A Comparison of the Wear Resistance of Normal, Degenerate, and Repaired Human Articular Cartilage

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ABSTRACT

In our aging population, arthritis is becoming an increasingly common problem. Pain, loss of joint function and other negative affects make arthritis a major health problem. The most common form of arthritis, osteoarthritis, is caused by the "wear and tear" of articular cartilage on the surface of bones in synovial joints. It is a chronic problem that is slowed with different types of therapies, including pharmaceutical, nutritional and surgical, but to date the wearing down of the cartilage cannot be stopped or reversed.

Normal, mature, articular cartilage does not spontaneously repair itself after an injury. In light of this, several surgical techniques are being developed to repair degenerate and/or osteoarthritic cartilage. One such approach uses Autologous Chondrocyte Implantation (ACI). Dr. Mats Brittberg, and associates at Goteborg University in Sweden began using this cartilage repair procedure in 1987. Other techniques attempt to stimulate the subchondral bone to generate cartilage, such as Abrasion Arthroplasty. Still others use tissue grafts to attempt to repair lesions in cartilage. The surface biomechanics of these repaired tissues have not yet been studied. How well does the repaired cartilage resist wear? How long will it last? How does the repaired cartilage compare to "normal" cartilage in terms of wear-resistance? It is the goal of this research to gain initial knowledge to help answer these questions. Dr. Brittberg has provided 17 sample of cartilage, from 9 Swedish patients, including repaired and normal pairs using the aforementioned repair techniques and others, as well as a degenerate and normal cartilage pair. The intention of this paper is to report the findings of experiments performed using these samples, and compare the wear-resistance of repaired and degenerate cartilage to that of normal cartilage.

Wear and friction tests were carried out on 2 mm diameter specimens using a biotribology device and a new, modified technique developed specifically for these small samples. The cartilage samples were mounted, using specially designed adapters, in our biotribology device for oscillating contact against polished stainless steel disks at a constant applied normal load, oscillating frequency, and test time. A buffered saline solution was used as the lubricant. Cartilage wear was determined from hydroxyproline analysis of the test fluid and washings from the wear test. Thin layers of transferred cartilage-like films to the stainless steel disks were also analyzed. Also, friction data was recorded throughout the tests.

The results of these experiments show that:

1) For the two pairs of ACI repaired cartilage, the repaired cartilage gave substantially less wear than that of normal cartilage.
2) For all other repair techniques tested, the repaired cartilage produced more wear than normal cartilage.
3) The single osteoarthritic cartilage tested produced similar wear to that of normal cartilage. This is surprising since the current thought is osteoarthritic cartilage is more susceptible to wear.
4) The hydroxyproline concentration, by weight, of cartilage increases after the wear test.
5) Friction levels were in the boundary lubrication regime, and had no correlation with the amount of wear.

To our knowledge, this research represents the first controlled "in vitro" study of an important unknown in cartilage repair, i.e., the wear-resistance of the repaired cartilage. It shows that ACI produces a cartilage with very good wear-resistance, better than that of other repair techniques, and possibly better than normal, healthy cartilage. ACI and its applications to the treatment of degenerate and osteoarthritic joints are promising, and studies will continue to investigate this and other types of cartilage repair.

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1 INTRODUCTION

1.1 Biotribology

The word "*Tribology*" comes from the Greek word Τριβω, meaning "to rub." It is concerned with surface interactions between two bodies moving relative to one another. Tribology is generally defined as the study of *wear*, friction, and lubrication of a surface [1]. This science can be applied to many areas including materials science, surface science and mechanics, chemistry, engineering, and even biology.

Biotribology is an area that concerns itself with tribology its applications to biomechanics, biomaterials, orthopedics, and other biological systems. It can be defined as the study of biological lubrication processes [1]. Mechanisms like the jaw, the eye, diarthrodial joints in humans and animals are examples of systems that undergo friction and wear of tissues. This study is concerned with the specific application of biotribology to the study of friction, wear and lubrication processes in load bearing synovial joints, such as the knee.

The Tribology/Biotribology Laboratory at Virginia Tech has explored connections between tribology and the mechanisms of synovial joint lubrication and degeneration for over twenty years. The experiments in the past focused on the effects of biochemistry on cartilage wear and damage. The findings have shown the importance of distinguishing between wear and friction, and that the two are not related. Currently the Biotribology Laboratory has been exploring the difference in the wear properties of normal human cartilage versus damaged cartilage and repaired cartilage.

1.2 Rationale of Research

Arthritis is a large problem today in our aging population. According to the Arthritis National Research Foundation, over 43 million Americans suffer from some form of arthritis. Osteoarthritis is the most common form of arthritis, affecting over 20 million Americans, and is the nation's most common crippling disease [2]. The pathology and accompanying symptoms are well known. The characterization of osteoarthritis is: *1. deterioration and detachment of the bearing surface of the joint; and 2. proliferation of new osteoarticular tissue at the margins and beneath the abraded surfaces* [3]. In other words, it is the degeneration of cartilage and the following formation of peripheral bone spurs. It is generally described as the "wear and tear" of articular cartilage. Normal, mature cartilage is incapable of healing itself, which has prompted scientists to develop surgical techniques to regenerate articular cartilage.

One approach that has been used to repair degenerate cartilage is *Autologous Chondrocyte Implantation* (ACI). Dr. Mats Brittberg and his colleagues at Goteborg University in Sweden began using this cartilage repair procedure in 1987 [4, 5]. Dr. Brittberg heads the Cartilage Research Unit at the university. They have done leading edge work on articular cartilage repair, including ACI. Recently, the unit has been trying to improve their cartilage repair techniques. Among the factors being tested are the use of scaffolding materials within the repaired cartilage to strengthen it, and the use of growth factors to stimulate chondrocyte reproduction. Some of the criteria used to determine if the cartilage repair tissue is successful are: 1) if the repaired tissue adheres to and fills the lesion and 2) if pain is reduced in the patient. The cartilage research unit would like to have more objective data to determine how durable the repaired cartilage tissue is, in order to estimate its ability to survive in the patient over time.

The Biotribology Laboratory at Virginia Tech, headed by Dr. Michael Furey, has carried out extensive research on wear and damage of articular cartilage. This research, discussed in more depth in section 2.4, was done in cooperation with Dr. Hugo Veit of the Virginia-Maryland College of Veterinary Medicine. These two groups working in

conjunction with the Cartilage Research Unit at Goteborg are capable of answering the questions mentioned in the abstract. The groups have formed a collaboration, illustrated in Figure 1, and have been working together to develop methods to determine the durability of cartilage.

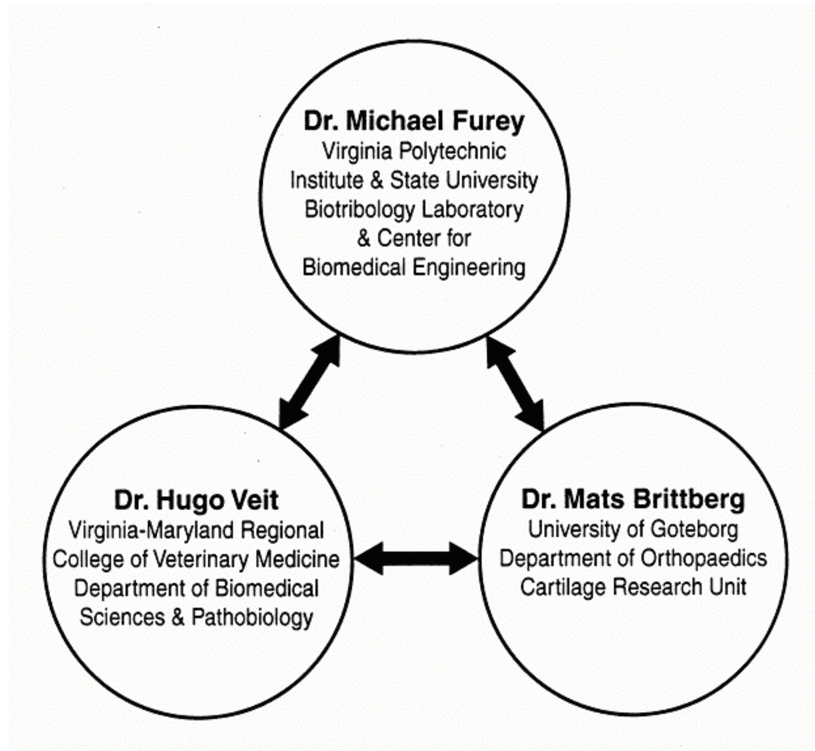

Figure 1. Collaboration in cartilage wear-resistance research

1.3 Objectives of this Research

The primary objectives of this research are to (1) develop methods to test the wear-resistance of human cartilage, normal, degenerate and repaired, using small, 2 mm diameter samples, and (2) determine the wear properties of normal, osteoarthritic and repaired cartilage, using ACI and other repair techniques. The results of this research should be useful as a guide to further research, and to selecting the most promising cartilage repair techniques.

2 BACKGROUND AND LITERATURE REVIEW

2.1 Synovial Joints

Synovial Joints, also called diarthrodial and true joints, are the most common form of joint in the body. The function of a synovial joint is to permit bone on bone articulation, giving a wide range of easy motion and supporting a substantial load. The basic makeup of the joint is articular cartilage and a synovial capsule. Figure 2 shows these and other basic components of a synovial joint. The encasing capsule secretes a fluid called *synovial fluid* which, among other tasks, acts as a lubricant. The cartilage and synovial fluid create an environment in the joint so that there is very little friction between the articulating surfaces, and also so that it is capable of sustaining a large load.

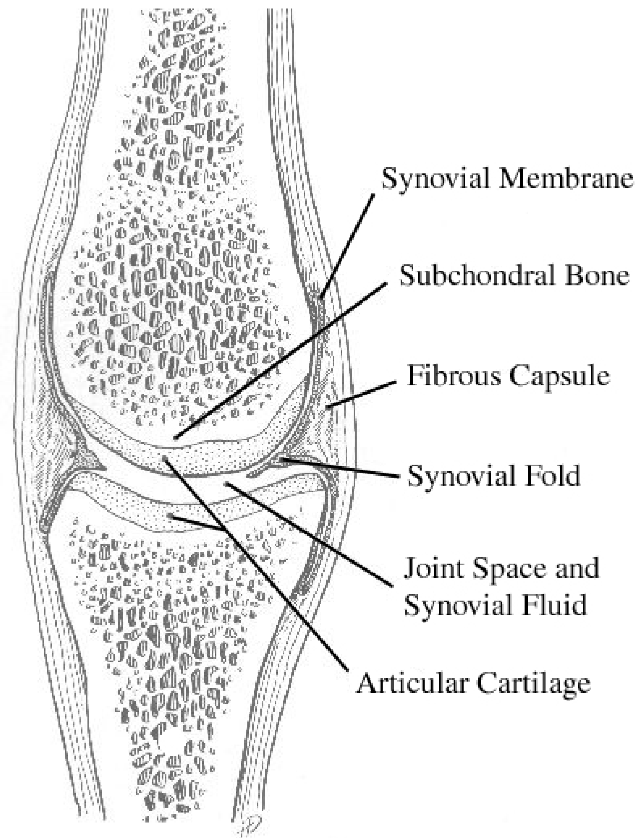

Figure 2. Diagram of a synovial joint [6]

The joint is enclosed by a fibrous capsule called a joint capsule. The inside of this capsule is lined by the synovium, a thin layer of cells that secrete the synovial fluid into the joint space. The synovium has plentiful vasculature and sensory nerves. Ends of the articulating bones are covered by *articular cartilage*. The cartilage is the weight bearing surface in the joint space, and with the synovial fluid provides for very low friction movement. Motion and flexibility of the joint system is provided by the surrounding ligaments and muscles of the joint [6].

2.1.1 Articular Cartilage

Articular cartilage is a specialized tissue that covers the bones of articulating surfaces in a synovial joint. Its function is to enable the joint to operate at high loads while keeping contact stresses low, and create a low friction environment for the bones to move on each other. The thickness and make-up of the cartilage varies from joint to joint, but its function is always the same. Its structure is complex, and creates a typically elastic, durable material, excellent for providing ease of motion in the joint, and absorbing impacts during load variations.

Articular cartilage is not innervated and is avascular, meaning there are no nerves, and no blood vessels in the tissue. It is comprised of cellular and extracellular materials. The cells are called *chondrocytes*, which account for about 5% of the cartilage by volume. The other 95% is the *extracellular matrix*, or ECM, which is secreted by the cells. The ECM contains a large amount of water, about 75% by volume, a network of *collagen* fibers, some structural proteins, and non fibrous filler, made of *proteoglycans* and *glycoaminoglycans* (GAG's). This ECM is a stiff gel, sometimes called 'gristle.' Much of the elasticity of cartilage is accounted for by the filler material and gelled water [7].

The organization of cells and the ECM varies with the depth of the cartilage, so typically the cartilage is subdivided into four zones that are aligned parallel to the articular surface. The zone closest to the surface is called the *Superficial Zone*. Cells in

this zone are long and flat, with their long axis oriented parallel to the surface. The collagen fibers are tight and arranged parallel to the surface. Proteoglycan and GAG content is typically low in the superficial layer. Figure 3 is a diagram of all four cartilage zones, showing the shape and orientation of the chondrocytes.

