The Effects of Freezing on the Mechanical Properties of Articular Cartilage

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Abstract

Studies have investigated and dismissed the effect of freeze-thaw cycles on both skeletal muscle and on trabecular bone, but have failed to properly address the effects of these storage methods on the integrity of articular cartilage. Preventing cartilage injury is important in minimizing the long term debilitating effects of osteoarthritis. Accurate subfracture injury prediction must take into account the possible effects that freeze thaw cycles may have on the mechanical properties of cartilage tissue. This paper addresses this concern with matched pair testing of various low temperature storage techniques against fresh control groups. Controlled mechanical indention tests were performed on bovine articular cartilage-on-bone specimens to compare stiffness, peak stress, and loading energy of the cartilage. Findings showed that a slow freeze thaw or flash freeze cycle caused cartilage stiffness to decrease by 37% and 31% respectively. Compressive stress at this strain was also lowered by 31% with a single freezing process. These results may be indicative of a weakened extracellular matrix structure caused by the freeze-thaw process. It is still unclear whether these changes in mechanical properties will result in a change in injury susceptibility for articular cartilage.

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Chapter 1: Literature Review

Introduction

Over 3 million occupants are injured in automobile collisions each year (USDOT, 2002). Concern for automobile safety has caused biomechanics to become an increasingly popular field for research. Many biomechanical experiments involve testing human or animal connective tissue. Tissue refers to a group of cells, fibers, and ground substance found in humans or animals that make up bone, cartilage, and ligaments in particular. The research conducted for this project focuses primarily on the storage of cartilage tissue and the implication it has on experimental results. Within this chapter, the reader will find justification for this research citing examples and giving some background information on experimental practice. Information about the anatomy and physiology of articular cartilage and bone will also be included. And finally, the reader will be presented with a thorough literature review summarizing and relating all relevant research to the topic at hand.

Purpose

The effects that freeze thaw cycles may have on the biomechanical properties are not well understood. The current study aims to elucidate the effects that various storage methods may have on articular cartilage tissue. Two storage protocols will be investigated in order to ascertain this information. The biomechanical properties of the cartilage will be compared using matched pair testing. In addition to biomechanical properties such as stiffness and hysteresis, the point of failure for the cartilage will also be determined and considered in the experimental analysis.

Justification

Post mortem tissue testing has many applications in automotive safety research, sports biomechanics, and military restraint design. Much of the current biomechanical research being conducted involves testing of both human and animal tissue. While some studies are conducted in vivo, on living subjects, most studies are performed using post mortem tissue.

In many of these research areas, especially those involving human tissue, circumstances may require frozen storage of the tissue for a period before testing (Atkinson, 2001; Funk, 2002; Duma, 1998, 2002). This is largely because cadavers are relatively difficult to obtain. The tissue must also be tested for HIV and Hepatitis requiring that the cadaver be held until these tests are processed. Because of these difficulties, laboratories often choose to procure the tissue when it becomes available as opposed to when it is required for testing. The tissue is then frozen and stored at subzero temperatures, usually around –20C. These specimens are left frozen until they are needed for testing, this timeframe can range from a few days to months at a time. Many labs also freeze animal tissue prior to testing (Oyelede, 1992; Jurvelin, 1987; Silyn-Roberts, 1990; Bursac, 2000).

Studies have investigated and dismissed the effect of freeze-thaw cycles on skeletal muscle (Van Ee, 1998), trabecular bone (Linde, 1993), and ligament tissue (Woo, 1986), but have failed to properly address the effects of these storage methods on the

integrity of articular cartilage. As a result, conflicting opinions exist on the proper freezing protocol for testing cartilage (Keifer, 1998; Hori, 1976). While some researchers assume the freeze thaw effects on mechanical properties of cartilage are negligible (Kerin, 1998; Athanasiou, 1991) and proceed with conventional freezing methods, others make a concerted effort to test the tissue while it is still fresh. Still others have adopted methods designed to alleviate the effects of freezing. At the University of Virginia: Center for Applied Biomechanics, researchers subject cadavers to a flash freezing process involving extreme low temperatures of -80^{o}C. Once the cadaver has been frozen, it is stored in a conventional freezer at -20^{o}C until needed. To our knowledge, no tests have been conducted to assess the effectiveness of such a process.

Several experiments by researchers at Michigan state have focused on cartilage injuries and providing additional information for modification of injury criteria for the lower limb (Atkinson PJ, 1995-2001; Newberry, 1996; Haut, 1995). Injury criteria are a way of quantifying the risk of injury for a given degree of impact trauma. Injury criteria have implications in the design of safety devices ranging from the knee bolsters on dashboards of automobiles to sports equipment. Some of the specimens in the studies were tested fresh and others were frozen prior to testing.

The matter of whether or not freezing plays a role in altering the likelihood of cartilage injury is important and should be considered in the experimental use of previously frozen tissue. The potential alteration of material properties should also be considered in the development of injury criterion for joints such as the knee and elbow. The current study addresses these concerns by using a series of matched pair compression tests and several storage protocols to assess any possible effects of freezing on failure characteristics of articular cartilage.

Anatomy

To begin experimenting with bone or cartilage, one must become familiar with the makeup and structure of these tissues. Like most biological materials, the adage that "form follows function" holds very true for articular cartilage. Articular cartilage is found on the surface of bones in articulating joints. An articulating joint consists of two or more bones that make contact and slide against each other. Articular cartilage exists to help absorb impact energy in the joint and provide a smooth, low friction surface for bones to slide on. Synovial fluid found in diarthrodial joints also aids in providing a low friction surface by acting as a lubricant. In patients with joint disorders such as osteoarthritis in which the articular surface is damaged, joint movement can be very difficult and painful. Clearly, healthy articular cartilage plays an important role in allowing normal, painless movement in people and animals.

Examples of diarthrodial joints in humans include the knee, hip, shoulder and elbow. The knee is actually comprised of two diarthrodial joints: the patello-femoral joint which joins the patella and femur and the tibial femoral joint. Mechanically, this arrangement allows for the larger moments necessary for knee extension.

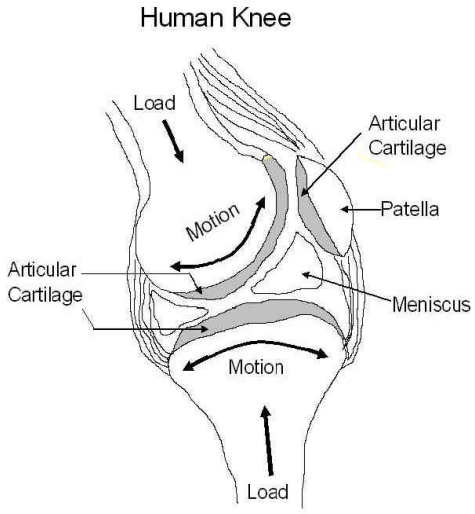

Figure 1.1: Illustration of the human knee

It is widely known that cartilage is a multiphase structure that consists of an extracellular matrix (ECM) and an interstitial fluid. An appropriate analogy is the sponge. In a sponge, fibers are tightly woven together and water is allowed to flow around the fibers when a sponge is compressed. The water flow around the fibers provides a resistance that increases as the sponge is compressed more rapidly. The resistance is known as a damping force. In articular cartilage, the dampening force is what allows the cartilage to absorb forces and dampen shocks that would otherwise be transmitted through the joints and into the bone.

Compared to a common household sponge, cartilage is much thinner and has a denser mesh of fibers. This results in a structure that is also relatively stiff. A layer of cartilage found on the end of a bone may only be about 1-2 mm thick but can support a load of 8 MPa while only displacing as much as 0.3 mm. These numbers were taken from pilot data in the current study with a bovine large metacarpal bone. The thickness of cartilage can vary depending on the joint and its location within the joint. Variations in thickness can influence the load magnitude and type of loading that the cartilage can withstand without sustaining damage.

The solid phase of cartilage is composed primarily of collagen type II fibers and a type of proteoglycan called aggregan. The network of collagen fibers embedded in a high concentration of proteoglycan gel form the extracellular matrix. Other minor constituents include proteins, other types of collagen, biglycan, COMP, decorin, fibromodulin, perlican, thrombosondin, and hyaluronan. It is believed that although these components only make up a small percentage of the weight of cartilage, they may serve important biological functions which are not yet fully understood. This solid phase composes 15-40% of the wet weight of articular cartilage.

The fluid phase, which consists makes up the remaining 60-85% of the wet weight is water with dissolved electrolytes Na^+, Ca^+, and Cl^-. A recent triphasic theory regards the dissolved electrolytes as a third phase. This has implications in cartilage swelling and water content within the collagen fibers.

Electron microscopy has revealed that cartilage is made up of three separate structural zones: the superficial tangential zone, the middle zone and the deeper zone. Researchers will commonly refer to the three different zones when describing localized damage in the cartilage. The primary difference between the three zones is the manner in which the collagen fibers are oriented. In the superficial zone, the fibers are oriented tangent to the articular surface. In the middle zone, the fibers assume a more randomized pattern. Fibers in the deep zone are oriented perpendicular to the underlying bone. Another difference in the zones is the level of collagen and proteogylcan content present. Collagen content is highest in the superificial zone at 85% (dry weight) and decreases to 68% in the middle zone (Mow, 1997). One the other hand, proteoglycan content is higher in the middle zone than in the surface zone.

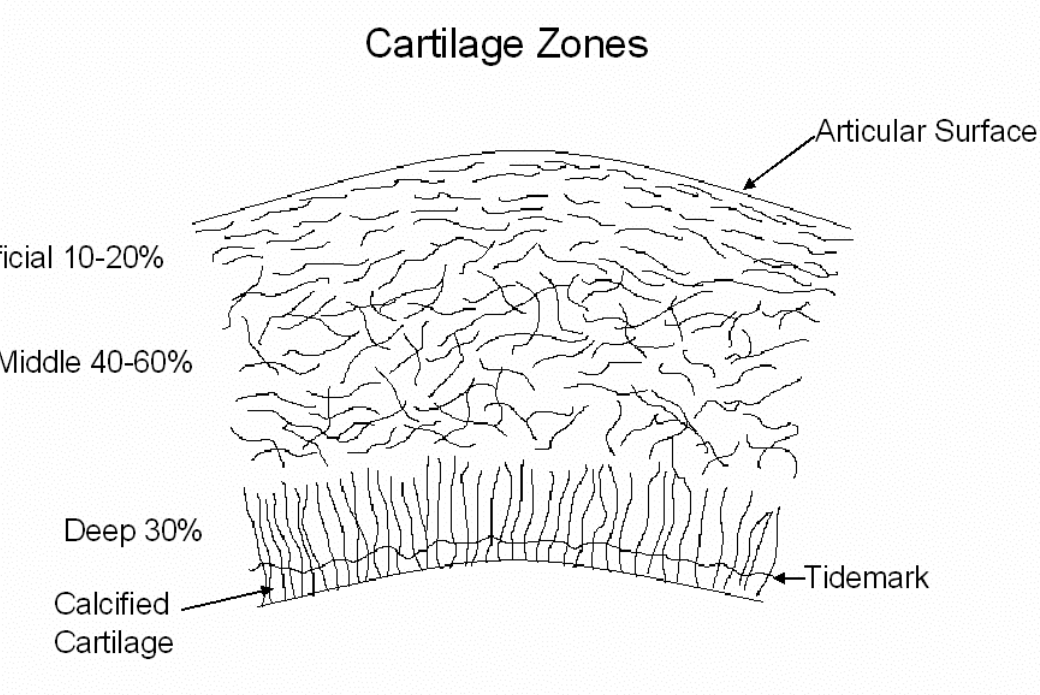

Figure 1.2: An illustration showing the 3 distinct layers of articular cartilage.

Viscoelasticity and Rate Dependencies of Articular Cartilage

As mentioned earlier, the resistive force of cartilage increases with the rate of deformation. Therefore, tissue is considered to be rate dependent (viscoelastic) material. This means that energy is both stored and absorbed by the tissue when it is compressed. The force displacement curve will show different patterns for loading and unloading, as well as different patterns for varying rates of deformation.

Cartilage has been modeled in different ways as the knowledge base has continued to increase. Early studies used a linear elastic theory to model cartilage (Hayes, 1972; Hori, 1976). The linear elastic model was unable to account for the creep and stress relaxation of the material because these artifacts are intrinsic to viscoelastic materials.

Later on, viscoelastic models consisting of various spring damper configurations were used to successfully describe creep and stress relaxation. However, these models failed to account for the effects of the interstitial fluid flow.

The latest theory is the biphasic model which does account for the drag effects of the interstitial fluid flow. It was developed by Mow and associates and continues to be a standard model used by other workers in the field of orthopedic biomechanics (Athanasiou 1991, 1995, Suh 1995, Wang 2000). The biphasic model describes cartilage as consisting of an elastic solid matrix with an interstitial fluid that flows through the mesh in compression causing drag and a resistance to compression. The biphasic theory specifies three intrinsic properties of AC: permeability, aggregate modulus and Poisson's ratio.

Previous Studies

The compressive strength of Articular Cartilage

Published data by Kerin (1998) presents a technique for measuring the compressive strength of articular cartilage. The cartilage used in this experiment comes from previously frozen (-20^{o} C) femoral condyles, tibial plateaus, and patella grooves of skeletally mature cows. The specimens were extracted using a band saw and then mounted using dental plaster. The resulting specimen was approximately 15 mm square and 5 mm deep.

Testing was conducted using a hydraulic uniaxial materials testing machine to compress the cartilage while force data was recorded. The indenters used were plane ended as opposed to hemispherical for the purpose of creating an even stress distribution over the indented area. Several difference sizes of indenters were investigated both with and without a beveled edge.

A linear displacement ramp with a period of 1 second was used as a loading cycle for all specimens. The maximum displacement was gradually increased for each cycle until the cartilage ruptured according to the following criteria used by the author to determine failure:

- An obvious decrease in the force-displacement gradient during loading.
- A large increase in hysteresis in the failure cycle compared to previous cycles. This is attributable to the failure mechanism.
- A residual displacement in the loading part of post-failure cycles.

The author reasons that once these criteria have been satisfied, it means that the cartilage integrity has been compromised due to its reduced resistance to high compressive forces. He also notes that this definition of damage is similar to that used by researchers testing bone and intervertebral joints. The damage was also confirmed using an ink staining technique.

The exact point of failure was measured subjectively be picking out the point where the force-displacement gradient began to decrease. To validate the subjective approach, a 6th order polynomial curve was fit to the force-displacement curve and then differentiated twice to accentuate the changes in gradient. The new point of failure was

also picked by a "blind" observer. The mean difference in points of failure was 5% which is not significant in this case. Therefore, the subjective test was determined to be an acceptable method for determining point of failure.

Kerin also investigate the possibility that specimen extraction from the joint disrupts the collagen network and alters the mechanical properties being measured. This possibility was investigated by reducing the effective specimen size and comparing stiffness measurements for each new effective specimen size. It was not mentioned whether any consideration was given to the possibility of the cartilage being preconditioned in each preceding testing interval. The stiffness was unaltered by changing the effective specimen size.

The author provides a repeatable technique for quantifying the failure stress and failure strain of articular cartilage. This technique serves as a model for the experiments later conducted to determine the effects of sustained loading on the compressive strength of cartilage. It also paves the way for the current research being pursued to determine the effects of freezing on cartilage.

Sustained loading increases the compressive strength of articular cartilage:

The research presented in this paper follows a procedure similar to the previously mentioned study. The study was conducted by Adams (1998) at University of Bristol, UK. Here, the authors subject a number of cartilage specimens to creep loading and compare the results to a control group of specimens. Whereas the previous study presented a method recognizing cartilage damage and quantifying the point of damage, this work applies the method in a comparative study to evaluate the effect of creep loading cartilage prior to cyclic loading to the point of rupture.

The creep loaded specimens were compressed under a stress of 2 Mpa for a period of 30 minutes. This group of specimens exhibited a denser mosaic of fissures in the visual examination following the failure test and ink staining procedures. Damage could also be seen in the control group although it was not as extensive. Histology also revealed damage with fissures that extended to approximately 15-25% of the cartilage depth. Fraying in the superficial zone and splitting along the bone-cartilage interface were also observed and recognized as signs of damage. The damage observed through histology was the same for both groups.

The results were quantified in terms of failure strength and failure displacement. The failure strain was calculated by dividing the failure displacement by the initial cartilage thickness as defined by the average thickness measured at each of the four corners of the specimen. Failure energy was also calculated as the area under the force-displacement curve up to the point of rupture.

The failure strain was found to be 0.30 +/-.07 for both groups. So failure strain was unaffected by the creep loading process. The failure strength was found to increase from approx 35.7 Mpa to 43.3 Mpa in creep loaded specimens. This represents an increase of 21% (P value of .01). Stiffness was also found to increase by 100% in creep loaded specimens.

Justifications similar to the previously discussed article are presented in this study. The author also addressed the issue of bone compliance, the amount of deformation that occurs in the specimen that is due to the subchondral bone. By removing the cartilage from the specimen and applying similar loads to the bone and

testing apparatus alone, they were able to determine that these deformations account for less than 7% of the total deformation being measured during the tests. The possibility of bone failure in the test is also dismissed as a rare case with evidence of occurrence in just two out of 40 specimens which had both been creep loaded.

Effect of Cryopreservation on the Biomechanical Behavior of Bovine Articular Cartilage:

In a study conducted by Keifer (1989) at University of Alberta, Canada, the short term effect of cryopreservation on specific mechanical properties of articular cartilage was investigated. The study uses bovine articular cartilage for testing. Cryopreservation is a technique used to preserve tissue primarily for the purpose of transplantation. The process of transplanting cartilage from another human referred to as allografting has become an increasingly common procedure and an attractive alternative to artificial joint replacement. The purpose of this study was to investigate whether or not mechanical properties are compromised after undergoing the cryopreservation process. The cryopreservation process generally involves freezing the specimens to ultra low temperatures of -79^{o}C. Usually dimethylsulfoxide (DMSO) is used as a cryoprotectant to help prevent damage from occurring in the freezing process.

Specimens are divided into three groups prior to testing: thickness controls, DMSO controls, and cyropreserved samples. In short, the thickness controls group is tested, measured, tested, frozen and thawed and then tested again. The DMSO controls group is tested, measured, immersed in DMSO, and tested. The cryopreserved group is tested, immersed in DMSO, frozen and thawed and then tested again.

Kiefer uses a nondestructive indentation test to assess the mechanical properties for each of the specimens both before and after freezing. Instead of undergoing compression to the point of failure as in Kerin's test, specimens are loaded to a defined amount of 16 N while displacement and force data are recorded. The resulting displacement is small relative to the point of failure. The indentation process was repeated until two consecutive load curves were identical. They defined the resulting condition as a "limit cycle." This justifies comparing mechanical properties both before and after freezing on the same specimen because the specimen is already preconditioned.

Kiefer was unable to measure any differences in stiffness, load energy or hysteresis between any of the three groups. Important differences to note between this study and research presented now are storage conditions and testing method. Instead of ultra low temperatures, we are primarily investigating storage at -20^{o}C, a common storage temperature for most laboratories. Also, we are examining failure data as opposed to just investigating mechanical properties of cartilage.

Tissue storage studies

Other studies have also examined the effects of freezing on other types of biological tissue such as skeletal muscle (Van Ee, 1998), trabecular bone (Linde, 1993), and ligaments (Woo, 1986).

Van Ee et al at Duke University used 12 rabbits to test the effects of freezing and rigor mortis on muscle stiffness. The tibialis anterior muscle was tested using a servo

hydraulic MTS machine to produce a 1 Hz haversine force signal with a maximum force of 4.6 N. Force and displacement data was collected using a strain gage based load cell and a linear voltage displacement transducer (LVDT). The data was filtered using a 100^{th} order finite impulse response filter with a cutoff frequency of 25Hz.

The specimens were divided into 4 testing groups: a continuous test group, a single test group, a pre-rigor freeze and a post rigor freeze. Preconditioning, referred to as mechanical stabilization, was performed for all test groups. They concluded that stiffness was not affected by a freeze and thaw cycle.

Linde (1993) examined the effect of various storage methods including freezing on the mechanical properties of trabecular bone. Using human bone from two male amputees, cylindrical core bone specimens (n = 74) were removed from the legs and tested nondestructively within 2.5 hours of removal. They were also tested 24 hours later to evaluate post mortem changes. After the second test, the specimens were divided into several storage protocol groups and then stored accordingly. Specimens were either frozen for 1 day (n=19), 10 days (n=18), or 100 days (n=19) at a temperature of $-20^{o}C$.

Specimens were tested in compression with an Instron test machine between a lower load of 5 N and an upper strain level of 0.45%. Specimens were also preconditioned to achieve steady state load-deformation curves. The quantities calculated were loss tangent, a ratio of hysteresis energy to total loading area.

