** are micrographs of 1-*inch* bottom plugs treated with 50 u/ml collagenase-3 and tested with synovial fluid and saline, respectively.

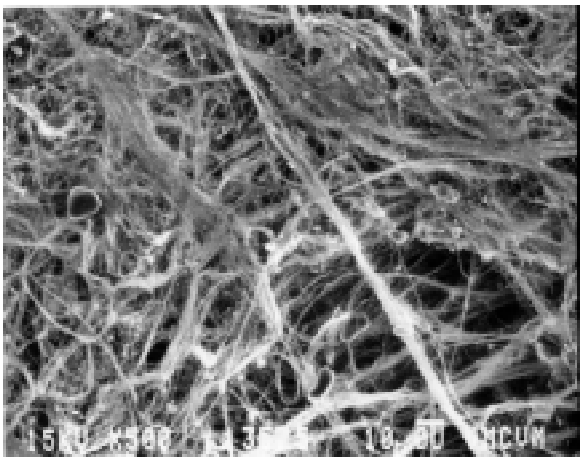

Figure 4.13 SEM of 1-*inch* cartilage plug treated with 50 u/ml collagenase-3, tested with synovial fluid

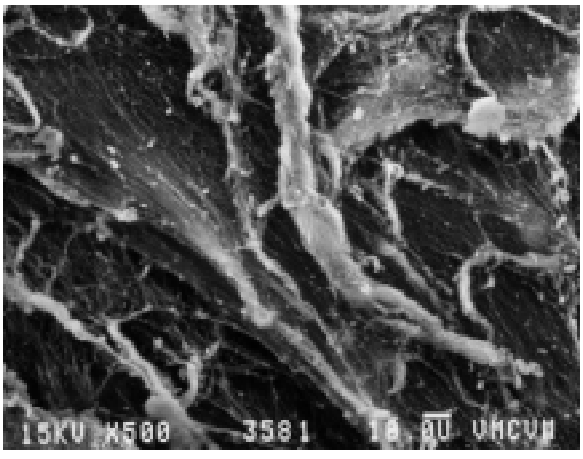

Figure 4.14 SEM of 1-*inch* cartilage plug treated with 50 u/ml collagenase-3, tested with buffered saline

Increasing the collagenase-3 concentration to 100

u/ml produced the surfaces shown in **Figures 4.15 - 4.18**. There were a few detectable differences between surfaces tested with the two lubricants. The saline tests seemed to produce collagen fibers with more directionality than the synovial fluid tests, which were still rather randomly oriented. The ¼-*inch* plugs still produced thicker collagen fiber bundles than the 1-*inch* plugs in both cases of lubrication.

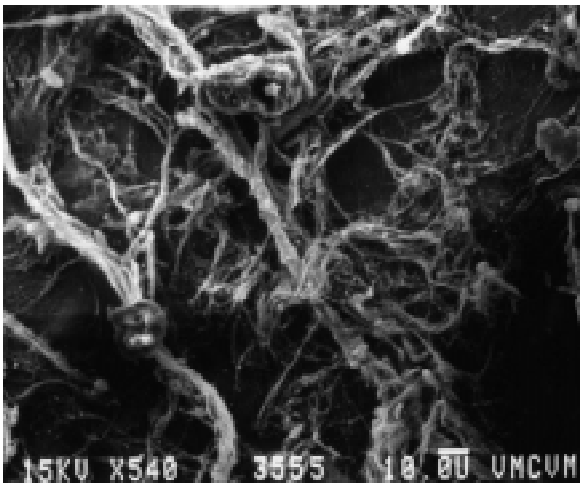

Figure 4.15 SEM of ¼-*inch* cartilage plug treated with 100 u/ml collagenase-3, tested with synovial fluid

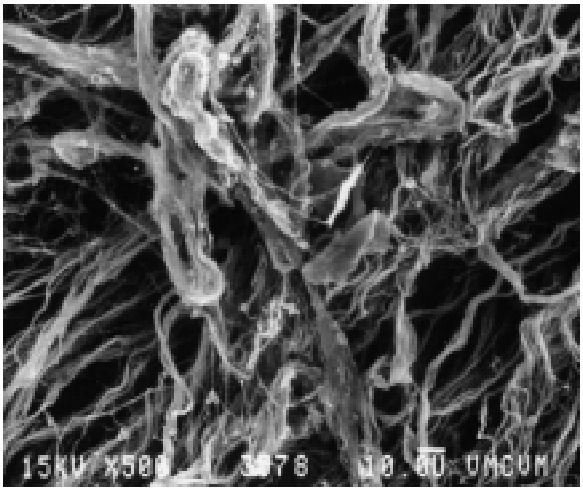

Figure 4.16 SEM of 1-*inch* cartilage plug treated with 100 u/ml collagenase-3, tested with synovial fluid

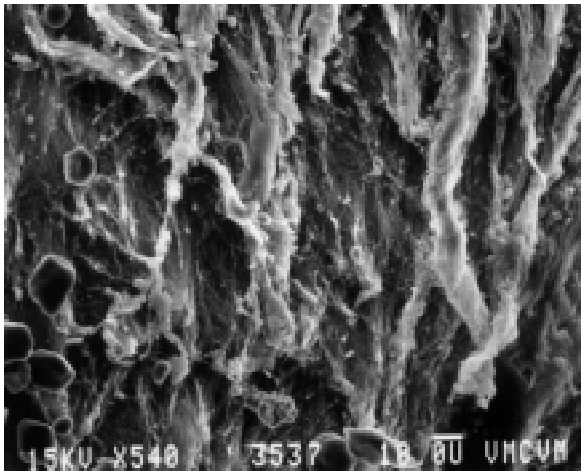

Figure 4.17 SEM of ¼-*inch* cartilage plug treated with 100 u/ml collagenase-3, tested with buffered saline

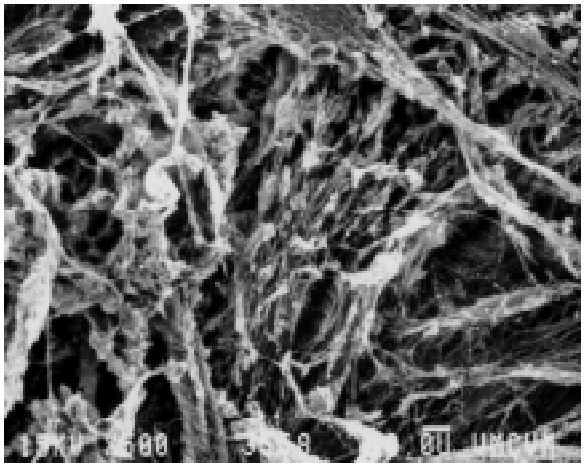

Figure 4.18 SEM of 1-*inch* cartilage plug treated with 100 u/ml collagenase-3, tested with buffered saline

4.5.4 *Histology*

After testing, selected cartilage plugs were prepared with histochemical techniques for histologic evaluation. The ¼-*inch* and 1-*inch* plugs from the tests were stained with hematoxylin and eosin (H&E) and alcian blue as outlined in **Section 3.3.3**. Surface and subsurface damage, as well as biochemical content and distribution were evaluated and compared according to lubricant and enzyme treatment.

For the control cases with no collagenase-3 exposure, the synovial fluid and saline tests yielded similar results. For the tests done with synovial fluid, the ¼-*inch* plugs showed normal staining with both stains and little to no surface damage to the superficial layer. The 1-*inch* plugs showed a lighter intensity of basophilic staining under that area of contact. **Figures 4.19** and **4.20** show slides of ¼-*inch* and 1-*inch* plugs of cartilage tested with synovial fluid.

In control tests done with buffered saline, the ¼-*inch* plugs again, showed little to no damage or abnormal cell alignment with either stain. The superficial layer remained intact and there was no sign of excessive wear or loss of the surface cartilage. The 1-*inch* plugs had a more detectable loss of cartilage in the contact area. There was also a loss of affinity for basophilic stain under the contact area. Example of both ¼-*inch* and 1-*inch* plugs can be see in **Figures 4.21** and **4.22**.

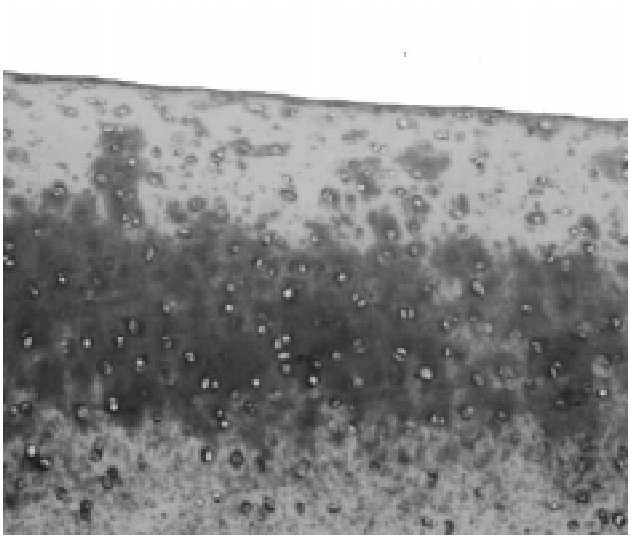

Figure 4. 19 Section of ¼-*inch* plug tested with synovial fluid, 0 u/ml collagenase-3

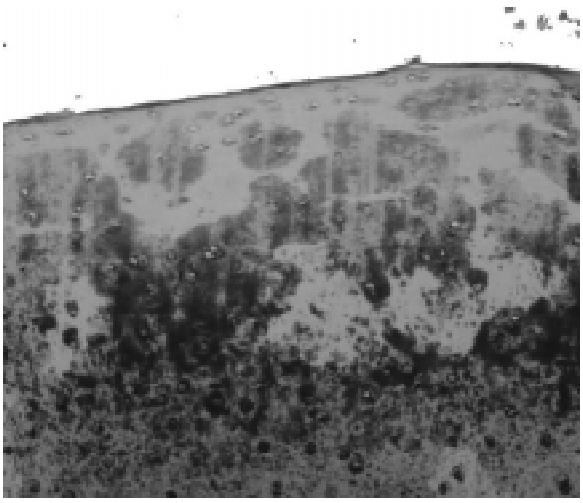

Figure 4.20 Section of 1-*inch* plug tested with synovial fluid, 0 u/ml collagenase-3

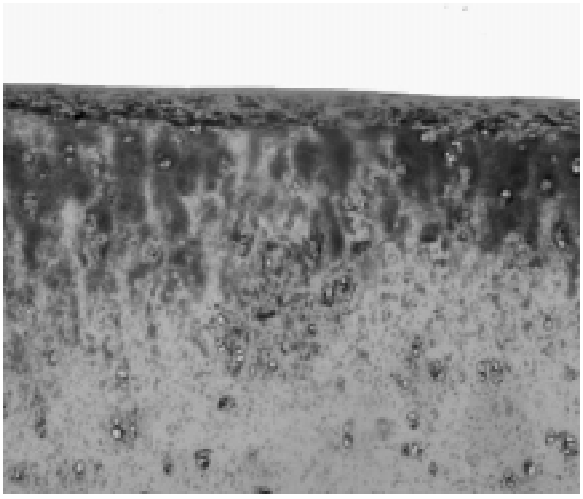

Figure 4.21 Section of ¼-*inch* plug tested with buffered saline, 0 u/ml collagenase-3

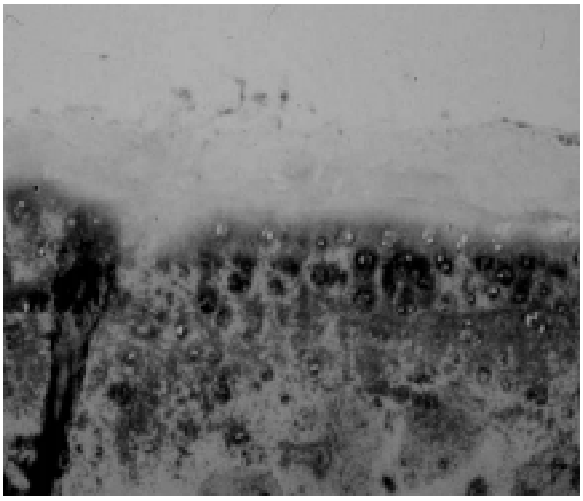

Figure 4.22 Section of 1-*inch* plug tested with buffered saline, 0 u/ml collagenase-3

After exposure to collagenase-3 at the 50 u/ml concentration level, increased damage to the cartilage was observed. In synovial fluid tests, the superficial layer of the ¼-*inch* plugs was disrupted and the surface topography appeared rough and bumpy. Some small areas were completely devoid of superficial layer. The cell alignment was slightly abnormal and there was a distinct loss of basophilic staining in the middle zone of the cartilage matrix.

The 1-*inch* plugs showed further damage in the contact area, although some of the superficial layer was still intact. There was a lot of subsurface disruption and damage underneath the area of contact. **Figure 4.23** is the alcian blue slide of a ¼-*inch* cartilage plug tested with synovial fluid.

The saline tests exhibited more severe histologic change and damage. The superficial layers were completely degenerated and devoid of cells. There was an almost complete loss of basophilic staining in the middle zone of the cartilage denoting a loss or removal of GAGs. A section of a ¼-*inch* plug for these tests is shown in **Figure 4.24**.

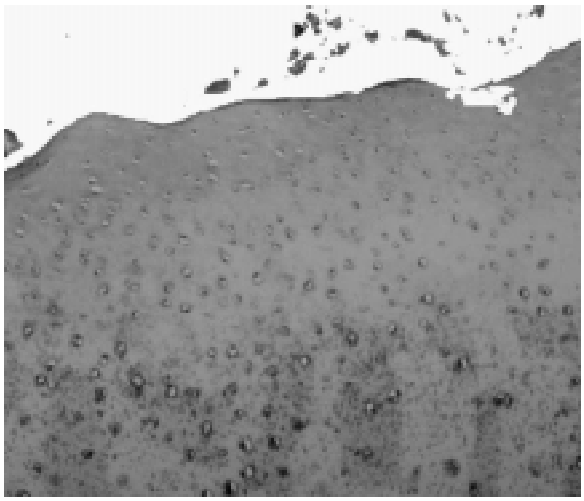

Figure 4.23 Section of ¼-*inch* plug tested with synovial fluid, 50 u/ml collagenase-3

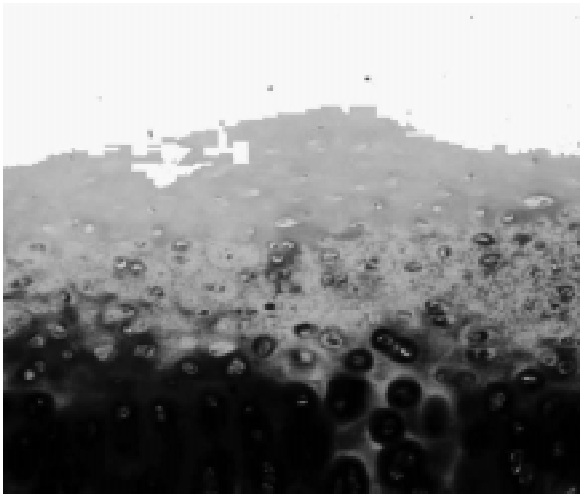

Figure 4.24 Section of ¼-*inch* plug tested with buffered saline, 50 u/ml collagenase-3

The cartilage treated with 100 u/ml concentration of collagenase-3 showed the same features of the saline tests exposed to 50 u/ml. There was the same loss of superficial layer and non-discrete surface. The removal of GAGs was also evident by a lack of basophilic stain in the middle zone, this was seen with both test lubricants and both top and bottom plugs.

4.7 Discussion

The results of this study are discussed in the following section. In addition, some of the major sources of error and improvements for further study are suggested.

4.7.1 *Wear*

The wear of articular cartilage increased significantly after treatment with collagenase-3 in comparison to normal untreated cartilage. As shown in **Figure 4.10**, collagenase-3 treated cartilage remained intact with no wear or irregularity. The treatment alone did not produce a loss of cartilage, but after tribological testing under normal load, wear and damage of the cartilage were observed.

The increase in wear was seen with both the synovial fluid and buffered saline lubricants. The saline tests on average produced more wear than the synovial fluid, but this difference was found to be statistically insignificant. In the collagenase treated samples, the lack of difference between the lubricants can be attributed to the severity of the damage created by the collagenase treatment. In these cases the damage was so severe, the effect of the lubricant was much less significant in comparison and difficult to detect in a small study such as this. However, the controls failed to show a difference between lubricants as well. In previous work done with cartilage-sliding-on-steel [3], cartilage plugs tested with saline lubricant produced more wear than those tested with natural synovial fluid by a factor of 10. In the cartilage-sliding-on-cartilage configuration, the conditions are much less severe and may result in no difference between synovial fluid and saline lubricants. First, there is much more variability in two cartilage surfaces as opposed to one. The cartilage imbibes fluid during decompression cycles and expels it during compression. This repeated cycle in both surfaces may add biochemical constituents to the saline that help prevent wear and damage of the surfaces. In this study, the cartilage surfaces were soaked in their respective test fluid prior to test in order to reduce this effect. Unfortunately, it cannot be completely removed. In addition, in cartilage-on-steel tests, the steel surface is significantly harder than the cartilage. Therefore, deformation occurs solely in the cartilage surface. The viscosity of the lubricating fluid is more relevant in reducing the compressive load

experienced by the cartilage in this case than it is in the cartilage-on-cartilage cases where both surfaces are deforming. The increased compressive stress produced in cartilage-on-steel tests with the saline lubricant may be responsible for the increased levels of wear observed. In the tests of this study, the lubricating fluid viscosity is less significant in the compressive stresses in the cartilage and may lead to a lack of difference between the saline and synovial fluid lubricants. The results indicate that other factors such as biochemical constituents of the fluids, or lack of, had no resulting effect on the wear of the cartilage surfaces, under the conditions of this study.

Surprisingly, there was no difference between the 50 and 100 u/ml concentrations of collagenase-3 treatment. The absence of an increase in wear with the 100 u/ml concentration over the 50 u/ml concentration may be due to the fact that the damage done by the lower concentration was already so significant that additional enzyme made little difference in the wear. Hence, after the initial damage to the cartilage matrix, the wear rate became independent of enzyme concentration and was dependent only on the system and parameters. Also, since u/ml is a measure of how many bonds are cleaved in the cartilage as a function of mass and time, during the enzyme exposure, a terminal amount of collagen bonds within the cartilage may have been cleaved and adding additional enzyme did not affect this result because the mass of cartilage remained relatively unchanged in each case. It is also possible that there was a physical limit as to how deep the enzyme could penetrate the cartilage. Therefore, both the 50 and 100 u/ml collagenase treated cartilage had the same amount of degradation created by the enzyme, leading to no difference in the wear between the two treatments.

There was no significant increase in wear detected between cartilage of the bovine knee and shoulder. The two types of cartilage exhibited macroscopic differences in appearance and texture. As cartilage is variable from location to location within the same animal, they may have different structural characteristics such as differing collagen fiber alignment and constituent dependence on depth. Nonetheless, these variations did not result in observable differences in wear in this study. In addition, there were no significant interactions of factors detected that affected the wear of cartilage.

4.7.2 *Scanning Electron Microscopy*

Observation of the cartilage surfaces with SEM after testing allowed for determination of surface damage created by each treatment. No damage or surface irregularities were seen in the ¼-*inch* plugs of the control tests with either lubricant. This indicates that the tribological wear test has little effect on the cartilage. The conditions of testing are not excessively exaggerated as to produce high levels of wear with normal cartilage. The wear observed is from the superficial layer of the cartilage surfaces which, in an *in vivo* situation, may be regenerated. The 1-*inch* bottom plugs of the control tests experienced more damage than the top plugs due to the fact that they are dynamically loaded and their geometry was more irregular. The tensile stresses experienced by the 1-*inch* plug were greater and led to some damage to the surface.

In the collagenase treated samples, excessive surface damage was observed relative to the untreated samples. Damage and removal of the superficial layer was seen with exposure and disruption of the underlying collagen fibers. The mode of wear is difficult to determine, but indicates that there was an abrupt removal of the surface and fatigue failure of the collagen network. The damage seen differed slightly in accordance to the lubricant used. The synovial fluid seemed to provide some increased protection over buffered saline. In control tests, synovial fluid resulted in a slightly less severely damaged surface than saline. In collagenase treated cases, the synovial fluid produced less damaged collagen fibers that were randomly oriented, while the saline tests showed more disruption of fibers aligned in the direction of sliding. This indicates that synovial fluid protects cartilage surfaces and collagen fibers better than saline. The method by which it does this is undetermined. It may be due to constituents of synovial fluid that bind to cartilage and collagen providing some barrier to contact, or it could simply be a function of viscosity.

There was no difference in the micrograph features of the 50 and 100 u/ml treatments. Samples treated with 100 u/ml of collagenase-3 had a more complete removal of superficial layer in some cases, but there was no consistent increase in the severity of damage. This is in agreement with the wear data.