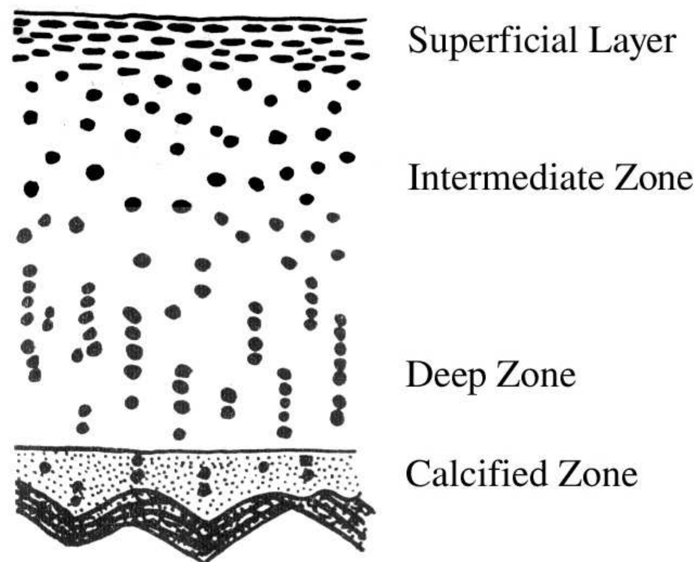

Figure 3. Drawing of articular cartilage zones [7]

The zone beneath the superficial layer is called the *Intermediate Zone*, which is much larger in volume than the superficial zone. Here the collagen fibers are randomly oriented, are loosely packed, and are thicker. The cells are typically spherical and are evenly distributed. The proteoglycan and GAG content is much higher in this zone.

The next zone beneath the intermediate zone is called the *Deep Zone*. Chondrocytes are produced in the deeper layers, and one usually sees the cells in tight columns on a radial axis to the bone. The collagen is tight and dense in this zone, oriented perpendicular to the bone, keeping the cells in their columns. Proteoglycans and GAG content is highest in this layer, while water concentration is low.

The junction between the cartilage and the *subchondral bone* is called the *Calcified Zone*. There are only a few cells in this zone, and collagen fibers penetrate from the deep layer directly into the calcified cartilage, anchoring it to the underlying bone. So its main purpose is to provide the attachment of the cartilage to the subchondral bone. This layer is present in humans, but in some animals the collagen from the deep zone attaches directly to the subchondral bone.

Since cartilage tissue is avascular, it relies on diffusion from the synovial capsule for nutrition. Metabolites from capillary beds in the synovium find their way to the chondrocytes via the synovial fluid and diffusion through the surface layer. Any maintenance of the cartilage ECM is the job of the chondrocytes. Inactivity of the joint results in decreased diffusion, and loss of cell activity. Also, in adult cartilage these cells become less active over time and lose the ability to secrete ECM or to proliferate. Typically any damage to the superficial layer can only be repaired by compensatory secretion by the chondrocytes. If this ability is limited or absent, the cartilage is unable to repair itself. If a defect of the cartilage extends all the way to the deeper zones, the tissue sometimes tries to repair itself by replacing the cartilage with fibrocartilage or bone. This degenerate condition is called osteoarthritis.

2.1.2 Synovial Fluid

Synovial fluid is secreted into the joint space by the synovial cells and as a dialysate of blood. The synovial cells of the joint lining, synoviocytes, add GAG's and hyaluronic acid to the fluid. Normal synovial fluid is comprised mainly of water (85%), *hyaluronic acid* and protein. These low molecular weight proteins contribute to the very high viscosity of synovial fluid. The functions of synovial fluid are nutrition, protection, and lubrication of the articular cartilage, as well as to carry away waste products.

Nutrition is carried out by the diffusion of material between the fluid and the articular cartilage and the blood vessels of the synovial membrane. Nutrients are carried

from the blood vessels to the cartilage by the fluid, and waste products from the cartilage cells are returned back to the blood via the fluid. The surfaces of the joint are protected by the enzymes and phagocytes present in the synovial fluid. These protect the joint surfaces against cell debris, foreign bodies, and microbes [8].

The extraordinary lubrication properties of the synovial joint have long been studied. Well over thirty models of joint lubrication have been presented [1]. In one theory, the low friction was attributed to the high viscosity of the synovial fluid, and therefore to the hyaluronic acid which makes the fluid so viscous [9]. However, the lubrication of the joint surfaces is very complex and not yet completely explained. The sheer number of models shows the lack of complete understanding of joint lubrication. This work will focus on wear of normal, degenerate and repaired human cartilage using a standardized load with a saline solution as a lubricant.

2.2 Osteoarthritis

Osteoarthritis is a disease of the synovial joint. It is characterized by the degeneration and *fibrillation*, or splitting and fraying, of the articular cartilage and the eventual loss of articular cartilage from all or a portion of the joint surface. In severe cases, there is exposure of underlying bone and the bone undergoes abrasive wear and deep proliferation, called eburnation. At the perimeter of the joint surface, new growth of tissue is usually seen as the joint attempts to repair itself. This tissue develops into bony outgrowths called osteophytic lipping, usually referred to as bone spurs. Sometimes this extraneous bone will extend over the articular cartilage around the joint perimeter [10, 11].

The term osteoarthritis is the name this disease is well known by, but it is somewhat technically and pathologically incorrect. Osteoarthritis refers to a primarily inflammatory problem, which is not the case. The pathogenesis described above is the

end result of multiple injuries to the joint surface with minor inflammation to the joint capsule also occurring. The more accurate term for this disease is osteoarthrosis (OA), implying a primarily degeneration of the joint. This paper, however, will continue to use the common term of osteoarthritis or OA.

The causes of osteoarthritis have been subject to a number of theories. Most theories show that early in the progression of the disease, damage to the superficial layer and subsequently deeper layers are the first changes in the cartilage to be seen [10]. Theories propose that the degeneration of cartilage occurs along with other changes in the joint which include bone remodeling. The relationship between cartilage lesions and bone remodeling has been difficult to establish, but likely relates to the common load effects on the joint.

Some theories [12] suggest that as osteoarthritis progresses to the point where fibrillation is apparent, the collagen network loses integrity and this may lead to increased wear and damage of the cartilage compared to normal cartilage. To our knowledge the tribological properties of osteoarthritic human cartilage have not been examined in an effort to test the validity of this hypothesis, until this study.

Other theories of the pathogenesis of OA suggest that the stiffening of the subchondral bone is the initial cause of OA [13, 14]. Perhaps 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 a greater proportion of the shock and stress and this results in increased damage. Another theory posed is that biochemical changes in the cartilage reduce proteoglycan and GAG content, which reduces the elasticity of the cartilage and leads to more rapid collagen degradation [12, 15]. In addition, there are still other theories that point to genetic factors involved in OA that can predispose the patient to or produce osteoarthritic lesions [16].

It has not yet been determined exactly which mechanism causes OA or what is the initial trigger. It is likely that there are multiple pathways. Continuing efforts of research

are being made to gather more information about the early stages of OA and the chain of events that occur during the progression of the disease.

2.2.1 Treatments of Osteoarthritis

Attempts to slow the progression of osteoarthritis have been made using nutritional and pharmaceutical supplements, physical therapy, and other approaches. These treatments have been shown to reduce pain, but do little to stop the progression of the disease and cannot reverse its effects [1, 17, 18]. This paper is does not address these treatments of symptoms, but will examine a few surgical attempts to repair the cartilage lesions. Repair techniques are in use that attempt to replace damaged cartilage with a similar biological tissue, or to cause the cartilage to repair itself. Some of the current techniques in use, including the aforementioned ACI, are discussed in the next section.

2.3 Cartilage Repair Techniques

Chondral and osteochondral injuries are a common result of trauma to the joints. It has been well documented that articular cartilage is unable to repair itself back to normal *hyaline* cartilage after a traumatic injury. Continuous wear on a damaged joint surface usually results in impaired joint function, pain and patient disability, progressing to chronic osteoarthritis. Scientists have been developing ways to restore or repair cartilage, and many promising techniques have emerged in the last 10 years. This paper does not discuss every cartilage repair technique currently in use, but will give an overall summary, and brief descriptions of the repairs that were tested in this study. More comprehensive reviews of cartilage repair techniques are found in references [17-22]. There are two main methods of repairing cartilage: 1) Tissue grafts and 2) cartilage regeneration [17]. The objective of both is to repair the damaged area of articular cartilage to restore a more normal joint function.

2.3.1 Tissue Grafting Techniques

Tissue grafts, or Allografts, include procedures such as Mosaicplasty. These are techniques in which cartilage plugs are taken from non-weight bearing areas of the patient's joint and used to replace the cartilage in the damaged area. First the damaged area is abraded down to the subchondral bone, encouraging the generation of fibrocartilage. Then a graft from a healthy, low weight bearing area, like the patella, is placed in the hole left where the damaged cartilage used to be. In the case of mosaicplasty, several small plugs of cartilage are used as a graft, instead of one large plug [17, 23]. This technique was wear tested in this study.

2.3.2 Cartilage Regeneration Techniques

Regeneration techniques focus on stimulating the regenerative properties of the existing cartilage, or to add extra chondrocytes to form more cartilage. The techniques do not rely on a donor site as in tissue grafting, and are generally less invasive to the patient. The current treatments of regeneration use one or more methods that 1) stimulate the bone marrow to form a repair tissue, 2) implant cultured autologous chondrocytes, and 3) use resorbable scaffolding [18].

Abrasion Arthroplasty is an example of a treatment that stimulates bone marrow stem cells to form repair tissue [24]. In this technique a burr is used to remove the damaged cartilage, all the way down to the vasculature and bone marrow within the subchondral bone. The surface is reshaped and made smooth, free of bony spurs. The thought is that blood vessels and bone marrow in the bone provide stem cells to stimulate cartilage growth. The resulting tissue is generally *fibrocartilage*, though sometimes hyaline cartilage will form as well [17]. The durability of this regenerated tissue is not well known, but is thought to be less than that of normal cartilage. Another technique,

tested in this study, called Spontaneous Repair, is similar to abrasion arthroplasty, yet occurs naturally. It occurs occasionally with severe chronic OA when the damage is severe enough that the cartilage defect extends all the way to the subchondral bone, allowing stem cells to migrate into the area and form fibrocartilage [25].

Regeneration techniques that incorporate scaffolding use a variety of materials which attempt to maintain chondrocytes in a more stable extracellular matrix. The scaffold acts as a substrate to which chondrocytes can adhere. The current scaffolding research has been using resorbable polymers that will stimulate collagen growth and degrade over time, leaving just the cartilage tissue [26, 27]. Some of the scaffolding techniques add no cells to lesions, hoping to encourage cell migration [28, 29]. Others use undifferentiated mesenchymal cells instead of chondrocytes [30], because they will form chondrocytes and can be expanded many times. If chondrocytes are expanded many times the tissue formed tends to be more fibrous in nature [31]. A regenerated cartilage specimen tested in this study uses carbon fiber scaffolding [32].

Autologous Chondrocyte Implantation

Autologous Chondrocyte Implantation, ACI, is a promising method that uses a small biopsy of normal cartilage cells, expanded in vitro, to be used for cartilage regeneration. The expanded cells are implanted in lesions of articular cartilage to form a cartilage tissue that is similar to normal hyaline cartilage

ACI has been used in Gothenburg, (Goteborg), since 1987 by the Cartilage Research Unit. The method has since developed into a reliable method of treating deep cartilage defects or lesions by forming regenerated hyaline cartilage using cells that have been biologically expanded ex vivo [5]. A diagram of the procedure is seen in Figure 4.

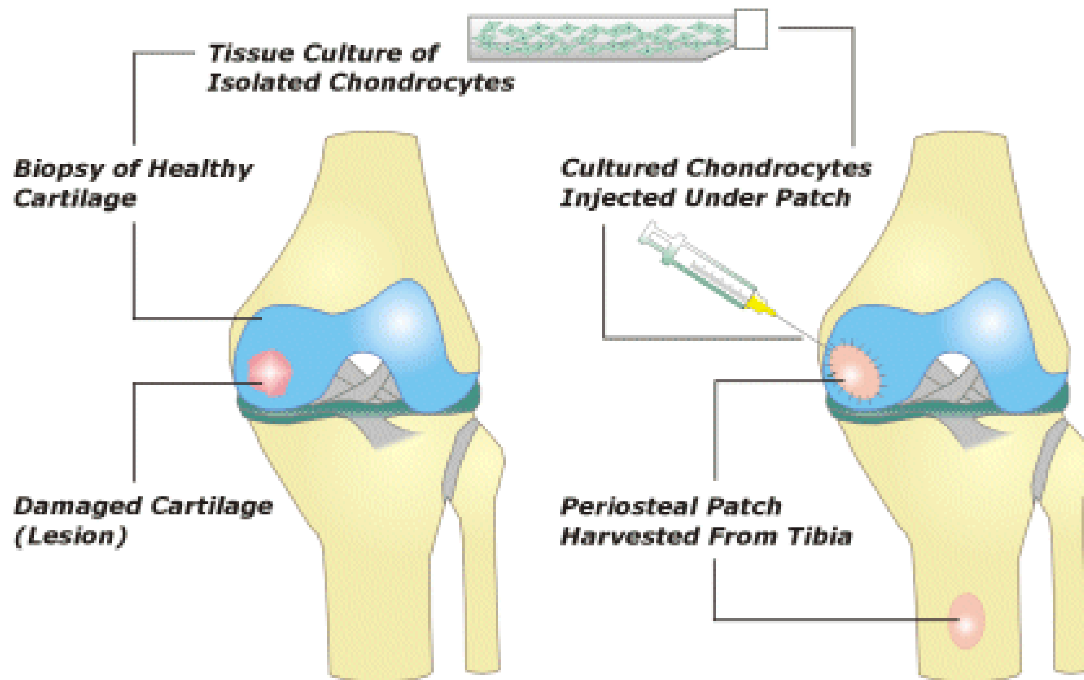

Figure 4. Diagram of the ACI procedure [33]

ACI is an established treatment to repair localized chondral and osteochondral lesions in the joint. Autologous chondrocytes are harvested from a healthy area in a low weight bearing area of the patient's joint, using an arthroscopic procedure. The chondrocyte cultures are isolated and then expanded in vitro for about two weeks. The implantation procedure is begun by debriding the defect to the edge of normal cartilage. A full thickness, down to the bone, area is cleared away. A patch is obtained from the *periosteum* and is sutured over the defect as a cover. The expanded cells are then implanted in high numbers, about 6-18 million, into the defect beneath the periosteal patch. A complete discussion of the ACI operative technique may be found in references [34-36].