The tests revealed that there was a 10% decrease in stiffness and unloading energy and a 10% increase in loss tangent between 2 hours and 24 hours post mortem. Storage in ethanol resulted in a statistically significant increase in hysteresis energy and an increase in loss tangent. Results showed a significant increase in loss tangent for specimens frozen for 100 days. There was also a trend towards increasing modulus at 0.45% strain with increasing freeze time. However, the trends were not statistically significant. These changes in the viscoelastic properties of trabecular bone were concluded to be only minor changes by the investigators.

Woo et al examined the effect that storage at $-20^{o}C$ had on the mechanical properties of the medial collateral ligament (MCL) of rabbits. They found no differences in the cyclic stress relexation properties between fresh and frozen samples. Ultimate tensile strength and ultimate strain were also unaltered by the storage process.

Atkinson Studies

Recent studies conducted at Michigan State by Roger Haut and Patrick Atkinson appears to be reshaping the way that lower extremity injury criteria are defined. A lower extremity injury criterion was adopted by the government in 1976. It was based upon a seated cadaver blunt impact test and designed to prevent bone fracture is a result of axial loading. The criterion requires all new automobiles to limit axial loads in the femur to 10 kN. Atkinson and Haut have conducted a variety of experiments to evaluate this injury criterion and suggest that changes be made to it.

A particularly important contribution made to the advancement of lower extremity injury criterion was a study conducted to investigate subfracture injury in the patellofemoral joint. Subfracture injury refers to injury in the joint aside from a gross fracture. Many researchers feel that these types of injury including cartilage fibrillation and occult microcracks in the bone-cartilage interface can be just as debilitating as a gross fracture, sometimes leading to degenerative joint diseases such as osteoarthritis.

Atkinson (1995) was able to show that 45% of the impact energy required to sustain a patella fracture will likely produce occult fractures at the cartilage-subchondral bone interface. They concluded that injury criteria of 10 kN in the lower extremity may not be sufficiently conservative to prevent joint damage. Damage to the patellofemoral joint can lead to posttraumatic osteoarthritis.

Another study by Atkinson (2001) investigates the effectiveness of a deformable impactor at mitigating joint injury due to impacts. The knee flexion angle is varied to examine the interface effectiveness at different knee positions. The deformable material used is designed to more evenly distribute the impact load across the anterior surface of the patella. Use of the deformable impact surface was effective in reducing bone fractures at 5 kN. However, the incidence of subfracture injuries was not reduced with usage of the deformable interface. These results have implications in knee bolster design as well as lower extremity injury criterion. The joints used were frozen at -20^{o} C until needed.

Atkinson et al also conducted a separate study to evaluate the effects of age on fracture tolerance and occult injury. This research has implications with lower extremity injury criterion as well because the original tests were derived from the impact tests of aged specimens.

The procedure used in these tests is dubbed the "Haut protocol" and is the same procedure used in similar Haut and Atkinson studies. Tissue was either stored in a culture medium or frozen prior to preparation and testing. A knee is isolated from the cadaver body after removing the superficial tissue. The proximal end of the femur is potted in epoxy and mounted. A 4.5 kg impact mass is accelerated to a predetermined speed and strikes the joint in a direction that is axial to the femur. The impact velocity is sequentially increased until a visible bone fracture is realized.

For the comparative age study, the specimens were divided into two groups: the aged group with an average age of 71 and the young group with an average age of 47. 10 pairs of joints for each group were tested. Contralateral limbs were impacted with a percentage of the fracture energy to produce subfracture injury as seen in earlier experiments.

They hypothesized that the incidence of occult microfractures between the cartilage and both interface would be lower for the aged specimens. The reasoning behind this is that osteoarthritis (OA) is commonplace in many older people. OA can cause calcification at the cartilage-bone interface resulting in the highest shear stress deep the trabecular bone. The results showed that older specimens (pathological) showed fewer incidences of microcracking. Another finding was that the older specimens actually required higher loads to produce a gross fracture. These findings led to the conclusion that lower extremity injury criterion based on older pathological specimens does not accurately reflect the impact tolerance of the general population.

Mechanical properties of cartilage in different species

Studies by Athanasiou (1991, 1995) revealed that biomechanical properties of articular cartilage can vary between different species. Using a creep indention test, the mechanical properties from 5 different species (bovine, canine, human, monkey, and rabbit) were measured. The creep test was used in conjunction with the biphasic creep theory to calculate aggregate modulus, a measure of the overall material stiffness,

permeability, a measure of how easily water can flow through the material, and poison's ratio, a measure of the compressibility of the material. These properties were compared for several different sites on the knee joint. Another study examined biomechanical properties of the hip joint.

Athanasious's findings indicated that significant differences were present among the various species and also varied according to location on the location. For example, aggregate modulus tended to be lower in the patellar groove then compared to the femoral condyles. This is because the pateller groove is a low weight bearing region that experiences intermittent loading. Low weight bearing areas have lower amounts of proteoglycan (PG) and higher collagen content. PG was also found to be a contributing factor in the stiffness of cartilage. Varying opinions exist on the effect that PG has on stiffness of cartilage (Jurvelin, 1987). Athanasiou was also able to show that biochemical makeup differs between medial and lateral condyles so it would not be wise to assume equal properties.

With regards to variations between species, they found that aggregate modulus was the same for each animal but poison's ratio and permeability varied between species. The bovine had the closest permeability to the human followed by the dog. In a comparison of poison's ratio, human cartilage was significantly lower than all other specimens. They concluded that these differences should be recognized and care must be taken in the development of experimental animal models for studies on disease and injury. However, limited access to fresh human knee joints will require that animal tissue be used in substitution.

Comparison of Testing Methods

There are many different methods used by researchers to test biological tissue. Cartilage is typically tested in compression using one of the following three methods: creep, controlled indention and impact. Below is a discussion of these three methods and their respective application in biomechanical testing of AC.

Biphasic Creep

Biphasic creep is a nondestructive type of indention test used to measure intrinsic properties of articular cartilage. These properties are calculated by measuring the displacement response of the cartilage under a constant pressure imparted by a porous indenter and then applying the biphasic theory. This test yields aggregate modulus, permeability, and poison's ratio as previously discussed.

What the test does not reveal are failure properties such as failure strain and failure stress. To our knowledge, no studies have correlated any of the intrinsic properties to failure stress or strain. This would be an interesting study to pursue. The test typically involves relatively low stresses applied for a relatively long period of time. The loading patterns used for the test don't appear to simulate real world conditions but instead they are used as a standard to compare properties of different specimens.

An advantage of the biphasic creep test is a high repeatability and a relatively simple procedure. (Athanasiou,1991; Athanasiou,1995; Mow, 1995). The fact that the test is non destructive is an asset because multiple tests can be performed on a single specimen to evaluate the effect of a treatment on that specimen. For example, if the biphasic creep test were to be used to evaluate the effect of freezing on articular cartilage,

a single specimen could be used for a fresh and frozen test instead of matched pair testing required by destructive testing.

If a strong correlation could be established between the intrinsic properties of cartilage as measured by the biphasic creep test and failure, future testing could be simplified a great deal.

Indenter testing

Indenter testing is another common approach used by researchers to test biological tissue. It is typically accomplished by using a force or displacement single to control the indenter. The machines can be electromechanical, mechanical, or servo-hydraulic. Each type of machines has advantages and disadvantages associated with them. Maximum load capacity and loading rates can vary among machines. Direction reversal times can also vary dramatically between different types of machines. For the current study, an MTS 810 machine was chosen for its low direction reversal time, and relatively high accuracy.

Indention tests can be both destructive and nondestructive in nature. Kiefer et al used a nondestructive displacement controlled indention test in his investigation of cryopreservation effects on mechanical properties of cartilage. Hori et al used nondestructive testing as well to compare the properties of articular cartilage with samples of polyurethane. Other workers choose a destructive test (Borrelli, 1997, Kerin, 1998; Adams, 1999)

An advantage of controlled indention tests is a high degree of accuracy and control. Exact stresses and displacements can be measured during the process. Loading energy and hysteresis can be calculated from the resulting stress strain curves. Hysteresis is known as the amount of energy absorbed by the specimen during a loading-unloading cycle. This is a valuable piece of information used by many biomechanists in their analyses. Variable loading rates can also be used to simulate different conditions. For example, a displacement loading cycle with a period of 1 second was used by Kerin and Adams. It was reasoned that this is representative of resulting forces experienced by a person walking. There are upper limits to the loading rates that can be imparted by a materials testing machine.

Impact tests

A third common type of test is impact testing. These tests are performed when an extremely high loading rate is desired by the investigator. Impact tests can be performed in a variety of different fashions. Atkinson, Haut, and Ewers et al used a missile system impart an impact force onto potted cadaver knees in various studies. Time to peak loading was on the order of 5ms for this approach. Loading energy was varied by changing the impact velocity of the missile.

One problem with this approach is that exact stresses and strains are very difficult to measure. Pressure sensitive film has been used to gain an approximation of the peak stresses experienced in various areas (Atkinson). Nevertheless, without continuous measurement of stress and strain, hysteresis cannot be determined. Repeated impacts with increasing impact energy were used to produce subfracture joint injury. It was unclear whether or not the repeated impacts played a role in weakening the structure and altering the subfracture injury force.

Various other methods have been developed for impact testing on extracted cartilage-bone specimens. (Repo and Finlay, 1977; Oloyede, 1992; Silyan-Roberts, 1990; Jeffrey, 1995). Using a special impact device and mounting method, Repo and Finlay were able to record stress and strain measurements during testing. Stress levels 25 N/mm^2 corresponded to strains of around 20-30%. A rather complicated setup is used by the investigators to record all the data. Stress strain graphs were produced and they were able to control strain by limiting the amount of travel in the impacting mechanism. The point of failure is easily recognizable on the stress-strain diagram as a sharp decrease in stress-strain gradient. This method of recognizing failure is similar to the analysis performed by Kerin et al.

Jeffrey (1995) used a similar type of drop tower apparatus to impact test AC. By varying drop height and mass she was able to control impact energy and impact velocity. Chondrocyte viability was used as a dependent variable and was found to decrease linearly with increasing impact energy.

Histology

Many researchers use histology in the analysis of cartilage specimens (Jeffrey, 1995, Kerin, 1998; Malinin, 1984, Marco, 1990, Meachim, 1972, Atkinson, 1995-2001). The current study uses histology to confirm damage and to assess the manner in which it occurred. For instance, the compressive loads may cause fissuring in the surface, fissures extending into the middle zone or occult microfractures at the cartilage bone interface. Histology can be used to investigate the possibility that the mechanism of failure may be altered by the storage method. Figure 1.3 shows an example of a histological slide taken from a cartilage and bone sample.

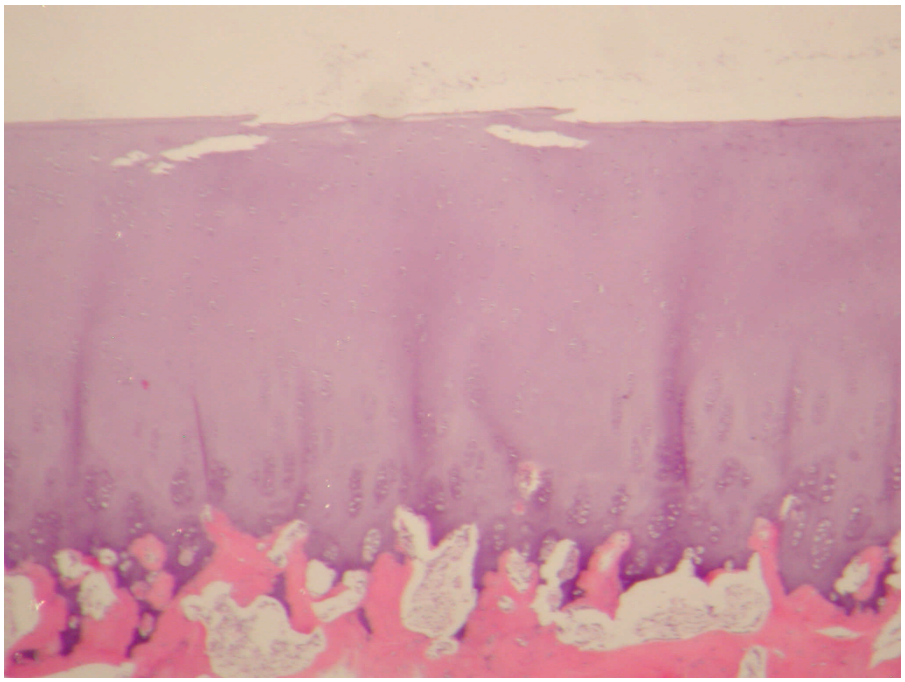

Figure 1.3: Histological section of a damaged articular cartilage-bone specimen. Dyes are used to distinguish between cartilage and bone. Note the fribrillation present on the superficial surface.

At the microscopic level (10^{-7}m-10^{-4}m), chondrocyte cells are visible and various dyes can be used to differentiate between the cartilage and bone tissue. In order to see the collagen and proteoglycan matrix, it is necessary to use an electron microscope with magnification on the order of 65,000x (10^{-8} to 10^{-5}). This is referred to as the ultra magnification level.

Some researchers have used a scanning electron microscope to achieve ultra magnification in order to study the collagen structure. Kaab et al examined collagen fiber deformation under mechanical load. Malinin et al examined the collagen matrix of cryopreserved specimens.

Most studies analyze histological sections at the microscopic level. Atkinson (2001) was able to determine that most damage was occurring at the cartilage bone interface as a result of blunt impact trauma. Kerin et al used histology as a visual confirmation of failure in extracted cartilage-bone specimens.

Cryopreservation Cartilage Studies

Many researchers have conducted nonbiomechanical studies to examine the effects of storage methods on cartilage. Most of these experiments concentrated on the use of cryopreservation techniques used to preserve chondrocyte cells for transplant and other applications (Muldrew, 1994, 2000, 2001; Marco, 1990; Malinin, 1984; Vazquez, 2001; Tomford, 1984; Tavakol, 1993). Cryopreservation is a special type of freezing that is designed to help preserve biological tissue. Cryopreservation methods typically involve much lower storage temperatures than conventional freezing. It is also common to use a chemical called a cryoprotectant to increase survival of the chondrocyte cells.

A study was conducted by Muldrew et al to investigate the effects of cooling rate, storage temperature and cryoprotectant on chondrocyte viability. Pairs of osteochondral dowels taken from femoral chondyles of sheep were subjected to slow cooling (1^{o}C/min) to various subzero temperatures with and without a cryoprotectant of 10% DMSO. At designated temperatures, one specimen was rapidly warmed and the other was rapidly cooled in liquid nitrogen. Injury was assessed for the plasma membrane using fluorescent dye. Two mechanisms of injury were found to exists, solutions effects injury occurred at slow cooling rates, and rapid cooling injury that occurred during temperature plunging.

A later study by the same author investigated the possibility that the difference in cell recovery between the intermediate and surface zones could possibility be due to biological difference in the chondrocyte cell in different layers noting that in previous experiments, middle depth cell recovery had been poor compared to surface recovery. Cuts were introduced into the cartilage to give intermediate cells the same proximity to the outer surface. Muldrew (2000) observed that intermediate cell recovery was dramatically improved and concluded that cell injury depends on proximity to the cartilage surface and not on biological differences between the chondrocyte cells in surface and middle zones.

Conventional freezing methods are harmful to tissue as a result of the formation of ice crystals. Cryopreservation attempts to reduce ice crystal formation through rapid cooling and the use of cryoprotectants. Freezing without the formation of ice crystals is

called vitrification. A study by Tavakol examined the ultrastructural changes to cryopreserved cartilage. Since the ultrastructure plays an important role in determining the mechanical properties exhibited by the cartilage, it is reasonable to hypothesize that unaltered ultrastructure would result in unchanged mechanical properties of cartilage. Their findings showed no difference in matrix organization between fresh and cryopreserved samples. However, cryoprotectant use is not a practical solution for the preservation of large body parts or organs because it must permeate entirely through the tissue to be effective (Best, 2002). With regards to preserving mechanical properties of cartilage, a low temperature freeze protocol similar to cryopreservation but without the cryoprotectant may still be a better alternative to conventional freezing techniques currently used by laboratories.

Objectives

Many studies have attempted to measure properties of articular cartilage including stiffness, permeability and compressibility as well as failure characteristics. Many have also done so without regard to possible consequences of freezing cartilage prior to testing. These experiments can have important implications involving the development of new injury criterion for automobile safety and prevention of injury in sport biomechanics. Accurate knowledge of articular cartilage is also necessary in the research and prevention of various debilitating joint disorders such as osteoarthritis.

While it is fairly well known that bone, muscle, and ligament tissue appear to be unaffected by frozen storage, varying opinions still exist concerning the possible alternation of strength characteristics of articular cartilage. Thus far, opinions have been speculation and to our knowledge this study is the first to directly investigate the possible effects of conventional storage protocol. Using the work and techniques of previous authors, this study will attempt to:

- Use an established testing method to elucidate any possible effects of conventional freezing methods
- Investigate several possible low temperature storage protocols including experimental approaches.
- Provide insight and recommendation of future storage methods for cartilage and whole body storage.

In the following chapter the reader will find a detailed explanation of the testing methods used, the experimental results and a discussion of the results with recommendations for future testing protocol.

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Chapter 2: The Effects of Freezing on the Mechanical Properties of Articular Cartilage

Abstract

Studies have investigated and dismissed the effect of freeze-thaw cycles on both skeletal muscle and on trabecular bone, but have failed to properly address the effects of these storage methods on the integrity of articular cartilage. Preventing cartilage injury is important in minimizing the long term debilitating effects of osteoarthritis. Accurate subfracture injury prediction must take into account the possible effects that freeze thaw cycles may have on the mechanical properties of cartilage tissue. This paper addresses this concern with matched pair testing of various low temperature storage techniques against fresh control groups. Controlled mechanical indention tests were performed on bovine articular cartilage-on-bone specimens to compare stiffness, peak stress, and loading energy of the cartilage. Findings showed that a slow freeze thaw or flash freeze cycle caused cartilage stiffness to decrease by 37% and 31% respectively. Compressive stress at this strain was also lowered by 31% with a single freezing process. These results may be indicative of a weakened extracellular matrix structure caused by the freeze-thaw process. It is still unclear whether these changes in mechanical properties will result in a change in injury susceptibility for articular cartilage.

Introduction

Post mortem tissue testing has many applications in automotive safety research, sports biomechanics, and military restraint design. Much of the current biomechanical research being conducted involves testing of both human and animal tissue. While some studies are conducted in vivo, on living subjects, most studies are performed using post mortem tissue.

In many of these research areas, especially those involving human tissue, circumstances may require frozen storage of the tissue for a period before testing (Atkinson, 2001; Funk, 2002; Duma, 1998, 2002). This is largely because cadavers are relatively difficult to obtain. The tissue must also be tested for HIV and Hepatitis requiring that the cadaver be held until these tests are processed. Because of these difficulties, laboratories often choose to procure the tissue when it becomes available as opposed to when it is required for testing. The tissue is then frozen and stored at subzero temperatures, usually around $-20^{o}C$. These specimens are left frozen until they are needed for testing, this timeframe can range from a few days to months at a time. Many labs also freeze animal tissue prior to testing (Oyelede, 1992; Jurvelin, 1987; Silyn-Roberts, 1990; Bursac, 2000).

Studies have investigated and dismissed the effect of freeze-thaw cycles on skeletal muscle (Van Ee, 1998), trabecular bone (Linde, 1993), and ligament tissue (Woo, 1986), but have failed to properly address the effects of these storage methods on the integrity of articular cartilage. As a result, conflicting opinions exist on the proper freezing protocol for testing cartilage (Keifer, 1998; Hori, 1976). While some researchers assume the freeze thaw effects on mechanical properties of cartilage are negligible (Kerin, 1998; Athanasiou, 1991) and proceed with conventional freezing

methods, others make a concerted effort to test the tissue while it is still fresh. Still others have adopted methods designed to alleviate the effects of freezing. At the University of Virginia: Center for Applied Biomechanics, researchers subject cadavers to a flash freezing process involving extreme low temperatures of -80^{o}C. Once the cadaver has been frozen, it is stored in a conventional freezer at -20^{o}C until needed. To our knowledge, no tests have been conducted to assess the effectiveness of such a process.

Several experiments by researchers at Michigan state have focused on cartilage injuries and providing additional information for modification of injury criteria for the lower limb (Atkinson PJ, 1995-2001; Atkinson TS, 1998; Newberry, 1996; Haut, 1995). Injury criteria are a way of quantifying the risk of injury for a given degree of impact trauma. Injury criteria have implications in the design of safety devices ranging from the knee bolsters on dashboards of automobiles to sports equipment. Some of the specimens in the studies were tested fresh and others were frozen prior to testing.

The matter of whether or not freezing plays a role in altering the likelihood of cartilage injury is important and should be considered in the experimental use of previously frozen tissue to ensure accurate experimental results. The potential alteration of material properties should also be considered in the development of injury criterion for joints for the upper and lower extremities. The current study addresses these concerns by using a series of matched pair compression tests and several storage protocols to assess any possible effects of freezing on failure characteristics of articular cartilage.