4.7.3 *Histology*

Histologic examination of the cartilage cross sections after testing showed varying degrees of damage and histologic change. The untreated controls were normal with no surface damage or abnormal histologic features. The results coincide with both the wear and SEM results. The 1-*inch* plugs showed slight damage to the surface in the contact area and a slight loss of GAGs under the contact area. This is due to the compression of the tissue and was easily differentiated from the damage due to treatment effects.

The collagenase-treated cartilage showed damage and loss of the superficial layer, in addition to a significant depletion of GAGs in the middle zone of the cartilage. It appears that the collagenase enzyme cleaved the collagen fibers, leading to the release of proteoglycans from the middle layer of the cartilage. The molecular components of the ground substance could have diffused through the tissue and exited through the damaged superficial layer.

The synovial fluid and saline lubricants produced different features in both the collagenase treated and control cases. The synovial fluid provides some form of added protection from surface damage and subsurface histologic change. It is suspected that in these cases of severely damage cartilage, the biochemical constituents and the viscosity of synovial fluid may play a role in the damage of the cartilage. The lower viscosity of the saline leaves more compressive load for the cartilage to support. After enzyme treatment, compressive stresses can lead to the loss of proteoglycans from the compressive load bearing region of the cartilage. The loss of the GAGs leads to the loss of compressive strength and the subsequent fatigue of the cartilage surface and subsurface. The higher viscosity of the synovial fluid supports more of the compressive load and can lead to less loss of GAGs and less damage of the cartilage. The biochemical constituents may protect exposed fibers from further damage.

4.7.4 *Proposed Mode of Wear and Damage*

It is proposed here that cartilage wear is greater when exposed to enzyme treatment because when bonds are broken within the collagen matrix via enzymatic degradation, biomechanical

wear testing makes it easier to remove cartilage from the surface or superficial layer. The collagenase enzyme can cleave the collagen triple-helical matrix across all three chains or a single strand. In either case, the cleavage, which usually occurs between the Y- Gly bond, leads to the uncoiling of the of the collagen conformation. The breakage of these bonds and uncoiling of the matrix lead directly to the loss of the proteoglycans within the matrix by way of diffusion through the disrupted matrix and the damaged or removed superficial layer.

To evaluate this possibility, an ELISA assay for detection of keratan sulfate was performed on samples of the normal, control and enzyme-treated test fluids [100]. As discussed in **Section 2.1**, keratan sulfate is a biochemical constituent of proteoglycan aggrecan and can thus be used as a measure of proteoglycan content. If the proteoglycans diffused through the cartilage matrix and exited the cartilage through a damage surface layer, they should be detected in increased levels in the lubricating fluid.

Increased levels of keratan sulfate were detected in the fluids from the enzyme treated tests. The normal synovial fluid before testing and fluid from control tests exhibited the same levels of proteoglycan, while the fluid from a collagenase treated test exhibited higher levels of proteoglycans than the untested fluid. **Table 4.7** illustrates a comparison of concentrations found in each fluid. This supports the theory that the proteoglycans are lost through damaged cartilage surface layers and enter the synovial fluid. Since the proteoglycans give the cartilage its elasticity and compressive strength, their loss is directly implicated in the increase in wear seen with the collagenase-3 treatment.

Table 4.7 Comparisons of Keratan Sulfate Concentrations

Lubricant	Normal untested synovial fluid	Synovial fluid tested with normal cartilage	Synovial fluid tested with collagenase treated cartilage
Keratan Sulfate Content (μg/ml)	18	18	57

Although wear was not significantly affected by the lubricant used, differences in the severity of damage were observed. Synovial fluid provides increased protection of cartilage surfaces and collagen fibers from damage. The inability to detect differences in wear may be due to the variability found among biological tissues. It may also indicate that the synovial fluid protects cartilage from damage, but that damage did not lead to loss of cartilage in the saline test cases.

4.7.5 *Relevance of Study to In Vivo Conditions*

The collagenase-3 enzyme is an enzyme actively released by the chondrocytes of OA cartilage [89,90]. The wear and damage they produce in the articular cartilage is relevant to understanding the possible modes of wear in OA. It is noted that intra-articular temperatures are generally below 34^{o}C *in vivo* and the incubation with collagenase-3 was done at 37 ^{o}C. This may suggest that the treatment is not representative of possible *in vivo* conditions. However, during instances of stress and trauma, the intra-articular temperature can rise to 37^{o}C and above [101] and the enzyme is a catalyst that maintains activity over a range of temperatures. Therefore, if the chondrocytes are releasing the collagenase-3 enzyme, the damage observed in this *in vitro* experiment, could be seen *in vivo*.

4.7.6 *Sources of Error*

There were several sources of error in this study. Some sources were unavoidable and others were not recognized before the study was begun.

First was the variability of results. Most of variability within a certain treatment group is attributed to the variability of the biological materials used. The articular cartilage varies from location to location within the same joint, from joint to joint within the same animal, and from

animal to animal within the same species. The variability due to the animals was taken into account in the statistical design, but the remaining variability could not be eliminated. This factor not only affects the wear data, but the SEM and histology results as well.

For the purpose of statistics, larger treatment groups were necessary. If the treatment groups were large enough to see random distribution within each group, the statistical analysis would have had more power for detecting differences between treatments. The variability between joints and individual plugs would have been less significant. Unfortunately, the size of the groups was limited by the available materials. The number and uniformity of cartilage samples was limited by what was currently available.

The other source of error was in the hydroxyproline assay. Each set of cartilage from different animals was used in a batch of tests. That batch was tested and the hydroxyproline analysis done separately from the other sets. Each run of the assay yields different results and has to be compared to a standard. The variability of each set of hydroxyproline data was a source of error that could have been avoided if all tests were completed first and the lubricants assayed together in one batch. Most of the error was reduced by the block design of statistical analysis.

4.7.7 *Improvements for Further Study*

The following are several improvements that can be made for a more detailed study on the effect of collagenase-3 on cartilage wear and damage:

- Acquire a uniform set of animals and test cartilage closer to time of death of animal
- Fully characterize cartilage via SEM, histology and other techniques prior to testing
- Perform a separate study to test the variable resistance to wear of cartilage from different animals
- Perform a separate study to test the tribological properties of knee vs. shoulder cartilage
- Use one concentration of collagenase-3 found in OA cartilage and study effect on normal cartilage from similar locations in the same joint

- Use one composite/uniform synovial fluid as a test lubricant to reduce variability between fluids.
- Modify test device for temperature and humidity control
- Test the effectiveness of inhibitors at impeding the action of the enzyme *in vitro*

4.8 Conclusions for Phase I Study

It is concluded from this study that exposure to the collagenase-3 enzyme increases the biomechanical wear of articular cartilage during *in vitro* tribological wear testing. The damage to the surface of the cartilage increases as well with collagenase-3 exposure, as seen with SEM. Both SEM and histologic evaluation confirmed damage to or removal of the superficial layer of cartilage exposed to the collagenase-3 enzyme. The method by which the enzyme increases wear and damage is undetermined. However, evidence exists that collagenase-3 cleaves the bonds of the collagen matrix, thereby releasing the proteoglycans and other ground substances, which are in turn exuded from the matrix during compression. The proteoglycans are then easily lost through the damaged or removed superficial layer. Loss of proteoglycans to the lubricating fluid was substantiated by their increased detection in post-test fluids by keratan sulfate ELISA. With the loss of the proteoglycans and destruction of the collagen matrix, the cartilage loses its elasticity, tensile and compressive strength and is more susceptible to wear and damage.

The normal synovial fluid of the joint seems to provide some increased protection against damage over the saline lubricant. The exact method by which synovial fluid acts to protect the cartilage surface is not known. The effects of the lubricant type, joint type and interactions of these factors on wear were all found to be statistically insignificant, but a larger test design, with a larger n in each treatment group, is recommended to validate these results. In addition, further investigation is needed to confirm the method by which collagenase-3 exposure increases cartilage wear and the method by which synovial fluid protects cartilage surfaces.

This study confirms that the collagenase-3 enzyme has the ability to play a significant role in the increased wear and damage of articular cartilage seen in osteoarthritis. The proposed methods by which wear and damage are increased by exposure to the enzyme provide new research questions related to joint pathology. Methods to inhibit the action of the enzyme may be crucial in the treatment of the disease. The electron microscopy and histologic results have proved a useful method of comparison of different cartilage treatments and may be useful in additional *in vitro* studies that examine possible *in vivo* causes of osteoarthritis.

5 PHASE II: Wear of Osteoarthritic Lapine Cartilage

5.1 Theory and Background

As previously discussed, the initial causes and progression of OA remain undetermined. There are several theories that have been proposed as possible pathways of the disease. Among them are theories of adverse biochemical conditions and mechanical instability that contribute to the loss and damage of the articular cartilage. Once OA begins, some patients see an accelerated decline in the health of their synovial joints while others do not. This has raised the question as to whether or not fibrillation results in a change in the mechanical properties of the cartilage, resulting in increased wear and damage. The fact that some joints exist with fibrillated cartilage and never experience any complications associated with OA has led researchers to discuss the role of initial cartilage fibrillation and degeneration in the subsequent wear and damage associated with the disease [31,102,103].

Although it is widely accepted that fibrillation increases in severity during the progression of osteoarthritis, the effect of fibrillation on the subsequent biomechanical wear and damage of the cartilage has yet to be determined. Review of current literature does not reflect any studies done that investigate the tribological properties of osteoarthritic cartilage in comparison to those of normal cartilage. However, several studies have been done to investigate the *in vivo* characteristics of OA with animal models. One model in particular involves the surgical induction of osteoarthritis into laboratory rabbits. OA is induced through meniscectomy of the lateral meniscus and transection of the lateral collateral ligament of the knee. The rabbits develop clinical OA within six weeks. These studies provide a means to compare the properties of normal and OA joints. It is therefore of interest to examine the tribological properties of OA cartilage in comparison to normal cartilage in an effort to determine whether these changes contribute to the progression of the disease.

5.2 Objective

The objective of the Phase II study was to determine whether osteoarthritic cartilage has different tribological properties than normal cartilage. Specifically, to evaluate if OA cartilage has

different wear characteristics and friction coefficients than normal cartilage. Wear tests were performed using lapine (rabbit) cartilage from specimens with surgically induced OA in one knee joint. Cartilage from the remaining joint was tested as a control and the tribological properties of the cartilage from both knees was compared.

5.3 Materials and Methods

5.3.1 *Cartilage Preparation*

The surgical and trimming procedures performed on the lapine cartilage specimens were described in **Section 3.1.3**. After the specimens were prepared for mounting on the test device, they were soaked, cartilage side down, in phosphate buffered saline which served as the lubricant for all the Phase II testing. The specimens were then refrigerated until testing.

5.3.2 *Stainless Steel*

Highly polished stainless steel disks were made from a 1-*inch* diameter 303 steel rod. The disks were cut, ground and polished to an average surface roughness of approximately 100 Angstroms. A separate disk was used as the lower specimen for each test in this study.

5.3.3 *Lubricant Preparation*

Phosphate buffered saline was removed in 1 ml aliquots from a bottle stored at room temperature and put between the lapine femoral condyle and the stainless steel disk during testing.

5.4 Design

Due to the limited number of samples available for this study, a very simple design was employed to determine if a significant difference exists between the wear of osteoarthritic and normal cartilage. Three surgical rabbits were used in the test design. Each rabbit had one joint with surgically induced osteoarthritis and one normal joint. The lateral femoral condyle of both the surgical and normal legs were dissected and prepared according to the procedure described in **Section 3.1.3**. A total of 6 specimens were prepared: 3 OA condyles and 3 normal condyles, which served as controls. The tests were organized in a fully balanced block design to be analyzed with analysis of variance procedure.

5.5 Wear Tests

After the cartilage was allowed to soak in the saline lubricant, the specimen was removed and mounted, along with the steel disk in the upper and lower specimen holders, respectively. Fresh saline was added between the contacting surfaces and translation of the lower disk begun. A load of 39.2 N was added via dead weight to the shaft. This load is representative of what the joints of the 1.5-1.6 kg rabbits would experience. The test was run for a total of 60 minutes with data acquisition taken at 30 minute intervals. Tangential load was measured through the strain gage system which was balanced before the start of the test. The test parameters are shown in **Table 5.1**.

Table 5. 1 Test parameters

Phase II Test Parameters	
Configuration	cartilage-on-stainless steel
Load	39.2 N dead weight
Duration	60 minutes
Lubricant	phosphate buffered saline
In Situ Data Acquisition	tangential force
Post-test Analysis	SEM, histologic evaluation, hydroxyproline assay

Upon completion of the tests, the cartilage and steel specimens were rinsed thoroughly to remove any loose wear debris. The cartilage was sectioned perpendicular to the sliding direction and half was fixed for histological sectioning and staining, the remaining half was fixed for viewing with scanning electron microscopy. The lubricating fluid and rinses were collected and frozen until analyzed by hydroxyproline assay. The steel disks were stored in petri dishes for possible future analysis with Fourier Transform Infrared (FTIR) spectrometry.

5.6 Results

After all tests were completed, analysis was performed according to the protocols outlined in **Section 3.3**. Wear, friction and damage of the cartilage specimens were measured and related to the test treatments. Hydroxyproline assay of test lubricants was used to quantitatively determine wear of the cartilage surface. Information obtained from scanning electron microscopy and

histological sectioning and staining was combined and used to qualitatively determine surface damage and biochemical changes in the cartilage.

5.6.1 *Wear Results*

The buffered saline lubricant was collected after wear testing and analyzed for hydroxyproline content. Using the assay procedure, cartilage wear was determined for the six wear tests: three with OA cartilage and three with normal cartilage. In each of the six tests, the fluids were assayed in triplicate and the average used as the hydroxyproline content.

The wear results obtained from the six tribological tests are shown in **Figure 5.1**. The average wear values for each test treatment are shown in **Table 5.2** and graphically represented in **Figure 5.2**. (See **Appendix A** for data tables.)

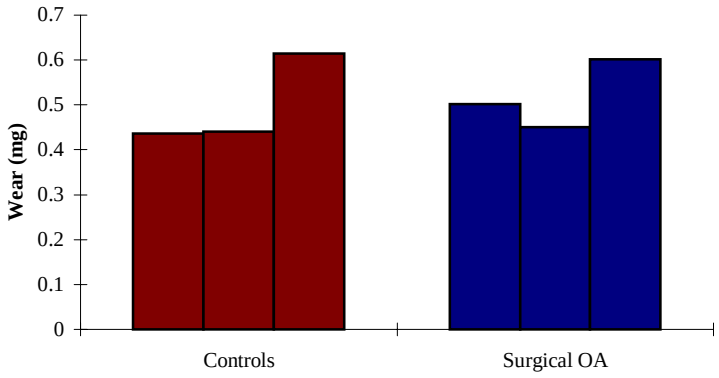

Figure 5. 1 Wear values for Phase II study

The average wear of the osteoarthritic cartilage increased by 4% over the normal cartilage. The standard deviations are included in **Table 5.2** and it appears that there is no statistical difference between the mean wear of the two types of cartilage.

Table 5. 2 Average wear values for Phase II study

Cartilage Type	Cartilage Wear (mg)
Normal	0.497 ± 0.102
Osteoarthritic	0.518 ± 0.077

The wear of cartilage from one animal, used in tests II06 and II07, exhibited the highest values in its respective treatment groups. A full statistical analysis is performed in the following section.

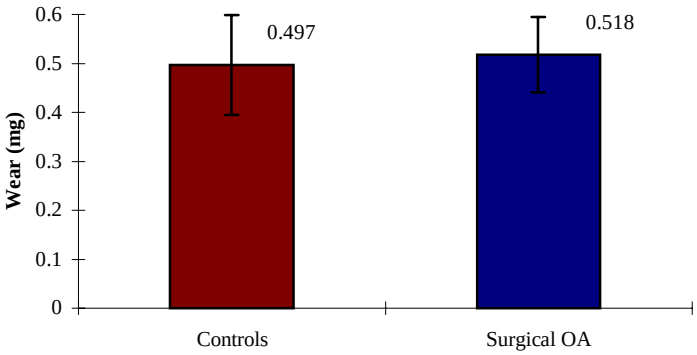

Figure 5. 2 Average wear for Phase II study

5.6.2 *Statistical Analysis*

The testing in Phase II was done in a randomized complete block design. Each animal was used as a block because there is an expected variation between cartilage from different animals. The only treatments or variables involved were the source of cartilage which were the surgically induced OA cartilage or non-surgical controls. Statistical analysis of the wear data was done using the SAS System software. Differences between the means were analyzed using analysis of variance at the $\alpha = 0.05$ confidence interval. The mean wear for the controls was 0.497 mg with a standard deviation of 0.102 mg. The mean wear for the osteoarthritic cartilage was 0.518 mg with a standard deviation of 0.077 mg. Analysis of variance showed no significant difference

between the controls and the osteoarthritic cartilage, p= 0.4652. (See **Appendix B** for SAS code and output.)

5.6.3 *Scanning Electron Microscopy*

After testing, the lapine condyles were cut through the middle of the contact area perpendicular to the sliding direction. Half of the condyle was then prepared for viewing with scanning electron microscopy. Damage to the surface of the articular cartilage was evaluated and compared according to the cartilage source: normal or osteoarthritic.

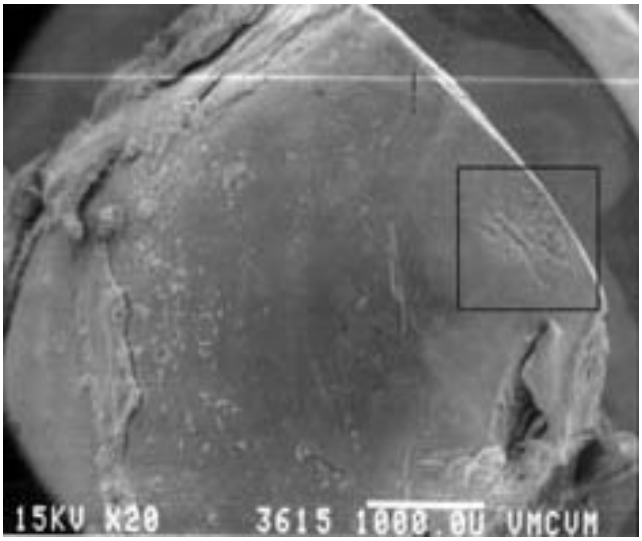

Figure 5.3 SEM of non-surgical condyle after wear testing (box indicates contact area)

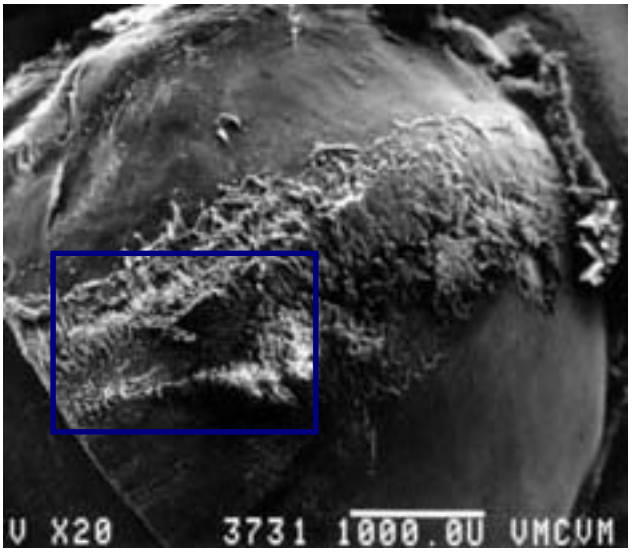

Figure 5.4 SEM of surgical condyle after wear test (box indicates wear test contact area)

The normal cartilage tested from the control (non-surgical) knee showed no damage other than that created by the tribological testing, **Figure 5.3**. In the contact area, the cartilage was mostly intact with some scratches and cuts due to plowing of the surface by steel asperities, shown in **Figure 5.5**. These cuts were all parallel to the sliding direction. There was some pitting and ridges perpendicular to the direction of sliding, as a result of tensile failure of the tissue during sliding contact. All damage appeared to be isolated to a thin layer of the surface tissue and there was no visible fibrillation. The matrix was grossly intact aside from the overt tears due to asperity plowing.