This method, ACI, has been used as a treatment for deep lesions in cartilage, but its usefulness for treating osteoarthritis is still in question [37]. However, Dr. Brittberg and the Cartilage Research Unit have seen very promising results. In a survey of 94 patients that underwent ACI, good to excellent results were seen in many of the patients (Up to 92% in the femoral condoyle group). The tissue was considered a good repair if

there was 1) good repair tissue fill, 2) good adherence to underlying bone, 3) seamless integration with adjacent cartilage, and 4) hardness close to that of the adjacent tissue [38].

2.4 Connections Between Arthritis and Biotribology

As previously discussed, arthritis is sometimes referred to as the "wear and tear" or articular cartilage. Despite the prevalence of the disorder, the early pathogenesis of osteoarthritis has yet to be fully understood. It is generally thought, however, that greater susceptibility to wear of articular cartilage is among the first changes occurring in osteoarthritis [12].

Many studies have been devoted to studying the connections between tribology and the function of a synovial joint. These theories are generally preoccupied with friction between the articulating surfaces. Well over thirty theories have been presented on the lubrication of synovial joints [1]. 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. Furthermore, they do not discuss *wear*, which is not related to friction. It may be a natural inclination when considering a connection between arthritis and tribology, that the initial wear and degradation of the joint may be due to poor lubrication and high friction, but this is not necessarily true. According to Swanson [9], "there exists at present no experimental evidence which certainly shows that a failure of lubrication is or is not a causative factor in the first stages of cartilage degeneration." Much more can be known about osteoarthritis if the wear properties of normal articular and osteoarthritic cartilage were known. For example, does arthritic cartilage wear at a higher rate than normal cartilage? The Biotribology Laboratory at Virginia Tech is trying to answer this question, and other related ones in order to give some further insight into osteoarthritis, and hopefully take steps to reduce its incidence and severity [39].

2.5 Biotribology Research at Virginia Tech

The Biotribology Laboratory at Virginia Tech, headed by Dr. Michael Furey in the Department of Mechanical Engineering, is involved in continuing research concerned with the lubrication and wear of joint surfaces. Not only have the tribological properties of normal, healthy cartilage been studied, but also research has been focused on what causes the joint degeneration seen in osteoarthritis. In the past, the Laboratory has used *in vitro* experiments with bovine and lapine cartilage, and emphasized the effects of fluid biochemistry on cartilage wear and damage [1, 40, 41].

Several graduate students have contributed to the advances made in this area. Ms. Bettina Burkhardt's research led to the design and development of the Biotribology Device, an experimental device for wear testing of cartilage [42]. A modification of this device was used in the current study, and is described later in section 3.2.1. Mr. Schroeder's work 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 [41]. This work showed that synovial fluid produced less wear than either buffered saline or a bovine serum. It also showed that the amount of wear and damage increased with an increased duration of the wear test.

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 [40]. His work confirmed that the amount of wear and damage was affected by the lubricant used, with synovial fluid being the best. Also it was found that the friction of the system is affected by load, but not significantly by lubricant. Ms. LaShaun Berrien followed this with Ph.D. work that studied wear and damage of cartilage in biochemical environments that simulated conditions in clinical cases of osteoarthritis [15, 43]. One of her findings was that exposing bovine cartilage to an enzyme suspected of causing cartilage degeneration in osteoarthritis greatly increased the wear and damage of

the cartilage. Also it was discovered that there was no increase in cartilage wear in cases of surgically induced osteoarthritis in lapine cartilage.

The biotribology research done at Virginia Tech so far has focused on lubrication of the joint to assist in the prevention of wear and damage of articular cartilage, as well as the biochemical environment and changes associated with degenerative joints. The current research looks to investigate the wear-resistance of degenerate and repaired cartilage itself as it compares to “normal” cartilage, keeping the environment controlled and constant [44, 45]. The collaboration formed between Virginia Tech and the Cartilage Research Unit in Sweden will likely continue in these efforts in the future.

3 EXPERIMENTAL METHODS

3.1 Materials Tested

3.1.1 Bovine Samples

Due to the limited number of samples from human patients coupled with their small dimensions, several detailed “dress rehearsals” of the wear tests were conducted using bovine samples cut with the same 2 mm biopsy needles used by Brittberg. The samples were run through each procedure, including thawing, the wear test, hydroxyproline analysis, histology and scanning electron microscopy. It was from these tests that major decisions were made on how to treat the human samples, including applied load, test duration, and analysis techniques.

3.1.2 Human Samples

Dr. Brittberg provided 8 pairs of human cartilage specimens from 8 Swedish patients to the Biotribology Laboratory for wear testing. A pair consists of a biopsy of cartilage taken from an area of regenerated cartilage, and a biopsy from a normal, healthy area of cartilage from the same joint surface. The specimens provided included several different cartilage regeneration techniques that were performed by the Cartilage Research Unit. The specimens include two pairs of ACI treated cartilage, two pairs of Abrasion Arthroplasty treated cartilage, and other samples of induced cartilage regeneration. Also included was a sample of degenerative cartilage and its normal counterpart. Table 1 is a complete list of the samples tested in this study. These specimens were shipped to us frozen, packed in dry ice. Each specimen was coated with Tissue-Tek®, a material that protects the cartilage from any damage from freezing, contact with other materials, or shipping trauma.

Table 1. Human cartilage samples tested in this study

Specimen	Gender	Age	Description
1a	Male	65	Biopsy from spontaneous repair tissue, medial *femoral condyle*
1b			Biopsy from normal surrounding cartilage, control biopsy, notch, lateral condyle
2a	Female	39	Biopsy from ACI transplant, femoral condyle
2b			Normal control biopsy
3a	Female	40	Biopsy from fibrous repair surrounding an osteochondral plug, mosaicplasty
3b			Biopsy from the osteochondral plug, centrally taken
5a	Male	36	Biopsy from an ACI transplant. Femoral condyle. Centrally harvested.
5b			Biopsy from normal tissue, control biopsy
6a	Female	62	Biopsy from a carbon fibre induced cartilage repair area. Femoral condyle.
6b			Normal control biopsy.
7a	Female	54	Biopsy from a cartilage repair induced by abrasion arthroplasty. Femoral condyle.
7b			Normal control biopsy
8a	Female	35	Biopsy from an area of degenerative cartilage
8b			Normal control biopsy.
9a	Male	49	Biopsy from cartilage repair induced by abrasion arthroplasty, patella.
9b			Normal control biopsy

Each specimen provided by Dr. Brittberg was a 2 mm cylindrical plug of cartilage and subchondral bone. An example is shown in Figure 5. The biopsies were taken using a TrapLok™ Bone Marrow Biopsy Needle. The 2 mm size is used because it has been acknowledged to cause minimal clinical affect on the patient [Brittberg 2003].

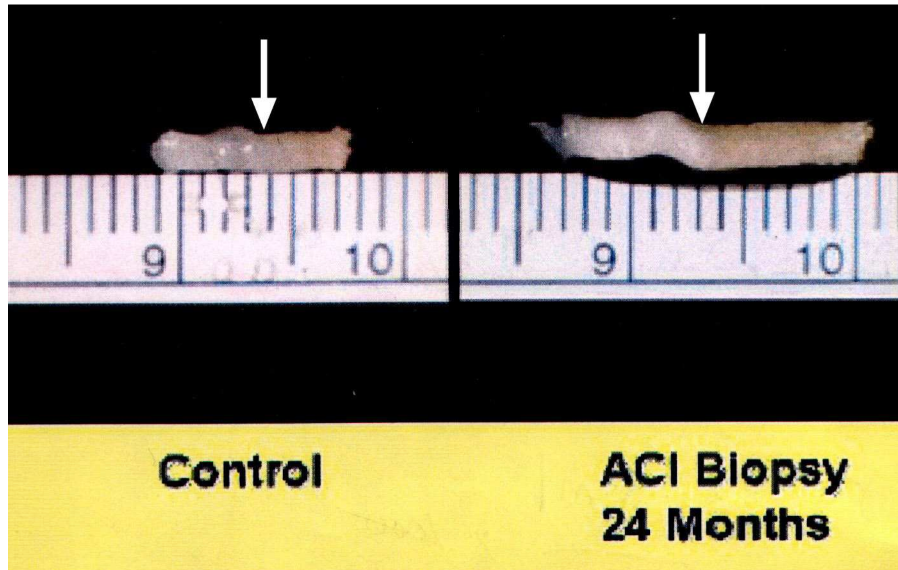

Figure 5. Picture of 2 mm human cartilage specimen

A sample pair of human cartilage specimens is shown in Figure 5, with the repaired cartilage on the right and the normal counterpart on the left. For each specimen the white arrow marks the transition of bone to cartilage, with cartilage on the left side of the arrow.

3.1.3 Stainless Steel Disks

The cartilage specimens used in this wear study were placed in sliding contact with 303 stainless steel disks. How this sliding contact is accomplished is described in the next section. The disks have a 25.4 mm diameter and a 6.35 mm thickness. The surface used for the wear test was ground and polished to a mirror finish. A profilometer was used to determine that the disks had a Center Line Average (CLA) roughness of 0.023 μm. A sample output from the profilometer is shown in Appendix A.

3.2 The Wear Test

3.2.1 Biotribology Device

The method of "wear testing" the cartilage specimens is to use our Biotribology Device to wear the cartilage. The device applies a load and a sliding motion to the sample. This device was originally developed by Ms. Bettina Burkhardt [1], a former student in the Biotribology Laboratory at Virginia Tech and modified and improved in later studies. It was developed to study cartilage deformation, wear, damage, and friction under conditions of tribological contact. Figure 6 shows a diagram of the device, including a new adapter to accommodate small specimens, and Figure 7 is a picture of the device, showing its specimen holders close-up.

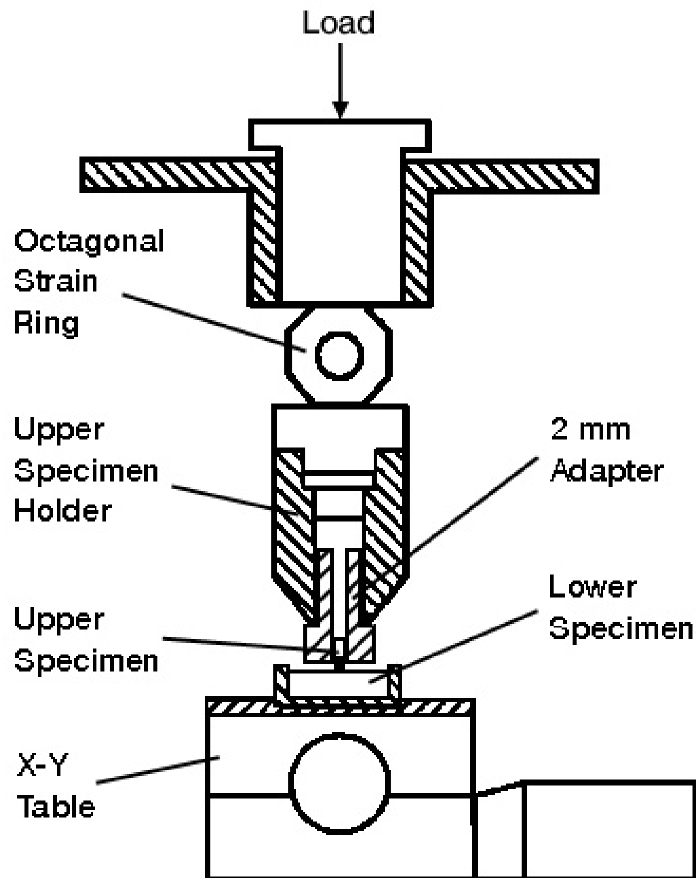

Figure 6. Diagram of Biotribology Device

The device applies a load in the downward direction on the upper specimen. This is done by adding weights to the top of the device. Motion of the lower specimen is controlled by the X-Y table, which is capable of creating a simple oscillating motion in one direction or more complex motions in any direction. The octagonal strain ring gives a measurement of normal and tangential loads, from which the friction between the specimens can be calculated. This device can accommodate different contact systems, including cartilage on cartilage, or cartilage on another solid, such as steel. The geometries of the specimens used include flat-on-flat, or even irregular-on-irregular. The upper specimen size can be up to 6 mm in diameter. A 15 – 25 mm diameter lower specimen can be used. An LVDT can be used with the device to measure the deformation and gross topography of the cartilage. The entire device is enclosed by an incubator to control the temperature and humidity of the environment. A complete list of the key features of the Biotribology Device is shown in Table 2.

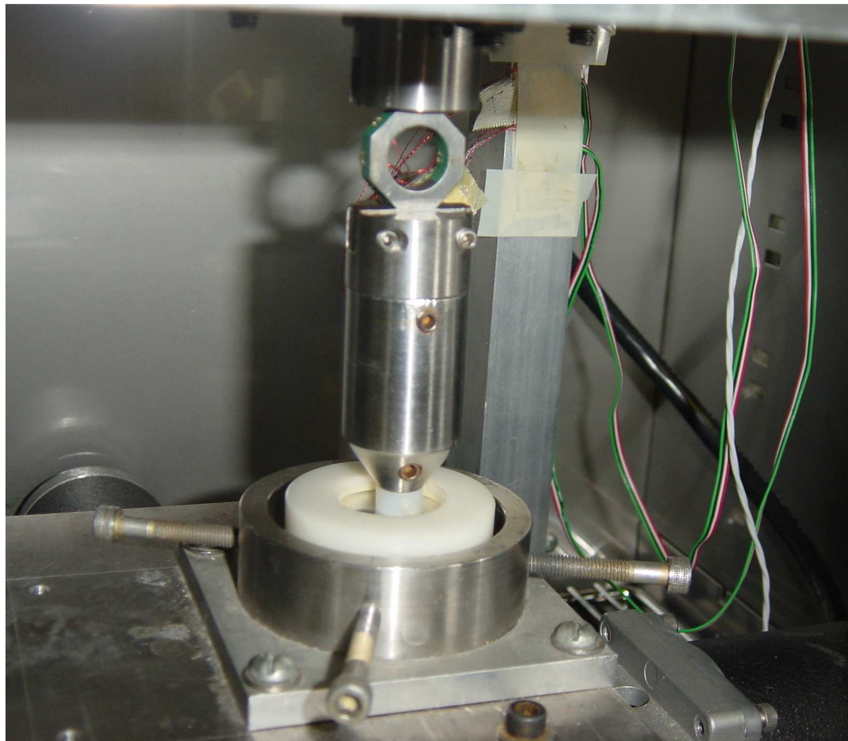

Figure 7. Close-up picture of the Biotribology Device

Table 2. Device specifications (adapted from [42])

Test Configuration	Cartilage-on-cartilage, Cartilage-on-stainless steel
Contact Geometry	Flat-on-flat, Convex-on-flat, Irregular-on-flat, Irregular-on-irregular
Cartilage Source	Bovine, Lapine, Human, etc.
Cartilage Type	Normal, Repaired, Degenerate, etc.
Lubricant	Synovial Fluid, Saline Solution, Other Fluids
Upper Specimen Size	6 mm diameter max, Smaller with adapters
Lower Specimen Size	15-25 mm diameter
Load	3.2 – 600 N
Type of Motion	Linear, Oscillating, Circular, Constant velocity, Complex Patterns
Sliding Velocity	0 – 20 mm/sec
Environment	Ambient, or controlled temperature and humidity

3.2.2 Chosen Test Conditions

There were not enough samples in this study to test many factors, such as load or lubricant used. The conditions were set and kept constant during the wear tests so that the only variable in the test was the source of the cartilage, e.g. normal or repaired.