Materials and Methodology

For this study, fresh mature bovine knee joints were obtained from Animal Technologies Inc (Tyler, Tx). The tissue was stored at +5 ^{o}C for a period less than 48 hours post mortem at which point tissues were both prepared and tested, or subject to one of the following low temperature protocols: single -20 ^{o}C freeze, or -80 ^{o}C flash freeze.

Bovine knees were used because they have served as popular models for human tissue in past studies (Kerin, 1998; Kiefer 1998) so results can be compared with previous studies. Although Athanasiou et al found that differences exist in biomechanical properties between animal and human cartilage, this study focuses on the effects of the storage process. Any differences observed in the bovine knee would also be expected to manifest in human cartilage but further investigation involving human articular cartilage would be necessary to quantify this. Another reason for choosing bovine is the requirement that fresh knees be used for direct comparison. Fresh bovine knees are cheaper and more obtainable than a human knee.

Storage Protocols

Two low temperature storage protocols were used for this study: a -20 ^{o}C slow freeze and a flash freeze to -80^{o}C. The knee joints and surrounding soft tissue were wrapped in a plastic bag and stored. Joints subject to the slow freeze were stored for a period of 7 days. The flash freezing process involved using dry ice to freeze the knee joints at -80^{o}C for 24 hours followed by storage -20^{o}C for 6 days. All frozen specimens were allowed to thaw overnight prior to testing. Both fresh and frozen tissue had reached room temperature at the time of testing.

The two specific storage protocols used in this study were chosen to simulate experiments and storage methods used by most laboratories. The most common type of

freezer operates at a temperature of -20^{o}C. The flash freeze process was designed to replicate an alternative storage method practiced by researchers at the University of Virginia: Center for Applied Biomechanics where the subject is rapidly cooled to -80^{o}C and then stored for long periods at -20^{o}C. Differences between the flash freeze process and a single freeze would indicate that cooling rate plays an important role in determining the extent to which mechanical properties may be altered.

Tissue Preparation

The bovine knee joints arrived from animal technologies sealed in plastic bags with the hide already removed. The rest of the surrounding superficial tissue was excised and the tibia was separated. Using a bone saw, a 1" x 1" by 3/4" deep specimen was extracted from the joint. Figure 2.1 illustrates the section on the lateral tibial plateau that was extracted for testing.

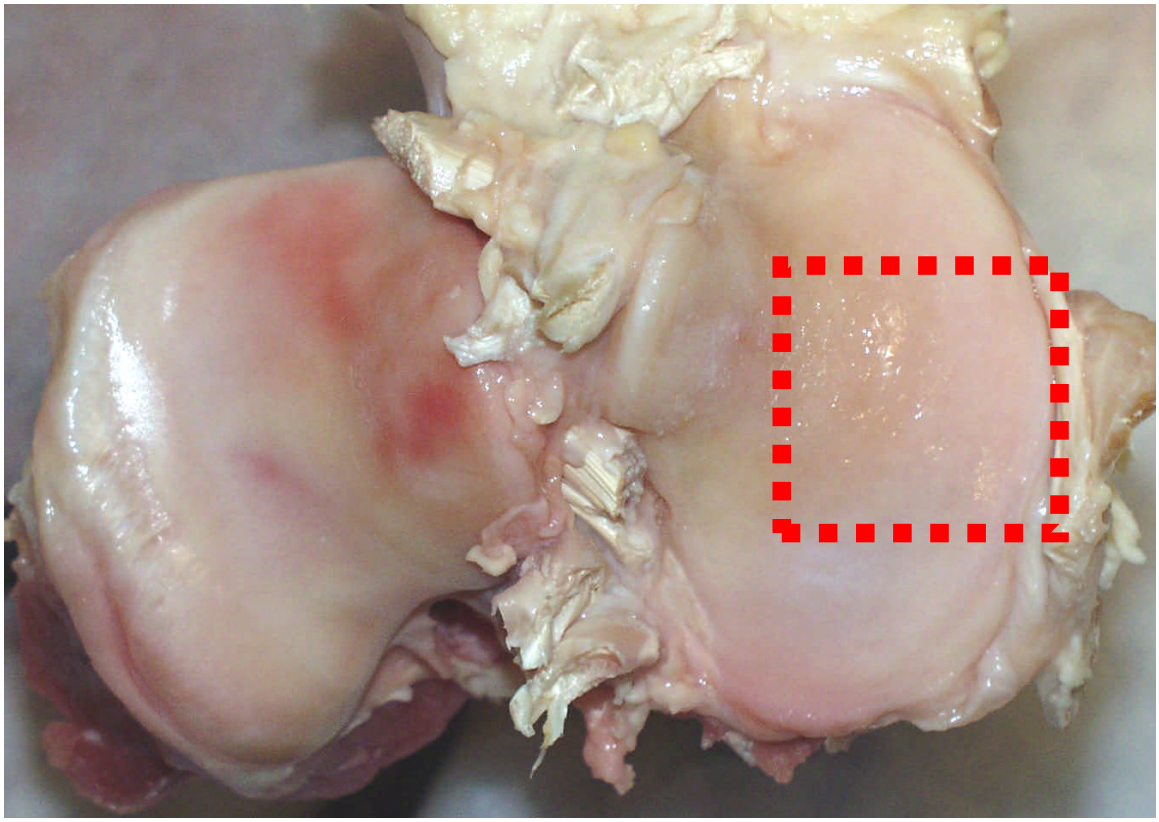

Figure 2.1: The specimens were extracted from the center of the lateral tibial plateau of a bovine knee.

The specimen thickness was reduced to approximately 8 mm using a low speed diamond saw (South Bay technologies, Arlington, VA). The specimen size was comparable to those used in the test conducted by Kerin et al.

Plaster was mixed with water, poured into Petri dishes, and allowed to cure to a semi-hard state. The specimens were then placed in the plaster and adjusted so that the flat target surface was parallel to the indenter surface. This was confirmed by balancing

the unfastened indenter piece on the cartilage surface. The target indenter area was then marked using a black permanent marker. The specimen was then covered in moist gauze while the plaster was allowed to fully cure.

Test procedure

Specimens now in dishes were placed on a flat mounting plate on the lower clamp of a hydraulics materials testing machine (Model 810, MTS, Raleigh, NC). The indenter and load cell were bolted to an upper mounting fixture which was clamped into upper mounting grips of the MTS machine (Figure 2.2).

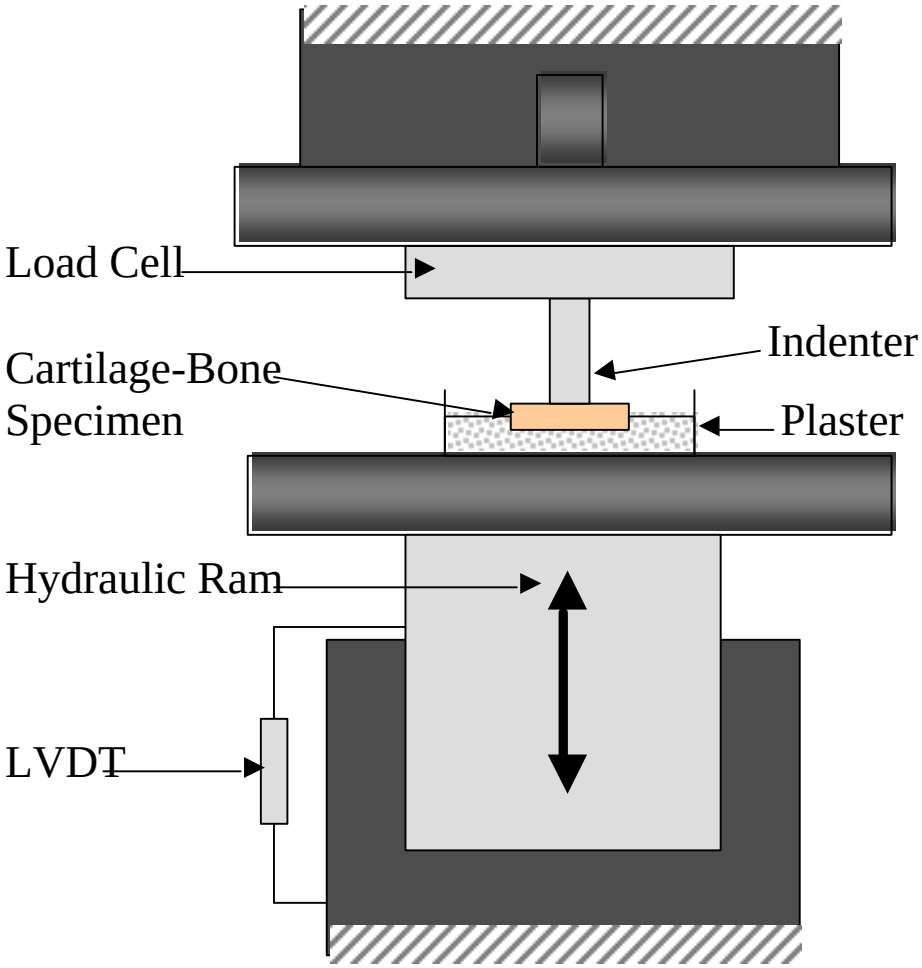

Figure 2.2: A hydraulic MTS machine was used to conduct dynamic mechanical tests on potted articular cartilage-bone specimens.

The indenter was aligned with the target lines marking the flat level area on the specimen. The load cell was zeroed and the platform was then lifted until a small force was realized by the load cell. This force was typically around 0.3 lb. For the loading cycle, a Microcontroller (Model 458, MTS, Raleigh, NC) generated voltage signal was sent to the MTS machine operated in displacement control mode. A saw tooth waveform with a peak displacement of 0.6mm and a period of 1 second was the loading cycle used

to deliver a single insult to each specimen. The indenter used was an 8 mm plane ended cylindrical indenter chosen to be similar in size to the indentor used by Kerin (1998).

Data from the 500lb load cell (1210AF-500, Interface, Scotstsdale AZ), microprofiler (Model 418.91, MTS, Raleigh, NC) and LVDT (MTS systems corporation, Eden Prairie, MN) , were all recorded 1000 Hz through an iotech wavebook 16 bit 1 MHz data acquisition system operated by Waveview software. The data was later filtered using Diadem with a 2nd order low-pass Bessel filter at a cutoff frequency of 30 Hz. Similar cutoff frequencies have been used by other researchers conducting similar tests involving biological material (Van Ee, 1998).

After indention, the specimens were kept moist by gauze and refrigerated for post-test analysis for a period of 24 hours while tissue was allowed to reach equilibrium. An ink test was used to highlight any damage caused by the indention test. A mixture of 10 parts water 1 part India ink was applied to the cartilage surface with a q-tip and then gently wiped clean with a moist cotton swab. This process allows the ink to collect in any fissures that are present while superficial surface ink is wiped away. Each specimen was photographed after this process. Next, the diamond saw was used to section each specimen to 3 mm thick slices. Using a digital micrometer (IP54, Mitutoyo, Japan), the cartilage thickness and overall thickness were measured at the location of testing. The slices were fixed in a 10% buffered formalin solution and later treated with a hematoxylin and eosin stain and sliced to 0.06mm for histological examination.

Many of the methods used in this study were adapted from testing performed in previous studies. Kerin (1998) and Adams (1998) used a similar test apparatus consisting of specimens potted in plaster. They also used a hydraulic materials testing machine operated in displacement control to test the compressive strength of articular cartilage. However, Kerin used multiple increasing displacement cycles until failure was realized to validate his methods. In order to eliminate any concerns of preconditioning specimens the current study used just a single insult with a 0.6 mm displacement. This displacement was chosen to initiate failure in most specimens without damaging the subchondral bone. Previous studies have also shown the failure strain of cartilage to be 30% with little variation. Given an average cartilage thickness of approximately 1.5 mm, 0.5mm would be the average failure displacement and 0.6mm would be large enough to see most failures without compromising the subchondral bone.

Adams et al performed validation testing for the apparatus by measuring the displacement of just the apparatus and subchondral bone under similar loading conditions. He found that these displacements accounted for less than 7% of the total displacement of tests conducted with articular cartilage.

An indention test was chosen because it provided a repeatable loading cycle with accurate stress and displacement measurements. It would be more difficult to achieve this using an impact test. A destructive indention test was performed for the purpose of investigating cartilage response for the entire loading spectrum to the point of failure. A single insult was used to avoid any effects that preconditioning may have on the tissue.

Cooling rates of storage protocols

A wide range RTD digital thermometer (PT-100, Fisher Scientific) with an 8” probe was used to measure the rate of cooling at the location of the cartilage. A pair of bovine knees with intact superficial tissue was frozen while the temperature was

monitored. The right limb was placed in a conventional -20°C freezer and the left limb was packed in dry ice. Both limbs were cooled from room temperature.

Results:

The resulting time-temperature data (Figure 2.3) indicated large differences in the cooling rates of dry ice freezing and conventional freezing. In the conventional freezing process, freezing of the cartilage at the test site occurred after 390 minutes. The flash freezing process was much faster, requiring just one hour for freezing to occur.

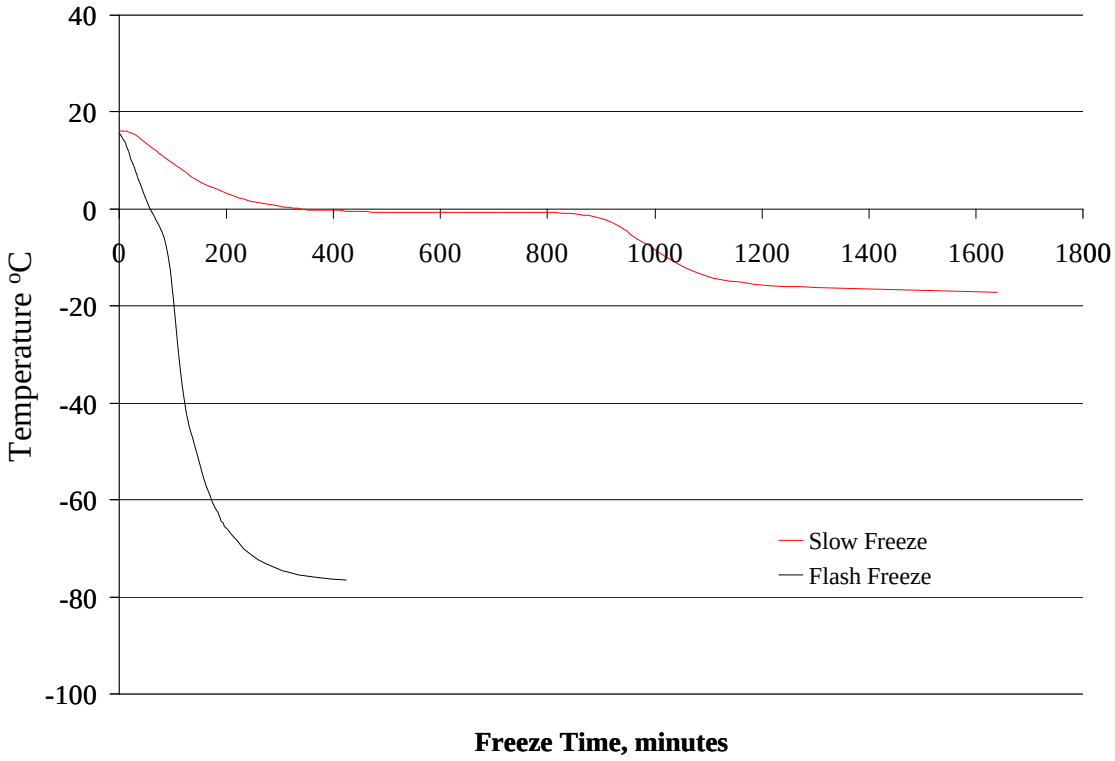

Figure 2.3: Temperature profile data for flash and frozen specimens. The flash freeze specimens cool at a much higher rate than conventional freezing techniques.

Force and displacement data from the load cell and LVDT were filtered and normalized to yield stress as a function of strain. Stress strain data plots were used as a basis for comparison between fresh and preserved specimens. Figure 2.4 is representative of the data collected in this experiment.

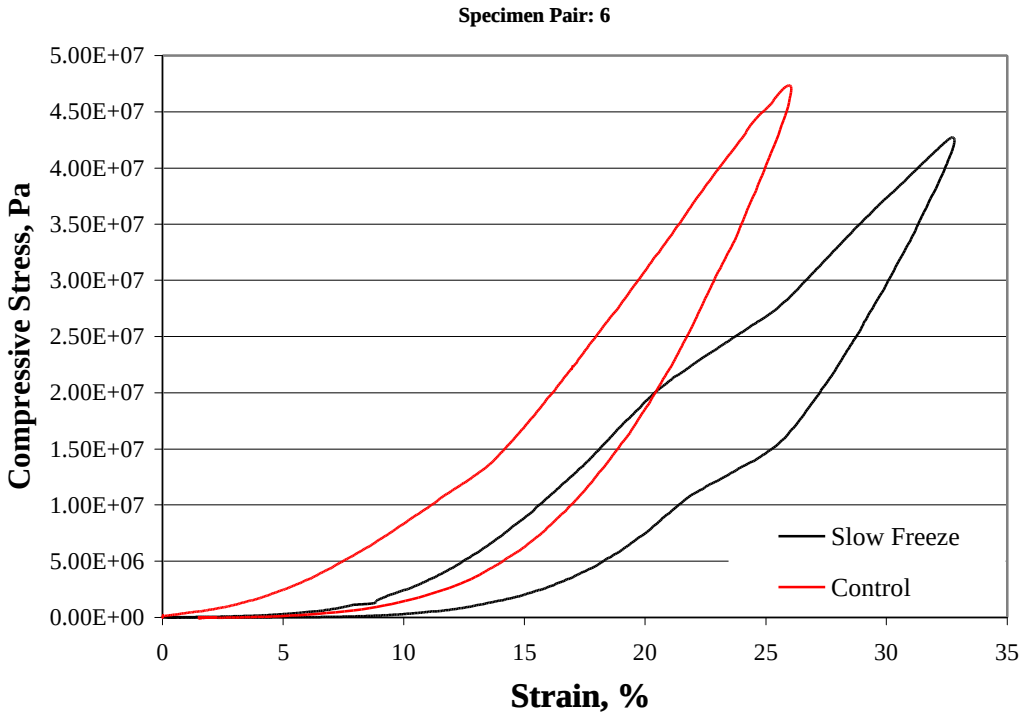

Figure 2.4: Typical stress-strain comparative plot for a frozen and fresh specimens.

The stiffness modulus was defined as the slope of the stress-strain curve between 20 and 22.5% strain. This point was chosen for three reasons. (a) It represented stiffness for the cartilage at the point slightly below the expected failure strain of 30%. (b) 22.5% represented the largest strain values in some cases so it was also chosen for uniformity. While all specimens were displaced the same distance during testing, the maximum strains varied due to thickness fluctuations between specimen pairs and even specimens of the same pair. (c) It represented a linear region of the curve in most cases. A wider range stiffness modulus from 15-22.5% was also calculated as additional comparison between the two groups.

To further characterize the stress strain curves, stress strain data was integrated to the yield strain energy density (SED) required to load the specimen to 20% strain. The energy dissipated in the cartilage is the area between the loading and unloading curves. Because this energy is both rate and history dependent, it could not be directly compared between specimens because all specimens were not strained to the same degree.

Table 2.1: Curve characteristics of individual stress strain curves

	Specimen Pair Number	Strain Energy Density at 20% strain (Pa)		Modulus 20-22.5% (Pa)		Modulus (15-22.5%) (Pa)		Stress at 20% (Pa)	
		Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Slow freeze	6	2.10E+06	1.03E+06	3.01E+08	1.67E+08	2.86E+08	1.92E+08	3.09E+07	1.92E+07
	7	8.49E+05	7.85E+05	1.76E+08	1.08E+08	1.26E+08	9.37E+07	1.27E+07	1.11E+07
	8	1.01E+06	9.03E+05	2.86E+08	1.93E+08	2.43E+08	1.88E+08	1.85E+07	1.69E+07
	9	5.78E+05	8.35E+05	2.19E+08	1.32E+08	1.95E+08	7.82E+07	1.46E+07	9.70E+06
	10	1.80E+06	1.00E+06	2.84E+08	1.91E+08	2.72E+08	1.53E+08	2.84E+07	1.52E+07
Flash freeze	16	1.82E+06	8.50E+05	2.38E+08	1.07E+08	1.94E+08	8.23E+07	2.30E+07	1.11E+07
	18	1.76E+06	2.25E+06	2.66E+08	2.45E+08	2.47E+08	2.23E+08	2.53E+07	2.97E+07
	19	2.10E+06	1.11E+06	3.00E+08	1.76E+08	2.44E+08	1.51E+08	2.64E+07	1.65E+07
	20	1.60E+06	1.39E+06	2.55E+08	1.70E+08	2.55E+08	1.96E+08	2.58E+07	2.20E+07
	21	7.96E+05	7.18E+05	2.55E+08	2.09E+08	2.08E+08	1.43E+08	1.59E+07	1.19E+07

A two tailed t-test was used to determine the level of significance in differences between fresh and frozen groups. Our results indicate that stiffness modulus at 20-22.5% strain decreased by 37% (p=0) in single freeze groups and 31% (p=0.01) in flash frozen specimens. Modulus between 15% and 22.5% was also significantly lower in slow freeze (p=0) and flash freeze groups (p=0).