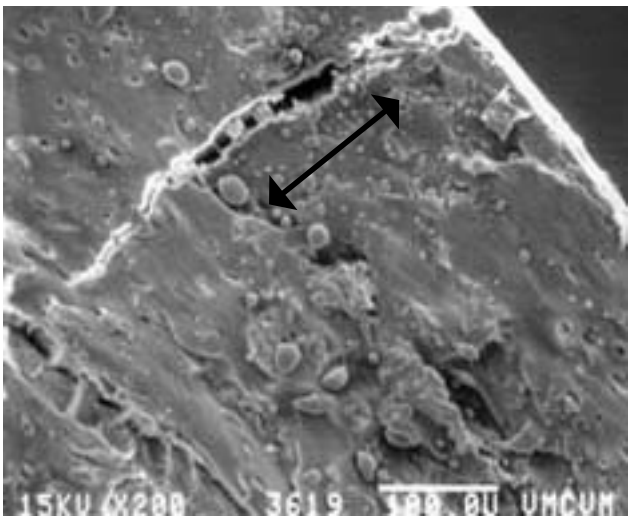

Figure 5. 5 Plowing damage by steel asperities in non-surgical condyle
(arrow indicates sliding direction)

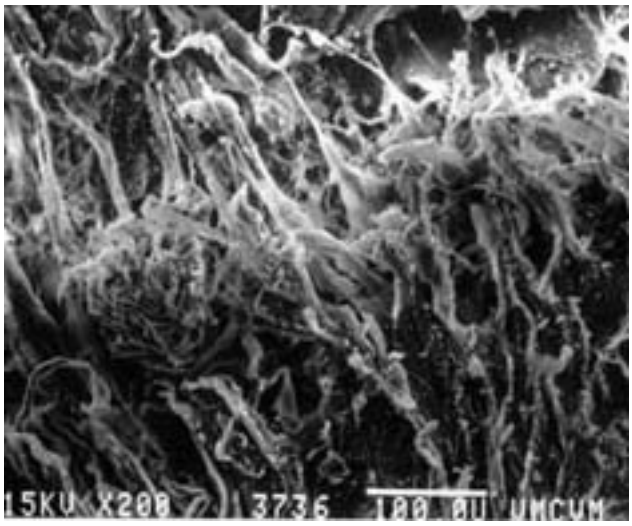

Figure 5. 6 Collagen fibers in surgical condyle after wear testing

After tribological testing, cartilage with surgically induced osteoarthritis exhibited extensive disruption of the cartilage matrix, **Figure 5.4**. Collagen fibers were visible in bundles of < 10μm in diameter within the contact area, as shown in **Figure 5.6**. There was a clear loss of the proteoglycans and interstitial material within the contact area. The contact area is visible as a deep groove (shown in **Figure 5.4**) within the pre-existing wear scar created by the surgical procedure. Outside of this groove (contact area) the collagen fibers are in thicker bundles > 10 μm in diameter. The fibers are aligned perpendicular to the sliding direction, both inside and out of the contact area created by testing. In one case, the fibers were randomly oriented throughout the wear scar and the contact area. These fibers were also < 10 μm in diameter. The micrographs for tests II06 and II07 showed the most extensive damage in their respective treatment groups. Test II07 (**Figure 5.6**) shows collagen fibers similar to those seen in collagenase studies.

Cartilage from the surgical knee of the rabbit, with induced osteoarthritis, exhibited substantial disruption of the matrix prior to tribological testing, **Figure 5.7**. A clear wear track is visible with loose collagen fibers and cracks at the margins of the wear scar. Inside the scar there is a

roughening of the surface, but the proteoglycans and interstitial material are still present. The surface inside the wear scar is obviously at some intermediate stage of degeneration. **Figures 5.8** and **5.9** are additional micrographs of this specimen.

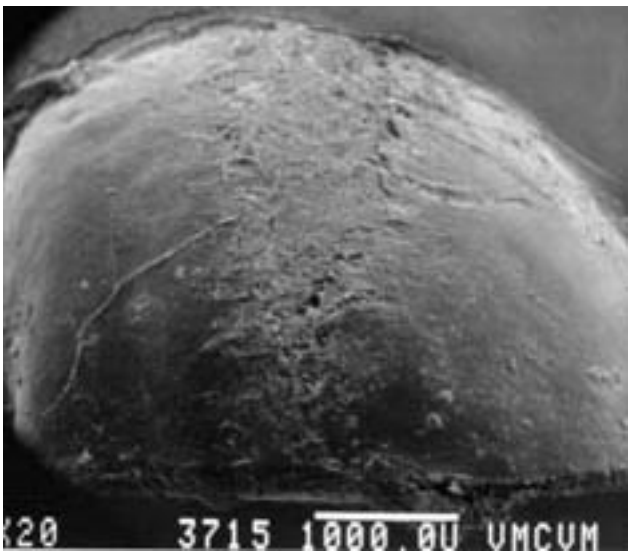

Figure 5. 7 SEM of surgical condyle before testing

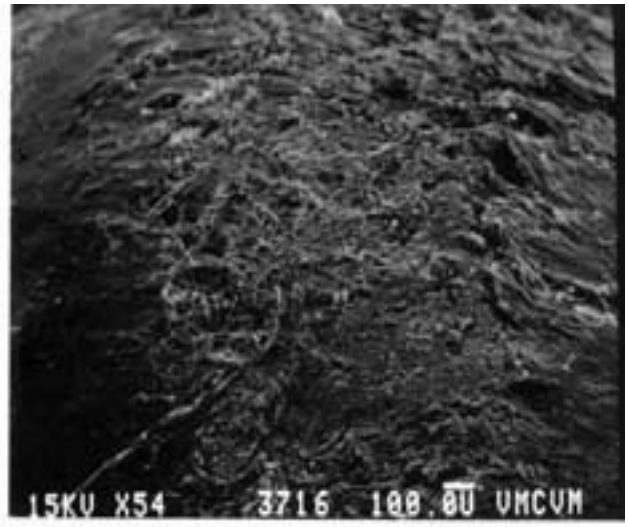

Figure 5. 8 SEM of surgical condyle before wear testing

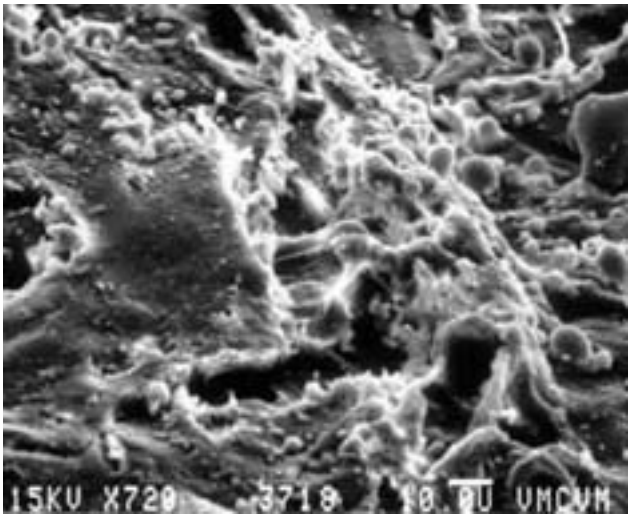

Figure 5. 9 SEM of surgical condyle before testing

5.6.4 *Histology*

Histological examination of cartilage sections after wear testing revealed similar information as the electron microscopy. Cartilage sections were stained with both alcian blue and hematoxylin

& eosin. In order to distinguish the damage created from the surgically induced OA and the tribological wear testing, a sample of cartilage was taken from a condyle with induced OA before testing and a section and stain prepared. The cartilage section stained with alcian blue is shown in **Figure 5.10**.

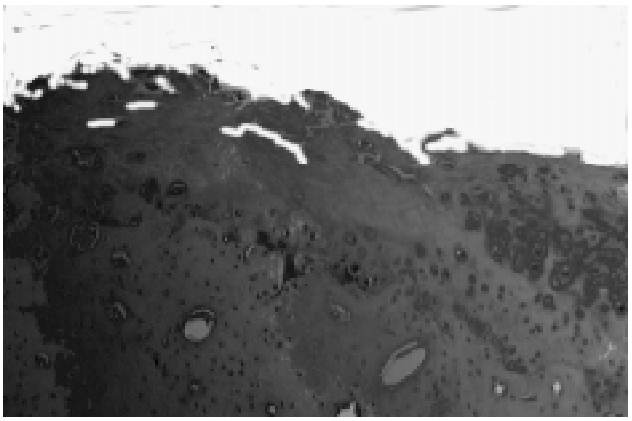

Figure 5. 10 Section of surgical condyle before wear testing

The cartilage from the non-surgical lapine condyles showed little damage to the superficial layer in the area of contact. The cartilage was intact for the most part and showed only minimal disruption, as shown in **Figure 5.11**. The cell alignment was normal and staining was within the normal variation for this type of connective tissue. There was increased damage in test II06 affecting the surface layer and some of the deeper layers. The other controls exhibited damage restricted to the surface layer.

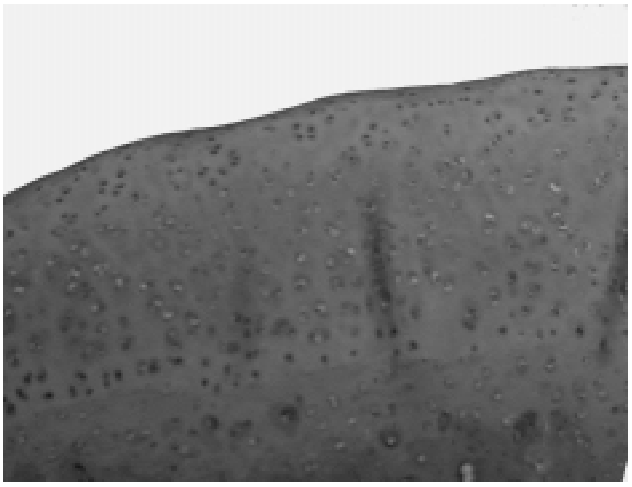

Figure 5. 11 Section of non-surgical condyle after wear testing

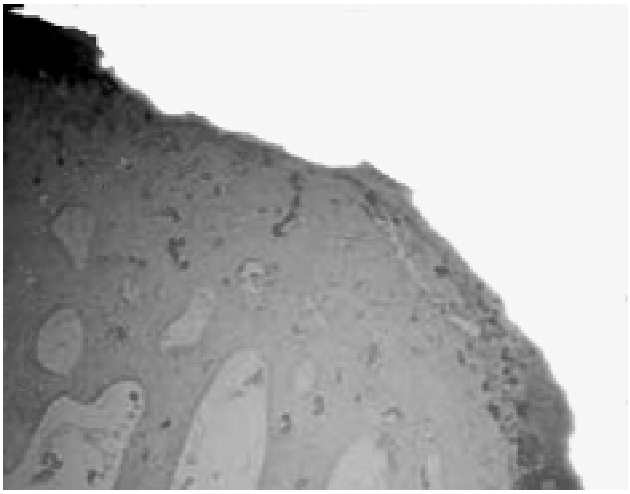

Figure 5. 12 Section of surgical condyle after wear testing

In the cartilage with surgically induced osteoarthritis, the cartilage damage was much more severe. In all three cases, the cartilage was worn all the way to the bone, with test II07 showing the worst damage. The damage to any remaining cartilage was severe with the loss of cells and clustering near the area of contact. There was also a depletion of GAGs around the area of contact. **Figure 5.12** shows a surgical condyle stained with the alcian blue stain after wear testing.

5.6.5 *Friction*

The data acquisition was analyzed to determine the coefficient of friction during the tests. Data were taken at the beginning of the test (0 min.), at 30 minutes and at 60 minutes. The acquisition was done in three second intervals with a frequency of 250 Hz. The data collected were in the form of voltage change in the strain gages. The voltage data were used to determine the tangential force applied to the octagonal strain ring. These data were then divided by the applied normal load to obtain the coefficient of friction. The friction coefficient plots shown are 3 second sampling intervals that include 2 full cycles of motion.

There was variation over time and treatment in the characteristics of the friction plots. The plots exhibited the standard cyclic plateaus seen during oscillating tribological tests. The absolute values were taken of all data to remove the negative values resulting from the reversal of motion. The resulting positive plateaus represent the friction of the cartilage surfaces during sliding. **Figures 5.13** and **5.14** are friction coefficient plots of control and surgical cartilage at 30 minutes of testing, respectively.

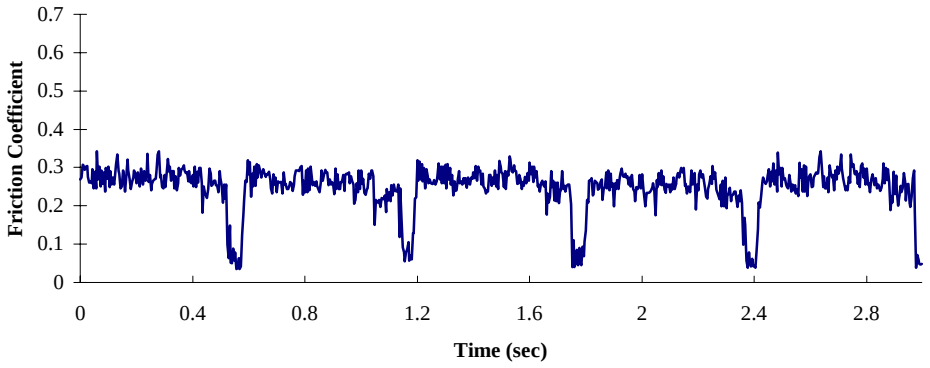

Figure 5. 13 Coefficient of friction at 30 minutes for a non-surgical control specimen

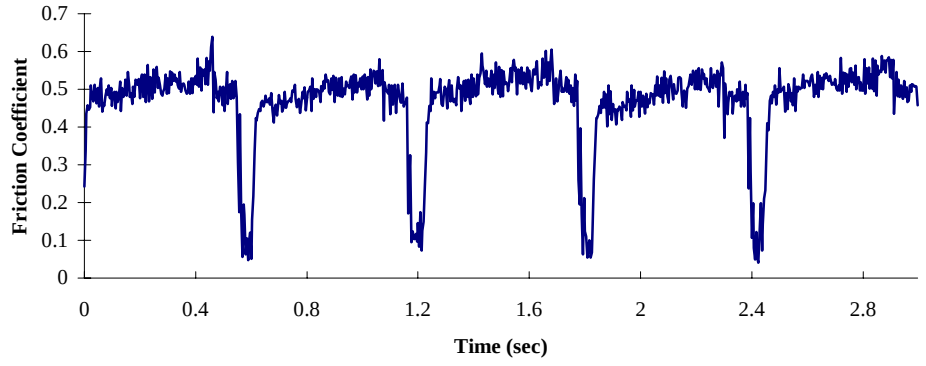

Figure 5. 14 Coefficient of friction at 30 minutes for a surgical OA cartilage specimen

It is evident from these plots that the OA cartilage has a higher friction coefficient than the normal control in this animal and that friction in both sliding directions is relatively the same. At

60 minutes of testing, all the OA and control tests showed variation in the magnitude of friction in the opposing sliding directions. **Figure 5.15** and **5.16** are friction coefficient plots at 60 minutes for both treatments.

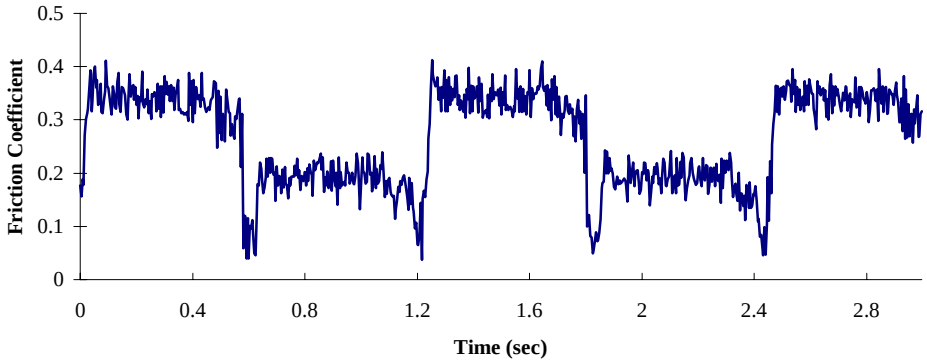

Figure 5. 15 Coefficient of friction at 60 minutes for a non-surgical control specimen

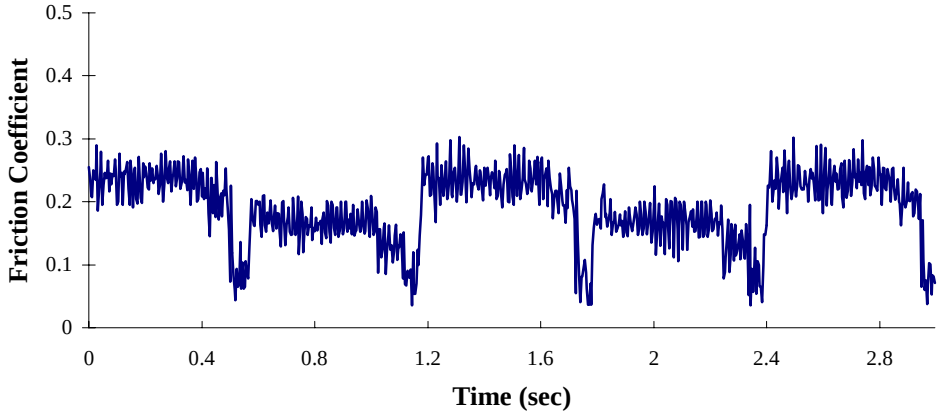

Figure 5. 16 Coefficient of friction at 60 minutes for a surgical OA cartilage specimen

The average coefficient of friction was obtained by averaging the values in the plateaus and disregarding the values approaching zero during the reversals of motion. The average friction coefficient of the OA cartilage increased significantly by an average of 30% over the non-

surgical controls. **Table 5.3** displays the average friction coefficients for each animal and treatment over the 3 sampling intervals.

Table 5. 3 Average friction coefficients (μ_{ave})

Time	Non-surgical control cartilage		Surgical OA cartilage	
*	0.204		0.191	
0 min.	0.224	μ_{ave} = **0.214**	0.232	μ_{ave} = **0.293**
	0.213		0.457	
	0.259		0.256	
30 min.	0.221	μ_{ave} = **0.255**	0.237	μ_{ave} = **0.350**
	0.284		0.559	
	----		----	
60 min.	0.259	μ_{ave} = **0.312**	0.249	μ_{ave} = **0.400**
	0.366		0.551	

* cartilage from the same animal source is found in rows within each time interval

A complete series of friction coefficient data for a surgical cartilage specimen and its corresponding control is included in **Appendix C**.

5.7 Discussion

5.7.1 *Wear*

Cartilage with severe fibrillation due to surgically induced OA did not exhibit increased wear rate in comparison to normal cartilage from the same animal. Damage to the cartilage matrix alone did not accelerate the rate at which the cartilage was removed from the surface in these one hour tribological tests. This was an unexpected result because in the previous phase of study it was concluded that the loss of tensile and compressive strength of the cartilage led to increased wear and damage of the tissue. It would seem that once an *in vivo* condition resulted in wear and damage of the cartilage, its properties would be sufficiently altered resulting in the continued wear of the cartilage. However, this was not the case in this study and it raises the question as to whether or not there is a difference in the properties of cartilage based on the manner in which OA has been established. In the surgical procedure performed, OA was induced via mechanical

instability. The stresses were increased within the tissue leading to its fatigue and breakdown. This did not lead to an increase in the subsequent wear of the cartilage once it was removed from that environment. On the other hand, the exposure of the cartilage to an enzymatic environment detected in OA joints, led to the increased wear of cartilage even after it was removed from the adverse environment. This is something that may need further investigation in order to determine which conditions or pathways to OA result in the accelerated wear of the cartilage.

There was a difference in wear observed between the cartilage from different animals. The wear from two of the three animals was very similar, but the third exhibited significantly higher wear than the others. This indicates that in health and disease, cartilage from different specimens may have a different resistance to wear and damage. This has an effect on all studies that investigate the properties of biological materials and can not be addressed in a tangible manner within this research.

5.7.2 *Scanning Electron Microscopy*

The SEMs of the normal lapine cartilage showed minimal damage to the surface of the cartilage. Cuts and grooves parallel to the sliding direction appeared to be a result of plowing of the surface by steel asperities. There was also some ridges and tearing visible perpendicular to the sliding direction. These were due to tensile failure of the surface as a result of the reciprocating sliding. This is common when an elastomeric material is slid against a hard metal [104]. Cartilage is a very complex material that has properties similar to a highly elastic, composite material. Therefore, it is not unusual that it would behave like an elastomeric material. All damage to the normal cartilage appeared to be isolated to a thin layer of the surface tissue and there was no visible fibrillation. The matrix was grossly intact aside from the overt tears due to asperity plowing.

Prior to testing, the cartilage with surgically induced OA showed significant damage to the surface and subsurface cartilage. A wear track was already present with significant fibrillation. There was interstitial material left within the underlying cartilage matrix and the matrix fibers did not look severely disrupted. The specimens were good because it was evident that OA had been

clearly established within the joint, but it was not at an end stage where there was excessive damage and an absence of cartilage along the wear track.