A special adapter for the Biotribology Device, seen in Figure 6, was designed and constructed for use with the 2 mm specimens provided by Dr. Brittberg. For the purposes of this study the device was set up to run with a lower specimen of a 1 inch stainless steel disk, using buffered saline solution as a lubricant. The temperature of the environment was ambient, measured to be 25°C in the incubator.

The load and duration were chosen to be 6.08 N for 3 hours. It was decided to use this load because it translated to a pressure of 1.94 MPa. If the typical walking cycle is examined, it is found that during parts of the cycle the joint surfaces are in a Hydrodynamic Regime of lubrication, where there is no contact between the surfaces, and no wear. In other parts of the walking cycle there is contact and the joint surfaces are in the Boundary Lubrication Regime, where wear and damage is more likely [46]. For this study, it was desired to study an extreme condition of contact, to achieve relatively high levels of wear and damage. This chosen load and pressure is comparable to the highest pressures seen in the walking cycle, putting the cartilage in an area of maximum load, and in this boundary lubrication area of contact. This load and duration will create an environment of continuous contact, and produce a measurable amount of wear.

3.2.3 The Wear Test Procedure

The following is a summary of the treatment of the cartilage specimens during the wear test. The detailed procedure for the wear test can be found in Appendix B.

Each human cartilage sample, having been stored in a freezer at -20 ^{o}C, was thawed in 100% humidity by placing it in a Petri dish with a buffered saline soaked gauze pad. The dish was covered and the sample left for 20 minutes, or until the Tissue-Tek® was thawed. Using distilled water, the Tissue-Tek® was rinsed off the sample, which is then set back in a Petri dish to wait for the wear test. A clean 25.4 mm diameter polished stainless steel disk was then placed in the lower specimen holder, which was then mounted on the X-Y table. Then the cartilage specimen was placed in the special 2 mm plug adapter. It was adjusted so that just about 1 mm of cartilage protruded from the holder. The special 2 mm plug holder was then mounted in the upper specimen holder with the set screws lining up. This was so the direction of sliding could be determined later. The upper specimen holder was mounted so that its set screws were aligned perpendicular to the direction of motion of the X-Y table, not yet allowing the upper and lower specimens to touch. Then, 0.5 ml of saline solution was added to the stainless steel

disk for lubrication. A load of 6.08 N was applied by adding weights to the top of the device.

Next the sliding motion program was begun. A reading from the strain gage was taken immediately and then after 5 minutes, after that every 20 minutes until the end of the 3-hour test. During the test the cartilage specimen had to be adjusted so that enough would protrude from the holder (described further in section 4.2.1). The test was continued until 3 hours had elapsed and then stopped. Upon stopping the test, the load was removed and the upper specimen holder was removed from the device.

The wear debris was collected by rinsing the plug off into a vial using distilled water. Then the disk holder was removed and the disk was taken and rinsed off into the same vial. About 7 ml of water in total was used to collect the wear debris. Using a razor, a small section of cartilage was taken from the remains of the plug and set back in the freezer for later testing. The disk was placed in a jar, also to be tested later for cartilage deposit on its surface.

3.3 Analysis

The amount of wear of the human cartilage specimens is relatively small, on the order of 1 mg or less, and cannot simply be weighed accurately. The techniques used to determine the amount of wear of each specimen include: 1) determining the concentration of hydroxyproline in each cartilage specimen, 2) measuring the amount of hydroxyproline in the washings from the wear test, and 3) measuring the amount of hydroxyproline in the material transferred to the stainless steel disk during the wear test. Friction measurements were also made throughout the test.

3.3.1 Material Transferred to Disk

After a wear test is complete, a thin film of material is often present on the stainless steel disk. The film is a material from the cartilage plug transferred to the disk during the test. The total wear of the cartilage specimen should include the debris collected by washing the specimens, and also any material present on the disk. The material on the disk is collected by gently scrubbing the surface of the disk with a Teflon scraper and a small amount of HCl. The material on the disk becomes suspended in the HCl, and is then rinsed with 3 ml of HCl into a vial. This material can then be analyzed in the hydroxyproline analysis along with the debris in the washings to determine the total amount of wear.

3.3.2 Hydroxyproline Analysis

Hydroxyproline Analysis was the main method of determining the wear of the cartilage plugs. The collected wear material was run through the colorimetric assay to produce a color yield that is accurately proportional to the hydroxyproline concentration of the solution. Hydroxyproline is a unique amino-acid of collagen, and is found in consistent proportion in collagen; therefore this hydroxyproline assay can be used to measure the amount of collagen debris in the solution, as well as the amount of collagen on the stainless steel disk. Hence, hydroxyproline amounts found are directly related to the total wear of the cartilage specimen tested.

The procedure for the hydroxyproline assay was originally developed by Neuman and Logan [47]. A version modified by Dr. E.M. Gregory of the Biochemistry Department of Virginia Tech and LaShaun Berrien of the Biotribology Laboratory was used for these experiments. The detailed procedure used in this study is in Appendix C. In the test, solutions with known amounts of hydroxyproline are run through the assay, producing a color yield that is linearly proportional to the amount of hydroxyproline. A

comparison can then be made of the color yield after running our cartilage wear samples through the assay to determine the amount of hydroxyproline in the solutions.

The hydroxyproline assay was performed on 3 sets of samples for each cartilage specimen that underwent a wear test. First, a section of cartilage was removed from the cartilage plug after a wear test. The section's mass was measured using a digital scale accurate to a tenth of a milligram. After the analysis was complete, the amount of hydroxyproline in the section of cartilage was known. This allowed us to determine the cartilage's hydroxyproline concentration, in μg hydroxyproline/mg cartilage (μg/mg).

The other two sets of samples for each cartilage specimen were the debris from the washings, and the material transferred to the disk. The amount of hydroxyproline in the collection of wear debris can be determined by comparing the color yield to that of the standards. We then can use the amount of hydroxyproline found in the wear debris to calculate the weight of cartilage worn away, by using the determined hydroxyproline concentration of each specimen.

3.3.3 Friction Data

Friction was determined from the output of the strain gage unit discussed in the biotribology device section. The gage was calibrated by applying known amounts of normal and tangential load and then measuring the voltage output from the strain gage device. This gives a calibration curve showing the relationship between force and voltage. The voltage then acquired during the wear tests was translated into tangential force via these calibration curves. The normal load is the known applied load. The coefficient of friction was calculated as the ratio of tangential force to normal load. The friction data was measured at various times during the wear test and can be shown as a function of time.

4 RESULTS OF HUMAN CARTILAGE EXPERIMENTS

4.1 Introduction

The success of surgical repairs, such as ACI, on cartilage has been measured by how well the patient's symptoms of pain and discomfort are alleviated. In the case of ACI, techniques such as MRI and histology has been used to judge the quality of the repaired tissue [48]. Other studies have performed hardness tests on the repaired cartilage [38]. There has not been a study to our knowledge of the wear properties of repaired cartilage. To our knowledge, the present work is the first to determine the durability of repaired articular cartilage.

4.2 Observations from Wear tests

The wear tests were completed with the described procedure on all of the human samples provided by Dr. Brittberg, listed in section 3.1.2, with one exception. Sample 9a, a specimen treated by abrasion arthroplasty, was impossible to test. The specimen had no cartilage attached to the bone. It perhaps was lost in transport or storage at some point before it was received by the Biotribology Lab.

The completion of these tests in itself marks an accomplishment, and a step forward in the areas of biotribology and cartilage repair research. No other tests have been performed to measure and compare the wear resistance of human or animal normal and repaired cartilage. The procedure developed to test the wear of 2 mm specimens of human cartilage produced measurable amounts of wear under the load and duration selected. This procedure, using the biotribology device, can be used for future experiments on larger quantities of samples to further the exploration of the wear properties of normal, repaired and degenerate cartilage.

4.2.1 Physical Changes of Cartilage

The human cartilage samples provided by Dr. Brittberg had very thick cartilage. The entire plug was generally about 1 cm long, and 7 mm of that was cartilage. This was much thicker than the bovine cartilage, usually 2 -3 mm in thickness, previously examined by the Biotribology Laboratory. It was a concern that the cartilage would simply break off or wear away. The samples were carefully mounted so that this would be unlikely to happen, with only 1 – 2 mm of the cartilage protruding from the holder. A picture of the cartilage mounted in the holder is shown in Figure 8. The axial set screw, at the top, can adjust how much cartilage protrudes from the bottom, and the radial set screw holds the cartilage in place.

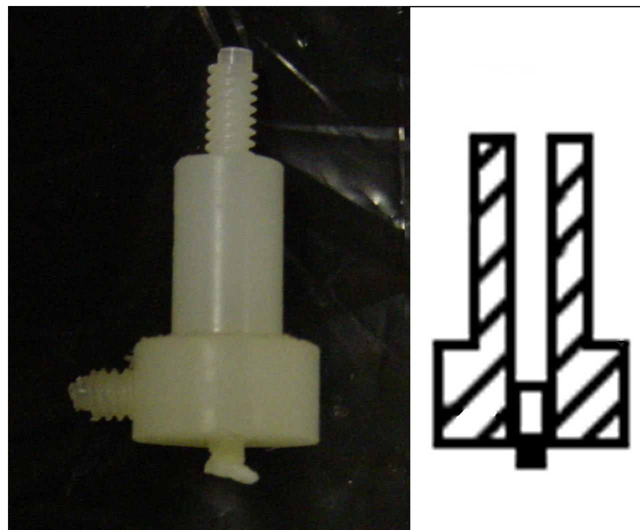

Figure 8. Picture of cartilage plug mounting

The cartilage did not break off quickly, as concerned, in any of the cases. However there were some interesting changes in the shape of the cartilage after the wear tests. Throughout the wear test the cartilage would often be compressed, and pushed back into the holder so that the holder would be contacting the stainless steel disk. This was corrected by periodically, about every 1 hour, stopping the test and turning the axial set screw of the holder so that more cartilage would protrude from holder. Also, as the test continued the cartilage would be “squeezed” out of the holder and flattened out

between the holder and the disk. This is probably due to adhesion between the disk and the cartilage. Due to the sliding motion, the cartilage was "pulled" out slowly by the adhesion, and flattened. Figure 9 demonstrates this phenomenon, showing a picture of sample 2a, before and after the wear test.

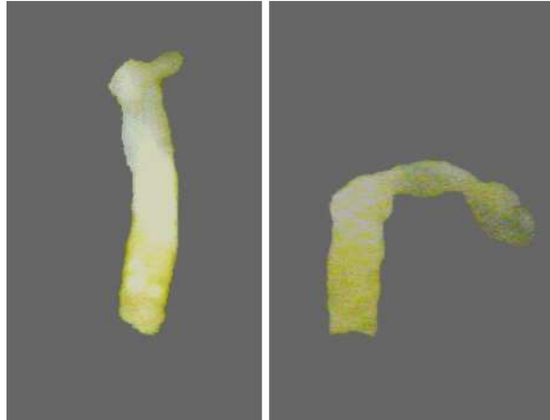

Figure 9. Comparison of cartilage specimen structure before and after wear test

The specimen on the left in Figure 9 is specimen 2a before the wear test occurred, and the same specimen after the wear test is shown on the right side. While this phenomenon was typical and happened to an extent in every case, the pictured sample 2a was the most extreme.

4.3 Analytical Results

4.3.1 Hydroxyproline Concentration

The hydroxyproline concentration of each specimen was determined by first removing a small section of cartilage from the plug after the wear test was completed and setting it aside. The mass of this section was measured using a scale accurate to a tenth of a milligram. The section was then tested for hydroxyproline using the procedure described in previous sections starting with step 3 of the procedure, *hydrolyzation*. After the amount of hydroxyproline was found, the concentration of each specimen could be

determined by dividing this amount by its mass in mg. So now there is a calculated hydroxyproline concentration for each specimen that can be used with the amount of hydroxyproline found to calculate the amount of cartilage wear. The hydroxyproline concentrations for each sample are reported in Table 3.