Stress in the cartilage at 20% strain was significantly higher for fresh specimens vs. slow freeze specimens (p = 0.028). Stress was also observed to be higher although borderline significant for fresh vs. flash freeze specimens (p=0.07).

Loading energy, defined as the strain energy density to 20% strain, also appeared to be higher for fresh versus frozen specimens although the differences were not statistically significant.

Table 2.2: Summary of overall differences between frozen and control groups

	Strain Energy Density (Loading to 20%) Mpa	STD DEV	Modulus 15-22.5% strain (MPa)	STD DEV	Modulus 20-22.5% strain (MPa)	STD DEV	Stress at 20% strain (MPa)	STD DEV
SF Controls	1.27	(0.65)	224.00	(65.10)	253.00	(53.70)	21.00	(8.21)
Slow Freeze	1.03	(0.11)	141.00	(52.90)	158.00	(37.40)	14.40	(3.95)
%Difference	-18.9		-37.1		-37.5		-31.4	
p-value	0.11		< 0.01		< 0.01		0.03	
FF Controls	1.61E+00	(0.49)	229.00	(27.10)	263.00	(23.40)	23.30	(4.33)
Flash Freeze	1.26E+00	(0.61)	159.00	(54.00)	181.00	(51.00)	18.20	(7.77)
%Difference	-21.7		-30.6		-31.2		-21.9	
p-value	0.14		< 0.01		0.01		0.08	

Cartilage Damage assessment: Ink staining and histology analysis

Each specimen showed signs of damage after being stained with India ink. Some specimens appeared to be more damaged than others but there didn't appear to be any strong correlation between plot shape, stress level or loading energy. Damage was most apparent along the circumference of the indented area. Figure 2.5 shows a representative photograph of damage sustained by the indention test.

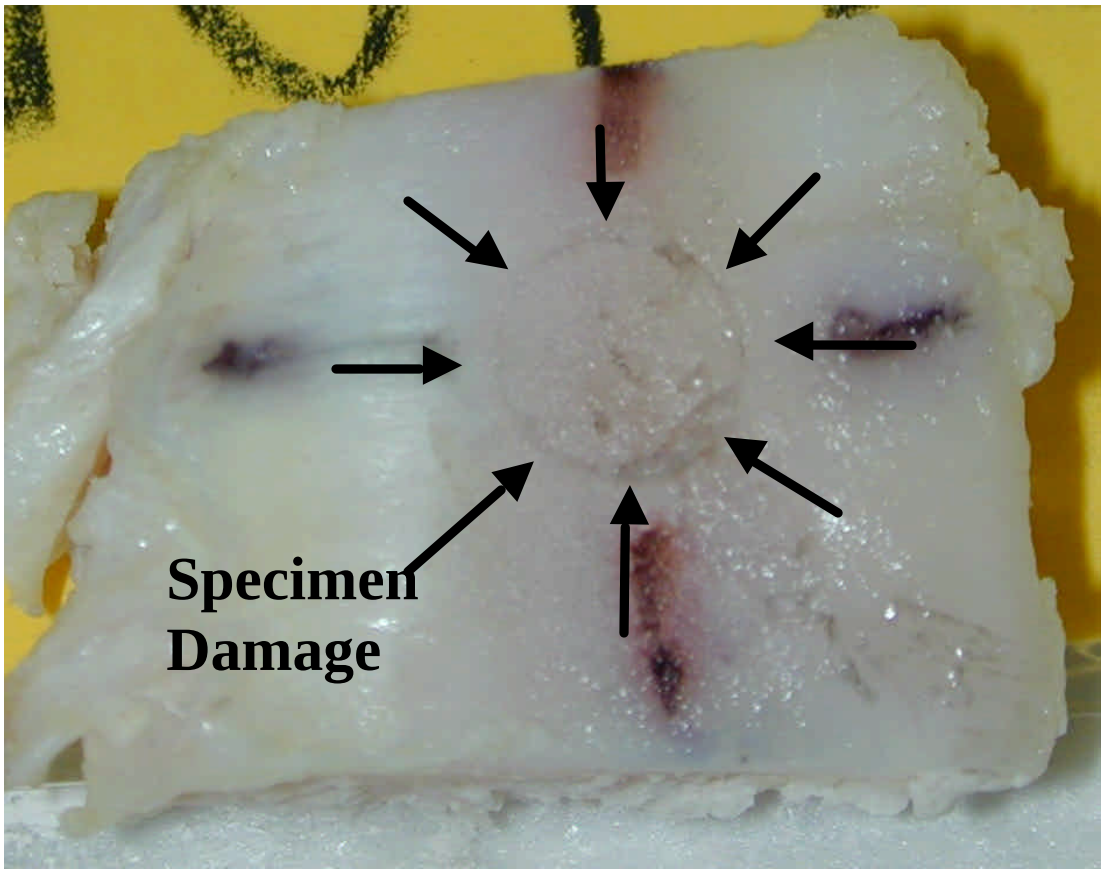

Figure 2.5: Damage sustained from indention testing as indicated by the india ink staining process. Specimen #10FL

The post-test histology slides showed varying degrees of damage ranging from no damage to fissures and separation between the cartilage and subchondral bone interface. Specimens were compared and visual inspections were conducted at 40X to assess the amount of specimen damage (Table 3). Surface and subsurface damage was rated on a scale of 1 to 3 with 3 being the most severe.

Table 2.3: Assessment of surface and subsurface damage as indicated from histology slides

	Specimen Pair #	Fresh		Frozen	
		Surface damage	Subsurface damage	Surface damage	Subsurface damage
Slow Freeze	6	2	3	1	0
	7	1	1	1	0
	8	1	0	1	3
	9	2	2	1	2
	10	2	0	3	0
Rapid Freeze	16	2	1	1	0
	18	1	0	1	2
	19	1	1	0	1
	20	2	1	1	0
	21	0	1	0	1

Histological inspections showed no obvious differences between fresh or frozen specimens. However, there does appear to be a direct relationship between superficial damage and peak stress. Additionally, subsurface damage appears to correlate well with loading SED. In several cases, one specimen was damaged more severely than another

Figure 2.6 illustrates a sample comparison between fresh and frozen specimens. Both specimens have superficial damage which was quantified by a damage index of 3 and 2 respectively. The surface damage was primarily located in proximity to the indenter edge.

Frozen Fresh

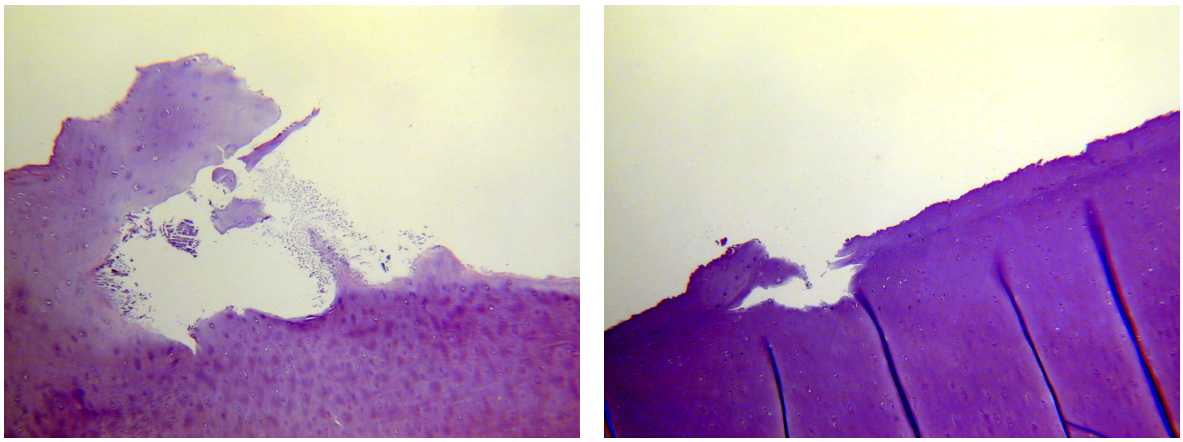

Figure 2.6: The frozen specimen (left) appears to have been damaged more extensively than the fresh specimen (right). (Specimens #10FL and #10-20R, 40X)

Subsurface damage usually took place at the cartilage and bone interface. The damage was characterized by fissures in the deep zone of the cartilage or gaps between the cartilage and bone (Figure 2.7).

Frozen Fresh

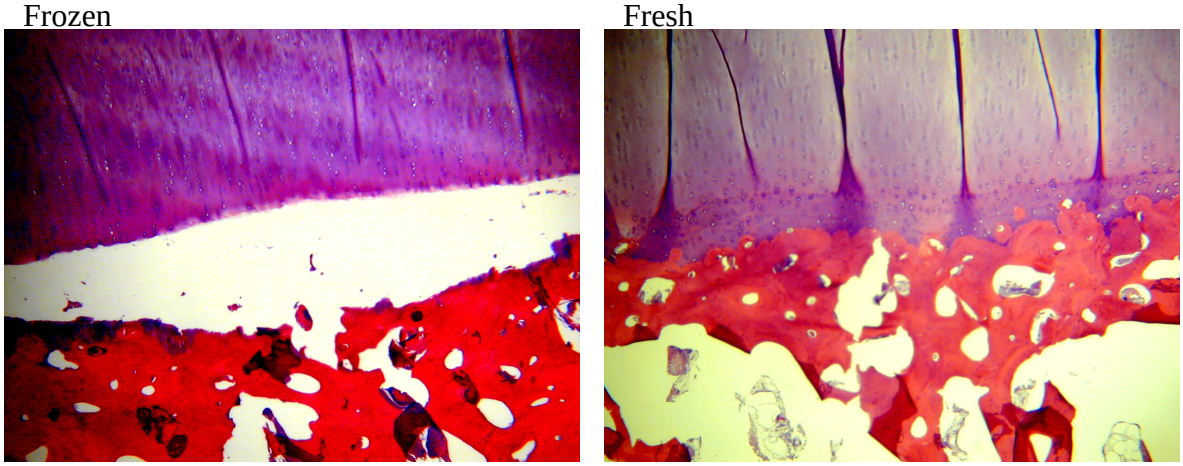

Figure 2.7: Comparison of subsurface damage incurred in specimen pair 8. The cartilage is clearly separated in the frozen specimen (left). Loading Energy was also much higher for the damaged specimen (Specimens #8FL and #8-20R, 40X).

Discussion

This study showed that in both test groups, the stiffness averaged from 20-22.5% strain was significantly higher for fresh specimens (p=0 slow freeze, p=0.01 flash freeze). An average stiffness modulus taken from 15% to 22.5% strain also proved to be significantly higher for fresh cartilage tissue (p=0 slow freeze, p=0 flash freeze). Results also indicated that stress at 20% strain is significantly higher for fresh specimens compared to slow freeze specimens. The results showed that these forces are also likely to be higher than the flash frozen group (p=0.07).

The results of the current study were different from those measured by Kiefer (1989) who was unable to measure any significant differences between fresh cartilage specimens and cryopreserved samples. Possible reasons for these differences are twofold: Keifer used a different storage protocol which maintained the specimens at -80^{o}C instead of freezing to -80^{o}C and storing at -20^{o}C as done in the current study. The testing methodologies were also different in the way that Kiefer used a relatively low compressive force (16 N) and a non-destructive repeated test.

An objective for this study was to be able to quantify the point of failure using this testing methodology and the failure criteria specified by Kerin (1998). Attempts to do so were unsuccessful because while some specimens exhibited obvious decreases in the force-displacement gradient, a strong indication of failure, others showed little or no signs of breakdown. Instead, it was necessary to compare values of stiffness, loading strain energy density, and stress. It is possible that strain rates of 0.6 +/-0.1 was too slow to cause an obvious graphical indication of failure. Repo and Finlay (1977) used a device capable of much higher loading rates to measure failure in articular cartilage. The force and displacement plots produced by their tests had noticeable points of failure for each specimen.

Stiffness plays an important role in the ability of articular cartilage to carry a load without being strained to the point of failure. The finding that cartilage stiffness is decreased 37% by slow freezing and 31% by flash freezing indicates a significant change in the biomechanical properties. These decreases in stiffness may be an indication of the microstructural damage sustained during the freezing process.

Cryonics research has shown that cell damage occurs as a result of certain types of freezing (Muldrew, 1993; Vazquez, 2001). Additional studies have also indicated extracellular matrix damage as a result of freezing (Muldrew 2000). Muldrew et al reported a greatly disrupted matrix with a large increase in matrix pore size as a result of ice crystal formation in cryopreservation. It is possible that the reduction in compressive stiffness being observed is a result of this damage. However, there are conflicting opinions on this subject (Tavakol, 1993). Tavakol did not observe any changes in ultrastructure with an electron microscope after cryopreservation.

At 60-85%, the fluid phase consisting of water and dissolved electrolytes make up the largest wet weight in articular cartilage. The extracellular matrix is largely responsible for the structural integrity of cartilage. Compressive resistance is exhibited through frictional drag of fluid flow through the pores of the collagen fiber-proteoglycan matrix (Mow, 1995). An increase in matrix pore size could account for the marked decrease in compressive stiffness observed in this study.

The proteoglycan gel is also responsible for the compressive stiffness of articular cartilage. The swelling pressure exerted by the fixed charge density (FCD) of the proteoglycans. Muldrew (2000) observed a diffusion of electrolytes away from the ice forming front. He also noted that this phenomenon is dependent on cooling rate. If the proteoglycan gel or its electrolytic distribution was disrupted during freezing this could explain a reduced stiffness in the cartilage tissue.

Tissue damage can be minimized in a process called vitrification, which is freezing without ice crystal formation (Best, 2000; Studer 1995). Vitrification is accomplished by rapid cooling on the order of 1000 K/s and extreme low temperature storage coupled with diffusion of chemicals called cryoprotectants into the tissue. It is also achieved by a rapid thawing technique designed to minimize the amount of time spent in the ice crystal formation temperature range. The temperatures used in the current study were still much higher than temperatures used in vitrification and no rapid thawing techniques were attempted or investigated. In order for vitrification to occur, the storage temperature must be below the glass transition temperature of the material (Tg) which is approximately 2/3 of the freezing temperature on the Kelvin scale or about -90^{o}C for water. (Best, 2002)

In this study, a smaller change in mechanical properties were observed for flash frozen cartilage compared to the slow freeze group indicating that the flash freezing studied may be less damaging to cartilage tissue. While complete vitrification of tissue on a macroscopic scale seems highly impractical, it may be possible to limit ice formation by lowering the long term storage temperature to below Tg, further increasing cooling rate and by employing a rapid thawing process.

Histological examination at the microscopic level showed no obvious trend differences between damage sustained in fresh and frozen tests. This could be a result of inconsistencies in peak strains delivered to different specimens which in turn could result in different levels of stress. While the displacement was kept constant it would have been

more difficult to maintain a constant peak strain level between specimens because the thickness variance between specimens. The peak stress appeared to be a good predictor of surface damage observed on histology slides. This could be explained by the parallel orientation of the collagen fibers to the articular surface. Failure of these fibers under high stress levels could result in surface damage as indicated by the histology slides. Deeper subsurface injury was usually indicated by a separation in the cartilage bone interface. The type of injury appeared in specimens with higher strain energy density values than their contralateral limb counterparts. Therefore, it seems likely that this type of injury mechanism consumes larger amounts of energy.

Ideally, the limbs used in this study would be prepared and tested immediately following death of the animal. Efforts were made to minimize the time between death and postmortem testing but the 48 hour time lapse may be a cause for experimental uncertainty. It was also noted that upon arrival, minor pathology such as fraying was present in several of the specimens (Figure 2.8). However, the degree of damage was not noticeably different in any of the testing groups and no correlations were attempted to assess this possibility.

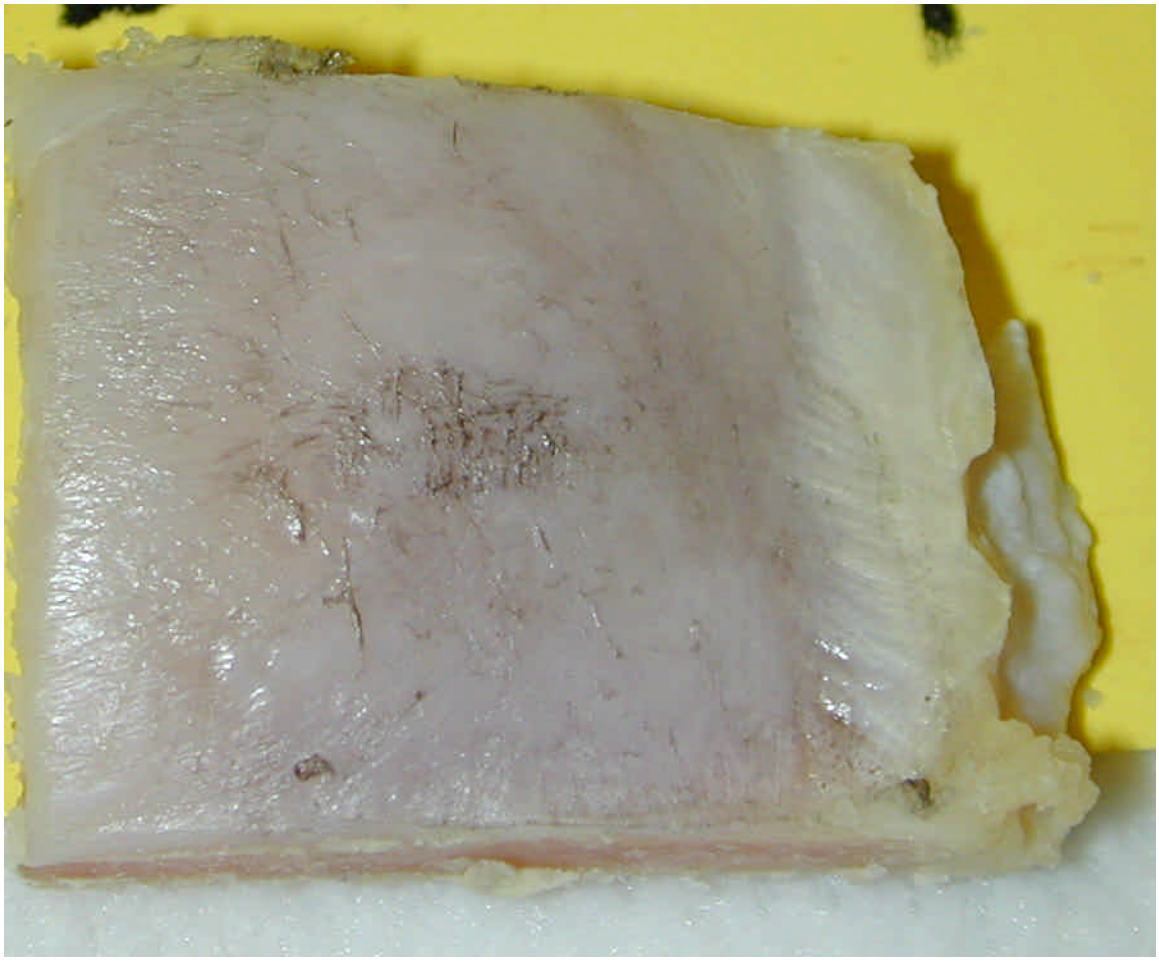

Figure 2.8: In some cases, minor damage was observed prior to indention testing. Ink staining indicates surface defects that are present. Specimen #6-20R

Another possible source of error was finding the location of a perfectly flat 8mm diameter surface for testing. If the surface was not completely flat, the area being indented could be reduced which alter force measurements and stress calculation. Although a relatively flat part of the tibial was chosen, and target lines were used to mark the flattest area, this will always be an issue with plane ended indenter testing.

Conclusion

Low temperature preservation and its effects on the mechanical properties of articular cartilage were studied. With a destructive controlled indention test, the stress-strain characteristics of cartilage on bone specimens were measured. It was determined that a single freeze thaw or flash freeze process caused a decrease stiffness modulus (as calculated at 20-22.5% strain) by 37% and 31% respectively. Compressive stress at this strain was also lowered by 31% with a single freezing process. No significant changes were measured in strain energy density for any of the treatments although fresh specimens appeared higher. These results may be indicative of a weakened extracellular matrix structure resulting from the freeze-thaw process.