After tribological wear testing, the OA cartilage showed extensive damage to the collagen fiber network. There was also a loss of interstitial material. The wear testing obviously continued to damage and separate the collagen fibers and release some interstitial ground substance and water. The fact that this did not result in increased wear is unexplained. It is noted that the source of the hydroxyproline may be a factor in the values measured for the OA cases. Due to the extensive loss of cartilage and collagen in the OA cases, the amounts of hydroxyproline measured during testing, although similar to the normal cases, may represent wear of non-cartilaginous, collagen containing tissue, such as bone. From this study it appears that the fibrillation of the cartilage may lead to increased damage of the matrix network, but the cartilage and collagen fibers in the OA joints are not necessarily lost from the surface as wear in amounts greater than normal joints, as measured by hydroxyproline.

5.7.3 *Histology*

Histologic evaluation of the controls showed no damage or abnormalities. This coincided with the wear data and indicates that the plowing seen in the SEMs was restricted to the superficial layer of cartilage along with any wear. This was unexpected considering the cartilage was in contact with a very hard steel and was lubricated with a buffered saline solution. The histology also indicates that the surgical procedure of one knee did not result in any histologic changes to the control knee, meaning there was not any increased stresses detected in the normal knee as a result of compensation by the animal.

The OA cartilage before testing showed damage and fibrillation of the cartilage matrix. This resulted in a thinner layer of cartilage on the joint surface than in the case of the normal joint. There was evidence of cell clustering and loss of chondrocytes. This was compared to the OA cartilage after test to establish what damage came from the wear testing.

The OA condyles after wear testing showed a loss of cartilage almost to the bone. In the histological sections, the magnification is only 33x and it is not possible to see the thin remaining layer of cartilage that is seen in the SEMs. It appears that the cartilage is completely worn to the bone, but in fact, there is a thin layer of radial collagen fibers present. There is nothing to evaluate in the area of contact on the histology sections because this thin layer is not visible. It does indicate that the amount of wear detected in these cases came from this deeper layer of cartilage, and that it closely coincided with the amount of cartilage left on the surface.

5.7.4 *Friction*

There was a significant increase in the average friction of the OA cases over the normal controls. There was also a difference in the friction of cartilage from one animal and the others, just as seen with the wear. The fact that despite the lack of difference in wear due to treatment, there was a difference in friction, provides more evidence that in these biological systems, wear and friction are not easily related. Conditions that increase the friction of cartilage are not necessarily related to increases in wear.

The friction plots were very uniform and indicate that there was little deformation of the cartilage over the duration of the tests. This is to be expected since the cartilage layer is very thin. It is clear from the data that there is an overall increase in friction over the duration of the tests for both the normal and OA cartilage, but this increase is not seen within each source of cartilage. The overall values were heavily influenced by the high values of one animal source. There was little difference between the friction values obtained with cartilage from two of the three animal sources used. However, the third animal's cartilage exhibited higher coefficient of friction values, in addition to higher wear values discussed previously. The increase in friction over the duration of the test has been previously observed [19] and was attributed to changes in thin films due to deformation. The friction coefficient values indicate that the system is operating in the boundary lubrication regime.

5.7.5 *Proposed Mode of Wear and Damage*

It is evident that the surgical procedure produced significantly more wear and damage than the tribological wear testing. The wear rate of the OA and normal cartilage remained the same, producing the same levels of wear in the 1-hour testing period. The wear of the normal controls originated from the superficial layer of intact cartilage and maybe from some plowing damage. The wear of the surgical OA cartilage came from the deeper layers of cartilage, which did not appear to wear at a faster rate. This contradicts the common view that the superficial layer which acts as a protective layer of cartilage, has a higher '*wear resistance*' than the deeper layer of cartilage.

The increased damage seen with SEM of the OA cartilage after testing may not have been damage at all. The histology shows that the cartilage was worn into the deep layer and what the SEMs showed is likely the radial collagen fibers of the deep zone of cartilage. They are arranged perpendicular to the joint surface and under compression are probably just pressed flatly against the surface. They were initially thought to be damage to the middle zone, but this is not the case.

It appears that the wear of the two treatment groups was the same and the pathologic cartilage has the same wear properties as normal cartilage. In addition, it appears that cartilage of the deep layers of cartilage are removed at the same rate as cartilage from the superficial layer. The increase in friction is likely due to the radial orientation of the fibers in the deep layer. They may offer more resistance to sliding in a direction perpendicular to their alignment. This would explain the observed increase in friction of the OA cases.

5.7.6 *Improvements for Future Study*

The lack of samples was the largest error in this study. There were only three surgical rabbits available for this work. In the future, more collaboration should be done with the Veterinary School to secure a larger treatment group of animals with surgically induced OA, if possible. Extensive investigation of the specimens should be done before and after to evaluate the impact of differences between the specimens. A larger set of tests could lead to a tremendous amount of information about the tribological properties of OA cartilage.

5.9 Conclusions for Phase II Study

The wear rate of normal cartilage and osteoarthritic cartilage appeared to be equivalent in this study. The surgical induction of OA resulted in significant wear and damage of cartilage, resulting in thinner cartilage layers on the OA condyles. The wear of the normal cartilage came from the superficial layer and was minimal when observed with SEM and histological sections. The wear of the OA cartilage came from the deep layers of cartilage and the radial collagen fiber network could be viewed with SEM. The friction of the OA cartilage was significantly higher than the normal controls and this was attributed to increased resistance of the radial fibers to sliding in a direction perpendicular to their alignment.

These results indicate that, unlike the enzymatic studies, when OA is induced via mechanical instability, the cartilage may not continue to experience accelerated wear once the pathologic condition is removed. This is significant toward this area of study because it contradicts a common and intuitive viewpoint that fibrillation of any kind, reduces mechanical strength of cartilage and results in accelerated wear. The findings do support the clinical observation that some joints can exist with fibrillation and yet not see the increased absolute loss of collagen presumed to be associated with OA.

These results suggest that in the later stages of OA, there is no measurable acceleration of the biomechanical wear of the cartilage. Existing *in vivo* conditions, such as enzyme exposure, must be present in order to alter the tribological properties of the cartilage, making it more susceptible to wear and damage. This is an important piece of information if methods of reversing or impeding the progression of the disease are going to be utilized.

6 PHASE III: The Effect of Exposure to White Blood Cell Lysate on the *In Vitro* Wear of Articular Cartilage

6.1 Theory and Background

Theories implicating a mechanical failure in the pathogenesis of OA include many that cite a breakdown in joint lubrication as a primary cause of increased cartilage wear and damage. A review of joint lubrication theories was discussed in **Section 2.2**. Among the theories of compromised lubrication, changes in the properties and composition of the natural synovial fluid are implicated. It has been proposed by Furey [3,8], that these changes could lead to a reduced "anti-wear property" of the synovial fluid, thereby increasing cartilage wear and damage.

During trauma or excessive loading of a diarthrodial joint, cartilage wear and inflammation of the synovium can occur. The body's natural response is for neutrophils and macrophages to be attracted to the damaged joint tissue and for phagocytic synovial cells to attempt to phagocytize any debris or dead tissue. If the damage is extensive, a large number of phagocytes (neutrophils and macrophages) can enter the joint space. Neutrophils have a short life span and upon their degeneration or death, they can lyse and/or release numerous phagocytic enzymes into the synovial fluid. Macrophages can survive many days, but often are injured or activated sufficiently to also release numerous enzymes and other bioactive substances into the synovial fluid. The activity of these enzymatic substances is increased under the conditions of increased joint temperature seen in inflammation. With cartilage damage and/or synovial inflammation, increased concentrations of white blood cell lysate entering the synovial fluid could have a significant effect on the lubrication, friction and wear properties of the articular cartilage. As a result, these tribological properties of the joint could be significantly and adversely altered, leading to the increased wear and damage of the articular cartilage.

Mild or low grade synovitis is a condition commonly seen in OA [31,105]. Synovitis is an inflammation of the synovium that can be caused by numerous factors including injury by wear particles from ulcerated cartilage or calcium crystals [105]. In joint fluids aspirated from patients with low grade synovitis increased numbers of white blood cells, in comparison to those of normal joints, have been detected [105]. Since this condition is common in OA, it is reasonable

to assume that increased numbers of white blood cells could be present in OA joint fluids at various stages of the disease. It is also not unreasonable to assume that during periods of prolonged exposure, these cells may lyse, releasing the lysate into the synovial fluid or release enzymes through intact but damaged cell membranes. Hence, an osteoarthritic joint fluid could contain excess enzyme/lysate/synovial fluid mixture, which is readily produced from bovine synovial fluid and lysed white blood cells.

The third phase of this project is concerned with determining the effect of white blood cell lysate on the lubricating ability of synovial fluid. A pathological fluid was created by combining a bovine white blood cell lysate (similar to what could be found in a low grade synovitis) with normal bovine synovial fluid. The resulting fluid was used as the lubricant in cartilage-on-cartilage tribological tests to determine any changes in the effectiveness of the pathologic synovial fluid as a boundary lubricating fluid.

6.2 Objective

The objective of Phase III of this research project was to create a synovial fluid comparable to that seen in osteoarthritis and determine if the altered biochemical content had an adverse effect on the lubricating properties, and hence, the tribological properties of the cartilage-on-cartilage system. This study provides some preliminary information on the theory that the condition of OA is caused, in part, by a failure of joint lubrication caused by constituent alteration of the synovial fluid.

6.3 Materials and Methods

6.3.1 *Cartilage Preparation*

Prior to testing, bovine cartilage plugs were thawed overnight at -2 to -4 ^{o}C. The plugs were previously frozen in vials of deionized water to maintain their moisture (see **Section 3.1.1** for complete description of bovine cartilage preparation). When thoroughly defrosted, the plugs were removed from refrigeration and tested.

6.3.2 *Lubricant Preparation*

The synovial fluid originating from the same joint as the cartilage specimens was removed and thawed overnight in a refrigerator at -2 to -4 ^{o}C . Blood from a healthy dairy cow was collected and centrifuged to separate the white blood cells from the red. The white blood cells were removed and lysed by sonication. A complete blood cell count was performed on the blood before separation to determine the number of white cells per volume of blood. The lysate was diluted with RPMI [1640 with L-Glutamine; MediaTech Cellgro; Lot#: 10040176] to a concentration of 1.5 x 10^6 cells/ml. The lysate solution was then combined with normal synovial fluid in a 1:1 ratio and incubated at 37 ^{o}C for 3 hours. A normal synovial fluid sample was incubated as well for use as a control. After incubation, the fluids were removed and allowed to cool to room temperature before testing.

6.4 Design

For this study, four sets of femoral (upper) and tibial (lower) cartilage plugs from three steers were used for a total of twelve tests. From each steer, two sets of cartilage plugs came from each knee for the total of four per animal. Synovial fluid with a white blood cell lysate mixture was used as a lubricant. The concentration of lysed white cells in the synovial fluid was 1.4 x 10^6 cells/ml, which is comparable to the concentrations of white blood cells often found in mild synovitis associated with OA [105]. There were a total of four control tests done with normal synovial fluid and eight with the lysate/synovial fluid mixture. A partially balanced block design was employed to allow for analysis of variance of the wear test results.

6.5 Wear Tests

After incubation and cooling of the lubricant mixture, cartilage specimens were removed from refrigeration in preparation for testing. The upper and lower specimens were put in their respective specimen holders and mounted on the test apparatus. The chosen lubricating fluid was added between the two specimens and the test begun. Tests were performed as described in **Section 3.2**. The specific test parameters for this phase of study are shown below in **Table 6.1**.

Table 6. 1 Test parameters

Phase III Test Parameters	
Configuration	cartilage-on-cartilage
Load	68.6 N dead weight
Duration	60 minutes
Lubricants	synovial fluid, ½ synovial fluid + ½ white blood cell lysate
In Situ Data Acquisition	tangential force
Post-test Analysis	histologic evaluation, hydroxyproline assay

At the end of the test, the cartilage specimens were immediately removed, rinsed, trimmed and fixed for further analysis. The rinses and lubricating fluid were collected, combined and frozen until analyzed by hydroxyproline assay.

6.6 Results

After all tests in the selected test design were completed, analysis was performed according to the protocols outlined in **Section 3.3**. Wear and damage of the cartilage specimens were measured and related to the test lubricants. Hydroxyproline assay of test lubricants was used to quantitatively determine wear of the cartilage surfaces. Information obtained from histological sectioning and staining was used to qualitatively determine surface damage and biochemical changes in the cartilage.

6.6.1 *Wear Results*

Using the assay procedure, cartilage wear was determined for twelve wear tests. The resulting design consisted of four tests done using normal synovial fluid as the lubricant and eight tests with the white blood cell lysate/synovial fluid mixture as the lubricant. In each of the twelve tests, the fluids were assayed in duplicate and the average used as the hydroxyproline content.

The wear results obtained from eleven of the twelve tribological tests are shown in **Figure 6.1**. The average wear values and standard deviations for each test treatment are shown in **Table 6.2** and graphically represented in **Figure 6.2**. (See **Appendix A** for data tables.)

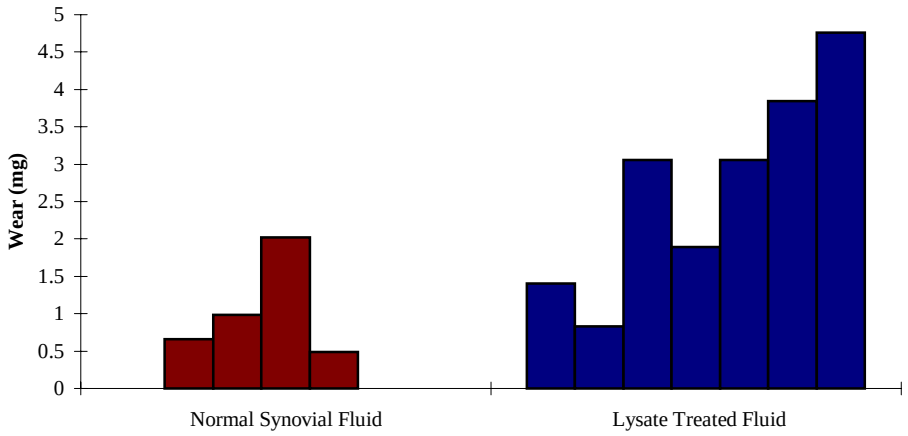

Figure 6. 1 Wear values for Phase III study

The average wear of tests done with lysate treated synovial fluid increased by a factor of 2.6 over the tests done with normal synovial fluid. The standard deviation for the lysate treated tests doubled in comparison to the deviation of the control tests. A full statistical analysis is provided in **Section 6.6.2**.

Table 6. 2 Average wear values for Phase III study

Lubricant	Cartilage Wear (mg)
Normal Synovial Fluid	1.04 ± 0.69
Lysate Treated Synovial Fluid	2.69 ± 1.39

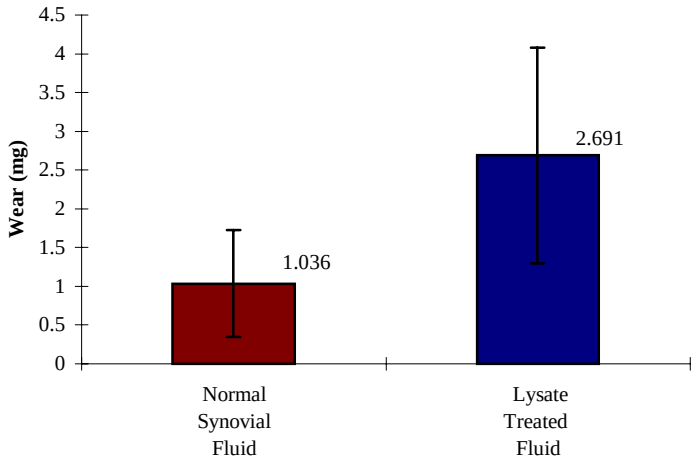

Figure 6. 2 Average wear values for Phase III study

6.6.2 *Statistical Analysis*

The testing in Phase III was done in an incomplete block design. Each animal was used as a block in order to eliminate the expected variation between cartilage from different animals. The two test treatments were the lysate treated synovial fluid and normal synovial fluid controls. Statistical analysis of the wear data was done using the SAS System software. Differences between the means were analyzed using analysis of variance at the $\alpha = 0.05$ confidence interval. The mean wear for the controls was 1.04 mg with a standard deviation of 0.69 mg. The mean wear for tests with lysate treated synovial fluid was 2.69 mg with a standard deviation of 1.39 mg. Analysis of variance showed a significant difference between the tests done with normal synovial fluid and those done with lysate treated synovial fluid, p= 0.002. (See **Appendix B** for SAS code and output)

6.6.3 *Histology*

The histology slides for this phase are substantially more difficult to read because the quality is not as good as in previous phases. In combination with the wear results, it seems that there is genuine damage to the cartilage tested with the lysate treated fluids There was considerable damage to the superficial layer of both the 1-*inch* and ¼-*inch* plugs. There was disruption to the

surface, loss of proteoglycan staining, and abnormal cell alignment in these cases. In one case there was moderate fibrillation of the cartilage with the superficial layer completely removed. **Figures 6.3** and **6.5** show the ¼-*inch* and 1-*inch* that were tested with white blood cell lysate treated synovial fluid. All magnifications are approximately 33X.

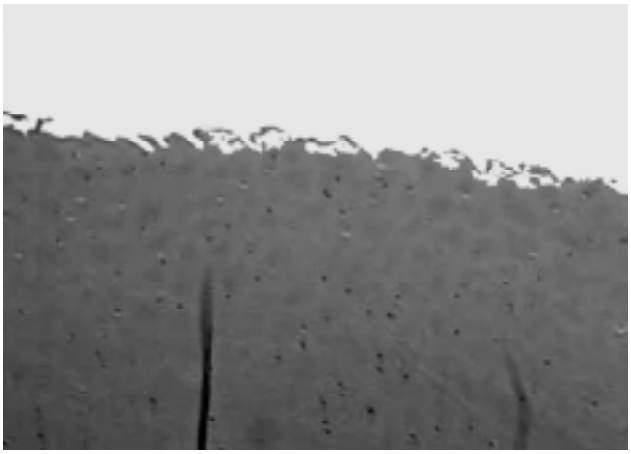

Figure 6. 3 Lysate-treated fluid test: fibrillation on 1-*inch* plug

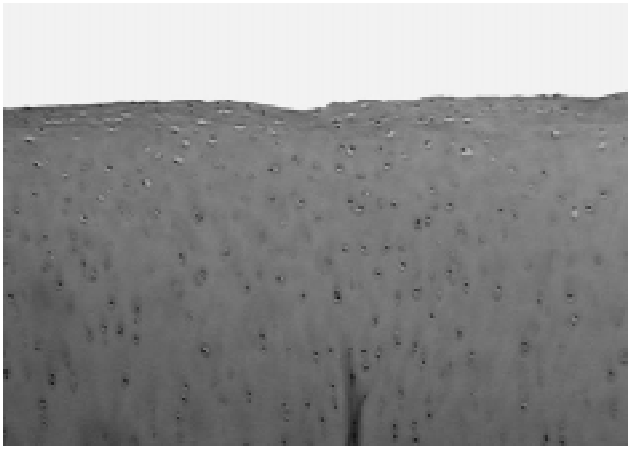

Figure 6. 4 Control fluid test: surface of 1-*inch* plug

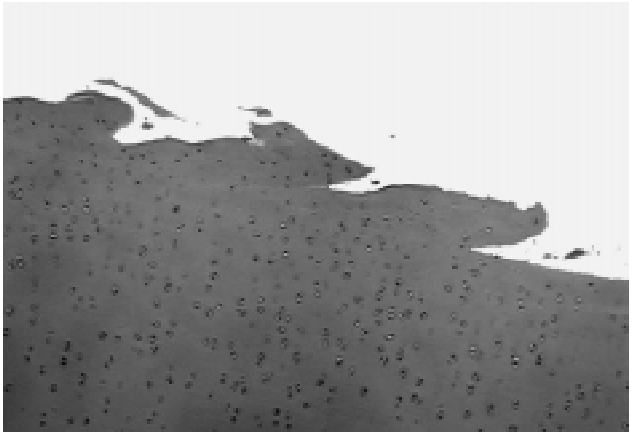

Figure 6. 5 Lysate-treated fluid test: fibrillation on ¼-*inch* plug

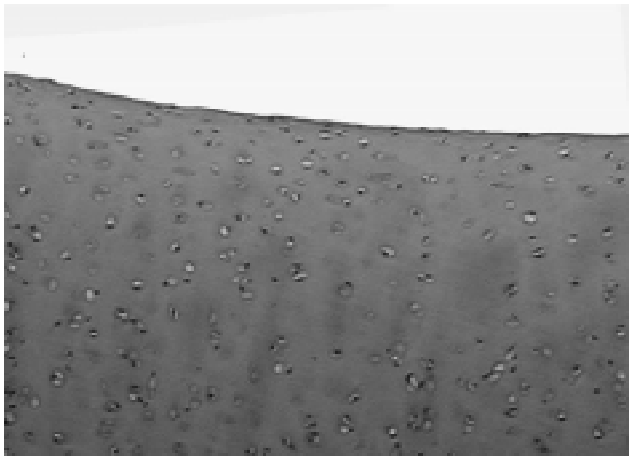

Figure 6. 6 Control fluid test: surface of ¼-*inch* plug

In the control cases there was some damage, some of which was genuine, most was artifact. The genuine damage consists of a slightly rougher than normal surface topography of the superficial layer, some loss of chondrocytes (which could have been preexisting), and some lower concentrations of GAGs in the contact area. **Figures 6.4** and **6.6** show the ¼-*inch* and 1-*inch* that were tested with normal synovial fluid.