Table 3. Hydroxyproline concentrations of each specimen

Cartilage Specimen	Type or Source	Hydroxyproline Concentration (μg/mg)
1a	Spontaneous Repair	5.88
1b	Normal	13.15
2a	ACI Repair	16.43
2b	Normal	6.85
3a	Mosaicplasty	13.13
3b	Normal	14.01
5a	ACI Repair	15.17
5b	Normal	17.13
6a	Carbon Fiber Induced	8.00
6b	Normal	19.07
7a	Abrasion Arthroplasty	14.53
7b	Normal	11.76
8a	Degenerate Cartilage	18.61
8b	Normal	29.72
9a	Abrasion Arthroplasty	N/A
9b	Normal	9.41

The values in Table 3 are the average results from tests done in triplicate. It was necessary to determine the hydroxyproline concentrations using cartilage taken after undergoing a wear test because, had a section of cartilage been removed for testing beforehand, there would not have been enough cartilage for the wear test, due to the small size of the samples, 2 mm in diameter. However, in pair 3, the original sample was larger than 2 mm. So a 2 mm plug was taken from the sample for the wear test, as well as a section to test for hydroxyproline concentration. A section was also taken after the wear test. This allowed for a comparison of the hydroxyproline concentration of the samples from patient 3 both after having gone through a wear test, and without going through a wear test. Table 4 shows this comparison.

Table 4. Comparison of hydroxyproline concentrations before and after wear test

Cartilage Specimen	Type or Source	Hydroxyproline Concentration (μg/mg)
3a before wear test	Mosaicplasty	9.21
3a after wear test		13.13
3b before wear test	Normal	10.74
3b after wear test		14.01

Again, these values are the average results of triplicate testing. Note that for both cases the hydroxyproline concentration is larger in the cartilage that underwent a wear test. This phenomenon is discussed in detail in later sections. These successful determinations of hydroxyproline concentration of cartilage have not been done in previous studies, and will make for a more accurate measurement of wear using the hydroxyproline analysis.

4.3.2 Material in Washings

Some of the debris from the wear test was collected by "washing" the cartilage plug and the disk after the wear test. Loose cartilage debris was rinsed into a vial using distilled water, as described in the wear test procedure. A solution containing water, saline from the lubricant, and cartilage debris is the result. The washing solutions were then prepared for the hydroxyproline analysis by removing all of the water in the solution. This was accomplished using a *lyophilizer* machine. Essentially this dry freezes and evaporates the water at -70°C for about 18 hours.

The sample is then treated to the rest of the hydroxyproline assay, in triplicate, as described in appendix C. The result is a solution with a color, generally pink or rose, that is proportional to the amount of hydroxyproline. The color is determined by measuring its absorbency coefficient at 540 nm using a spectrophotometer. The absorbency coefficient is then translated into an amount of hydroxyproline by comparing it to standards, known amounts of hydroxyproline, run in parallel with the samples, as

described in section 3.3.2. The amount of hydroxyproline is translated into an amount of cartilage wear using the hydroxyproline concentration of each cartilage specimen, described in the previous section. The cartilage wear found in the washings for each sample is shown in Table 5.

Table 5. Cartilage wear in washings.

Cartilage Specimen	**Type or Source**	**Cartilage Wear in Washings (μg)**
1a	Spontaneous Repair	2238
1b	Normal	785
2a	ACI Repair	202
2b	Normal	3610
3a	Mosaicplasty	508
3b	Normal	448
5a	ACI Repair	647
5b	Normal	650
6a	Carbon Fiber Induced	1624
6b	Normal	318
7a	Abrasion Arthroplasty	727
7b	Normal	231
8a	Degenerate Cartilage	470
8b	Normal	368
9a	Abrasion Arthroplasty	N/A
9b	Normal	1547

These results show that we can indeed obtain measurable amounts of wear with the developed procedure. The values of wear measure from 368 to 3610 μg of wear, which is easily measured using the modified hydroxyproline analysis.

The following is an example calculation using sample 7a, showing how cartilage wear is determined from this hydroxyproline analysis:

- The standards run during this hydroxyproline test had are shown in the following plot. They give a curve of $Y = 0.0074 \times X$, where Y is the absorbency coefficient and X is the amount of hydroxyproline in μg.

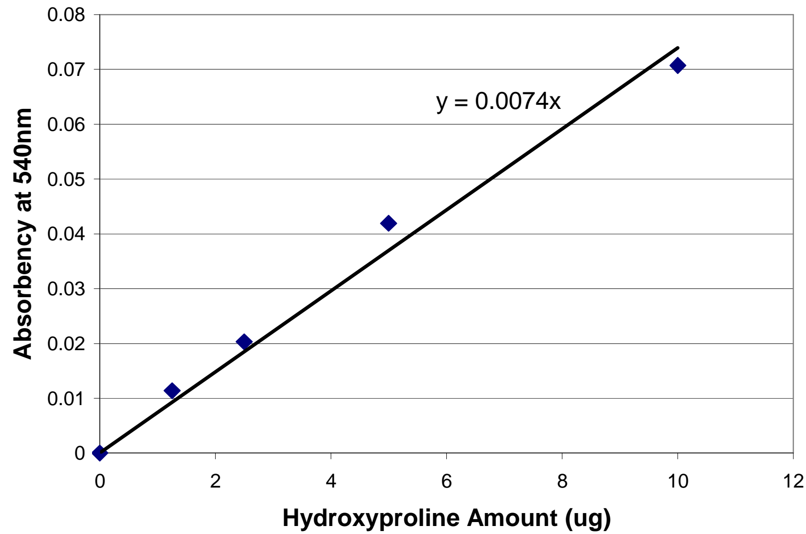

Figure 10. Example standard curve for hydroxyproline analysis

- The section of 7a used for hydroxyproline concentration had a mass of 1.0 mg, and yielded an absorbency of 0.036. Dividing by 0.0074 gives 4.84 μg of hydroxyproline. The tested sample was a 1/3 of the original amount so there was 14.53 μg of hydroxyproline. Dividing by the mass shows a concentration of **14.53 μg hypro/mg cartilage**

- The washings from sample 7a yielded an absorbency of 0.026. Dividing by 0.0074 gives 3.52 μg of hydroxyproline. Again this is only 1/3 of the original sample, so multiply by 3, and divide by 14.53 μg/mg gives 0.727 mg, or **727 μg** of cartilage wear in the washings.

4.3.3 Material Transfer

Other material that is considered wear is transferred to the stainless steel disk during the wear test. A thin film is deposited as a residue after the 3 hour test. The

nature of this material is unknown. In previous studies, FTIRM was performed on the film, showing a cartilage-derived material. In this study it was attempted to see if there was any hydroxyproline present in the material. We found that there was indeed hydroxyproline in the film, so there must be some collagen transferred to the disk. This must be included as part of the wear of the cartilage. An example of what the film looked like is shown in Figure 11.

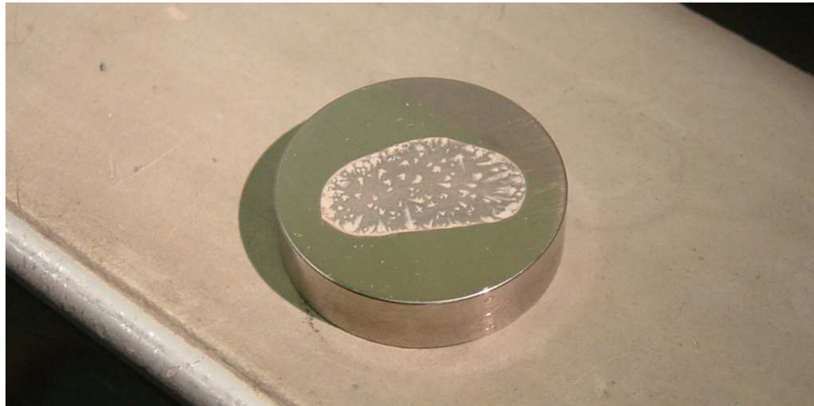

Figure 11. Picture of film on stainless steel disk

The film was removed from the disk using a Teflon scraper and a drop of 6N HCl. Gently rubbing the disk caused the material to be suspended in the HCl, and this solution was placed in a vial to be run through the hydroxyproline assay. There was no need to remove any water with this method, so the procedure was begun at the hydrolyzation step. The color yield from the material on the disk was compared to the standards and to the hydroxyproline concentration of the sample's cartilage as described previously. This was then translated into an amount of wear material on the disk in the same fashion as the results from the washings. The results for each sample are shown in Table 6.

Table 6. Cartilage wear seen in disk transfer material

Cartilage Specimen	Type or Source	Cartilage Wear on Disk (µg)
1a	Spontaneous Repair	516
1b	Normal	677
2a	ACI Repair	248
2b	Normal	14
3a	Mosaicplasty	108
3b	Normal	85
5a	ACI Repair	412
5b	Normal	730
6a	Carbon Fiber Induced	140
6b	Normal	242
7a	Abrasion Arthroplasty	219
7b	Normal	48
8a	Degenerate Cartilage	193
8b	Normal	168
9a	Abrasion Arthroplasty	N/A
9b	Normal	164

It is seen that there is a measurable amount of hydroxyproline in the film, and therefore there is cartilage wear on the disk. This contribution to total wear has not been measured in previous studies, and is significant.

4.3.4 Summary of Hydroxyproline Results

The total wear of each cartilage specimen is the total of the wear found by "washing" the cartilage plug and the stainless steel disk after the wear test, and that wear found in the material transferred to the disk. In some cases the wear material on the disk was almost as much as the debris in the washings, and in others it was much less. The total amount of wear varied greatly from as little as 450 µg to as much as 3,624 µg of wear. This may be a factor of the origin of the cartilage as much as the repair technique used. The following table gives a complete summary of the results of the hydroxyproline test, including gender and age of the patient from which the specimen was obtained.

Table 7. A summary of hydroxyproline analysis results

Cartilage Specimen	Gender	Age	Type or Source	Cartilage Wear (µg) Washings	Disk	Total	Hydroxyproline Conc. (µg/mg)
1a	Male	65	Spontaneous Repair	2238	516	2754	5.88
1b			Normal	785	677	1462	13.15
2a	Female	39	ACI Repair	202	248	450	16.43
2b			Normal	3610	14	3624	6.85
3a	Female	40	Mosaicplasty	508	108	616	13.13 [1]
3b			Normal	448	85	533	14.01 [1]
5a	Male	36	ACI Repair	647	412	1059	15.17
5b			Normal	650	730	1380 [2]	17.13
6a	Female	62	Carbon Fiber Induced	1624	140	1764	8.00
6b			Normal	318	242	560	19.07
7a	Female	54	Abrasion Arthroplasty	727	219	946	14.53
7b			Normal	231	48	279	11.76
8a	Female	35	Degenerate Cartilage	470	193	663	18.61
8b			Normal	368	168	536	29.72
9a	Male	49	Abrasion Arthroplasty	N/A [3]			N/A
9b			Normal	1547	164	1711	9.41

1. Pair 3 has available hydroxyproline concentration of cartilage that did not undergo a wear test. 3a was 9.21 µg/mg, and 3b was 10.74 µg/mg.

2. The “tail-like” elongation, typical with the thicker cartilage in pair 5, came off into the washings for 5b. It was removed and used for the hydroxyproline concentration test. It had a mass of 3300 µg

3. The specimen 9a did not have any cartilage available for a wear test.

4.3.5 Friction Results

During the wear test, data was collected on the normal and tangential loads. This was achieved using the octagonal strain gage and a continuous readout recorder, the AstroMed DASH II. The gage outputs a voltage that is proportional to the forces seen in both the normal, perpendicular to the sliding motion, and tangential, parallel to the sliding motion, directions. The continuous readout device displays the voltage output, with voltage on the Y-axis and time on the X-axis. For our purposes the voltage was only recorded for a short period once as the test was begun, once after about five minutes, and then about every 20-30 minutes for the duration of the wear test.

The voltage output from the tangential direction was translated into the tangential force by using the curve obtained during calibration, previously described in section 3.3.3. The calibration data used throughout these tests can be seen in Appendix D. The calibration curve was determined to be:

$$Voltage[mV] = 6.92 \times Tangential\ Force[N]$$

The normal load throughout the test is the constant, known applied load of 6.08 N. The coefficient of friction then can then easily be determined by the equation:

$$\mu_k = \frac{F_T}{F_N}$$

So, the determined tangential force is divided by the normal load of 6.08 N to obtain friction. The friction was determined for the beginning and end of the test. Also the friction coefficient at each 20 minute interval was found, and the average taken. The results for each specimen are shown in Table 8.

Table 8. Summary of friction results for each specimen

Cartilage Specimen	Coefficient of Friction		
	Initial	Average	Final
1a: Spontaneous Repair	0.05	0.22	0.32
1b: Normal	0.04	0.18	0.24
2a: ACI Repair	0.10	0.16	0.19
2b: Normal	0.03	0.17	0.24
3a: Mosaicplasty	0.11	0.18	0.20
3b: Normal	0.08	0.20	0.24
5a: ACI Repair	0.06	0.17	0.24
5b: Normal	0.06	0.14	0.31
6a: Carbon Fiber	0.12	0.16	0.19
6b: Normal	0.14	0.20	0.21
7a: Abrasion Repair	0.08	0.20	0.31
7b: Normal	0.05	0.13	0.17
8a: Degenerate Cartilage	0.12	0.15	0.19
8b: Normal	0.08	0.17	0.20
9a: Abrasion Arthroplasty	N/A		
9b: Normal	0.18	0.20	0.26

In every case the final friction was greater than the initial friction. Also, each of these friction coefficients show that the system is in the Boundary Lubrication regime, and that there is contact between the cartilage surface and the surface of the stainless steel disk, ensuring wear.

4.4 A Comment on Error

It is not possible to determine the error in the cartilage wear, due to the limited availability of human specimens. Based on our past experience with bovine cartilage, the

error in wear from specimens taken from a given joint of an animal is estimated to be approximately 10% [40, Furey 2004]. We can determine the error in hydroxyproline analysis since in this study, the analysis was carried out in triplicate. Each solution containing cartilage or wear debris from the specimen was split into three parts, and the analysis run on each. The result was an average error of 9.2%.

Each specimen received exactly the same treatment. Thus it is suggested that any error due to the procedure, would have little or no effect on the final comparison of results. Also, the error in the friction results can be estimated to be less than 10%, based on past experience [Furey 2004].