A primary objective in this investigation was to measure the failure characteristics of the articular cartilage for comparative purposes. However, only a few of the specimens produced a distinct decrease in force-displacement gradient indicating an exact point of failure. Consequently it was not possible to characterize all the specimens in this fashion. It is possible that a higher rate of loading may produce a more distinct failure characteristic but further investigation would be necessary to determine if this is the case.

The study presents evidence that cartilage mechanical properties are altered by a conventional freezing process and, to a slightly lesser extent by a flash freezing process. Laboratories performing high stress and strain testing on articular cartilage should be aware of these alterations and care should be taken when drawing conclusions from a test involving previously frozen cartilage tissue. It is unclear from histological examination whether these changes in mechanical properties will result in a change in injury susceptibility for articular cartilage.

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Appendix A: Force – Displacement Plots

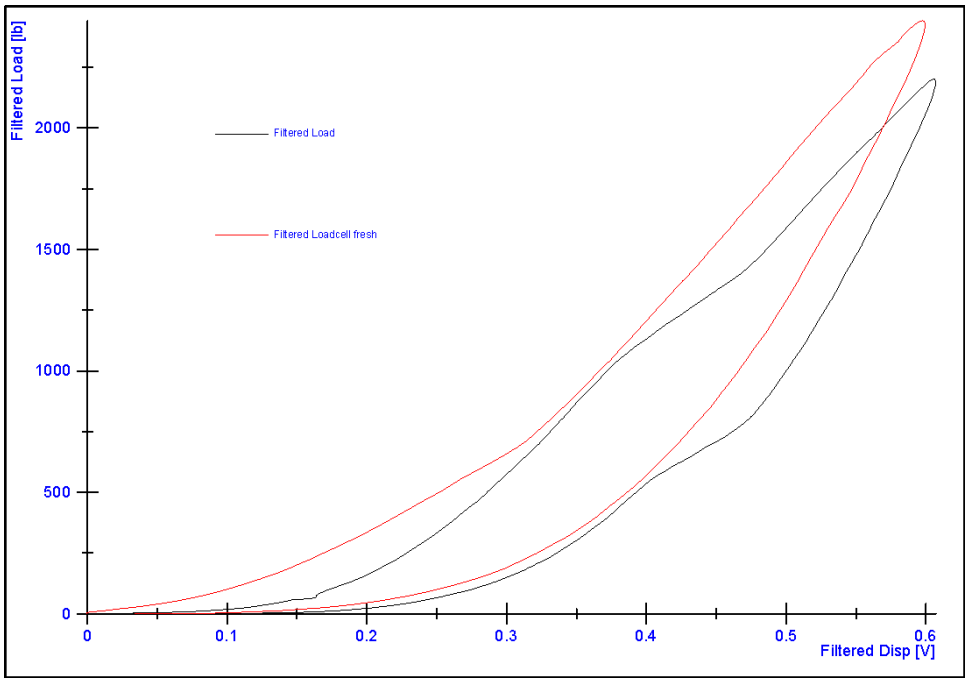


Figure A-1: Single Freeze (pair 6)

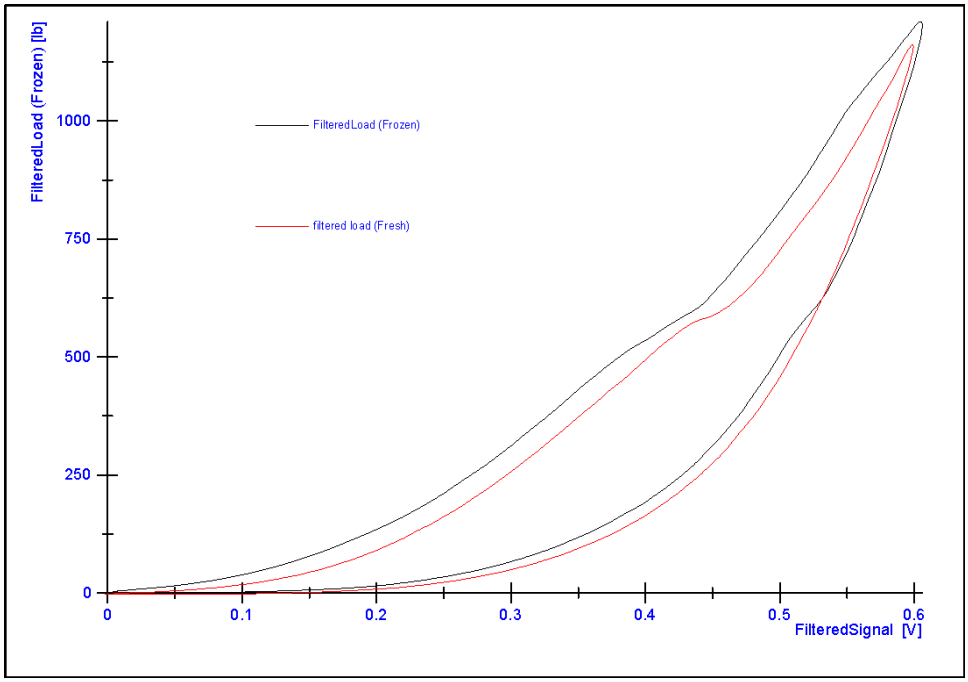


Figure A-2: Single Freeze (pair 7)

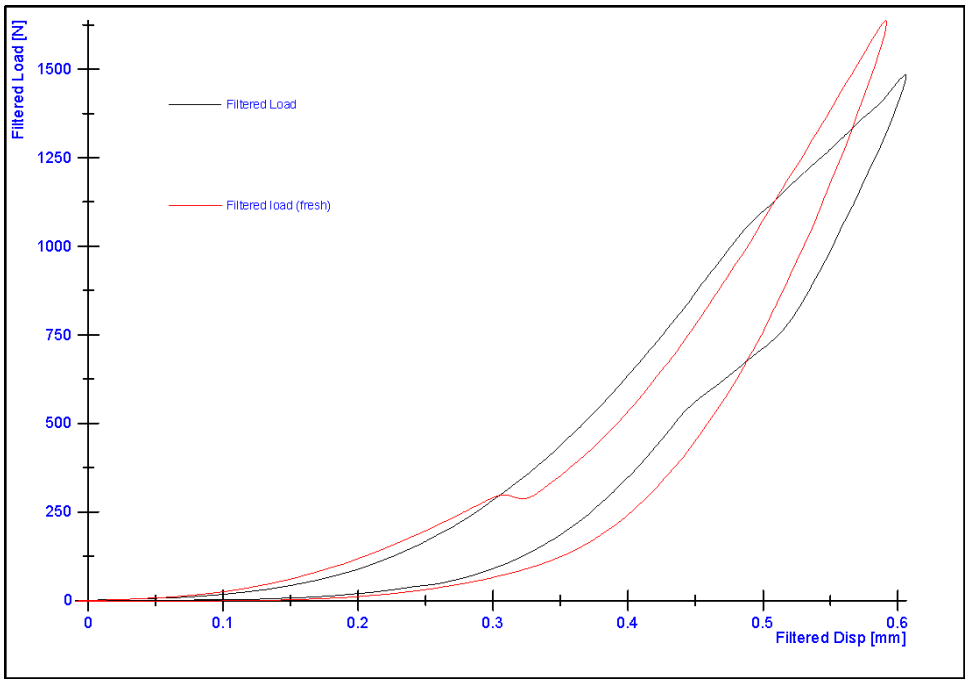
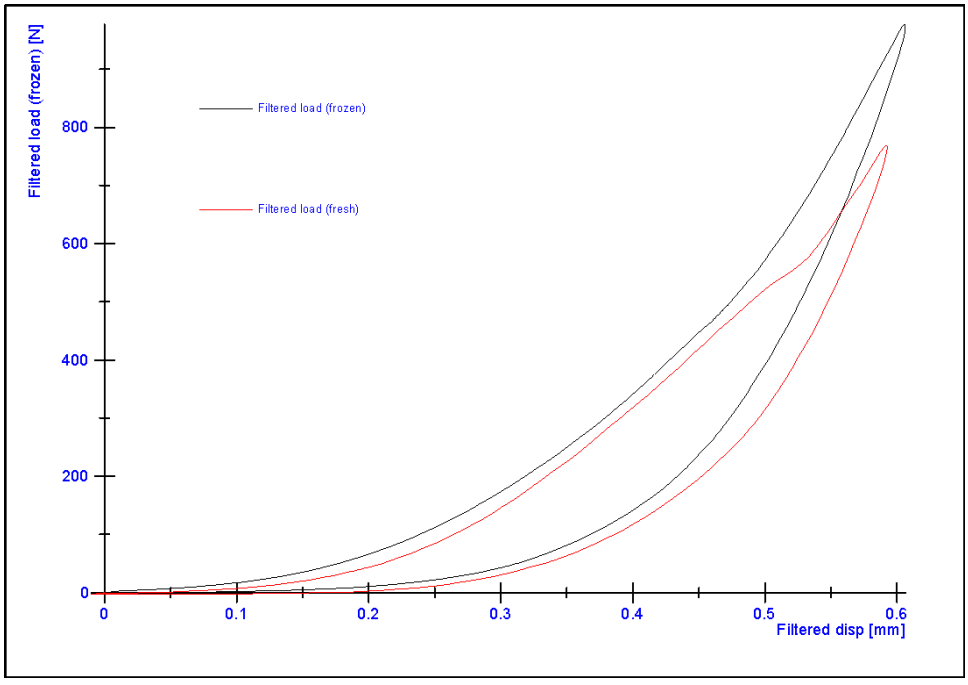


Figure A-3: Single Freeze (pair 8)



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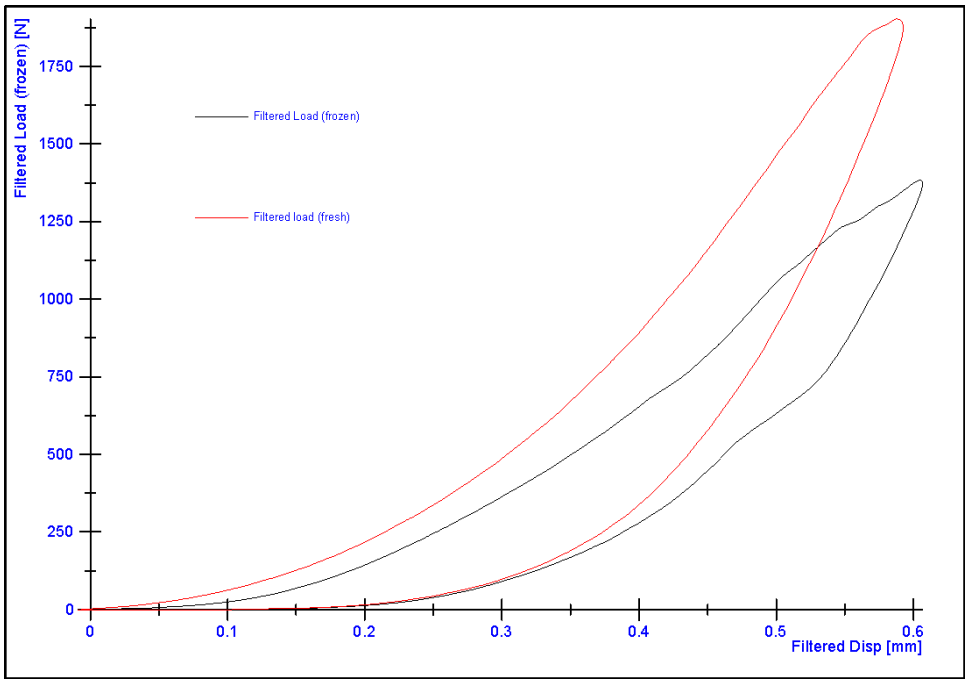


Figure A-5: Single Freeze (pair 10)

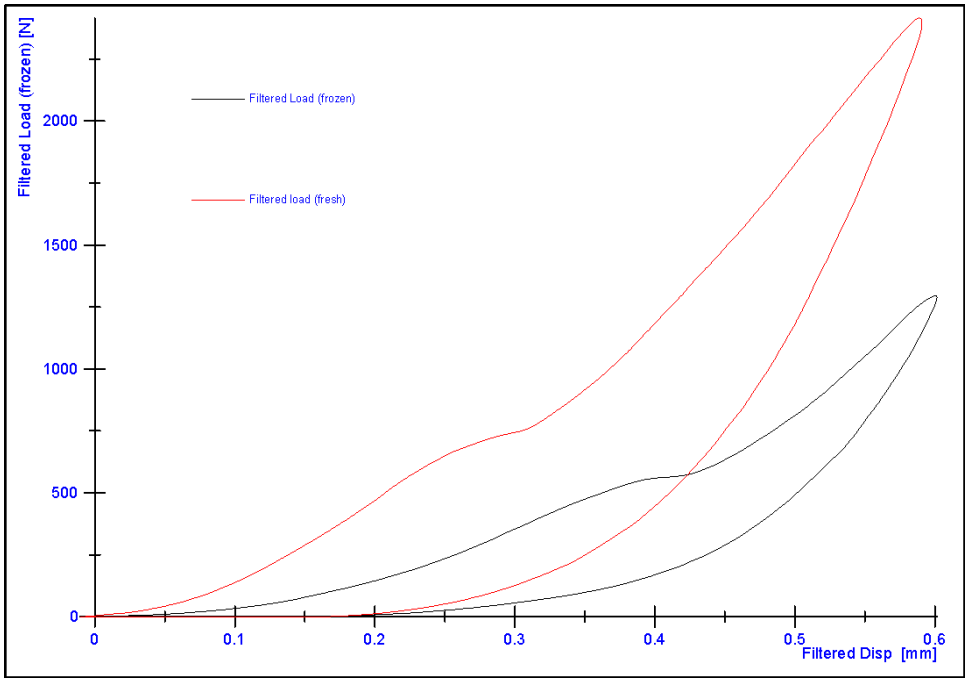


Figure A-11: Flash Freeze (pair 16)

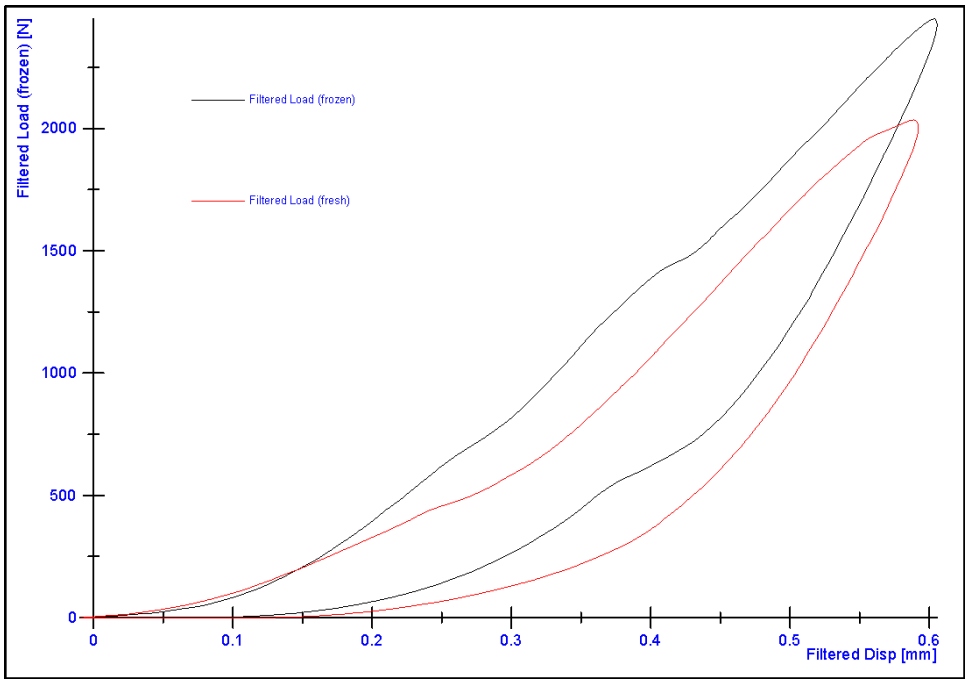


Figure A-12: Flash Freeze (pair 18)

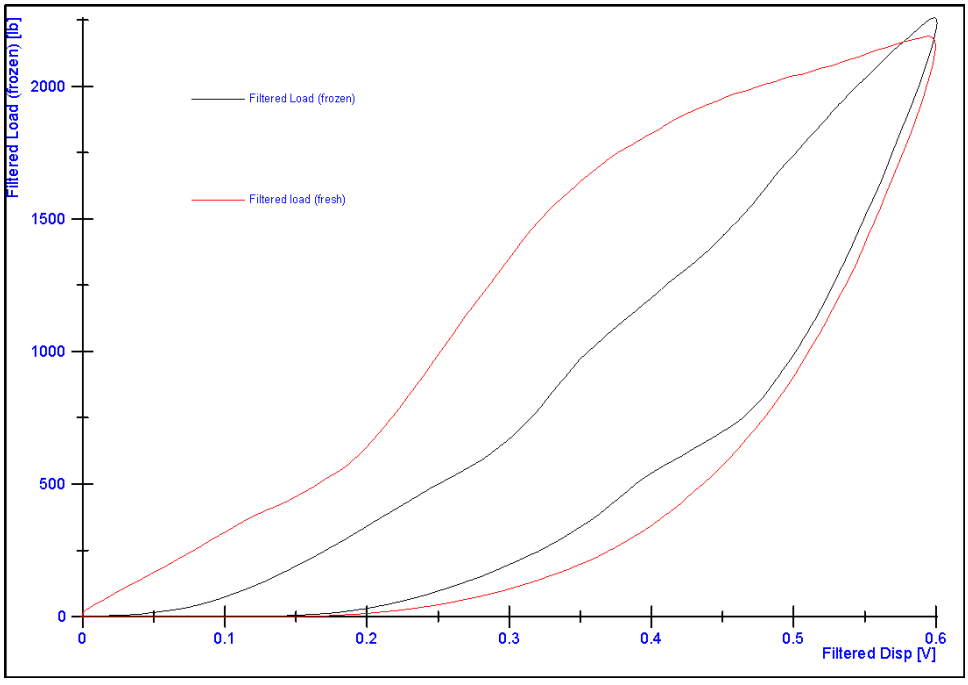
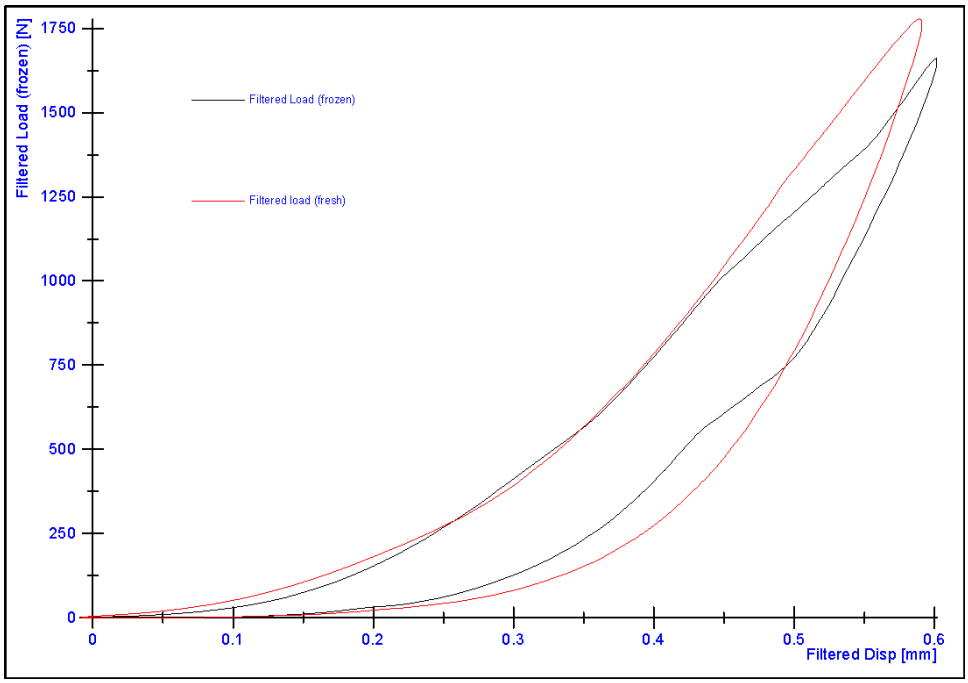


Figure A-13: Flash Freeze (pair 19)