6.6.5 *Friction Results*

The data acquisition was analyzed to determine the coefficient of friction during the tests. Data were taken at 0, 30 and 60 minutes. The acquisition was done in three second intervals with a frequency of 250 Hz. The data collected were in the form of voltage changes in the strain gages. The voltage data were used to determine the tangential force applied to the octagonal strain ring. These data were then divided by the applied normal load to obtain the coefficient of friction. The friction coefficient plots shown are 3 second sampling intervals that include 2 full cycles of motion.

The friction data obtained were very complex and had some interesting features. The first observation is that in all the plots, there was a large change between the 0 and 30 minute sampling times and little to no change between the 30 and 60 minute sampling times. This is illustrated in **Figures 6.7** - **6.12**. Most of the plots exhibited a difference in magnitude of friction in opposing sliding directions, shown in **Figures 6.11** and **6.12**. The only consistent difference between the lysate treated fluid tests and the controls was that the lysate treated cases had an initial fiction plot with a wavy sinusoidal characteristic **Figure 6.11**, while the controls had a more random periodic nature. Examples of the coefficient of friction traces are shown for both treatments at 0, 30 and 60 minutes of testing in **Figures 6.7** through **6.12**.

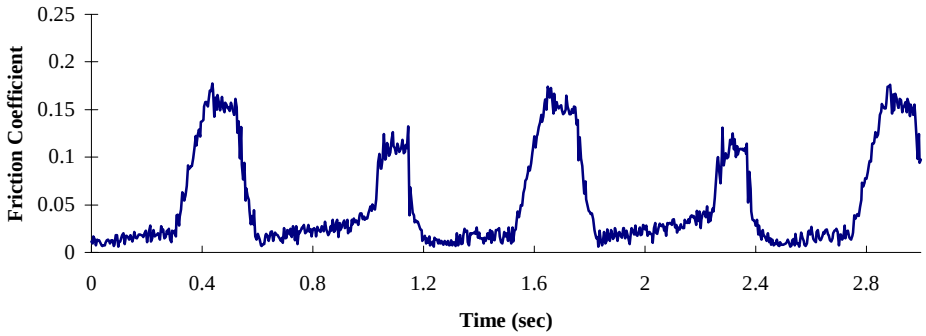

Figure 6. 7 Coefficient of friction plot at 0 minutes for normal synovial fluid

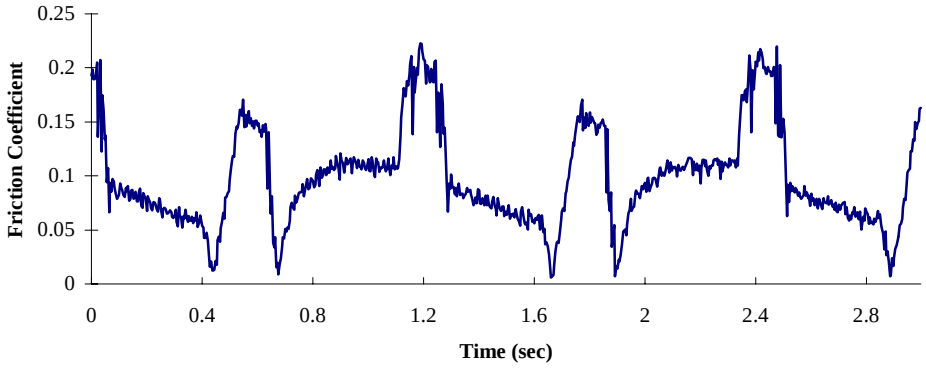

Figure 6. 8 Coefficient of friction plot at 30 minutes for normal synovial fluid

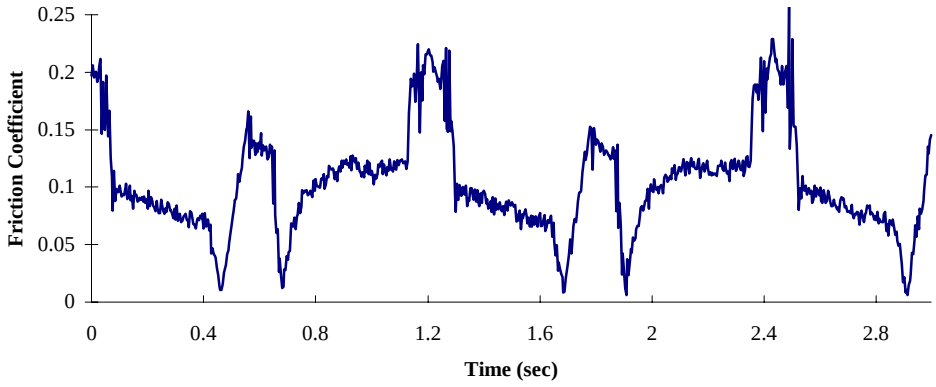

Figure 6. 9 Coefficient of friction plot at 60 minutes for normal synovial fluid

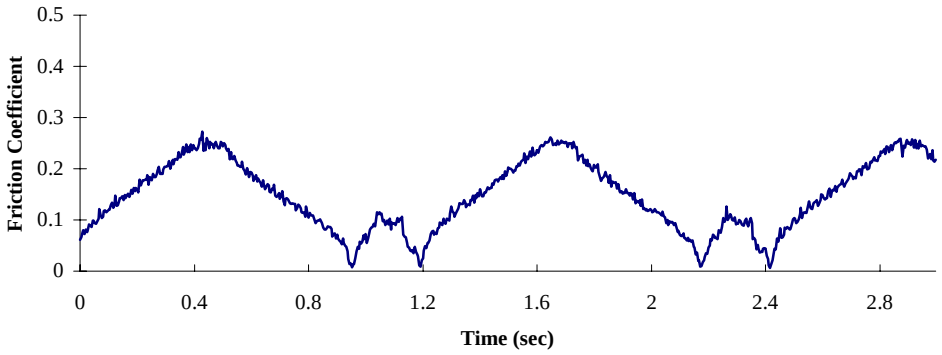

Figure 6. 10 Coefficient of friction plot at 0 minutes for lysate treated synovial fluid

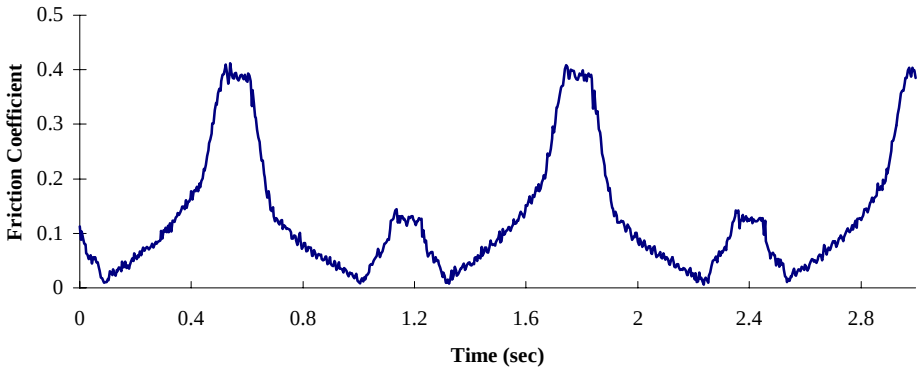

Figure 6. 11 Coefficient of friction plot at 30 minutes for lysate treated synovial fluid

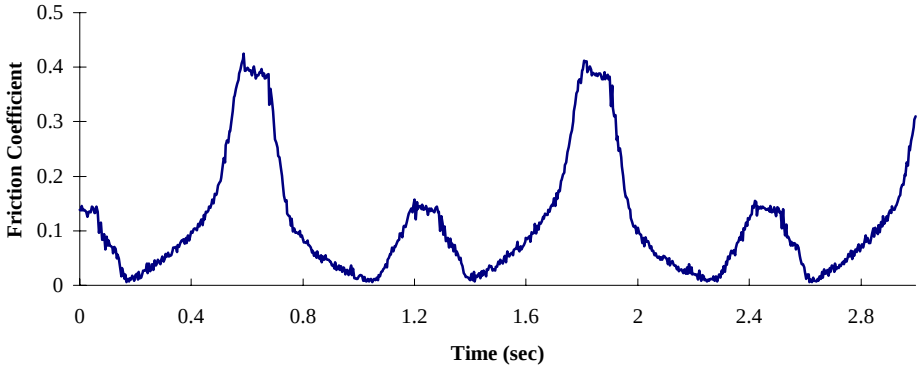

Figure 6. 12 Coefficient of friction plot at 60 minutes for lysate treated synovial fluid

The average coefficient of friction was obtained by averaging the peaks of the traces. There was no significant difference between the friction of the lysate treated and control tests, although the two highest friction levels recorded were from lysate treated tests. There was so much variance within each treatment group that it is not useful to present the average friction coefficient of each treatment. There was substantial overlap of the friction with the two treatments with most values falling in the range of 0.1 - 0.2, as shown in **Figure 6.13**. This figure also illustrates the lack of correlation between the friction and wear data over the range of values. There does appear to be a linear relationship between friction and wear at $\mu > 0.25$. In all of the control cases the average friction increased over the duration of the tests. In the lysate treated tests, five of the eight tests showed increasing friction over the test duration, while the remaining three showed a significant decrease in friction from the beginning of the test to the 30 minute interval.

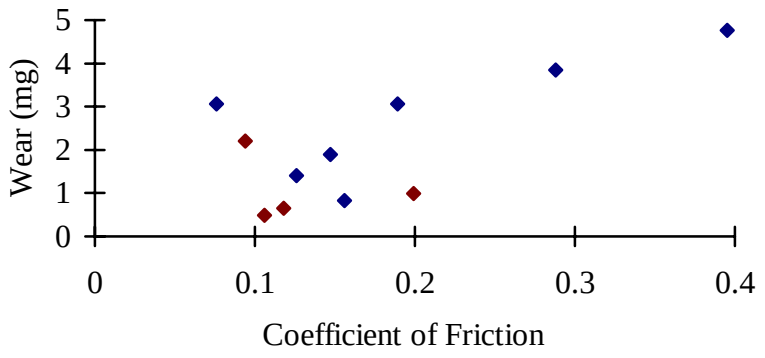

Figure 6. 13 Wear vs. Coefficient of friction

6.7 Discussion

6.7.1 *Wear*

The addition of a lysate, containing numerous phagocytes and macrophages, to the synovial fluid significantly increased wear of the cartilage in biomechanical wear testing. The wear of cartilage from the same joint consistently increased with the addition of the lysate to the normal synovial fluid. There are three possible explanations for this effect: 1) it could be a result of the enzymes in the lysate removing certain crucial elements of the synovial fluid that help to prevent wear and

damage, 2) it could be due to residual activated enzymes within the fluid reacting with the cartilage during the test and degenerating the cartilage matrix, or 3) the addition of the RPMI/lysate mixture may have caused a dilution of crucial constituents in the synovial fluid, resulting in increased wear and damage.

6.7.2 *Histology*

The control cases showed no histologic abnormalities while the lysate treated cases showed fibrillation and damage to the cartilage surfaces. In addition, the lysate treatment caused an abnormal depletion of GAGs in the middle zone of the cartilage similar to that seen in the collagenase-3 study. This loss of GAGs indicate that the constituents of the lysate contained enzymes that were capable of cleaving collagen fibers and/or proteoglycan aggrecan. The loss of proteoglycans led to increased wear and damage of the cartilage surface. The loss of the GAGs from the cartilage corresponded to their increased concentration in the synovial fluid lubricant, as detected by keratan sulfate ELISA [100].

Normal fluid samples were taken after incubation at 37 ^{o}C, after incubation and test with normal cartilage, after incubation with lysate, and after incubation with lysate and testing with normal cartilage. Results showed that the proteoglycan content of incubated fluids without lysate treatment remained constant, while lysate treatment significantly reduced the proteoglycan content. During tribological tests, the proteoglycan content of lysate treated fluid increased from the beginning of the test to the end. **Table 6.3** is a comparison of keratan sulfate concentrations found in the test fluids. These results indicate that proteoglycans entered the synovial fluid from the cartilage, likely from the middle zone based on keratan sulfate data and histologic impressions.

Table 6.3 Comparisons of Keratan Sulfate Concentrations

Lubricant	Normal untested synovial fluid	Normal synovial fluid after test	Lysate treated synovial fluid before test	Lysate treated synovial fluid after test
Keratan Sulfate Content (μg/ml)	18	18	10	16

6.7.3 *Friction*

There were no significant differences in the magnitude of friction detected. The values were variable and had no correlation between treatments or wear obtained. The change in the plot characteristics from 0-30 minutes is due to the large initial deformation of the surfaces. After the initial deformation, there was little change in the characteristics of the friction plots. The wavy nature of the lysate treated cases at the beginning of the tests remains unexplained.

In the control cases with normal synovial fluid, an increase was seen in the coefficient of friction over the duration of the test. This result has been observed in previous studies and has been attributed to the breakdown of thin film lubrication and changes in the contact area of the sliding surfaces over time [19]. As the cartilage surfaces deform, the contact geometry of these irregularly shaped specimens changes which could lead to increases in friction. In addition, the exuding of interstitial fluid that occurs over time also affects the friction. After all the fluid is exuded from the upper specimen, the cartilage properties change which could also result in frictional increases. There was a reduction in friction over the duration of the tests seen in some of the lysate treated samples. This reduction is suspected to be due to the increase in GAGs in the lubricating fluid over the duration of the test. If there were some degradative processes occurring within the cartilage during the test, components that were removed from the synovial fluid during incubation with lysate may have been restored to the lubricant as they were released from the cartilage matrix. The degeneration of the cartilage matrix that occurs with exposure to phagocytic and proteolytic substances can lead to the release of the ground substances, such as glycoaminoglycans and lipids, into the lubricating fluid. This has been the basis of several techniques to monitor the progression of osteoarthritis by evaluating the levels of cartilage ground substance found within the synovial fluid during the course of the disease [63-66].

Keratan sulfate ELISA showed that the lysate treatment reduced GAGs prior to testing and the testing of the fluids resulted in increased levels of GAGs in the lubricant. In the cases where friction was decreased, the constituents of the synovial fluid that may have been removed or

affected by the lysate treatment may have been restored to the fluid when GAGs were released from the cartilage and exuded through the damaged cartilage surfaces. In these select cases, the concentration may have increased enough to lead to decreased friction over the test duration.

6.7.4 *Proposed Mode of Wear and Damage*

It appears that the lysate affected both the fluid and the cartilage. The lysate treatment reduced the GAG content of normal synovial fluid. Whether this has an effect on cartilage wear is undetermined. The residual active enzymes in the lysate removed GAGs within the cartilage and this had an effect on wear in the same manner as the collagenase-3 study of Phase I. The wear of cartilage in this study was much lower than that of the collagenase-3 study where the cartilage was exposed to only one enzyme. In this case, there would be several enzymes present in unknown concentrations and activities. The exposure time here, however, was only for one hour at room temperature during the testing, which would reduce the activity of some of the enzymes. This may be why the wear was not as great as in the collagenase study, if in fact, the wear is a result of changes in the cartilage rather than the changes in the fluid, or both.

The dilution effect is something that was not properly considered during the study. However, in the case of an actual diseased joint, the presence of lysate in the synovial fluid would increase its volume and cause a dilution effect as well. Whether the changes in this case are due to the actual constituents of the lysate or the dilution of the synovial fluid is something that needs to be determined. Nonetheless, it is established that the presence of the lysate results in increased wear and damage of the articular cartilage.

During the test, proteoglycans were gradually increased in the synovial fluid lubricant and could have reduced the coefficient of friction in some cases. The method by which this is done is still not well understood. It could be a simple increase in viscosity or a more complex binding of biochemical constituents to cartilage surface material. Although the coefficient of friction is reduced, the continual breakdown of the cartilage matrix leads to increased wear. This theory provides and explanation for the reduction in friction and increase in wear seen in some of the lysate treated tests. It also provides more evidence that in these biological systems, friction and

wear are not easily related and biological factors producing a decrease in friction can cause increases in wear.

6.7.5 *Suggestions for Future Work*

It is suggested that now that the effect of the lysate has been determined, further study should be done to identify the constituents of the lysate that lead to increased wear. Improved methods of determining the amount of lysate present in inflamed joints would make a study more applicable to arthritis research. In addition, better characterization of the fluids after lysate treatment to determine what constituents have been affected is necessary.

6.9 Conclusions for Phase III Study

It can be concluded from this study that the presence of white blood cell lysate in normal synovial fluid leads to an increase in wear over normal fluid. The method by which the wear is increased appears to be similar to that seen in the Phase I collagenase-3 study. The results indicate that the enzymes or other degradative substances contained in the lysate degenerate the cartilage and lead to increases in wear through a loss of biochemical ground substances, such as proteoglycans. This was determined by the histology results which were similar to those seen in the Phase I study and results of synovial fluid analysis. The loss of ground substance in the cartilage corresponded to their increase in the lubricating synovial fluid. This suggests that the treatment of the synovial fluid with a white blood lysate did in fact have an effect on the tribology of the system by changing the constituents of the fluid and cartilage. The indirect effect of the lysate on the cartilage may have led to a reduction in friction over time, in some cases, by reversing the effects of the treatment on the lubricant. The effect of the residual enzymatic activity within the cartilage may have also resulted in the increased wear of the treated cases during the wear tests. Further investigation is needed to adequately evaluate the proposed mechanisms of cartilage wear. Also, additional methods of analyzing changes in the lubricating fluid are also necessary to confirm the affects of the lysate on the synovial fluid.

The significance of this study is that it implicates the products of joint inflammation in the loss of articular cartilage associated with osteoarthritis. Synovitis, which is commonly seen in cases of

OA, has been observed to result in elevated numbers of white blood cells in the synovial fluid. This study has shown that the presence of these white blood cells can lead to the increase in wear of the cartilage. Although the process by which wear is increased is speculated here, further study is necessary to determine the true mode of wear within the biological system.

7 PHASE IV: The Effect of Digestion of Synovial Fluid Phospholipids by Phospholipase A_2 on the Lubricating Properties of Synovial Fluid

7.1 Theory and Background

Recently, several articles have been published that suggest that surface-active phospholipids (SAPLs) are the biochemical constituent of synovial fluid that acts as the effective boundary lubricating element [106-109]. In the absence of fluid film lubrication conditions, boundary lubrication is accepted as the predominant mode of lubrication (see **Section 2.2** for a description of modes of joint lubrication). Much discussion has ensued regarding the effective boundary lubricating element of synovial fluid that leads to extremely low coefficients of friction between the cartilage surfaces. Hyaluronan was initially thought to be the primary element and studies have shown that it reduced wear of cartilage when added to a reference fluid [3,19]. However, hyaluronan was almost completely disregarded as the boundary lubricant in synovial fluid when it was discovered that its load-bearing capacity is greatly reduced at high loads. It is now generally accepted that hyaluronan's major role is in the lubrication of the synovium and articular cartilage contact [31]. It was later postulated that the boundary lubricating element of the synovial fluid was contained in the "glycoprotein layer" [110,111]. SAPLs have been cited as the constituent of this layer that provides the excellent boundary lubricating properties of synovial fluid [107-109, 112,113].

Phospholipase A_2 has been used to destroy the SAPLs in synovial fluid. Tests have been conducted where phospholipase A_2 (PLA_2) was used to digest phospholipids in synovial fluid and the resulting fluid was used in a four-ball wear test, along with normal synovial fluid. These tests concluded that the PLA_2 treated fluid produced more wear than the normal synovial, thereby making it a less effective boundary lubricant [108].

Unfortunately, these studies that have been done to examine the lubrication characteristics of synovial fluid and SAPLs are flawed. First, these tests assume that synovial fluid has certain lubricating properties, termed "lubricity," that are independent of the tribological system or environment. This is simply not accurate. Furthermore, a four-ball lubrication test with steel

balls was used to measure friction and wear properties of normal synovial fluid and synovial fluid with the SAPLs removed. These tests have no relevance on the effectiveness of these lubricants on the wear and damage of articular cartilage, which is a much more complex material.

In line with the tests done in Phase III, it is of interest to know if the presence or absence of any one constituent of synovial fluid has an effect on joint lubrication. In cartilage-on-cartilage tribological tests, this can be more accurately determined than previously demonstrated. The Phase IV study deals with the digestion, via PLA_2, of SAPLs in synovial fluid and how the resulting fluid affects the tribological properties of the cartilage-on-cartilage system.