5 DISCUSSION

5.1 Hydroxyproline Concentration Observations

It has been shown that, in humans, collagen accounts for about 10% of the weight of hydrated cartilage, and hydroxyproline makes up about 8% of dehydrated collagen [7, 49]. So typically in other work of this nature, including that by the Biotribology Laboratory [15], the hydroxyproline concentration of cartilage was assumed to be 8 μg/mg. However to our knowledge it has not been studied whether this concentration is consistent from patient to patient, or if it changes under extreme conditions, such as in our wear test. For this reason the hydroxyproline concentration of each specimen was determined in this study.

The hydroxyproline concentrations ranged from 5.88 to 29.72 μg/mg, and most, 12 out of 15, had higher concentrations than the standard of 8 μg/mg. It was not expected to see concentrations this high in human samples. Some light is shed on this by examining the specimens from patient 3, the patient from which we were able to test the concentration of the cartilage both with and without undergoing a wear test. Sample 3a had a concentration of 9.21 without undergoing a wear test, and a concentration of 13.13 μg/mg after the wear test. Sample 3b went from 10.74 to 14.01 μg/mg after the wear test. In both instances the original cartilage had hydroxyproline concentrations close to that of the standard 8 μg/mg, and then the concentration increased after the wear test (43% and 30% respectively). This large increase is probably due to the compression of the cartilage, and subsequent loss of water during the test.

The applied pressure of 1.94 MPa to the cartilage places it in a state of compression, squeezing it between the disk and the device's arm. Some of the water, proteoglycans and other ECM material are squeezed out. The collagen in the ECM, however, will not be lost simply due to compression. This leaves a cartilage with a

higher density of collagen after the wear test, explaining why such large hydroxyproline concentrations were found. It is inferred then that the hydroxyproline concentration, by weight, of the cartilage is not constant for the duration of the wear test. The hydroxyproline concentration determined after the wear test is higher, if not significantly higher, than the concentration of the original cartilage. However it is impossible to know by how much the concentration changes, since we cannot measure the amount of water and other material lost by the cartilage with this technique, only the amount of collagen worn off. Since it is not known by how much the hydroxyproline concentration of each cartilage specimen changed, the measured concentration, performed after the wear test, was used in all calculations as the best estimate available.

Again this large increase in hydroxyproline concentrations is due to the compression of the cartilage, and the subsequent squeezing out of water and other material, and is not necessarily related to wear. Any correlation between hydroxyproline concentration and wear and the durability of cartilage is discussed in section 5.3.

5.2 Wear Comparisons

5.2.1 Comparing Repair Techniques

In all the cases tested, the only type of repaired cartilage to have lower total wear than its normal counterpart is the ACI technique. In patient 2, the repaired cartilage had 8 times less wear than the normal cartilage, 450 compared to 3624 μg. In patient 5, the ACI repair had 23% less wear, 1059 compared to 1380 μg. For visual comparison of these amounts of wear, a bar graph is shown in Figure 12.

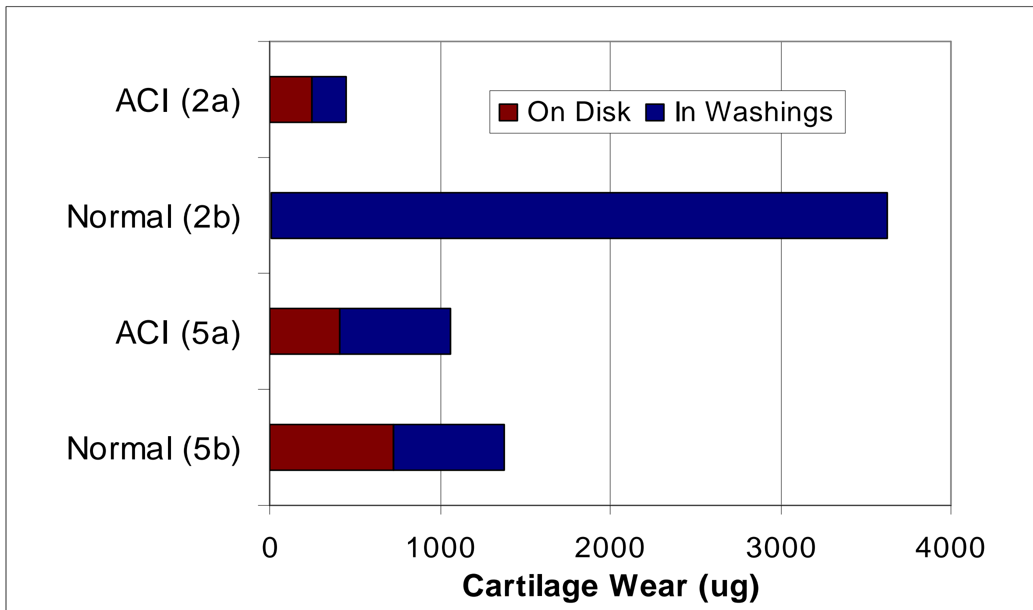

Figure 12. Wear comparison of normal and ACI repaired cartilage

The result from 2b is suspiciously high. It is noted that in one sample, 5b, a large piece of cartilage, with a mass of 3300 μg, detached from the plug and went into the washings. This was not considered as wear, and was treated separately. It is possible that a similar size piece of cartilage ended up in the washings of 2b and went unnoticed, explaining the large wear of over 3600 μg. However, the results do suggest that the ACI technique produces a cartilage with very good wear properties, perhaps even better than that of normal healthy hyaline cartilage.

The techniques that stimulate the subchondral bone to produce cartilage are "Spontaneous Repair," seen in patient 1, and "Abrasion Arthroplasty," patients 7 and 9 (results obtained from #7 only). These repaired cartilage samples all had higher total wear than their normal counterparts. Patient 1 had repaired cartilage with 88% more wear than normal, 2754 to 1462 μg, and in patient 7 the repaired cartilage had 3.4 times more wear than normal, 946 compared to 279 μg. Figure 13 shows a bar graph of these results.

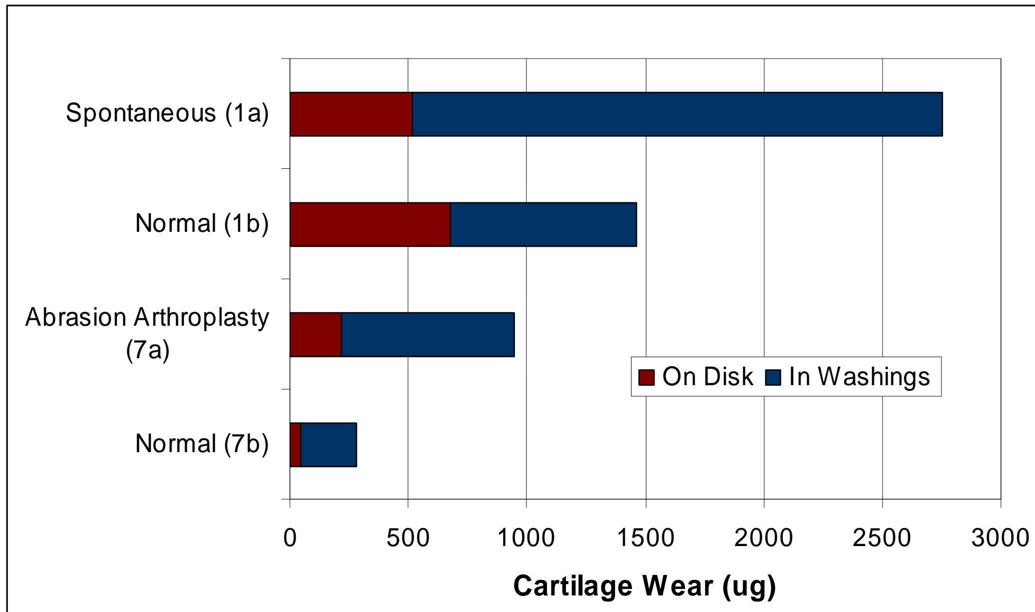

Figure 13. Wear comparisons between normal and repaired for Spontaneous Repair and Abrasion Arthroplasty

Both of these techniques rely solely on the ability of bone marrow and the cells it supplies to regenerate cartilage, and both of these repaired cartilage samples had higher amounts of wear than their normal counterparts. This suggests that techniques of this nature produce a less durable cartilage that wears easily.

Also the carbon fiber induced repair gave very high wear. It saw 215% higher wear, 1764 compared to 560 µg, than its normal counterpart. The mosaicplasty, and example of tissue grafting techniques, had slightly more wear than normal cartilage, 616 compared to 533 µg. These comparisons are shown in Figure 14.

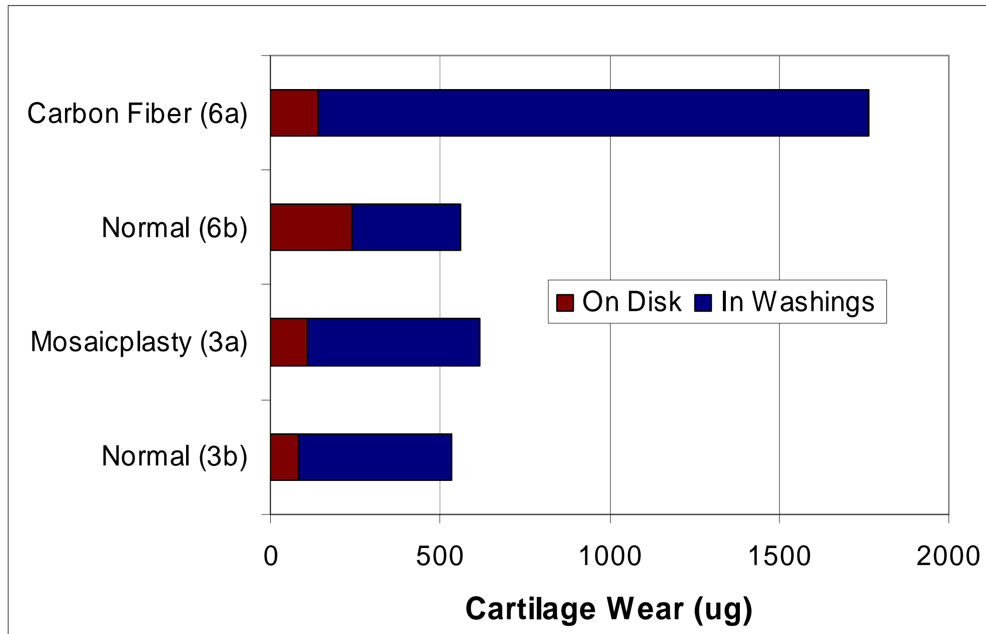

Figure 14. Wear comparison between repaired and normal cartilage for Carbon Fiber and Mosaicplasty

The carbon fiber induced repair tested in this study had poor wear-resistance compared to normal cartilage. The difference in wear is very small between the mosaicplasty repair and normal cartilage. With only one pair of samples using a tissue grafting technique it is difficult to speculate, but for this pair, the mosaicplasty produced cartilage tissue with wear properties very similar to normal cartilage.

It seems that the cartilage that is produced in a lesion using the ACI technique has very good wear resistance, much better than that of the techniques that only stimulate the bone marrow to form cartilage. The ACI repaired cartilage appears to have at least as good wear properties as normal cartilage, if not better. This fact, along with the successes of ACI discovered by Dr. Brittberg, including good adhesion to bone and seamless integration with surrounding cartilage [38], suggests that the ACI technique is indeed a very successful way to treat deep lesions and cartilage. Its applications to other joint injuries including osteoarthritis are very promising.

5.2.2 Degenerate Cartilage

As previously discussed, it is generally thought that osteoarthritic cartilage has a degraded collagen network, and is more prone to wear than normal cartilage [12], leading to the progression of the degeneration of the joint. So it was with much excitement that specimens from patient number 8, degenerate and normal cartilage, were tested. We know of no other study that has measured the wear resistance of degenerate cartilage. Only one pair was available for testing, but it was found that the degenerate cartilage had only slightly more wear than the normal cartilage. The degenerate cartilage had 663 μg of total wear compared to the 536 μg of wear of the normal specimen. This is 23% higher wear, but small compared to some of the other differences seen in the repaired pairs.

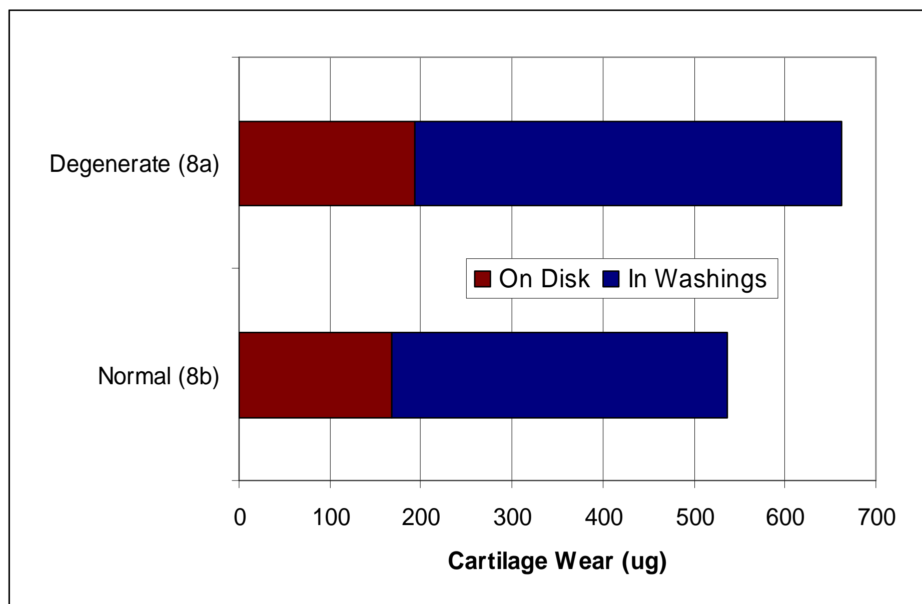

Figure 15. Wear comparison of degenerate and normal cartilage

There is not a large increase in wear in this degenerate cartilage sample. There could be several reasons for this. Perhaps the thought that degenerate cartilage is more susceptible to wear is incorrect. Or perhaps if we were able to test more than one degenerate and normal cartilage pair we would see more wear in the degenerate cartilage.