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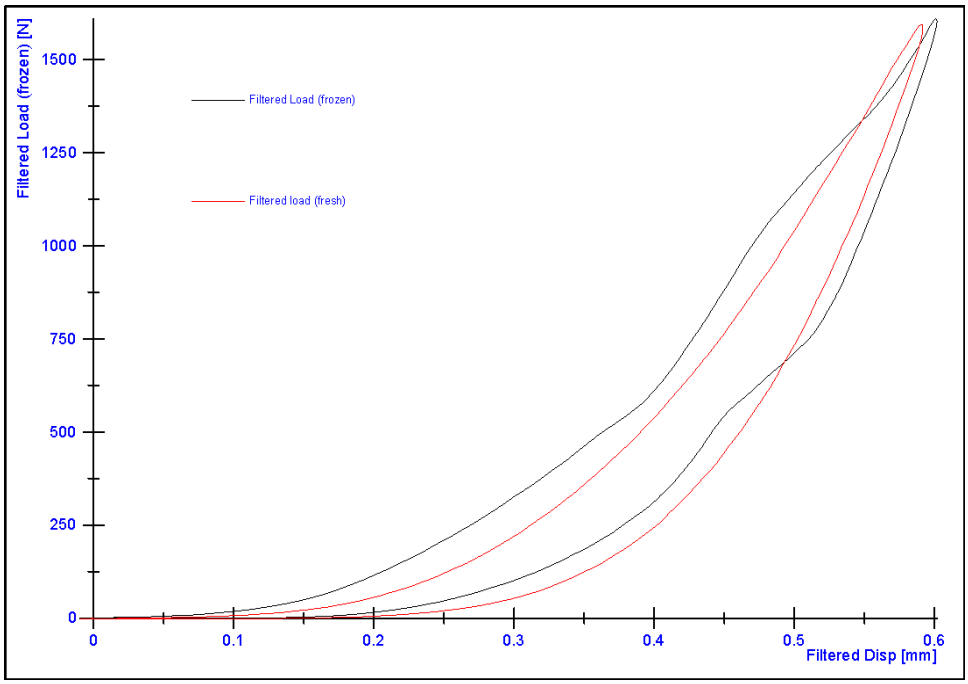


Figure A-15: Flash Freeze (pair 21)

Appendix B: Stress-Strain Plots

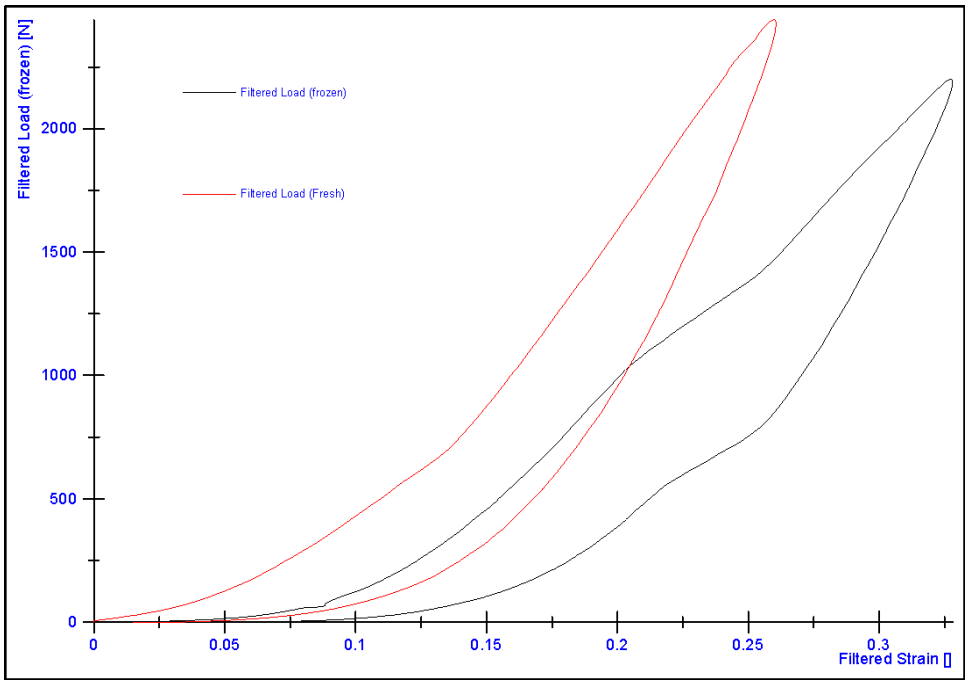


Figure B-1: Single Freeze (pair 6)

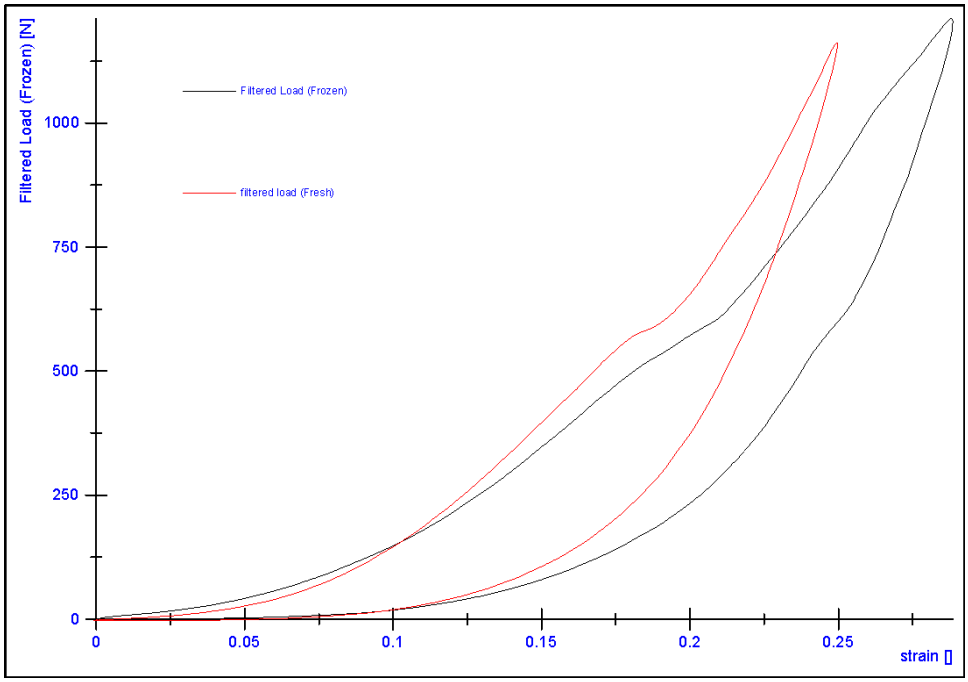
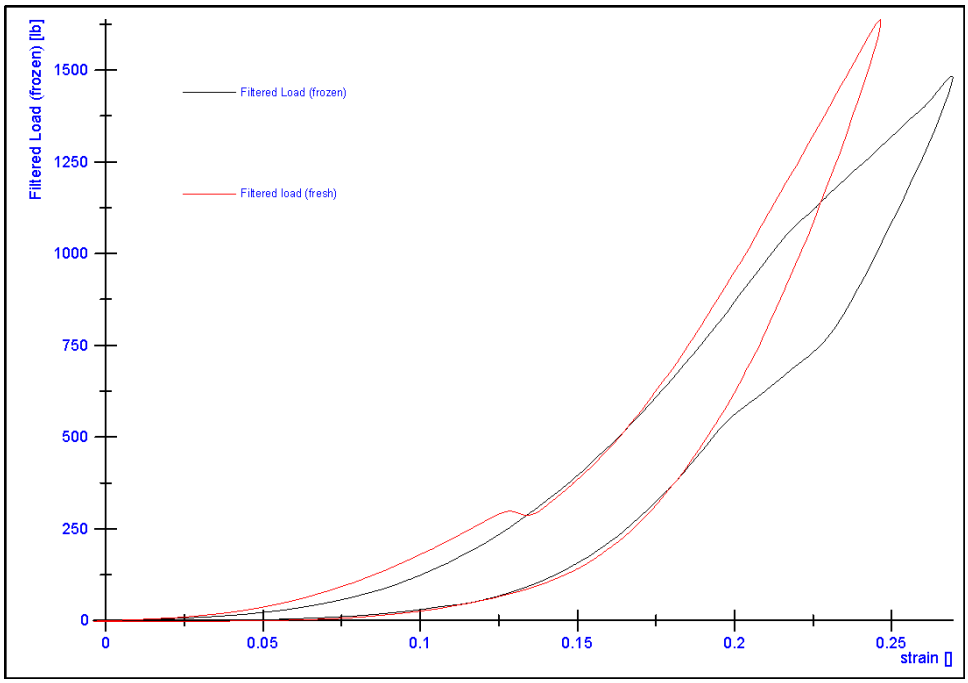


Figure B-2: Single Freeze (pair 7)



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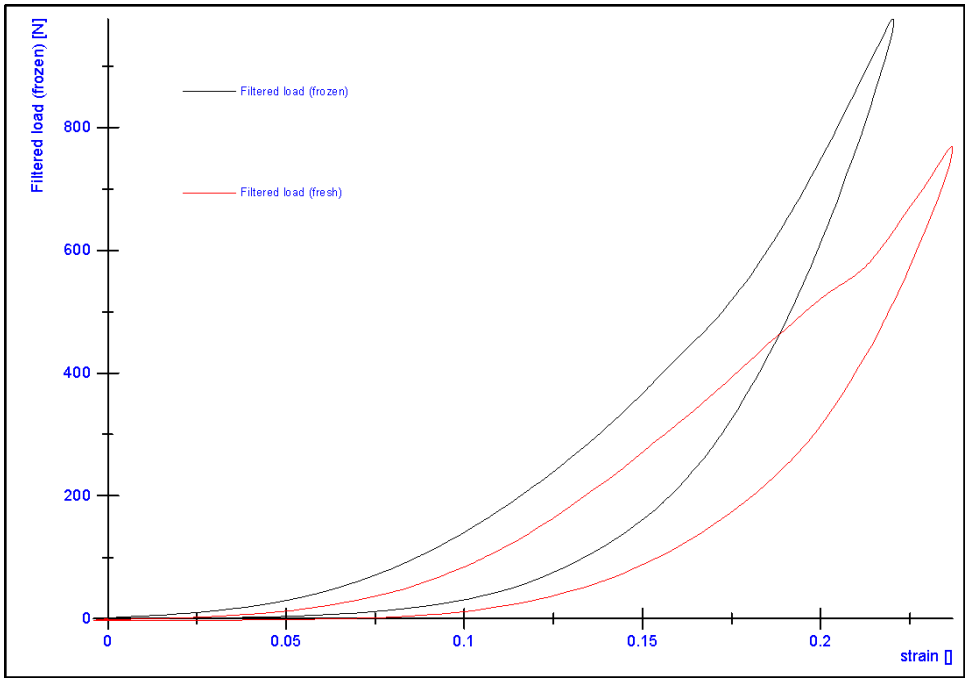

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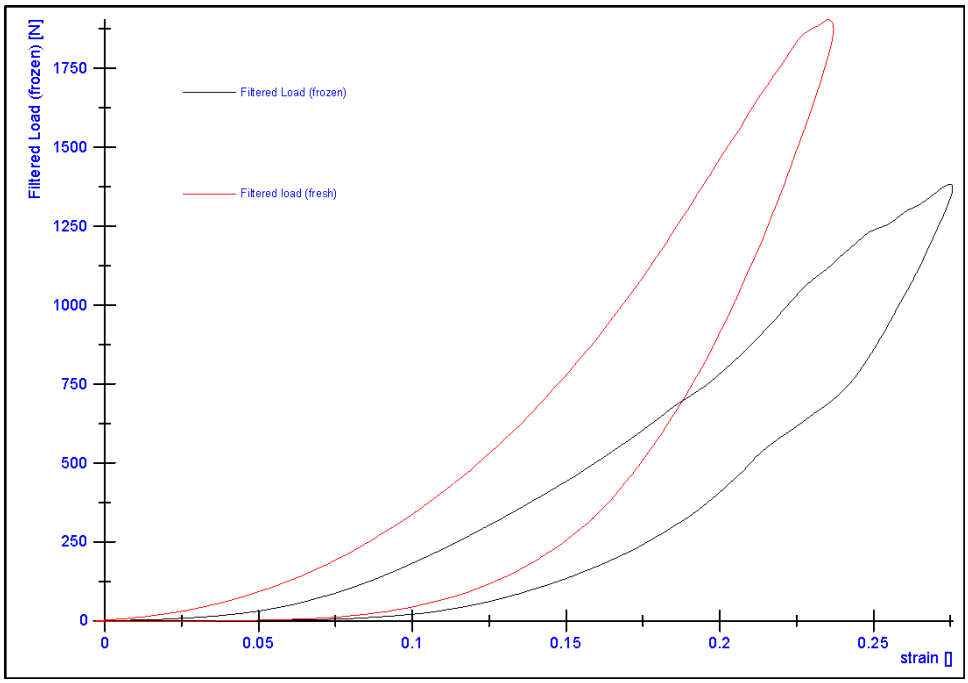


Figure B-5: Single Freeze (pair 10)

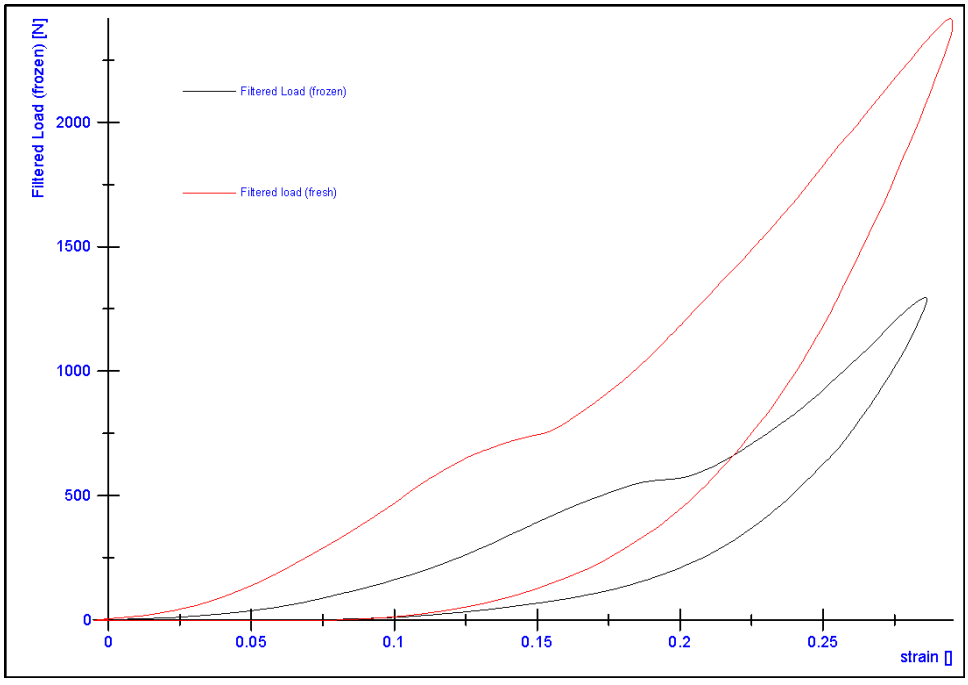


Figure B-11: Flash Freeze (pair 16)

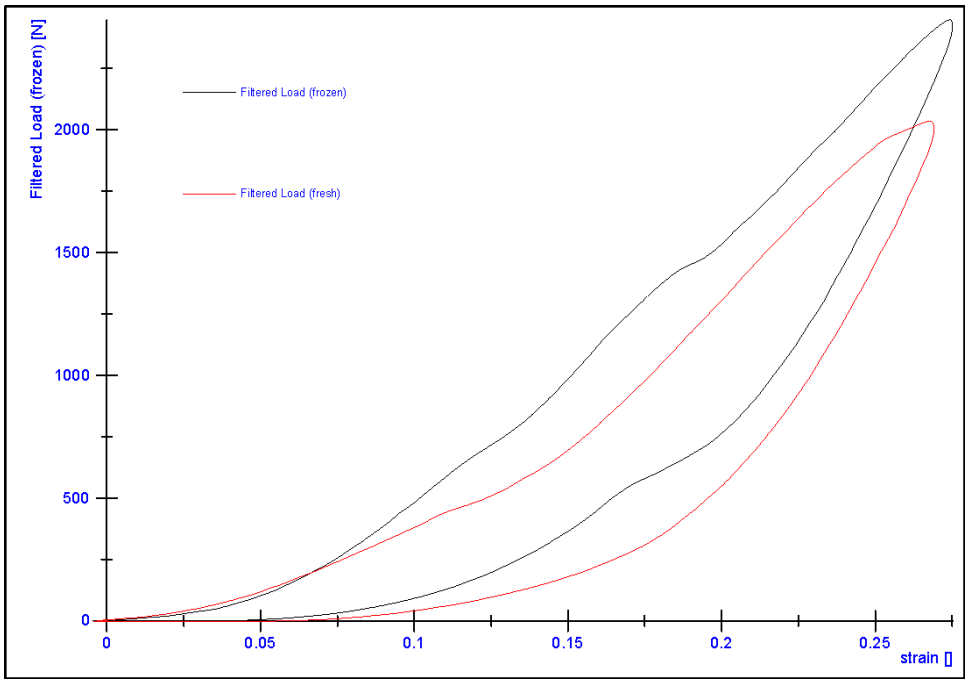


Figure B-12: Flash Freeze (pair 18)

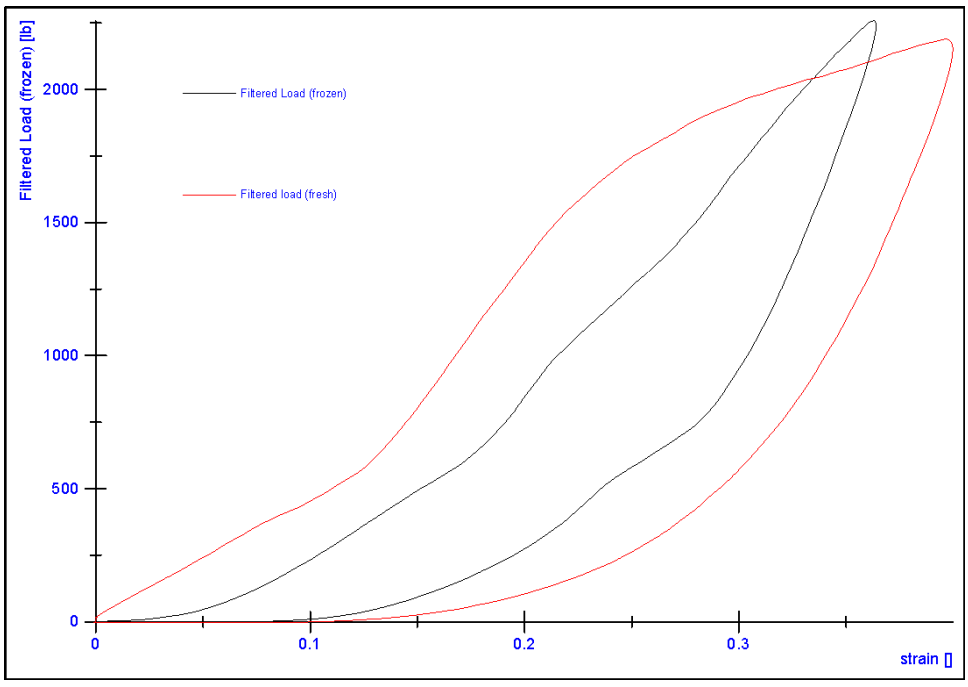
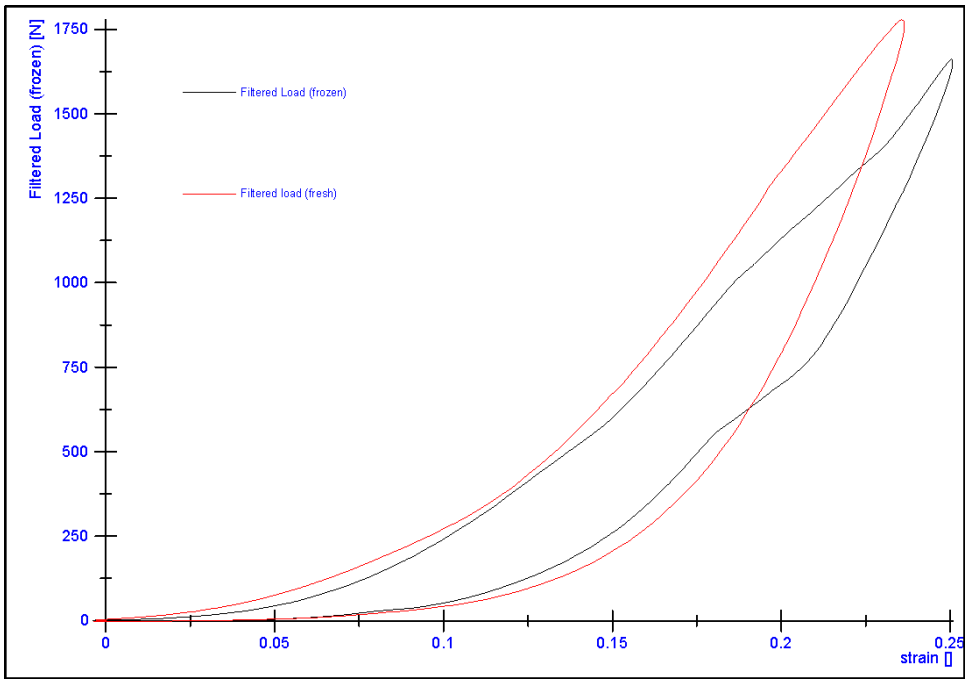