7.2 Objective

The objective of the Phase IV study was to determine if the removal of surface-active phospholipids from the synovial fluid altered its ability to act as an effective boundary lubricant for the cartilage-on-cartilage tribological system. This allows for the more realistic evaluation of SAPLs as boundary lubricating agents in the *in vivo* synovial joint. The study is aimed at addressing the theory that there are certain constituents of synovial fluid that act as natural "anti-wear" additives or agents.

7.3 Materials and Methods

7.3.1 *Cartilage Preparation*

Prior to testing, bovine cartilage plugs were thawed overnight at -2 to -4 ^{o}C. The plugs were previously frozen in vials of deionized water to maintain their moisture (see **Section 3.1.1** for complete description of bovine cartilage preparation). When thoroughly defrosted, the plugs were removed from refrigeration and tested.

7.3.2 *Lubricant Preparation*

The synovial fluids originating from the same joints as the cartilage specimens were removed and thawed overnight in a refrigerator at -2 to -4 ^{o}C. The fluids were combined to make one fluid mixture to use in all tests. This was done to reduce the variance created by different levels of phospholipids found in each individual fluid. Lyophilized Phospholipase A_2 (PLA_2) was ordered

from Worthington Biochemical (Lot #: 58N642P) and added to the aggregate synovial fluid in a concentration of 33μg/ml. This is similar to literature concentrations that were used in the digestion of SAPLs in synovial fluid [108]. The PLA_2 treated synovial fluid was then incubated for 3 hours at 37 ^{o}C. A normal synovial fluid sample was incubated as well for use as a control. After incubation the fluids were removed and allowed to cool and then frozen until the test day. On the day of testing, the fluids were removed and thawed to room temperature before testing.

7.4 Design

For the Phase IV study, four sets of femoral (upper) and tibial (lower) cartilage plugs from four steers were used for a total of sixteen tests. From each steer, two sets of cartilage plugs came from each knee for the total of four per animal. Normal and PLA_2 treated synovial fluid were used as lubricants. There were a total of four control tests done with normal synovial fluid and twelve with the PLA_2 treated synovial fluid. This design was a partially balanced block design to be analyzed by analysis of variance.

7.5 Wear Tests

After the thawing of lubricant samples, wear tests were begun. The upper and lower specimens were then put in their respective specimen holders and mounted on the test apparatus. The chosen lubricating fluid was added between the two specimens and the test begun. Test were performed as described in **Section 3.2**. The specific test parameters for this phase of study are shown below in **Table 7.1**.

Table 7. 1 Test parameters

Phase IV Test Parameters	
Configuration	cartilage-on-cartilage
Load	68.6 N dead weight
Duration	60 minutes
Lubricants	synovial fluid, synovial fluid + PLA_2 (33 μg/ml)
Post-test Analysis	histologic evaluation, hydroxyproline assay

At the end of the test, the cartilage specimens were immediately removed, rinsed, trimmed and fixed for further analysis. The rinses and lubricating fluid were collected, combined and frozen until analyzed by hydroxyproline assay.

7.6 Results

After all tests in the selected test design were completed, analysis was performed according to the protocols outlined in **Section 3.3**. Wear and damage of the cartilage specimens were measured and related to the test lubricants. Hydroxyproline assay of test lubricants was used to quantitatively determine wear of the cartilage surface. Information obtained from histological sectioning and staining was used to qualitatively determine surface damage and biochemical changes in the cartilage.

7.6.1 *Wear Results*

Using the assay procedure, cartilage wear was determined for 13 wear tests. The original test design called for 16 tests, but the test fluid from test IV12 leaked out of the contact area and was not recoverable. Test fluids from tests IV02 and IV14 were lost during the assay procedure. The resulting design consisted of three tests done using normal synovial fluid as the lubricant and ten tests with PLA_2 treated synovial fluid as the lubricant. In each of the thirteen tests, the fluids were assayed in duplicate and the average used as the hydroxyproline content.

The wear results obtained from the thirteen tribological tests are shown in **Figure 7.1**. The average wear values for each test treatment are shown in **Table 7.2** and graphically represented in **Figure 7.2**. (See **Appendix A** for data tables.)

The average wear of articular cartilage increased by 43% after the synovial fluid was treated with PLA_2. The standard deviation for the treated cases is 2½ times greater than for the control cases. This may be affected by the fact that there were substantially fewer control tests done.

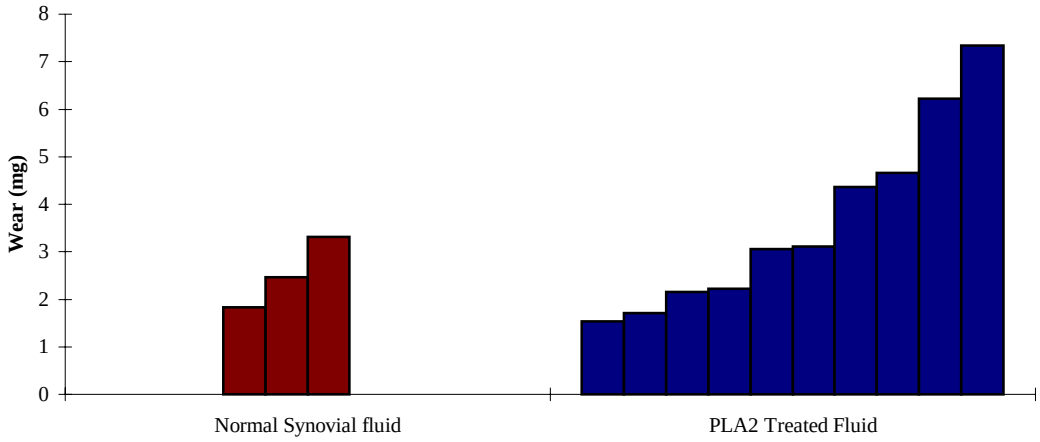

Figure 7. 1 Wear values for Phase IV study

Table 7. 2 Average wear values for Phase IV study

Lubricant	Cartilage Wear (mg)
Normal Synovial Fluid	2.54 ± 0.74
PLA_2 Treated Synovial Fluid	3.64 ± 1.97

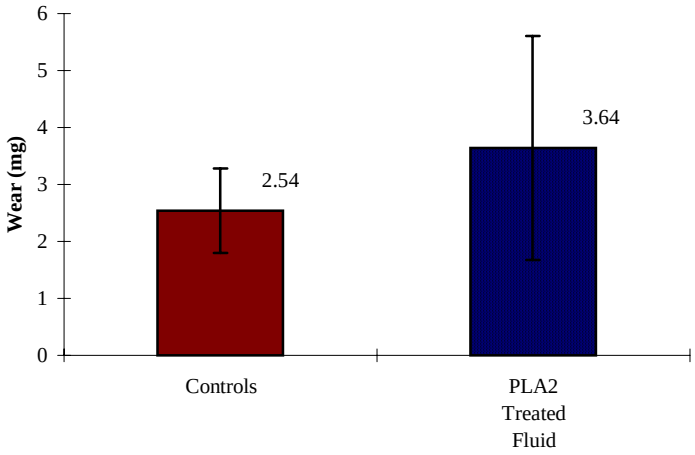

Figure 7. 2 Average wear for Phase IV study

7.6.2 *Statistical Analysis*

Phase IV testing was also done in an incomplete block design with each animal representing a block. The two test treatments were the PLA_2 treated synovial fluid and normal synovial fluid controls. Statistical analysis of the wear data was done using the SAS System software. Differences between the means were analyzed using analysis of variance at the $\alpha = 0.05$ confidence interval. The mean wear for the controls was 2.53 mg with a standard deviation of 0.74 mg. The mean wear for tests with lysate treated synovial fluid was 3.64 mg with a standard deviation of 1.97 mg. Analysis of variance showed no significant difference between the tests done with normal synovial fluid and those done with PLA_2 treated synovial fluid, $p = 0.436$. (See **Appendix B** for SAS code and output)

7.6.3 *Histology*

The control tests show some damage but the slides are difficult to read due to artifacts. The ¼-*inch* plugs look intact with normal staining, an example is shown in **Figure 7.3**. The 1-*inch* plugs have more damage as expected, but the damage seems to occur on the edge where the gasket was used to hold the plug in place and keep the lubricant in the contact area. No damage was seen in the actual contact area, as shown in **Figure 7.4**. There is, however, a depletion of the basophilic stain in the area of contact.

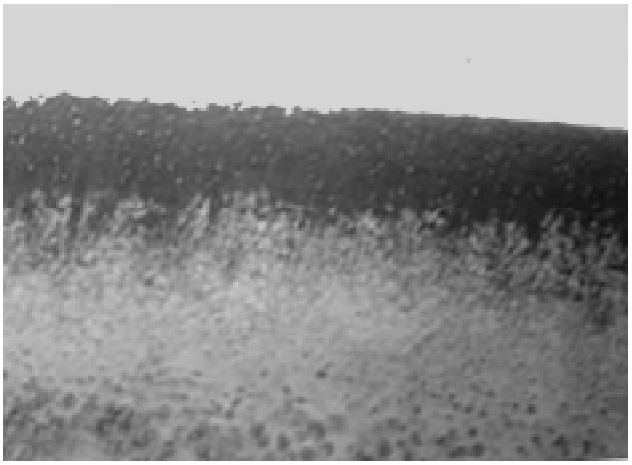

Figure 7. 3 Control test fluid: ¼-*inch* plug after wear testing

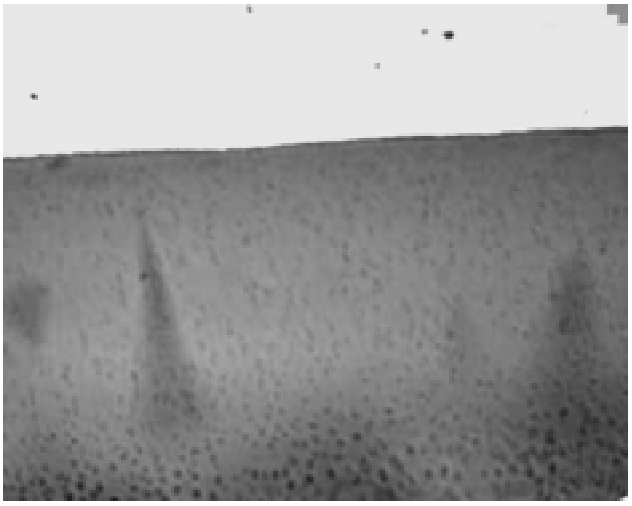

Figure 7. 4 Control test fluid: 1-*inch* plug after wear testing

Out of the four slides prepared from cartilage tested with PLA_2 treated synovial fluid, only one showed damage in excess of what was seen in the control cases. Most looked normal with various forms of artifact that resulted from processing. The slides from test IV05 showed an excessive amount of damage that is almost impossible to regard as legitimate. The entire cartilage surface was damaged, with strands of cartilage remaining parallel to the surface of the plug. The cartilage is totally destroyed in this manner in the ¼-*inch* plug and similar damage is seen in the superficial layer of the 1-*inch* plug. It is unlike any other damage seen in these tests or any of the other phases, so the cause of the damage is questionable. This unraveling or delamination of the cartilage may be another product of the processing of these histology slides. Examples of ¼-*inch* and 1-*inch* cartilage plugs tested with the PLA_2 treated synovial fluid are shown in **Figure 7.5** and **7.6**.

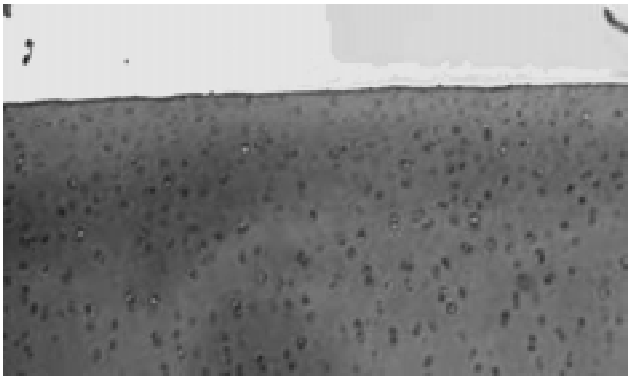

Figure 7. 5 PLA_2 -treated test fluid : ¼-*inch* plug after wear testing

Figure 7. 6 PLA_2 -treated test fluid : 1-*inch* plug after wear testing

7.7 Discussion

7.7.1 *Wear*

The results of this phase of study were very unexpected. The average wear of cartilage tested with PLA_2 treated synovial fluid increased by over 40% in comparison to cartilage tested with normal synovial fluid. However, this difference was found to be insignificant based on the test design used. This would indicate that SAPLs have no effect on the wear of the cartilage. This contradicts previous research done by Hills et al. [107-109,112,113] which has predicted that the removal of the phospholipids from the synovial fluid would increase the wear and damage. This seems intuitive because the lipids are fatty proteins that give the fluid viscosity and possibly provide an oily layer between the cartilage surfaces in the form of a thin film. It was suspected that the small number of controls in comparison to the number of treated cases reduced the ability to statistically determine differences between the treatments. Although there was a difference in the means, the variation within the treatment groups was so large that it made the difference in the means less significant.

In order to briefly test this hypothesis, the control tests from the Phase III study were considered with those of this study. Since the controls were tested under the same conditions and with the normal fluid, they should have yielded similar results. The pooling procedure increased the number of controls to seven. The statistical analysis was repeated and a difference was found

between the treatments. However, a statistical difference was also found between the controls of the two phases, with Phase III yielding lower wear than this phase. This contributed to creating a difference between the treatments. The pooling of the data indicates that the number of controls does have a significant affect on the power of the test and that it may be a factor in the results obtained. However, the significant differences found in the pooled test are not accurately representative of true differences in the means. This needs to be considered in future studies.

Another issue is that the SAPLs were not removed from the cartilage. The cartilage itself also contains phospholipids that may have been exuded from the matrix under compression and returned to the cartilage surface in a concentration lower than normal, but sufficient to protect the surfaces from wear and damage.

7.7.2 *Histology*

The histology showed that there was no difference in damage between the treatment groups. This was in agreement with the wear results. Histologic sections showed substantial amount of the damage done at the edges of the cartilage was a result of the pressure of the gasket used to keep the lubricating fluid in the contact area. In some cases where the 1-inch plug was irregular in shape, significant pressure was applied to the gasket in order to achieve a fluid tight seal. This pressure is also responsible for the lack of proteoglycan content around the edges of the plugs. There is no reasonable explanation for the variation in results of the histology for the treated cases. It was the first inclination to assume that the one extreme case, IV05, was just a processing artifact, however, the hydroxyproline results showed that the wear from this test was the highest of all the tests, therefore, the damage was compatible with the wear. This does not in any way prove that treatment itself was the cause of this excessive damage, it could have been some aspect of the test, although there was no recorded problem with the test and everything appeared normal. It was recorded that there was a lot of damage to the cartilage during the test. The high wear for this particular test was also suspected to be a product of this particular animal's cartilage, however, when looking at the wear of other cartilage samples from the same joint of the same animal with the same treatment, high wear and damage were not observed. The reason for such high wear and excessive damage in this one test case remains unexplained.

7.7.3 *Proposed Mode of Wear and Damage*

The wear and damage in these tests appears to come more from the testing method than the treatments. The damage to the bottom plugs is a result of the pressure of the gasket as a result of the irregular shape. There was no damage to the top plugs in either case and this is representative of the effect of the treatments. Wear detected in these tests came primarily from the damaged bottom plugs and secondarily from the superficial layer of the top plugs. The remaining phospholipids in the articular cartilage may have been effective in lubricating the surfaces of the PLA_2 treated cases.

7.7.4 *Relation of Results to Current Literature*

The results of this study contradict the predicted literature results [108,109]. The literature studies use non-cartilaginous materials to test the effect of removing SAPLs from synovial fluid. It is suggested here that if the phospholipids are surface active, they may have a different mode of action with a steel surface than they do with cartilage surfaces. This may result in the conflicting data obtained in these studies.

7.7.5 *Sources of Error*

The two main sources of error for this study were: 1) that the sample size was very small for the control cases and may have affected the power of the statistical analysis to determine differences between the treatments, and 2) there was no method employed to determine the extent to which the SAPLs were removed from the synovial fluid, or exuded from the cartilage.

7.7.6 *Improvements for Further Study*

For a more detailed and complete study on the role of SAPLs in cartilage lubrication, it is suggested that large sample groups of cartilage are used, preferably from the same animal. Both the cartilage and synovial fluid should be treated with PLA_2 and a method for determining the extent of SAPL removal should be employed. The SAPLs in the fluid should be measured before and after the test to see if they are being exuded from the cartilage matrix.

7.8 Conclusions for Phase IV Study

The results from this study lead to the conclusion that the removal of surface active phospholipids does not significantly affect the wear and damage of articular cartilage, except for one of the treated cases showing significant histologic damage over the untreated cases. The absence of a difference between the test treatments is suspected to be due to inadequate sample size and remaining phospholipids in the cartilage that were not removed. These lipids may have been exuded, via compression, to the surface of the cartilage where they acted as a boundary lubricant between the cartilage surfaces. Additional tests need to be performed that include the removal of phospholipids from both the cartilage and the synovial fluid in order to determine the role of phospholipids in boundary lubrication of cartilage.

8 DISCUSSION of PROJECT RESULTS

The results from each of the experimental phases provided new information on the biochemical and physical factors that influence the tribology of cartilage-on-cartilage systems. The analysis techniques proved useful in providing both quantitative and qualitative data on wear, damage and friction of articular cartilage. The following section includes a discussion of each of the experimental phases and a summary of the results obtained.

8.1 Phase I

The purpose of the Phase I study was to look at the role of the collagense-3 enzyme in the wear and damage of articular cartilage. The enzyme has been detected in the cartilage of osteoarthritic joints [98,99]. Although it has been established both *in vivo* and *in vitro* [86,87,89,90,97] that collagenases are capable of cleaving collagen bonds within articular cartilage, the affect of the enzyme on the subsequent wear of the cartilage under load and relative motion seen in the joint had not been previously evaluated. The exposure of cartilage to the collagenase-3 enzyme before tribological wear testing provided a means to evaluate the role of the enzyme in the cartilage destruction observed in clinical cases of OA, mainly, the loss or wear and damage to the cartilage.

The results of this study showed that exposure to collagenase-3, in concentrations similar to those detected in human osteoarthritic joints, yields a significant increase in cartilage wear detected by hydroxyproline analysis of lubricants after wear testing.

In addition to wear, increased damage to colagenase-3 treated cartilage was also observed via SEM and histologic sectioning. Damage and removal of the superficial layer was observed in collageanse-3 treated cases, while untreated cartilage was not substantially damaged by the wear testing. The increased damage to the surface correlates well with the levels of wear observed, hence excessive surface damage $\rightarrow$ high wear.

Histologic examination of cartilage after exposure to collagenase-3 and wear testing showed the distinct loss of proteoglycans and ground substance from the middle zone of the cartilage. Damage to the surface layers, and in some cases complete removal, was observed. Prior to tribological wear testing, collagenase treated cartilage showed no surface or subsurface damage and no removal or loss of proteoglycans.

Evaluation of keratin sulfate in test lubricants indicated an increase in proteoglycans in the post-test lubricants of collagenase treated cases, while keratin sulfate levels in control test fluids remained unchanged.

Based on the observed results, the increase in wear and damage of collagenase-3 treated cartilage was attributed to the cleavage of collagen bonds which resulted in the release of proteoglycans and other ground substance from the extracellular matrix. During compression and relative motion, these substances are thought to be exuded from the matrix and released to the lubricating fluid through damaged or removed superficial layers. It has been demonstrated that the proteoglycans give cartilage its compressive strength and provide the middle and deep layers of cartilage with the ability to carry compressive load. The loss of proteoglycans is associated with reduced compressive strength of the cartilage subsurface and increased damage to and wear of the surface.

The wear and damage of collagenase treated cartilage did not increase with an increase in concentration. This was unexpected but attributed to two factors:

1. The wear and damage created with the lower of the two concentrations was excessive making differences between the two difficult to detect,

2. The mass of cartilage used in each test was approximately the same and the activity of the enzyme was such that both concentrations yielded the same cleavage of all collagen bonds in the incubation period.

In addition, there was no significant statistical increase in wear between tests done with synovial fluid and buffered saline. There was, however, observable differences in the damage of cartilage

tested with the different lubricants. It is suggested that synovial fluid provides increased protection of cartilage surfaces from damage in relation to the buffered saline. The lack of statistical significance may be a factor of the variability of the materials, the small treatment groups, the excessive damage caused by the enzyme treatment and the exudation of GAGs or other protein substances into the saline lubricant.

There were also no differences detected between the wear of bovine knee and shoulder cartilage and there was no significant interactions of the treatment, lubricant or type of cartilage.

It has been demonstrated for the first time in this study that the collagenase-3 enzyme has the ability to play a significant role in the progression of osteoarthritis. The release of collagenase-3 from the chondrocytes of osteoarthritic cartilage can result in the increased wear and damage of the cartilage, thereby accelerating the course of the disease. It is suggested that additional studies be performed to determine the reason chondrocytes release the enzyme in pathologic situations and how the action of the enzyme can be impeded or reversed.