However, another possible answer is found in the timing of harvesting of the sample. The degenerate specimen is from an area of the patient's joint known to have an injury. Since the joint was already being degraded, it may be that the top layers of the degenerate cartilage had already worn away, and the biopsy only removed the cartilage that was left over. The cartilage would only be left over if it was durable, with a similar wear resistance to that of normal cartilage. The only way to truly test the durability of degenerate cartilage is to have a biopsy from a patient in the very early stages of OA. So early in fact that it would be very impractical, since the patient would have no symptoms, and no reason to have a biopsy taken.

5.2.3 Normal vs. Repaired Wear Averages

Since we have only 15 samples to draw conclusions from, it is difficult to draw conclusions based on averages. However, it may be of interest to examine all "normal" and "repaired" samples to see if there are trends. Table 9 shows the average results of normal and repaired samples in this study, and some interesting points are discovered.

Table 9. Normal vs. repaired averages for wear and hydroxyproline concentration

	Average Wear (µg)			**Average Hydroxyproline**
	Washings	**Disk**	**Total**	**Concentration (µg/mg)**
Repaired	916.6	262.3	1178.9	13.1
Normal	994.8	266.0	1260.6	15.1
ALL	958.3	264.3	1222.5	14.2

From these averages the following observations are made:

- The average Hydroxyproline Concentration of repaired cartilage from all types is 13.1 µg/mg, which is lower than the 15.1 µg/mg average of normal cartilage. This suggests that overall the repaired cartilage has a lower density of collagen.

- Wear (total, washings and disk) all averaged lower for repaired than normal.

The average wear and hydroxyproline concentration are not different statistically. There is not enough information to make conclusions based on the averages in this study, and it is better to examine each repair technique individually to compare wear-resistances.

5.3 Correlation Between Hydroxyproline Concentration and Wear

A trend that can be observed by looking at the results is that of a possible relationship between hydroxyproline concentration and total wear. It appears that there is a general trend that total wear decreases with increasing collagen concentration. Figure 16 is a plot of each tested cartilage sample's total wear versus its hydroxyproline concentration.

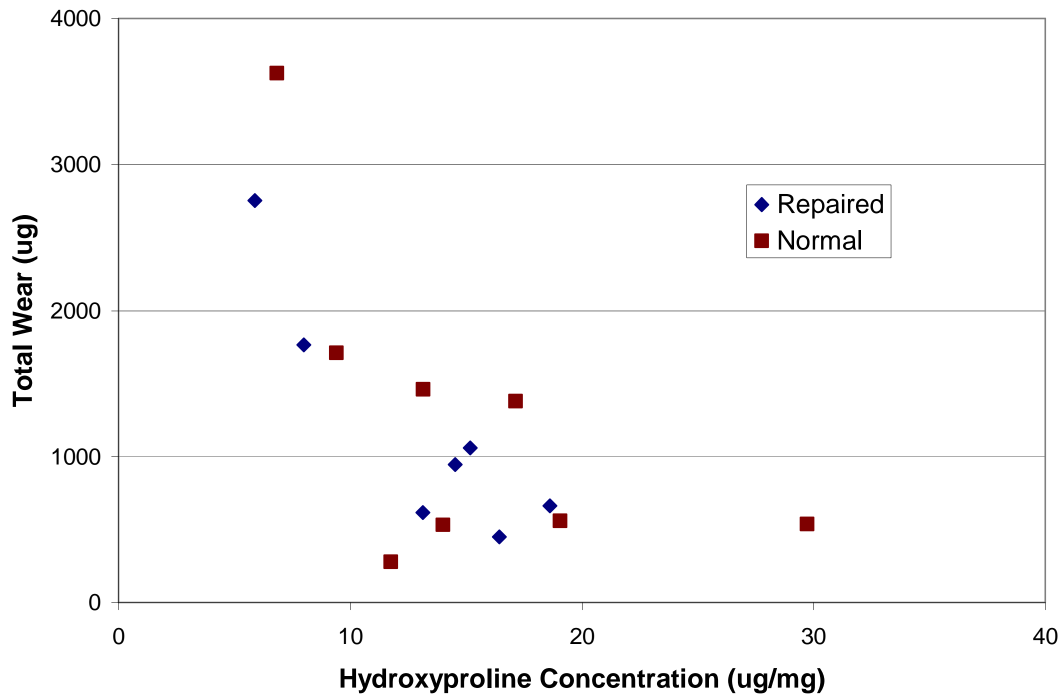

Figure 16. Plot of total wear vs. hydroxyproline concentration

Examining this plot shows that for both repaired and normal cartilage the total wear tends to increase with a decreasing hydroxyproline concentration. This trend may confirm what was expected. The amount of hydroxyproline concentration of a cartilage specimen is directly proportional to the density of the collagen in the cartilage. Cartilage that has a high hydroxyproline concentration would have a dense collagen network, and it should follow that this would make the cartilage more durable and wear-resistant. In converse, a low hydroxyproline concentration would indicate a less dense collagen network, and a higher susceptibility to wear.

It should be noted that an analysis of variance on this data shows no significant relationship between wear and hydroxyproline concentration. However, the general trend is visible in the plot, and seems to support the intuitive concept that the denser a cartilage's collagen network is, the more wear-resistant it is. It is once again cautioned against making hard conclusions, however, due to the small sample size, as well as the compression phenomena discussed in section 5.1. The high values of hydroxyproline concentration are not only explained by a dense collagen network, but also by the "squeezing" out of water and proteoglycans during the wear test. This must be taken into account.

5.4 Gender and Age Effects

A controlled study of only "repaired" versus "normal" cartilage would select cartilage samples from similar patients, but that was impossible in this research. So the patient's age and gender was a factor in this study, and how this might affect wear is of interest. The following table shows the average total wear and hydroxyproline (hypro) concentration of the 8 "normal" samples tested in this study, versus their age and gender. There were 5 females and 3 males; and "young" and "old" is defined as being born before or after 1950.

Table 10. Gender and age effects on "normal" cartilage wear and hydroxyproline concentration

	Average quantity	Young	Old	All
Female	Tot. Wear (μg)	1564.3	419.5	1106.4
	Hypro. Con. (μg/mg)	16.9	15.4	16.3
Male	Tot. Wear	1545.5	1462.0	1517.7
	Hypro. Con.	13.3	13.2	13.2
All	Tot. Wear	1556.8	767.0	1260.6
	Hypro. Cont.	15.4	14.7	15.1

From these data the following observations can be made, regarding "normal" cartilage:

- Males averaged lower hydroxyproline concentration than females: 13.2 to 16.3 μg/mg
- Males averaged higher total wear than females. 1518 to 1106 μg
- Older patients averaged lower hydroxyproline concentration than young ones. 14.7 to 15.4 μg/mg
- Young patients averaged more wear than older. 1557 to 767 μg
- Young females had highest hydroxyproline concentration. Old males lowest.
- Older females had lowest total wear. Young females the highest

The differences in all of the observations above are not found to be statistically significant, however these observations are interesting and do confirm some previously discussed trends. The men have a lower hydroxyproline concentration and higher wear, in line with the other findings of this study. However, the older patients have both lower hydroxyproline concentration and lower wear than younger patients, which is somewhat contradictory. Again with so few samples it is difficult to see trends simply looking at averages. Any future tests with larger sample sizes could take gender and age into account in a more controlled experiment.

5.5 Comment on Non-Random Samples

It should be noted that the samples are a non-random sampling of the population. All biopsies provided by Dr. Brittberg were from patients that had knee problems severe enough that they needed surgery. Hence, all of the "normal" samples are not truly a normal sampling of the population. It is unknown how this affects the results as a whole. The hydroxyproline concentrations, and wear resistance of "normal" cartilage from these clinical patients varied widely from patient to patient, and that may or may not be true for cartilage from a person with "healthy" joint tissues.

The fact that the samples are not truly normal adds emphasis to the argument that the results from one patient should not be compared to another patient. Each pair from one patient allows for a comparison of the repaired cartilage tissue to cartilage tissue in the patient that is as normal and healthy as possible in that joint. However, comparing normal or repaired tissue from one patient to normal or repaired tissue from another patient should not be done with this data, since the specimens are non-random. This fact, along with the observations from sections 5.2.3 and 5.4, show that the best way to examine this data is by comparing the repair techniques individually to their "normal" counterparts.

5.6 Discussion of Friction Results

In all cases the friction coefficients reflect that the system was in the boundary lubrication regime, with contact between the surfaces. Also in every case the friction was relatively low at the onset of the wear test, and gradually increased as the test continued. The increase may be due to the transfer of the material to the disk. The system began as cartilage-on-stainless steel, but as the test progressed became cartilage-on-"cartilage like" film. Further studies would have to be performed to understand this better. The average friction ranged from 0.13 to 0.22, but in general the variation is small between the

samples. Figure 17 is a plot that shows the average friction coefficients and the corresponding wear for both normal and repaired samples.

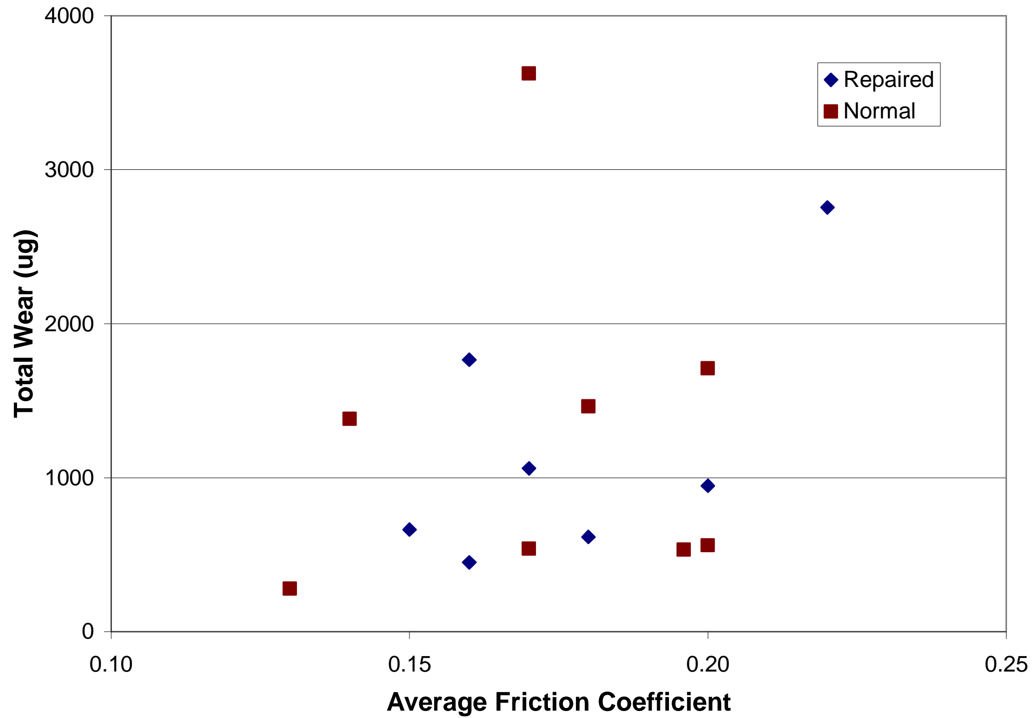

Figure 17. Plot of wear versus average friction for repaired and normal cartilage

From the plot in Figure 17, different observations can be made. First it can be seen that there is no correlation between the friction and the total wear. This is consistent with previous studies done by the Biotribology Laboratory, finding that wear and friction are unrelated. Also it is seen that whether the sample is normal or repaired has little influence on the friction coefficient. The data points are spread out for both types of cartilage.

6 SUMMARY AND CONCLUSIONS

In a collaborative venture of the Biotribology Laboratory at Virginia Tech, the Cartilage Research Unit in Goteborg, and the Virginia Maryland College of Veterinary Medicine the wear-resistance of repaired, normal and degenerate human articular cartilage was studied. Seventeen specimens of human cartilage from nine Swedish patients were obtained. These included two sets of normal and repaired cartilage using Dr. Brittberg's Autologous Chondrocyte Implantation (ACI) method, two sets of normal cartilage and biopsies from cartilage repair induced by abrasion arthroplasty, and several others.

Using a specially designed biotribology device, adapted to accommodate the small diameter (2 mm) biopsies, tests were successfully conducted on 15 of these specimens, to measure cartilage wear and friction. The tests consisted of a sliding the cartilage in a linear motion against a stainless steel disk for a 3-hour period at an average pressure of 1.94 MPa. Cartilage wear was determined from hydroxyproline analysis of washings plus material transferred to the stainless steel counterface.

This study, unlike previous studies done by the Biotribology Laboratory, not only measured the amount of hydroxyproline in the washings of the wear test, but also measured the hydroxyproline concentration of each cartilage specimen to accurately determine the amount of cartilage wear. Also, this study included the material transferred to the polished stainless steel disk during the wear test as part of the "total" wear. The amount of cartilage deposited on the disk had not been measured in previous studies, and turned out to have a significant contribution to total wear.

In the case the biopsies from Dr. Brittberg's ACI repair technique, the repaired cartilage specimens gave considerably less wear, one 88% less, one 23% less, than the normal cartilage specimen. The high wear seen with normal cartilage was surprising.

However these results suggest that the ACI repaired cartilage has very good wear-resistance, perhaps even better than that of normal, healthy cartilage.

Of the specimens that had been treated by Abrasion Arthroplasty, only one pair was able to be tested. In this case, the repair method gave higher wear by a factor of over 3 than the normal cartilage specimen. The cartilage repaired by bone marrow stimulation, or "spontaneous repair" and the cartilage repaired with carbon fiber stimulation both had significantly higher wear than their normal counterparts; 88% more and 215% more respectively. Even though only one pair was tested for each repair, these numbers suggest that these three techniques do not produce cartilage with good wear-resistance.