Figure B-13: Flash Freeze (pair 19)



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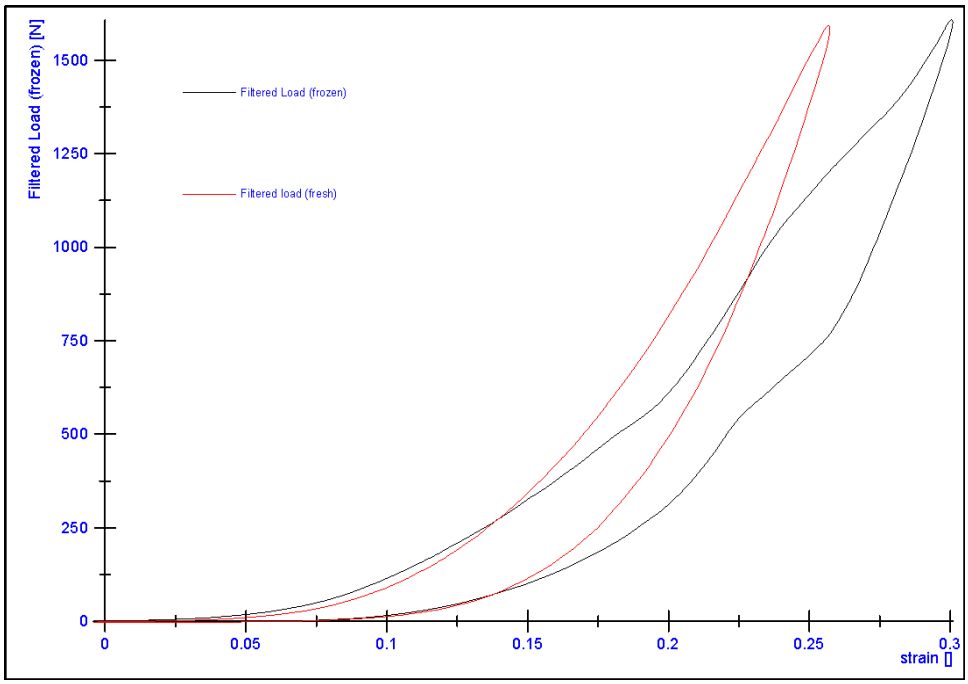


Figure B-15: Flash Freeze (pair 21)

Appendix C: Tabulated Data

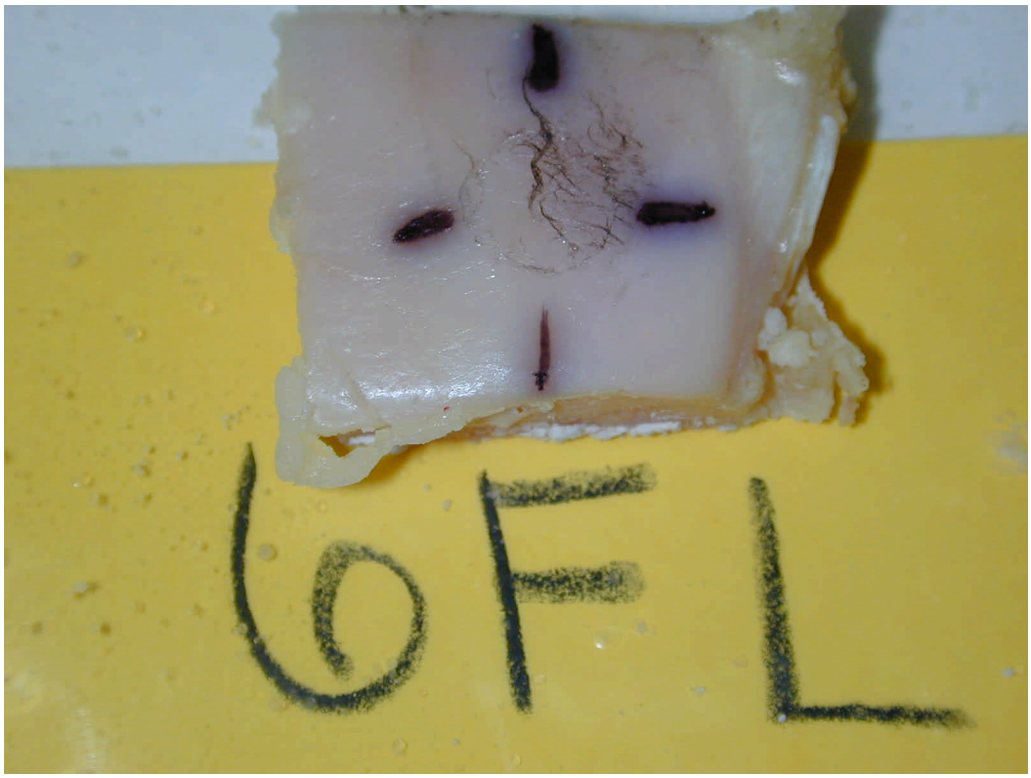
Specimen Number	Freeze Date	Thaw-Freeze Date	Test Date	Tested	Overall Specimen Thickness (mm)	Cartilage Thickness (mm)
Fresh Specimens						
6FL			3/20/2003	X	9.5	2.3
7FR			3/20/2003	X	8.2	2.4
8FL			3/20/2003	X	9.4	2.4
9FR			3/20/2003	X	9	2.5
10FL			3/20/2003	X	8.1	2.5
11FL			3/18/2003	X	7	2
12FL			3/18/2003	X	5.3	1.3
13FR			3/18/2003	X	5.2	1.15-1.8
14FR			3/18/2003	X	6.5	1.87
15FR			3/18/2003	X	6.35	1.8
16FR			3/20/2003	X	9.2	2
17FL			3/20/2003	X	9.3	2.7
18FR			3/20/2003	X	7.5	2.2
19FL			3/20/2003	X	6.6	1.5
20FR			3/20/2003	X	7.7	2.5
21FL			3/20/2003	X	9.2	2.3
Frozen once specimens						
6-20R	3/18/03		3/27/2003	X	7.9	1.85
7-20L	3/18/03		3/27/2003	X	9.7	2.1
8-20R	3/18/03		3/27/2003	X	8.3	2.25
9-20L	3/18/03		3/27/2003	X	9.6	2.75
10-20R	3/18/03		3/27/2003	X	8.5	2.2
Double Freeze						
11-20DR	3/18/03	3/21/03	3/27/2003	X	8.8	1.85
12-20DR	3/18/03	3/21/03	3/27/2003	X	5.8	1.7
13-20DL	3/18/03	3/21/03	3/27/2003	X	6.7	2
14-20DL	3/18/03	3/21/03	3/27/2003	X	8.1	1.85
15-20DL	3/18/03	3/21/03	3/27/2003	X	7.3	1.7
Flash Freeze						
16-80L	3/20/03		3/27/2003	X	7.4	2.1
17-80R	3/20/03		3/27/2003	X	6.9	2.8
18-80L	3/20/03		3/27/2003	X	8.4	2.2
19-80R	3/20/03		3/27/2003	X	7	1.65
20-80L	3/20/03		3/27/2003	X	8.3	2.4
21-80R	3/20/03		3/27/2003	X	8.1	2

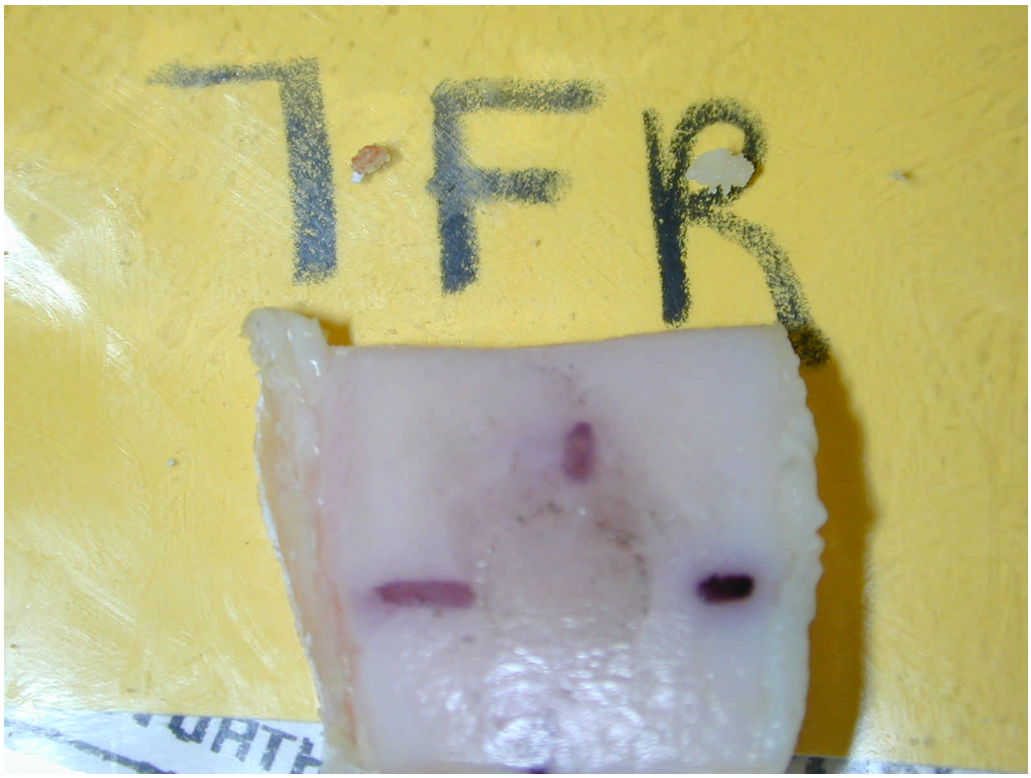
Specimen Number	Peak Force (N)	Peak Disp (mm)	Total Loading Energy (J)	Hysteresis Energy	Loss Tangent
Fresh Specimens					
6FL	2440	0.6	0.534	0.217	0.41
7FR	1161	0.6	0.211	0.0948	0.45
8FL	1636	0.59	0.267	0.0987	0.37
9FR	766	0.6	0.134	0.059	0.44
10FL	1900	0.59	0.393	0.185	0.47
11FL	2036	0.59	0.456	0.224	0.49
12FL	1669	0.59	0.487	0.326	0.67
13FR	1280	0.59	0.42	0.31	0.74
14FR	1341	0.59	0.305	0.16	0.52
15FR	1823	0.59	0.409	0.201	0.49
16FR	2415	0.59	0.534	0.268	0.50
17FL	582	0.6	0.117	0.055	0.47
18FR	2031	0.59	0.461	0.236	0.51
19FL	2188	0.59	0.73	0.502	0.69
20FR	1777	0.59	0.345	0.163	0.47
21FL	1593	0.59	0.25	0.086	0.34
Frozen once specimens					
6-20R	2200	0.606	0.446	0.19	0.43
7-20L	1210	0.605	0.247	0.115	0.47
8-20R	1482	0.606	0.29	0.101	0.35
9-20L	976	0.605	0.167	0.063	0.38
10-20R	1382	0.606	0.297	0.132	0.44
Double Freeze					
11-20DR	1701	0.605	0.345	0.145	0.42
12-20DR	2261	0.604	0.577	0.28	0.49
13-20DL	2076	0.604	0.427	0.16	0.37
14-20DL	1326	0.6	0.269	0.109	0.41
15-20DL	2576	0.605	0.702	0.419	0.60
Flash Freeze					
16-80L	1295	0.6	0.253	0.125	0.49
17-80R	1247	0.605	0.243	0.103	0.42
18-80L	2444	0.604	0.586	0.256	0.44
19-80R	2552	0.6	0.515	0.243	0.47
20-80L	1660	0.6	0.338	0.128	0.38
21-80R	1608	0.6	0.3	0.113	0.38

Specimen Number	Strain Energy Density (20%)	Strain Energy Density Loading	Strain Energy Density (Hysteresis)
Fresh Specimens			
6FL	2.1E+06	4.5E+06	1.8E+06
7FR	8.5E+05	1.7E+06	7.7E+05
8FL	1.0E+06	2.2E+06	7.9E+05
9FR	5.8E+05	1.0E+06	4.6E+05
10FL	1.8E+06	3.0E+06	1.4E+06
11FL	0.0E+00	0.0E+00	0.0E+00
12FL	0.0E+00	0.0E+00	0.0E+00
13FR	0.0E+00	0.0E+00	0.0E+00
14FR	0.0E+00	0.0E+00	0.0E+00
15FR	0.0E+00	0.0E+00	0.0E+00
16FR	1.8E+06	5.2E+06	2.6E+06
17FL	0.0E+00	0.0E+00	0.0E+00
18FR	1.8E+06	4.1E+06	2.1E+06
19FL	2.1E+06	9.4E+06	6.5E+06
20FR	1.6E+06	2.7E+06	1.3E+06
21FL	8.0E+05	2.1E+06	7.3E+05
Frozen once specimens			
6-20R	1.0E+06	4.9E+06	2.0E+06
7-20L	7.9E+05	2.3E+06	1.1E+06
8-20R	9.0E+05	2.5E+06	8.8E+05
9-20L	8.3E+05	1.2E+06	4.5E+05
10-20R	1.0E+06	2.6E+06	1.2E+06
	0.0E+00	0.0E+00	0.0E+00
Double Freeze	0.0E+00	0.0E+00	0.0E+00
11-20DR	0.0E+00	0.0E+00	0.0E+00
12-20DR	0.0E+00	0.0E+00	0.0E+00
13-20DL	0.0E+00	0.0E+00	0.0E+00
14-20DL	0.0E+00	0.0E+00	0.0E+00
15-20DL	0.0E+00	0.0E+00	0.0E+00
Flash Freeze			
16-80L	8.5E+05	2.3E+06	1.2E+06
17-80R	0.0E+00	0.0E+00	0.0E+00
18-80L	2.3E+06	5.2E+06	2.3E+06
19-80R	1.1E+06	6.1E+06	2.9E+06
20-80L	1.4E+06	2.7E+06	1.0E+06
21-80R	7.2E+05	2.9E+06	1.1E+06

Specimen Number	Load at 15% strain	Load at 20% strain	Load at 22.5% strain	Modulus 20-22.5%	Modulus 15-22.5%
Fresh Specimens					
6FL	873.9	1590	1978	3.012E+08	2.86E+08
7FR	395	654	881	1.762E+08	1.26E+08
8FL	382	952	1320	2.857E+08	2.43E+08
9FR	272	750	976	2.193E+08	1.95E+08
10FL	779	1465	1831	2.841E+08	2.72E+08
11FL		1060	1355	2.290E+08	
12FL		589	962	2.896E+08	
13FR		812	968	1.211E+08	
14FR		654	815	1.250E+08	
15FR		748	885	1.064E+08	
16FR	744	1186	1492	2.376E+08	1.94E+08
17FL		459	582	1.137E+08	
18FR	693	1305	1647	2.655E+08	2.47E+08
19FL	803	1358	1745	3.004E+08	2.44E+08
20FR	671	1330	1658	2.546E+08	2.55E+08
21FL	344	818	1147	2.554E+08	2.08E+08
Frozen once specimens					
6-20R	460	987	1202	1.669E+08	1.92E+08
7-20L	348	571	710	1.079E+08	9.37E+07
8-20R	391	870	1119	1.933E+08	1.88E+08
9-20L	368	500	670	1.320E+08	7.82E+07
10-20R	440	785	1031	1.910E+08	1.53E+08
Double Freeze					
11-20DR		638	789	1.172E+08	
12-20DR		1053	1229	1.366E+08	
13-20DL		967	1203	1.832E+08	
14-20DL		500	665	1.281E+08	
15-20DL		1289	1472	1.421E+08	
Flash Freeze					
16-80L	390	570	708	1.071E+08	8.23E+07
17-80R		1071	1247	2.176E+08	
18-80L	984	1531	1846	2.446E+08	2.23E+08
19-80R	493	848	1075	1.762E+08	1.51E+08
20-80L	597	1134	1353	1.700E+08	1.96E+08
21-80R	326	611	880	2.088E+08	1.43E+08

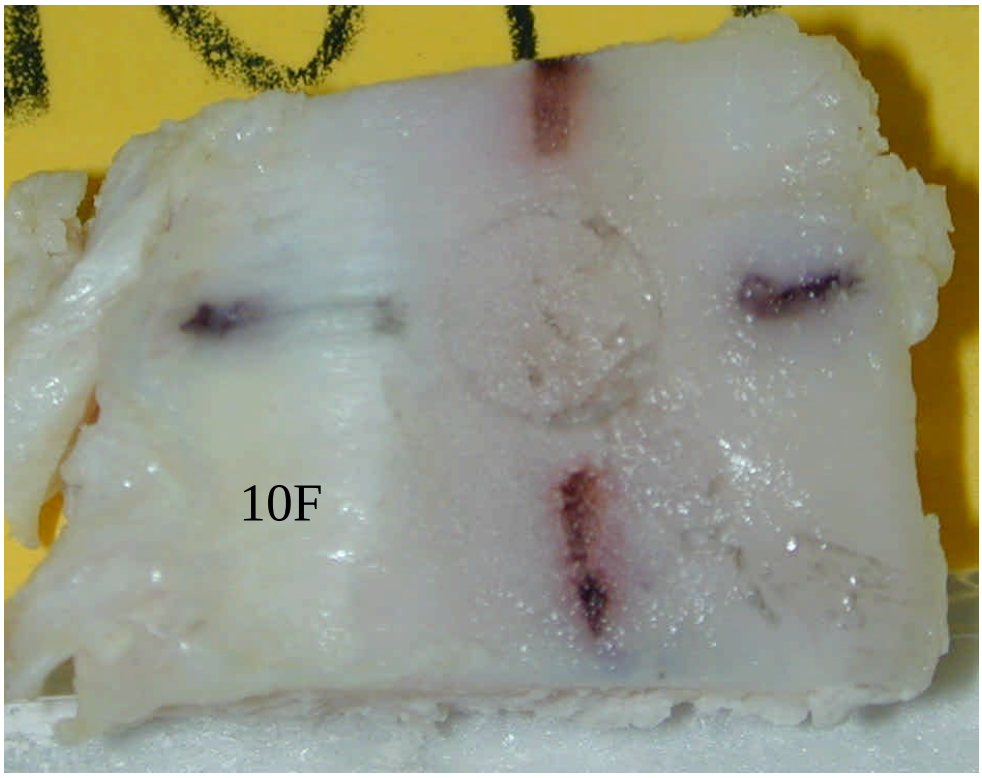
Appendix D: Photographs of Ink stained specimens