8.2 Phase II

The purpose of the Phase II study was to compare the tribological properties of normal and osteoarthritic cartilage. Due to the numerous biochemical, histologic and structural changes that occur during OA, it is of interest to determine whether OA cartilage has poor tribological properties, in relation to normal cartilage, which make it more susceptible to wear and damage. Wear, damage and friction of normal and OA lapine cartilage were investigated in cartilage-on-steel tribological wear tests.

Surgical induction of OA produced lapine cartilage surfaces with extensive wear and damage prior to tribological wear testing. The fibrillation of cartilage *in vivo* is a common indication of OA and the specimens obtained exhibited signs of well established disease. The *in vitro* wear of normal and fibrillated cartilage from the same animal exhibited no difference in wear during tribological wear tests.

SEM examination of cartilage surfaces after testing confirmed that the surgical procedure produced significant wear and damage prior to wear testing. The wear of the OA cartilage came primarily from the deep radial zone of the cartilage and the collagen fibers from this region were visible via SEM. In the normal control cases, wear originated from the plowing of the superficial layer by steel asperities. This was evident from the plowing grooves in the surfaces of the normal cartilage.

Histologic examination of cartilage before and after test for both treatments confirmed that the surgical procedure produced histologic change analogous to clinical OA, while there was no change or damage seen in the normal cases.

Friction data indicated a significant increase in friction of the OA cartilage over the normal cartilage. The increase is attributed to the collagen fiber alignment of the radial zone of cartilage. In this zone the collagen fibers are aligned perpendicular to the sliding direction rather than parallel, as in the superficial layer. This is thought to be responsible for the change in friction coefficient seen in the OA and normal cases.

These results indicate that there is no difference in the wear and damage of normal and OA, or fibrillated cartilage. This suggests that the method by which OA is induced may affect the subsequent progression of the disease. In this case, OA was initially induced by mechanical instability. Ensuing enzymatic or other biochemical change was not evaluated within the joint. The mechanically induced OA produced histologic change analogous to naturally occurring clinical OA, but did not result in increased wear and damage of cartilage collagen after tribological wear testing. Further investigation of the pathways to OA need to be evaluated, as this study implies, different pathways may result in different affects on the wear and damage of cartilage associated with the disease.

8.3 Phase III

The purpose of the Phase III study was to create a synovial fluid comparable to that seen in osteoarthritis and determine if the altered biochemical content had an adverse effect on the lubricating properties, and hence, the tribological properties of the cartilage-on-cartilage system. The effect of white blood cell lysate on the lubricating ability of synovial fluid was specifically examined. A pathological fluid was created by combining a bovine white blood cell lysate (similar to what could be found in a low grade synovitis) with normal bovine synovial fluid. The resulting fluid was used as the lubricant in cartilage-on-cartilage tribological tests to determine any changes in the effectiveness of the pathologic synovial fluid as a boundary lubricating fluid.

Mild or low grade synovitis is a condition commonly seen in OA [31,105]. Synovitis is an inflammation of the synovium that can be caused by numerous factors including injury by wear particles from ulcerated cartilage or calcium crystals [105]. In joint fluids aspirated from patients with low grade synovitis increased numbers of white blood cells, in comparison to those of normal joints, have been detected [105].

The addition of a lysate of white blood cells, containing numerous neutrophils and macrophages, to the synovial fluid significantly increased wear of the cartilage in biomechanical wear testing. The wear of cartilage from the same joint consistently increased with the addition of the lysate to the normal synovial fluid.

Tests done with normal synovial fluid showed no histologic abnormalities while the lysate treated cases showed fibrillation and damage to the cartilage surfaces. In addition, the lysate treatment caused an abnormal depletion of GAGs in the middle zone of the cartilage similar to that seen in the collagenase-3 study. This loss of GAGs indicate that the constituents of the lysate contained enzymes that were capable of cleaving collagen fibers and/or proteoglycan aggrecan.

Evaluation of keratin sulfate in lysate treated lubricants indicated their decrease in the synovial fluid before wear testing. After test, lysate treated lubricants exhibited an increase in keratin

sulfate content, while keratin sulfate levels in control test fluids remained unchanged before and after wear testing.

The results indicate that the presence of lysate of white blood cells in synovial fluid significantly increase wear and damage of articular cartilage. The method by which the lysate increases wear appears to be enzymatic, as in the case of collagenase-3. The loss of proteoglycans in the cartilage indicate that lysate contains constituents that are capable of releasing proteoglycans I the same manner as the collagenase-3 and this loss of ground substance results in damage and wear of the cartilage surface.

This study implicates the products of joint inflammation, or synovitis, in the loss of articular cartilage associated with osteoarthritis. Synovitis, which is commonly seen in cases of OA, has been observed to result in elevated numbers of white blood cells in the synovial fluid. This study has shown that the presence of these white blood cells can lead to the increase in wear of the cartilage. Although the process by which wear is increased is speculated here, further study is necessary to determine the true mode of wear within the biological system.

8.4 Phase IV

The objective of the Phase IV study was to determine if the removal of surface-active phospholipids from the synovial fluid altered the boundary lubrication of a cartilage-on-cartilage tribological system, as opposed to the steel system of previous studies. This allows for the evaluation of SAPLs as boundary lubricating agents in the *in vivo* synovial joint. Several articles have been published that suggest that surface-active phospholipids (SAPLs) are the biochemical constituent of synovial fluid that acts as the effective boundary lubricating element [106-109].

SAPLs were removed from normal synovial fluid and the resulting fluid used as a lubricant in cartilage-on-cartilage tribological wear tests. There was no increase in wear or damage of the cartilage tested with SAPL deficient synovial fluid in comparison to normal fluid. Determination of wear by hydroxyproline analysis and histologic examination showed no substantial differences

between the two treatment groups. Sample size is indicated as a significant factor in the results obtained.

The results indicate that lack of boundary lubrication by the SAPLs in the synovial fluid of synovial joints may not be important in the wear and damage associated with OA. It may be necessary to remove the SAPLs in the cartilage as well to create changes in the lubrication of the cartilage. There may also be other mechanisms within the cartilage and synovial fluid that provide the necessary protection against excessive wear and damage. This needs to be further investigated in a larger study.

8.5 Summary

Cartilage Wear

The wear of articular cartilage was found to be significantly affected by enzymatic biochemical environments. The mode by which wear was increased is suspected to be through a removal of collagen proteoglycans, GAGs and other ground substance from the compressive load bearing middle region of the cartilage matrix. The loss of these constituents leads to the loss of compressive strength and in concert with collagen fiber damage, results in increased wear of the cartilage surface.

Fibrillation of the cartilage associated with surgically induced OA did not affect the wear rate of cartilage in this study. In addition, alteration of the lubricating fluid by removing boundary lubricating elements also did not result in increased wear rates of cartilage.

The studies indicate that there are specific conditions that cause changes in cartilage that lead to at least transient accelerated wear rates, even after the condition is no longer present. Alteration of cartilage resulting in increased wear rate has been associated with enzymatic biochemical environments in these studies. The enzymatic environments have all been identified in cases of *in vivo* osteoarthritis.

Cartilage Damage

As with cartilage wear, cartilage damage was affected by enzymatic activity. Neither fibrillation, nor reduced boundary lubrication by synovial fluid phospholipids resulted in significant damage to articular cartilage.

Friction

Friction of osteoarthritic cartilage was found to have higher values than the normal cartilage. This was thought to be a result of the exposed cartilage layer. The deep layer of cartilage offers more resistance to sliding than the tangential surface. This would be the case in both normal and OA cartilage. In these studies, the deep layer was exposed in the OA cartilage.

There were differences in friction associated with different animal specimens. This indicates that cartilage from different animals, but not from different anatomical locations in the same animal, has different tribological properties and this needs to be addressed in future work.

There was little correlation between friction and wear values. This provides further evidence that particularly in these complex biologic materials, friction and wear are not simply related and care must be taken in predicting their tribological behavior under varying conditions.

The results of this research project contribute substantial information to this area of study. Biochemical environment was found to have a significant effect on the tribology of articular cartilage. Enzymatic environments were found to have a significant adverse effect on the wear and damage of cartilage. Enzymes found in lysate of white blood cells and the collagenase-3 produced by OA chondrocytes, were identified as having a significant role in the wear and damage of articular cartilage. The enzyme exposure causes a loss of proteoglycans which is implicated in the change in cartilage properties leading to increased wear. This indicates that: 1) although OA is not primarily an inflammatory disease, the low-grade synovitis associated with many cases of OA may play a critical role in the initial wear and damage of the cartilage, and 2) the collagenase-3 produced by OA cartilage may be a source of the accelerated loss of cartilage in the disease.

Numerous enzymatic studies have been done by researchers interested in the etiology of OA. Most have been biochemical studies which observe the action of enzymes in tissue culture. Although they prove collagenases have the ability to breakdown cartilage collagen, enzyme exposure alone does not result in increased wear or damage. Enzyme treated cartilage remains intact and only subsequent motion under load leads to wear or damage of the cartilage surfaces. The fibrillation that exists in OA induced by mechanical instability was not found to affect subsequent collagen wear rates. This indicates that a set of adverse physiologic conditions must exist for fibrillation to increase in articular cartilage and that collagen matrix damage alone does not produce increased wear rates. These conditions likely the activity of enzymes released into the synovial fluid. This contradicts some of the literature which suggests that fibrillation alone leads to a change in the mechanical properties of cartilage and results in the wear of cartilage seen in OA [102]

Tribological tests provide an inexpensive alternative to *in vivo* studies that can examine the role of suspected biochemical changes of both the cartilage and synovial fluid in the etiopathogenesis of osteoarthritis. These studies are the first to provide a measure of the extent of biomechanical wear and damage of cartilage as a result of enzyme exposure. They also present a new method of evaluating theories of joint pathology. The results suggest that methods of inhibiting the action of enzymes such as collagenase-3 *in vivo* may be critical in the effective treatment of the disease.

9 CONCLUSIONS

The overall objective of this research project was to examine the effects of biochemical environment on the wear and damage of articular cartilage. Several theories about the pathways of osteoarthritis were examined as part of this investigation. The four phases of study can be separated into those where the biochemical environment of the cartilage and the synovial fluid were examined independently. The role of several factors in the wear, damage and friction of cartilage were investigated. The following are the specific conclusions drawn from each of the experimental phases:

Phase I:

Cartilage-on-cartilage tests with normal and enzyme treated bovine cartilage

1. Exposure to the collagenase-3 enzyme significantly increases the wear and damage of articular cartilage ($p = 0.001$).
2. There was no significant difference between wear of cartilage exposed to the 50 and 100 u/ml collagenase-3 concentrations.
3. There is a distinct loss of proteoglycans from the middle layer of cartilage after exposure to collagenase-3 and wear testing. This loss is implicated in the wear and damage of the cartilage.
4. Synovial fluid provides enhanced protection of cartilage surfaces and collagen fibers in comparison to buffered saline.

Phase II:

Cartilage-on-stainless steel tests with normal and OA lapine cartilage

1. There is no difference between the collagen wear of normal and OA cartilage in cartilage-on-steel wear tests.
2. There is a significant difference between the friction coefficients of the superficial layer and the deep layer of cartilage in cartilage-on-steel wear tests.

Phase III:

Cartilage-on-cartilage tests with normal bovine cartilage

1. The presence of white blood cell lysate in normal synovial fluid significantly increases cartilage wear and damage ($p = 0.002$).
2. Cartilage after test with lysate treated synovial fluids exhibits loss of proteoglycans in the middle layer of cartilage.
3. Lysate treatment of synovial fluid does not affect the friction of the cartilage-on-cartilage system.

Phase IV:

Cartilage-on-cartilage tests with normal bovine cartilage

1. Removal of SAPLs in synovial fluid had no effect on the wear or damage of the articular cartilage.

From these conclusions, broader inferences can be made based on the objectives set forth at the beginning of the research project. These objectives were aimed at determining the types of conditions and environments that have a detectable effect on the wear and damage of cartilage. The objectives outlined in **Section 1.5** and the conclusions reached are outlined below.

Do certain biochemical changes in the pathologic synovial joint affect the wear and damage of articular cartilage?

Enzymatic biochemical environments were found to affect cartilage wear and damage. Specifically, environments containing the collagenase-3 enzyme and the contents of the white blood cells.

Do biochemical changes in the cartilage matrix lead to increased wear and damage?

Loss or destruction of proteoglycans and ground substance lead to increased wear and damage of the cartilage surface.

Do pathological environments, such as inflammation, affect cartilage wear and damage?

The addition of lysate of white blood cells found in mild synovitis to the synovial fluid results in increase levels of wear and damage to the cartilage. Hence, the inflammation that is often associated with cases of OA does have an effect on the wear and damage of cartilage.

Does osteoarthritic cartilage have different tribological properties than normal cartilage?

There was no difference detected in the wear and damage of normal and osteoarthritic cartilage under conditions of cartilage-on-steel wear testing.

Does initial fibrillation of cartilage lead to accelerated wear and damage?

Fibrillated cartilage seen in surgically induced OA experienced no increase in wear or damage in comparison to normal intact cartilage.

Do lipid constituents of synovial fluid provide boundary lubrication of the cartilage surfaces and protect them from excessive wear and damage?

Removal of suspected boundary lubricating elements of synovial fluid did not affect the wear and damage of articular cartilage.

Do phospholipids in the synovial fluid protect cartilage surfaces from wear and damage?

Removal of surface-active phospholipids from normal synovial fluid did not lead to an increase in wear or damage of the cartilage surface layer.

10 RECOMMENDATIONS

The preceding studies provide valuable information to the current state of knowledge of cartilage wear and damage. The results and conclusions provide a starting point on which much needed additional research can be based. The author would like to make several recommendations for further research in the areas covered as part of this project. The questions raised by this research are the potential topics for many related experimental studies.

1. More studies should be done to examine the effect of collagenase-3 on the wear of cartilage. It is suggested that one concentration of the enzyme is used in a study with cartilage from one source, if possible. One lubricant and one type of cartilage should be used to isolate the effect of the enzyme. The results of SEM and histologic sections should be compared to those of naturally occurring OA in the same species of animal. Proteoglycan content of the lubricating fluid should be measured before and after each test. Methods of characterizing wear particles should be employed in order to assist in determining the mode of wear. The effect of temperatureone the enzyme activity should also be examined.
2. Studies on the effect of white blood cell lysate should be continued. Future studies should include the characterization of the lysate components and isolation of those that produce the observed adverse effects on cartilage wear. Varying concentrations of the lysate should be examined. Proteoglycan content of the lubricating fluid should be measured before and after each test. The effect of the dilution of synovial fluid should be evaluated by diluting the synovial fluid with RPMI and testing it with the lysate treated fluids. The effect of temperatureone the enzyme activity should also be examined.
3. The properties of OA cartilage should be studied in more detail. A larger study should be done in conjunction with the Veterinary College. Both mechanical and tribological properties should be investigated in completely balanced statistical design.

4. Additional biochemical constituents of the synovial fluid should be isolated and their effect on cartilage wear and damage determined. The constituents could be added to a reference fluid or removed from the synovial fluid via specific enzymatic degradation, as in the Phase IV study.
5. Modifications or redesign of the tribological test device should be performed to reduce the vertical and horizontal translation of the shaft. Temperature and humidity control should also be included.
6. Further analysis of post-test fluids should be done to determine changes in content. In addition, characterization of wear particles should be performed to gain more information about the mode of cartilage-on-cartilage wear.

Continued research in these areas promises to lead to a more detailed understanding of joint tribology. The benefits include not only a better understanding of the exceptional function of normal joints, but also much needed information about the mechanisms and causes of joint pathology such as osteoarthritis.

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APPENDIX A
Data Tables for Tribological Wear Tests

Test #	Animal id & Joint	Treatment	Wear (mg)	Analysis
Phase I				
I-01	P5 rk	control	0.604	SEM
I-02	P5 rk	50 U/ml coll-3	2.93	Histology
I-03	W9 lk	control	1.37	SEM
I-04	W9 lk	100 U/ml coll-3	4.19	Histology
I-05	P5 ls	50 U/ml coll-3	9.32	SEM
I-06	P5 rs	100 U/ml coll-3	5.57	Histology
I-07	O10 rk	100 U/ml coll-3	24.75	SEM
I-08	O10 rk	100 U/ml coll-3	23.24	Histology
I-09	O10 lk	50 U/ml coll-3	24.66	Histology
I-10	O10 lk	control	2.71	SEM
I-11	O10 ls	50 U/ml coll-3	13.85	SEM
I-12	O10 rs	control	4.85	Histology
I-13	P12 rk	50 U/ml coll-3	26.86	Histology
I-14	P12 rk	control	6.24	SEM
I-15	P12 lk	100 U/ml coll-3	18.01	SEM
I-16	P12 lk	50 U/ml coll-3	38.93	Histology
I-17	P12 ls	control	11.12	Histology
I-18	O1 rs	100 U/ml coll-3	46.66	SEM
I-19	W11 rk	50 U/ml coll-3	14.0	SEM
I-20	W11 rk	control	8.93	Histology
I-21	W11 lk	100 U/ml coll-3	14.97	SEM
I-22	W11 lk	control	10.03	Histology
I-23	W11 ls	50 U/ml coll-3	23.42	SEM
I-24	W11 rs	100 U/ml coll-3	-----	Histology
Phase II				
II-02	98-072	control	0.436	SEM & Histology
II-03	98-071	surgical (OA)	0.502	SEM & Histology
II-04	98-472	control	0.440	SEM & Histology
II-05	98-471	surgical (OA)	0.450	SEM & Histology
II-06	98-232	control	0.615	SEM & Histology
II-07	98-231	surgical (OA)	0.602	SEM & Histology
Phase III				
III-01	G14 rk	wbc lysate	1.401	Histology
III-02	G14 rk	control	0.657	Histology
III-03	O10 lk	wbc lysate	0.829	Histology
III-04	O100 lk	control	0.984	Histology

Test #	Animal id & Joint	Treatment	Wear (mg)	Analysis
Phase III cont…				
III-05	G10 lk	wbc lysate	3.058	Histology
III-06	G10 lk	control	2.021	--
III-07	W5 lk	wbc lysate	1.896	--
III-08	W5 rk	wbc lysate	3.058	--
III-09	W1 lk	wbc lysate	---	--
III-10	G13 rk	wbc lysate	3.843	--
III-11	G13 rk	control	0.485	--
III-12	O1 rk	wbc lysate	4.757	--
Phase IV				
IV-01	P6 rk	PLA_2	4.66	--
IV-02	P6 rk	PLA_2	---	--
IV-03	P6 lk	PLA_2	2.15	--
IV-04	P6 lk	control	1.834	--
IV-05	O12 lk	PLA_2	7.335	Histology
IV-06	O12 rk	control	3.31	Histology
IV-07	O12 lk	PLA_2	2.226	--
IV-08	O12 rk	PLA_2	3.059	--
IV-09	O9 rk	PLA_2	1.534	Histology
IV-10	O9 lk	PLA_2	6.223	--
IV-11	O9 rk	control	2.465	--
IV-12	O9 lk	PLA_2	---	--
IV-13	W6 rk	PLA_2	4.369	Histology
IV-14	W6 lk	control	---	Histology
IV-15	W6 rk	PLA_2	1.714	--
IV-16	W6 lk	PLA_2	3.114	--

APPENDIX B

SAS Codes and Output for Phases I through IV

Phase I

code:

```
OPTIONS NODATE PS=80 LS = 64 NONUMBER;
data Phase1;
input animal position lub enzyme wear;
cards;
1 1 1 1 604
1 1 2 2 2391
1 1 2 1 1371
1 1 2 3 4190
1 2 1 2 9324
1 2 1 3 5574
2 1 2 3 24756
2 1 1 3 23238
2 1 1 2 24660
2 1 1 1 2713
2 2 2 2 13853
2 2 2 1 4852
3 1 1 2 26860
3 1 2 1 6238
3 1 1 3 18008
3 1 2 2 38927
3 2 1 1 11115
3 2 2 3 46657
4 1 1 2 13996
4 1 1 1 8933
4 1 1 3 14972
4 1 2 1 10034
4 2 2 2 23423
;
run;
data Phase1;
set Phase1;
lwear = log(wear);
run;
quit;

proc glm data=Phase1;
 class lub enzyme position animal;
 model wear= lub enzyme lub*enzyme position animal;
 output out=res r = resid;
contrast 'Linear effect in Enzyme' enzyme -1 0 1;
contrast 'Control vs. others' enzyme 1 -.5 -.5;
contrast 'Enzyme1 vs. Enzyme2' enzyme 0 -1 1;
means enzyme position lub enzyme*lub;
run;
quit;

proc univariate data=res normal;
var resid;
run;
quit;
```

output:

```
The SAS System

                General Linear Models Procedure
                    Class Level Information

                   Class    Levels    Values

                   LUB           2    1 2

                   ENZYME        3    1 2 3

                   POSITION      2    1 2

                   ANIMAL        4    1 2 3 4
```