Also tested was a pair treated by mosaicplasty. In this case the repaired cartilage had slightly more, but a comparable amount of wear to normal cartilage. This may suggest that techniques that employ tissue grafting have better success in terms of wear-resistance than techniques that only stimulate the subchondral bone and bone marrow to produce cartilage.

Degenerate cartilage was also tested and also gave slightly more, but similar wear compared to normal cartilage. This may contradict the previous thought that degenerate, osteoarthritic cartilage is more susceptible to wear. However it is also a possibility that, since the degenerate cartilage came from a patient already exhibiting osteoarthritic symptoms, the less durable cartilage had already worn away in the patient before the biopsy. So, the wear tested cartilage would be only the very durable cartilage left over after the "weaker" cartilage was worn off.

The coefficient of friction varied with the system and time, with levels indicating the boundary lubrication regime. This reflects the chosen conditions of an extreme load, and not just the average load of the walking cycle, which would put the cartilage in the hydrodynamic regime where there is no contact and no wear. In line with past experience, there is no correlation between friction and wear.

It is cautioned against making sweeping general conclusions based on the limited number of tests carried out in these experiments. Enough human samples of repaired cartilage were difficult to obtain in this study, making statistically significant conclusions impossible. It is the desire of the Biotribology Laboratory and the current collaboration, however, to continue studies on a larger scale. Funding is being sought to obtain larger quantities of repaired cartilage samples, and to develop the means to test them. It is recommended that any further tests using a larger sample size should have a controllable source for human cartilage, and also construct more biotribology devices for more efficient testing.

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GLOSSARY

The first occurrence of the following terms in the text is italicized.

Articular Cartilage: The specialized tissue that covers and protects the articulating ends of the bones in synovial joints

Arthritis: A disease of the joint causing inflammation, usually accompanied by pain, swelling, and stiffness, and resulting from infection, trauma, degenerative changes, or other causes.

Autologous: Coming from the same person; allogenous, from another person

Autologous Chondrocyte: A patient's own cartilage cells

Biotribology The Science that concerns itself with the application of tribology to biological systems

Calcified Zone: The layer of articular cartilage adjacent to the subchondral bone

Chondrocyte: The specialized cells of cartilage

Collagen: A fibrous protein constituent of cartilage and bone. It is responsible for the structure and strength of cartilage

Deep Zone: The layer of cartilage between the calcified and intermediate zone, where there is strong proliferation of chondrocytes and collagen fibers are arranged radial to the surface

Extracellular Matrix: The material secreted by chondrocytes that makes up 95% of the cartilage composition. It contains collagen fibers and other 'filler' material

Femoral Condyle: At the end of the long bones at the thigh, the bone forms into two half rounded structures called the condyles; the long bone is the femur – a femoral condyle.

Fibrillation: The splitting and fraying of the cartilage surface

Fibrocartilage: Cartilage that contains numerous thick bundles of collagen fibers

Hyaline Cartilage: Name and type of cartilage found in articular joints, different from elastic and fibrous forms.

Hyaluronic Acid: The proteoglycans are connected to a large molecule of hyaluronic acid produced by the synovial cells

Hydrolyzation: Process of digesting the cartilage debris in Hydrochloric Acid

Intermediate Zone: The cartilage layer beneath the superficial layer, where the collagen fibers are interlaced

Lyophilization: Process of isolating the debris material from water and lubricating fluid by freeze-drying

Osteoarthritis: A progressive disease in cartilage and bone leading to destruction of the articular joint cartilage and bone

Periosteum: The membrane overlying the bone

Proteoglycans: Components of cartilage consisting of long chains of glucosaminoglycans connected to a central core protein

Subchondral Bone: The porous bone beneath the articular cartilage covering

Superficial Layer: The topmost layer of articular cartilage that is exposed to the joint space

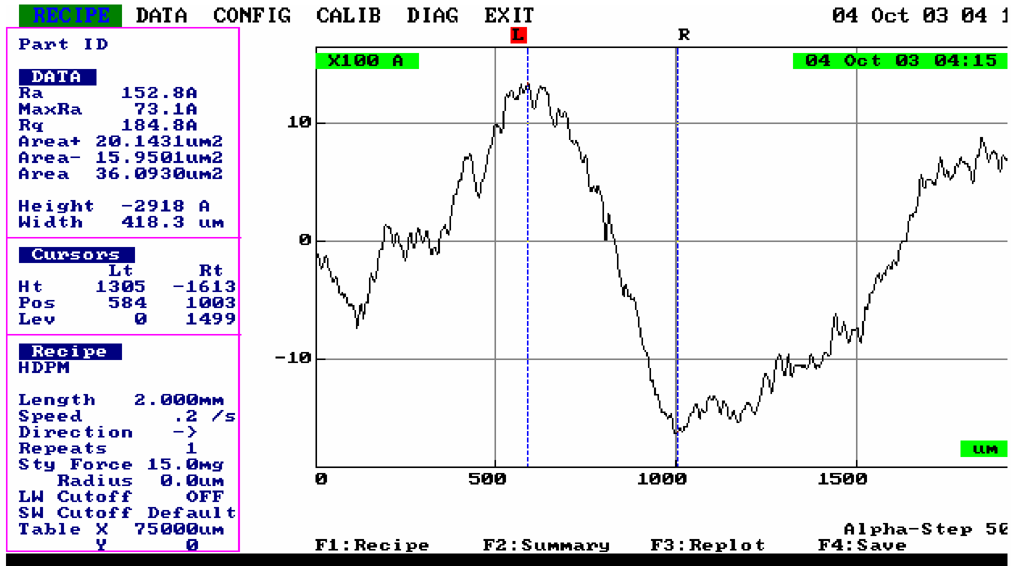
Synovial Fluid: The high viscosity, lubricating fluid that occupies the space between articulating surfaces in a synovial joint.

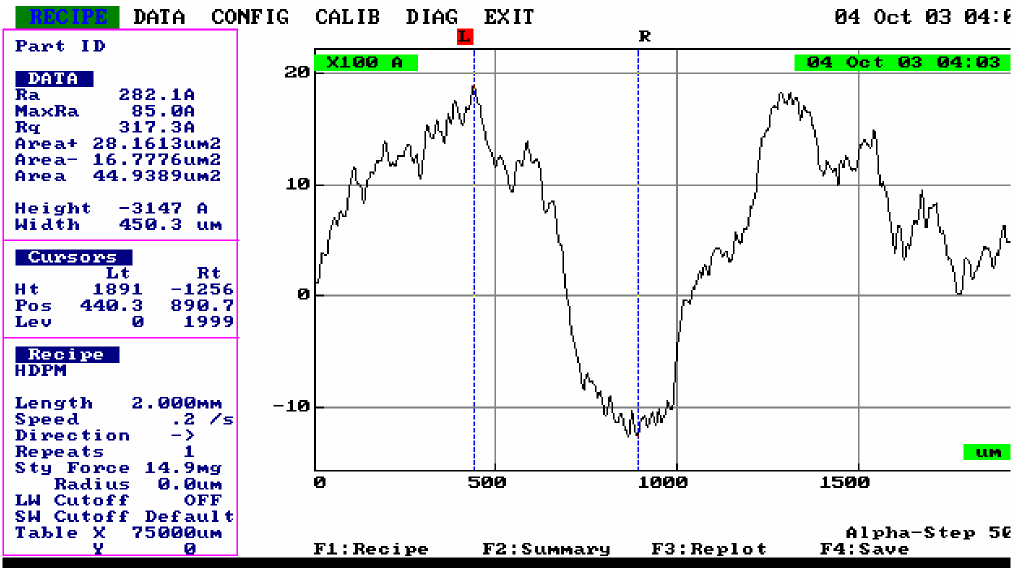
Synovial Joint: Type of joint in the body that permits bone on bone articulation. Examples include the knee, elbow, shoulder, etc.

Tribology: The science of the mechanisms of friction, lubrication, and wear of interacting surfaces that are in relative motion

Wear: The progressive loss of substance from the operating surface of a body as a result of relative motion at the surface.

APPENDIX A: Sample outputs from profilometer runs on stainless steel disks.





APPENDIX B. Detailed wear test procedure

A. Sample Preparation

1. Remove frozen cartilage specimen from covered with Tissue-Tek® using tweezers from vial
2. Place cartilage specimen in Petri dish with a buffered saline soaked gauze pad. Cover
3. Leave cartilage specimen in the Petri dish for 20 minutes, or until Tissue-Tek® is thawed.
4. Rinse Tissue-Tek® off plug with distilled water and set back in Petri dish

B. Wear Test Preparation

5. Obtain clean 1" polished stainless steel disk and place in disk holder
6. Mount holder on X-Y table
7. Obtain 2 mm plug holder and specimen holder
8. Remove cartilage specimen from Petri dish with tweezers
9. Place cartilage specimen in 2 mm plug holder
10. Tighten set screws so that 1mm bone is protruding from the specimen holder
11. Place 2mm plug holder in specimen holder and line up screws
12. Mount specimen holder on device

13. Tighten set screws so that they are aligned perpendicular to the direction of motion of the X-Y table
14. Note: Do not allow cartilage specimen to touch stainless steel disk.
15. Add 0.5ml of saline solution to the stainless steel disk using a dropper
16. Lower cartilage specimen on to stainless steel disk

C. Wear Test

17. Turn the power on for the strain gauges, readers, and motion controller
18. Place weights on device to 6 N load
19. Raise cartilage specimen from stainless steel disk
20. Initiate sliding motion program
21. Lower cartilage specimen on to stainless steel disk
22. Measure friction at 5 mm/sec.
23. Measure sliding friction at 10 m/sec at the following intervals: 5 minutes, 20 minutes, 40 minutes, etc.
24. Continue to make sliding friction measurements until 3 hours have elapsed
25. Stop test by turning off controller

D. Debris Collection

26. Remove the load
27. Raise the specimen and remove the holder from device
28. Place funnel in vial
29. Rinse plug into funnel with 2 ml of water using wash bottle
30. Remove disk holder and unfasten top piece
31. Rinse disk into funnel with 5 ml water

32. Place disk in a jar

33. Return remains of cartilage to vial in the freezer

E. Thin Film on Disk Collection

(Note: this section can be done later, before hydroxyproline analysis)

34. Wearing gloves, place bead of 6N HCl on disk

35. Using Teflon scraper, gently scrub entire surface of disk, removing thin film

36. Using a funnel, rinse scraper into vial with 2 ml 6N HCl

37. Rinse disk into same vial with 4 ml 6N HCl

38. Cap vial. Set aside for Hydroxyproline assay, beginning with procedure step 3.

APPENDIX C: Hydroxyproline Analysis Procedure.

REAGENTS:

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

PROCEDURE:

1. Put liquid samples in glass screw top vials, label and freeze at -70 ^{o}C.
2. Completely lyophilize frozen samples (approximately. 15-18 hours).
3. Hydrolyze dry samples with 1 ml of 6 M HCl for 24 hours at 100 ^{o}C.
4. Take .33 ml aliquot, add 0.67 ml of 3 N NaOH and shake.
5. Make trans-4-hydroxy-L-proline standards with total volume of 1 ml. Assay along with the neutralized samples.

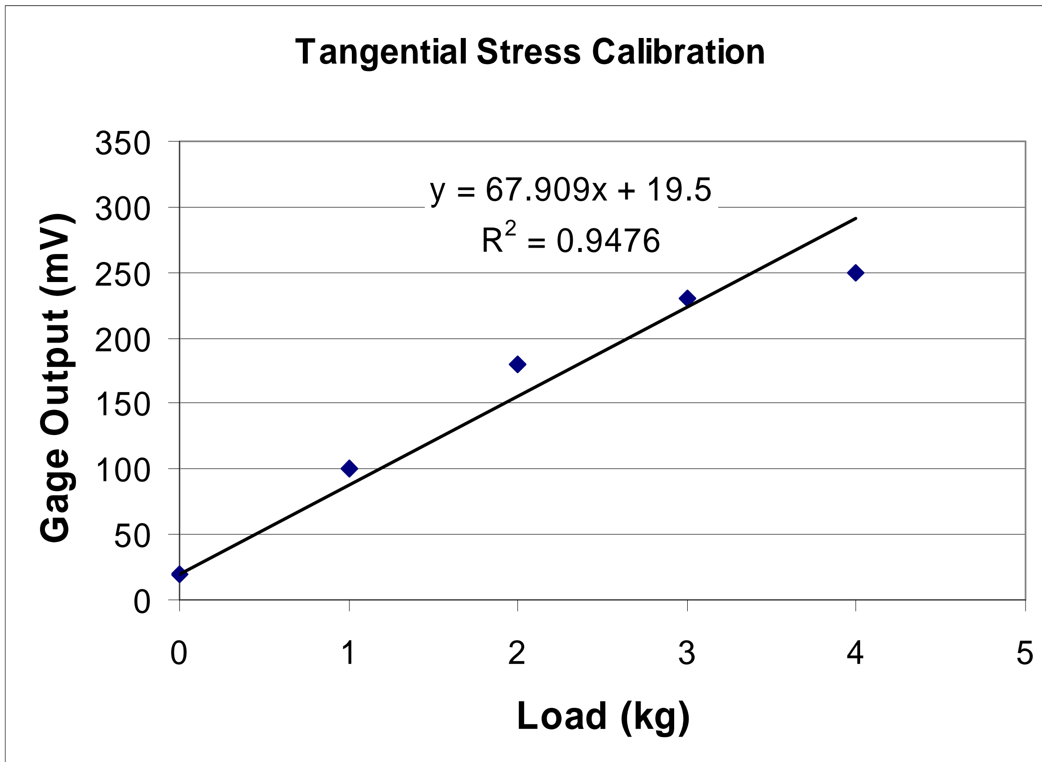
The colorimetric assay is as follows:

ASSAY:

1. Take the 1 ml of neutralized sample.
2. Add 0.25 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 1 ml of 3 N sulfuric acid.
7. Add 0.5 ml of 5% p-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.

APPENDIX D. Strain Gage Calibration Curve



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