Number of observations in data set = 23

The SAS System

General Linear Models Procedure

Dependent Variable: WEAR

Source	DF	Sum of Squares	F Value	Pr > F
Model	9	2451603153.35	4.45	0.0078
Error	13	796591121.87		
Corrected Total	22	3248194275.22		

R-Square	C.V.	WEAR Mean
0.754759	53.47428	14638.6522

Source	DF	Type I SS	F Value	Pr > F
LUB	1	42767705.39	0.70	0.4186
ENZYME	2	988691619.83	8.07	0.0053
LUB*ENZYME	2	107199203.50	0.87	0.4401
POSITION	1	4687356.21	0.08	0.7865
ANIMAL	3	1308257268.41	7.12	0.0045

Source	DF	Type III SS	F Value	Pr > F
LUB	1	68357032.20	1.12	0.3101
ENZYME	2	1011470242.90	8.25	0.0049
LUB*ENZYME	2	115663721.64	0.94	0.4143
POSITION	1	6548695.25	0.11	0.7489
ANIMAL	3	1308257268.41	7.12	0.0045

Contrast	DF	Contrast SS	F Value	Pr > F
Linear effect in Enz	1	792360626.18	12.93	0.0033
Control vs. others	1	1009772579.01	16.48	0.0014
Enzyme1 vs. Enzyme2	1	7769156.02	0.13	0.7275

The SAS System

General Linear Models Procedure

Level of ENZYME	N	WEAR Mean	WEAR SD
1	8	5732.5000	4021.1902
2	8	19179.2500	11533.9369
3	7	19627.8571	14318.0819

Level of POSITION	N	WEAR Mean	WEAR SD
1	16	13868.1875	11305.2545
2	7	16399.7143	14718.7826

Level of LUB	N	WEAR Mean	WEAR SD
1	12	13333.0833	8579.7686
2	11	16062.9091	15478.0162

Level of LUB	Level of ENZYME	N	WEAR Mean	WEAR SD
1	1	4	5841.2500	4986.1840
1	2	4	18710.0000	8409.1972
1	3	4	15448.0000	7415.3029
2	1	4	5623.7500	3582.7870
2	2	4	19648.5000	15463.0370
2	3	3	25201.0000	21236.9970

```
                          The SAS System

                       Univariate Procedure

Variable=RESID

                              Moments

            N                 23  Sum Wgts         23
            Mean               0  Sum               0
            Std Dev      6017.365  Variance   36208687
            Skewness     0.231547  Kurtosis    -0.9202
            USS          7.9659E8  CSS         7.9659E8
            CV                 .   Std Mean    1254.707
            T:Mean=0           0   Pr>|T|       1.0000
            Num ^= 0          23   Num > 0          11
            M(Sign)         -0.5   Pr>=|M|      1.0000
            Sgn Rank           0   Pr>=|S|      1.0000
            W:Normal     0.963483   Pr<W         0.5356


                       Quantiles(Def=5)

             100% Max      10767       99%      10767
              75% Q3    5755.773       95%   10427.06
              50% Med   -985.336       90%   7595.773
              25% Q1    -5383.77       10%    -6884.6
               0% Min   -9781.67        5%   -8535.48
                                        1%   -9781.67
             Range      20548.67
             Q3-Q1      11139.54
             Mode       -9781.67


                              Extremes

               Lowest    Obs     Highest    Obs
              -9781.67(       4)  6338.19(       1)
              -8535.48(      14)  7322.69(       3)
               -6884.6(      11) 7595.773(       8)
              -6589.73(      15) 10427.06(      16)
              -5752.33(      19)    10767(      18)
```

Phase II

code:

```
Options ps=80 ls=64;
data Phase2;
input trt wear block;
lines;

1 436 1
1 440 2
1 615 3
2 502 1
2 450 2
2 602 3

Proc anova;
classes trt block;
model wear= trt block;
means trt block;
run;
quit;
```

output:

```
The SAS System                          4
                                13:32 Tuesday, October 13, 1998

                 Analysis of Variance Procedure
                    Class Level Information

                   Class    Levels    Values
```

```
                  TRT           2    1 2

                  BLOCK         3    1 2 3


             Number of observations in data set = 6


                          The SAS System                          5
                                  13:32 Tuesday, October 13, 1998

                 Analysis of Variance Procedure

Dependent Variable: WEAR

Source                   DF    Sum of Squares  F Value   Pr > F

Model                     3     31840.5000000    12.86   0.0730

Error                     2      1651.0000000

Corrected Total           5     33491.5000000

                  R-Square              C.V.         WEAR Mean

                  0.950704          5.661382        507.500000


Source                   DF          Anova SS  F Value   Pr > F

TRT                       1       661.5000000     0.80   0.4652
BLOCK                     2     31179.0000000    18.88   0.0503


                          The SAS System                          6
                                  13:32 Tuesday, October 13, 1998

                 Analysis of Variance Procedure

          Level of       -------------WEAR------------
          TRT        N       Mean              SD

          1          3     497.000000       102.210567
          2          3     518.000000        77.252832

          Level of       -------------WEAR------------
          BLOCK      N       Mean              SD

          1          2     469.000000       46.6690476
          2          2     445.000000        7.0710678
          3          2     608.500000        9.1923882
```

Phase III

code:

```
Options ps=80 ls=64;
data phase3;
input trt wear block;
lines;

2 1.4  1
1 0.657 1
2 0.828 2
1 0.984 2
2 3.06  3
1 2.02  3
2 1.90  4
2 3.06  4
2 3.84  6
1 0.486 6
2 4.76  7

Proc anova;
classes trt block;
model wear= trt block;
means trt;
run;
```

```
quit;
```

output:

```
The SAS System                          1
                                13:32 Tuesday, October 13, 1998

                Analysis of Variance Procedure
                   Class Level Information

                Class    Levels    Values

                TRT           2    1 2

                BLOCK         6    1 2 3 4 6 7


            Number of observations in data set = 11


                        The SAS System                          2
                                13:32 Tuesday, October 13, 1998

                Analysis of Variance Procedure

Dependent Variable: WEAR

Source                   DF    Sum of Squares  F Value   Pr > F

Model                     6       19.88501849    89.90   0.0003

Error                     4        0.14746424

Corrected Total          10       20.03248273

                  R-Square              C.V.          WEAR Mean

                  0.992639          9.184862        2.09045455


Source                   DF          Anova SS  F Value   Pr > F

TRT                       1        6.97898626   189.31   0.0002
BLOCK                     5       12.90603223    70.02   0.0006


                        The SAS System                          3
                                13:32 Tuesday, October 13, 1998

                Analysis of Variance Procedure

          Level of       -------------WEAR------------
          TRT         N       Mean              SD

          1           4     1.03675000       0.68728906
          2           7     2.69257143       1.39262329
```

Phase IV

code:

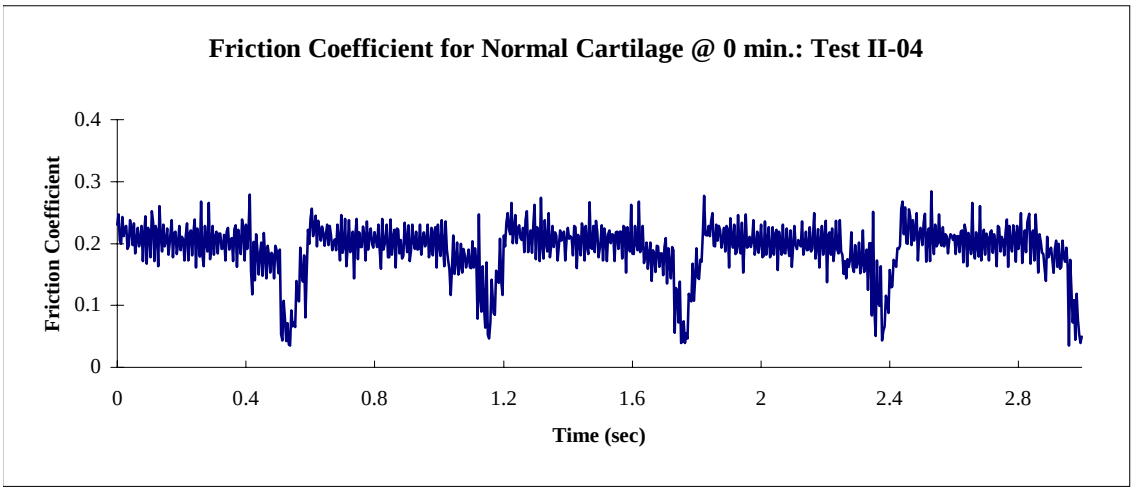
```
Options ps=80 ls=64;
data Phase4;
input trt wear block;
lines;

2 4.66  1
2 2.15  1
1 1.83  1
2 7.34  2
1 3.31  2
2 2.23  2
2 3.06  2
2 1.53  3
2 6.22  3
1 2.46  3
```

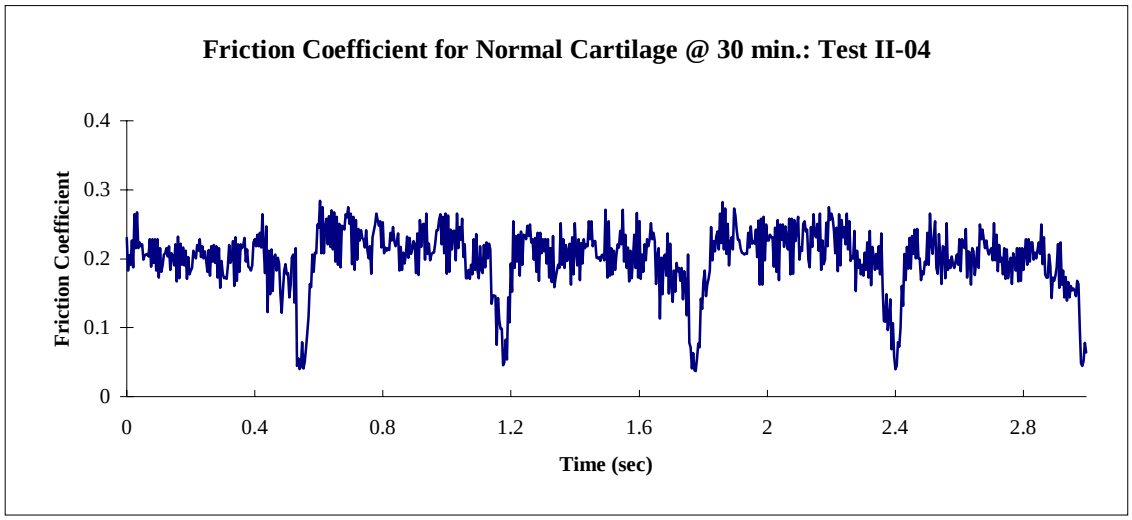
```
2 4.37  4
2 1.71  4
2 3.11  4

Proc anova;
classes trt block;
model wear= trt block;
means trt;
run;
quit;
```

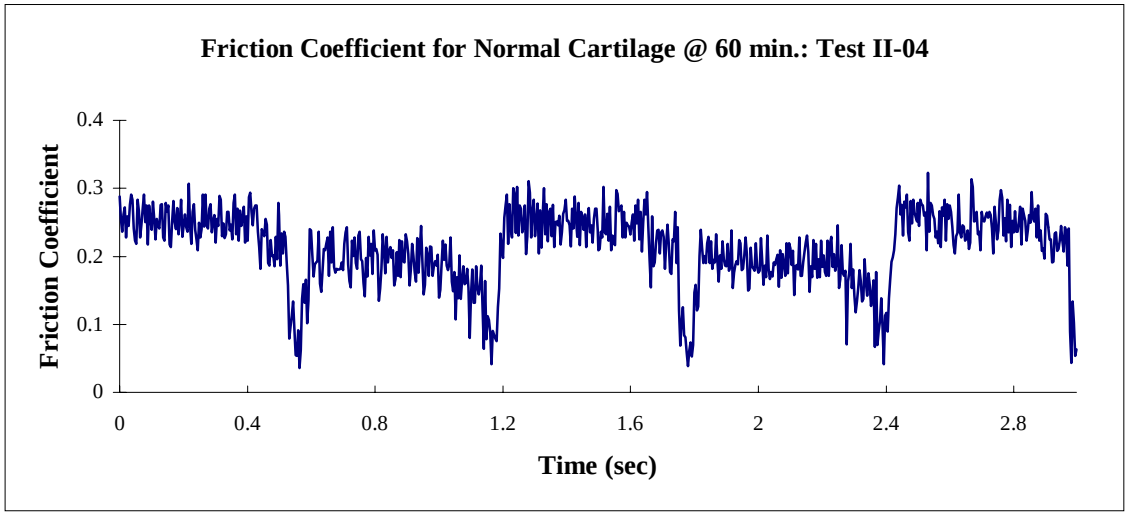
output:

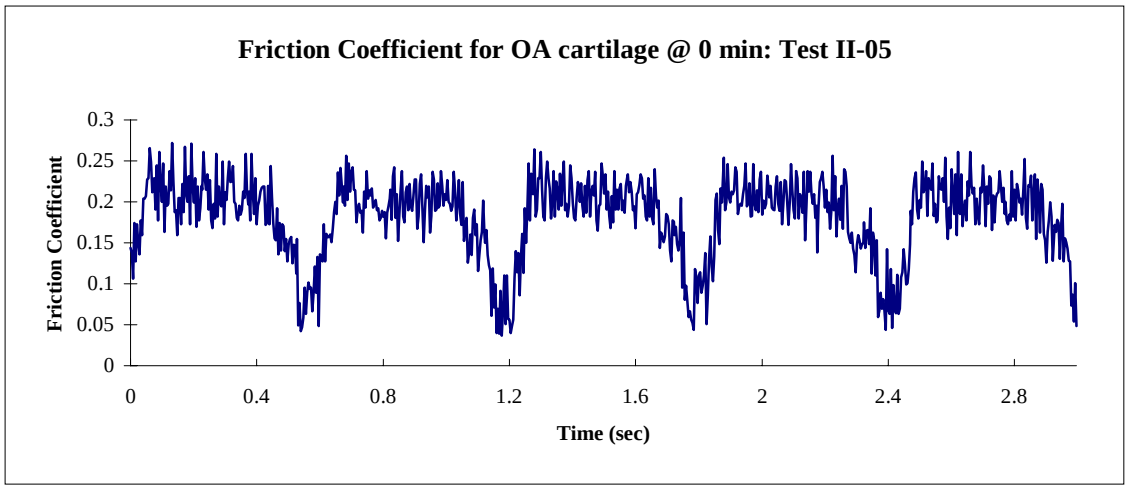
```
The SAS System                          7
                                13:07 Tuesday, October 13, 1998

                Analysis of Variance Procedure
                   Class Level Information

                  Class    Levels    Values

                  TRT           2    1 2

                  BLOCK         4    1 2 3 4


            Number of observations in data set = 13


                         The SAS System                          8
                                13:07 Tuesday, October 13, 1998

                Analysis of Variance Procedure

Dependent Variable: WEAR

Source                   DF    Sum of Squares  F Value   Pr > F

Model                     4        5.33249385     0.32   0.8581

Error                     8       33.50898308

Corrected Total          12       38.84147692

                  R-Square              C.V.         WEAR Mean

                  0.137289          60.49560        3.38307692


Source                   DF          Anova SS  F Value   Pr > F

TRT                       1        2.81605026     0.67   0.4360
BLOCK                     3        2.51644359     0.20   0.8933


                         The SAS System                          9
                                13:07 Tuesday, October 13, 1998

                Analysis of Variance Procedure

         Level of        -------------WEAR------------
         TRT          N       Mean              SD

         1            3     2.53333333       0.74272023
         2           10     3.63800000       1.96983248
```

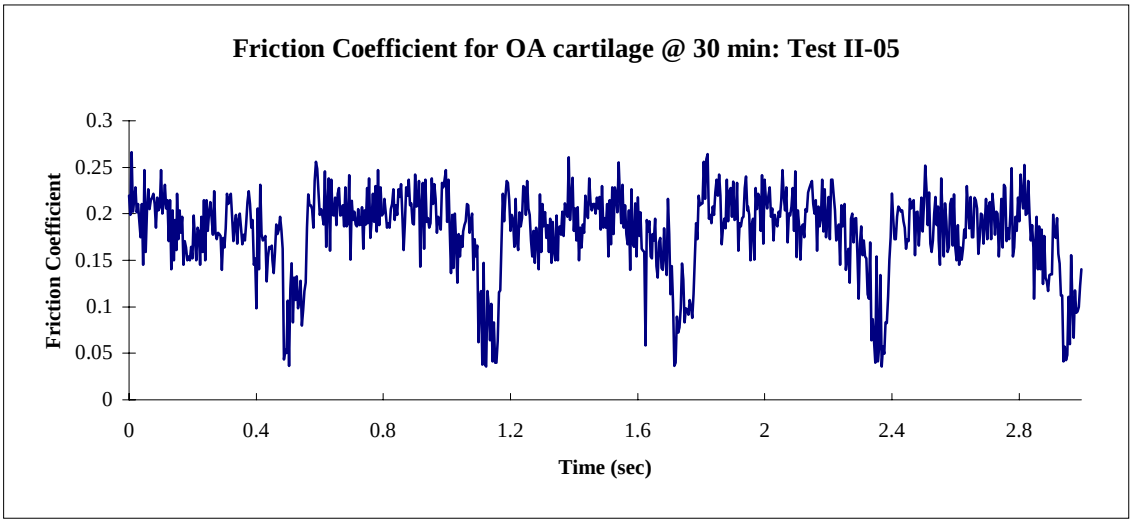
APPENDIX C
Coefficient of Friction Plots for Phase II

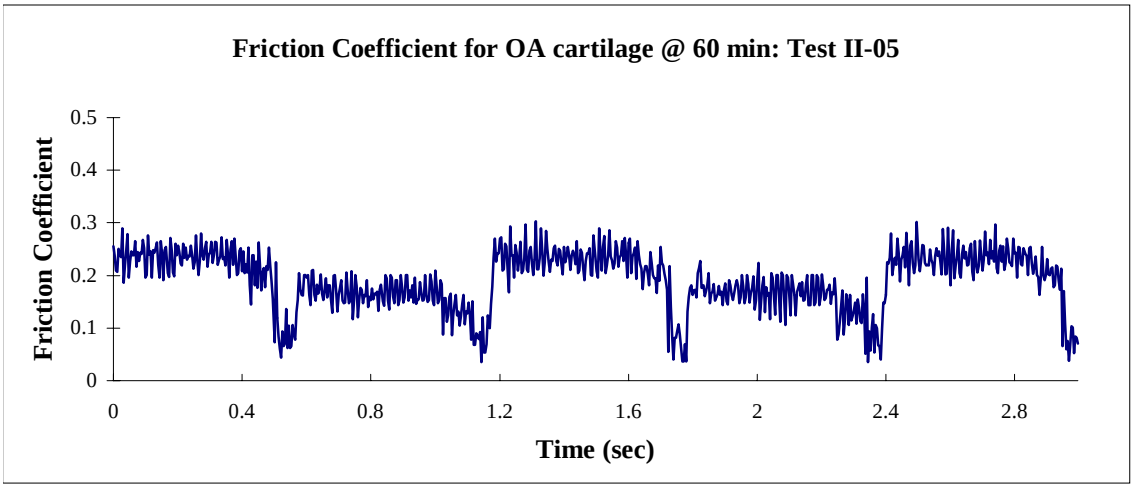







Friction Coefficient for OA cartilage @ 0 min: Test II-05
Friction Coefficient
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (sec)


Friction Coefficient for OA cartilage @ 30 min: Test II-05
Friction Coefficient
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (sec)


Friction Coefficient for OA cartilage @ 60 min: Test II-05
Friction Coefficient
0.5
0.4
0.3
0.2
0.1
0
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Time (sec)

Vita

La Shaun J. Berrien was born in San Diego, California to William and Deolinda Berrien. She received her B.S. in Mechanical Engineering from the Massachusetts Institute of Technology in 1995. Her undergraduate thesis, '*Formation of Tetanus Toxoid Microencapsulated PLGA Microspheres Using Supercritical Fluids as Solvents*,' was in the area of biomedical engineering and pharmaceuticals. Continued interest in the use of engineering concepts in biological and medical applications brought her to Virginia Tech to pursue graduate study in the tribology laboratory under Dr. Michael Furey. She will receive a Ph.D. from the Department of Mechanical Engineering at Virginia Tech in July, 1999. Her research and dissertation topic is in the area of biotribology, with a focus on the influence of biochemical environments on the wear, friction and damage of articular cartilage. Ms. Berrien is a Charles Minor Fellow and a three-time Texaco Scholarship recipient. She is an active member of Zeta Phi Beta Sorority, Inc and various graduate student associations.