8FL


9ER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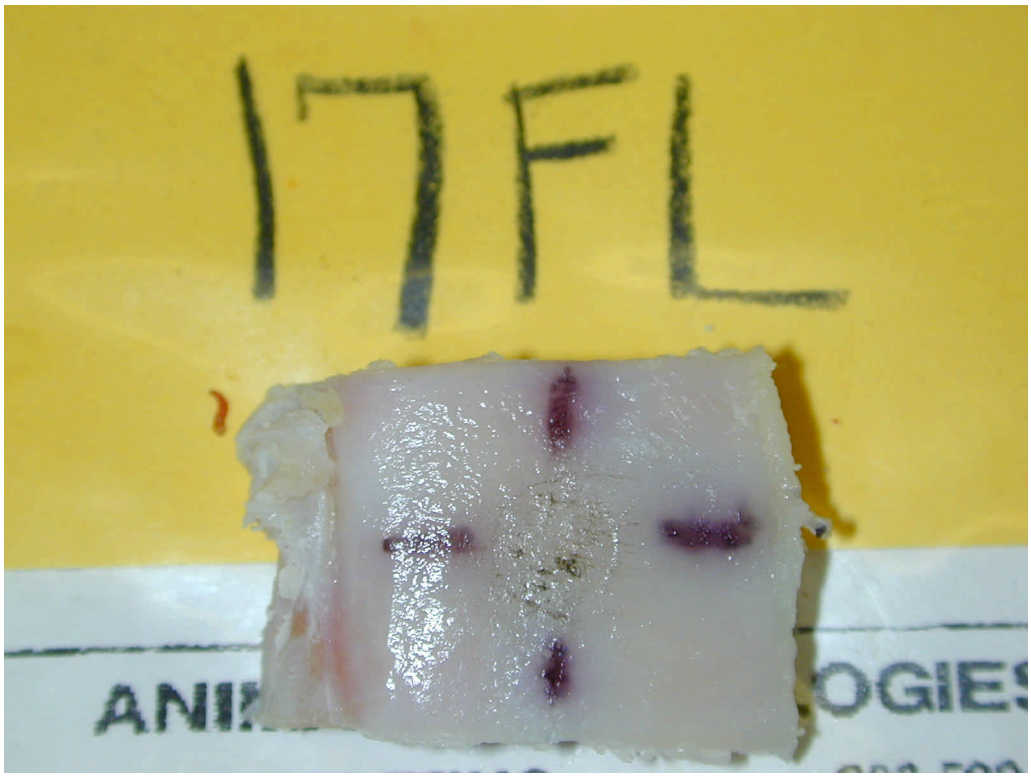
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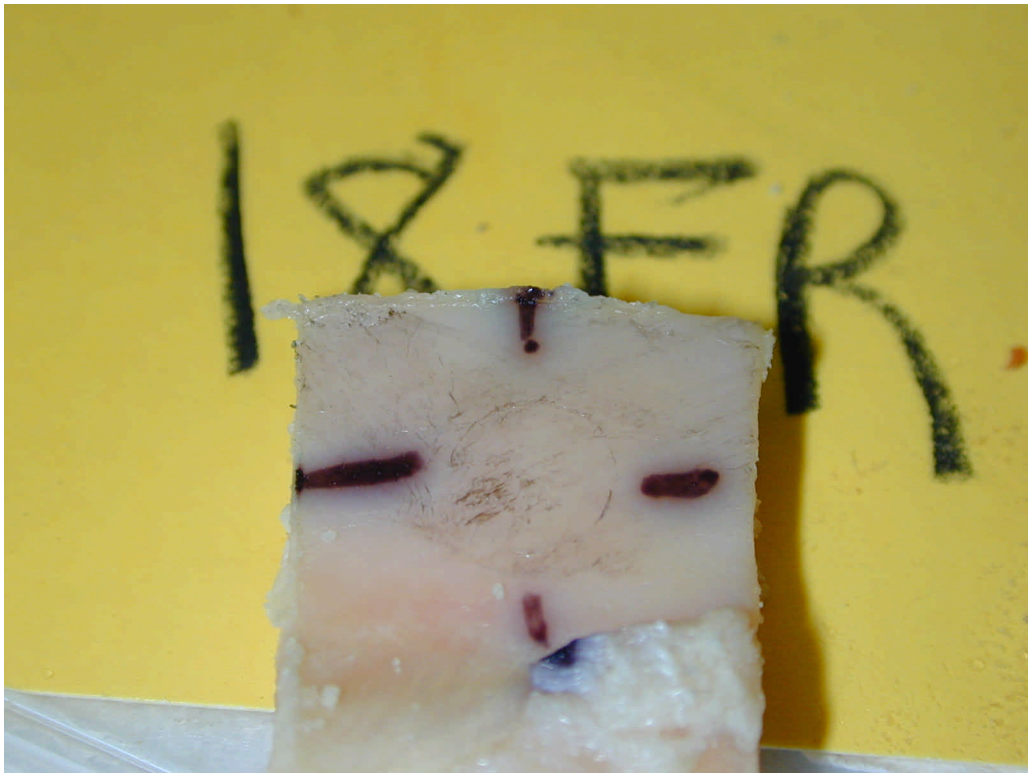
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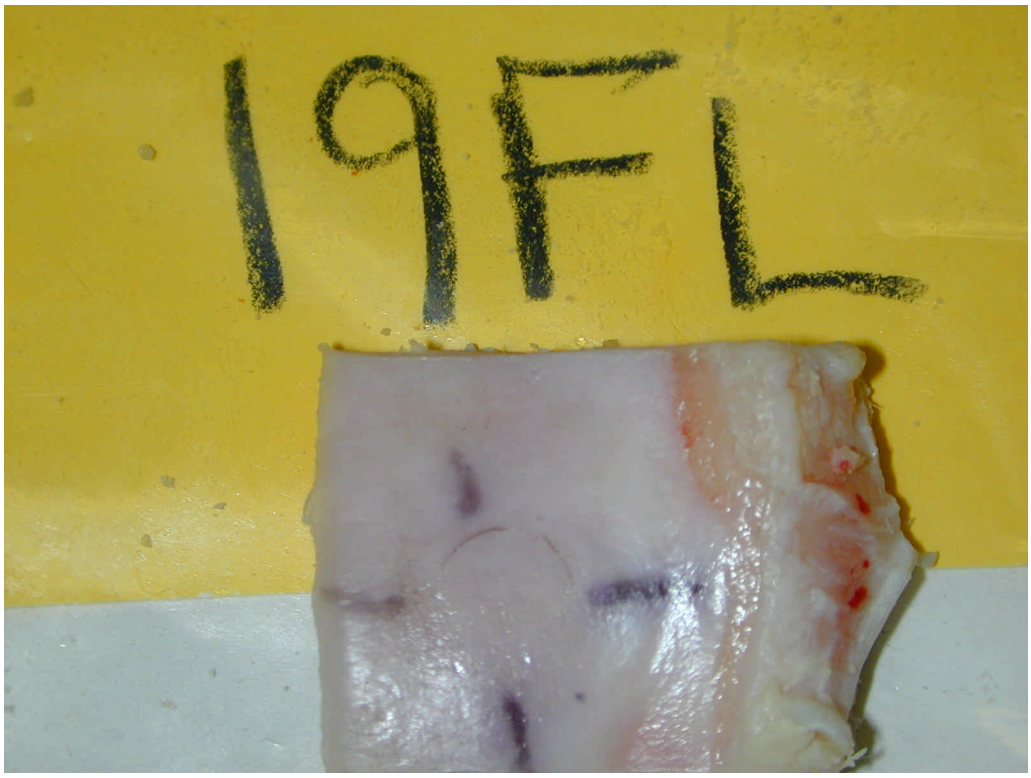
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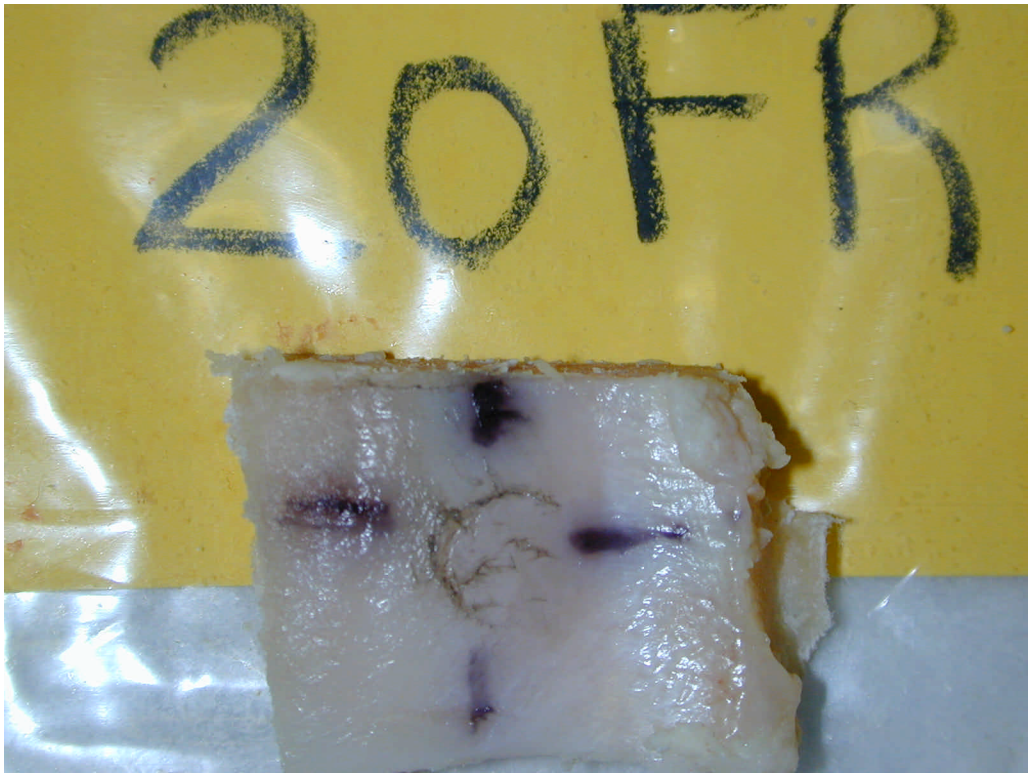
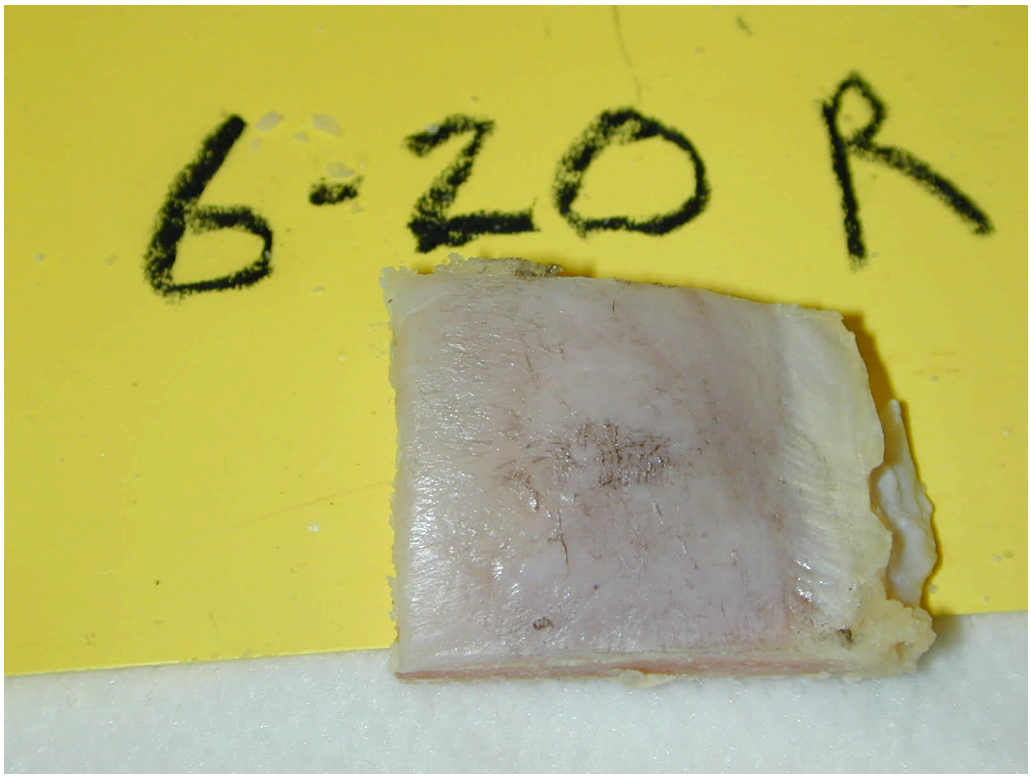
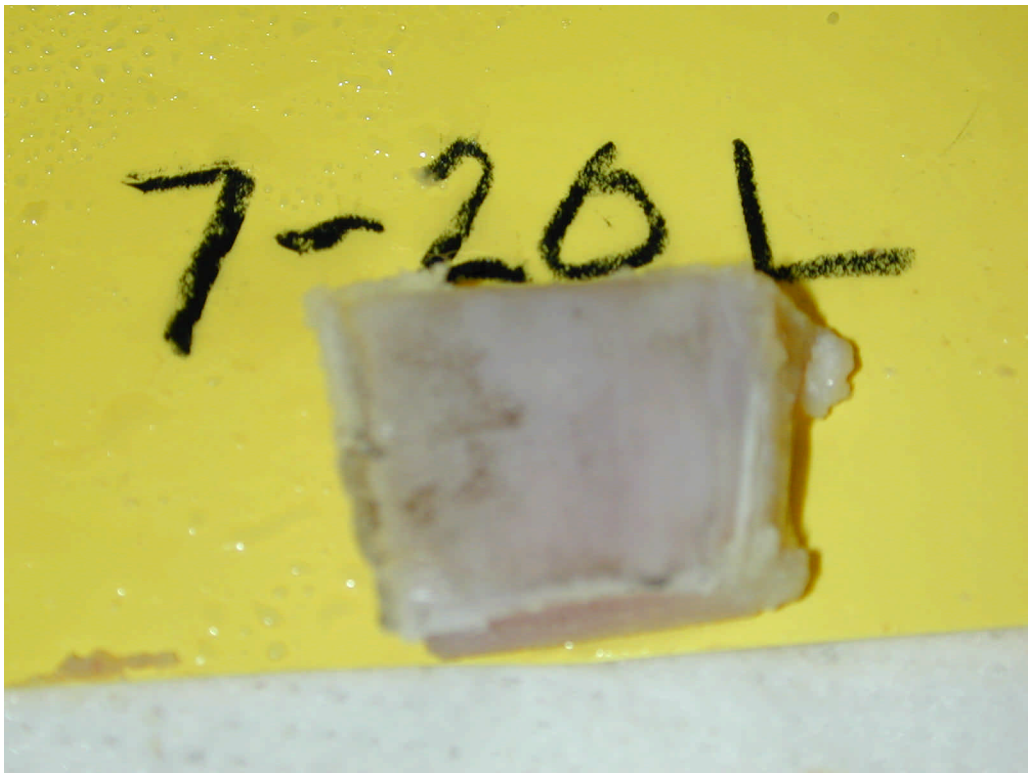

20FR

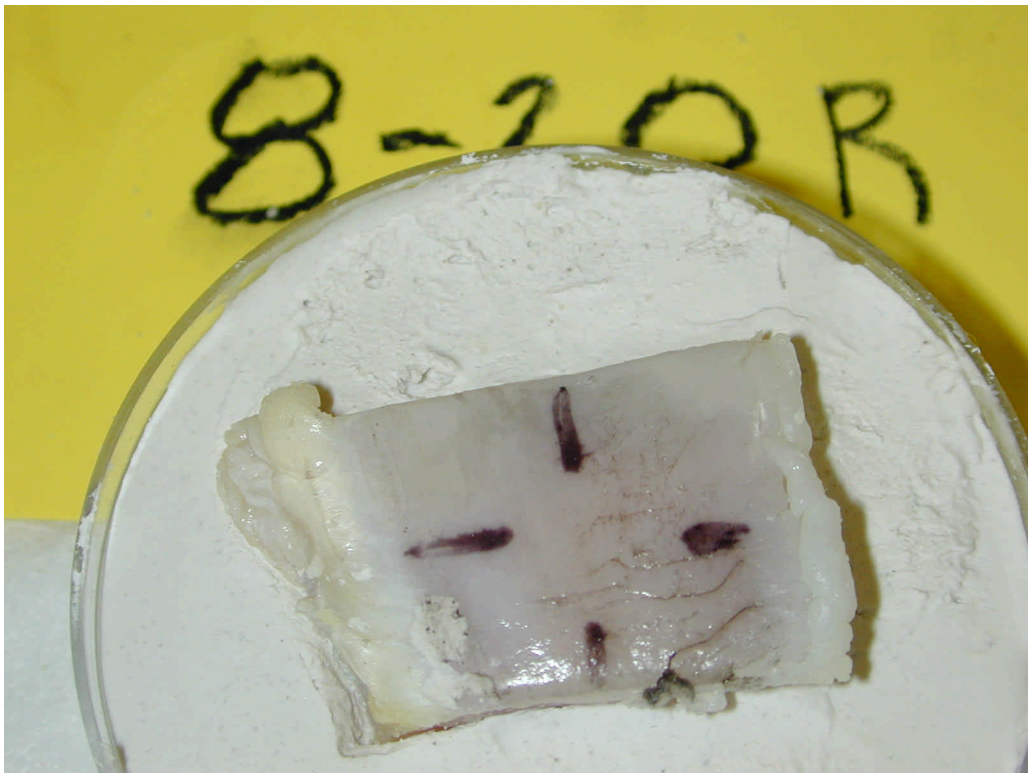

TYLER, TEXAS
FOR FURTHER MANUFACTURING AND/OR


6-20 R


6-20 R

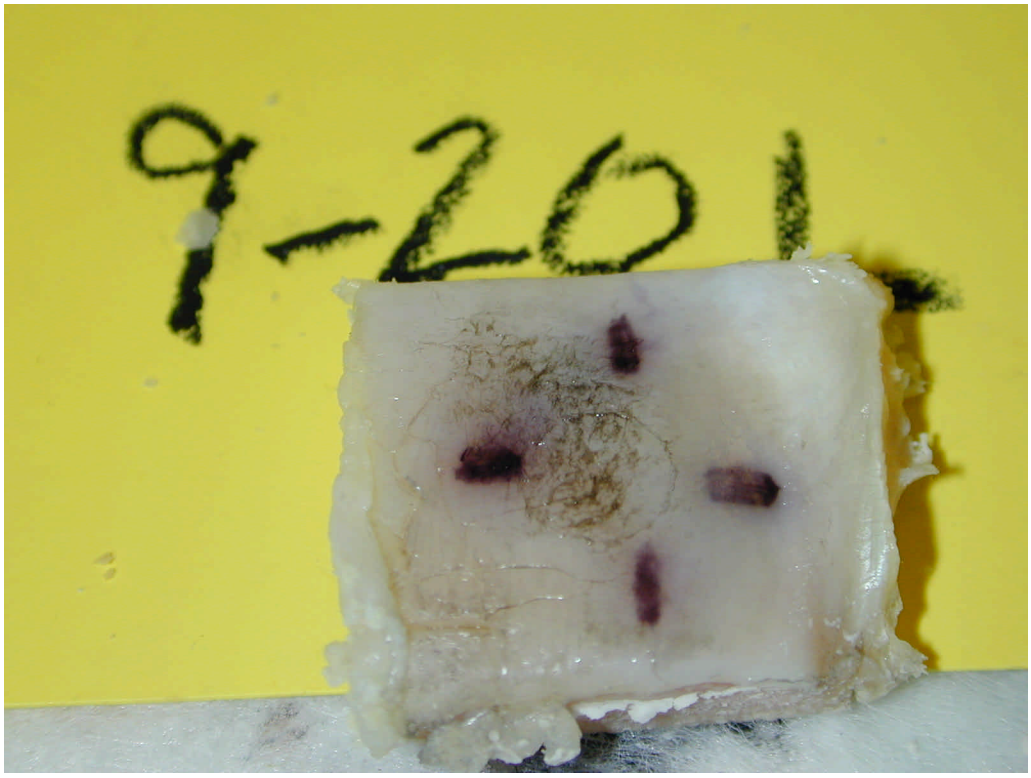

7-20L

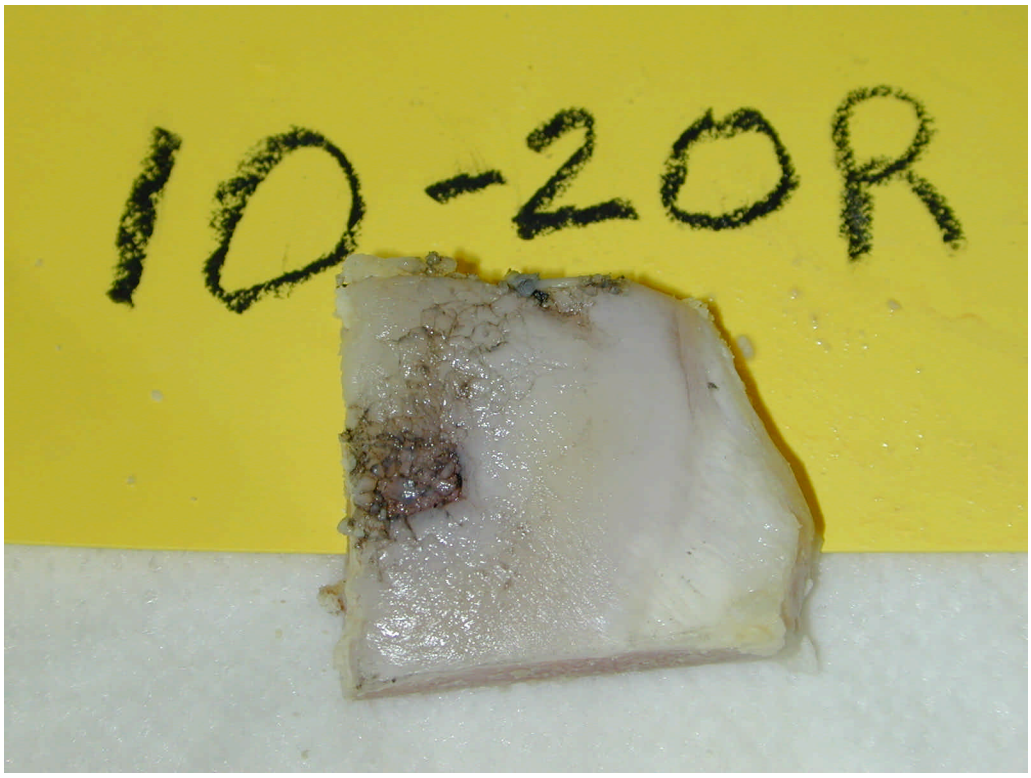

7-20L


8-20R


8-20R


9-20 L


9-20 L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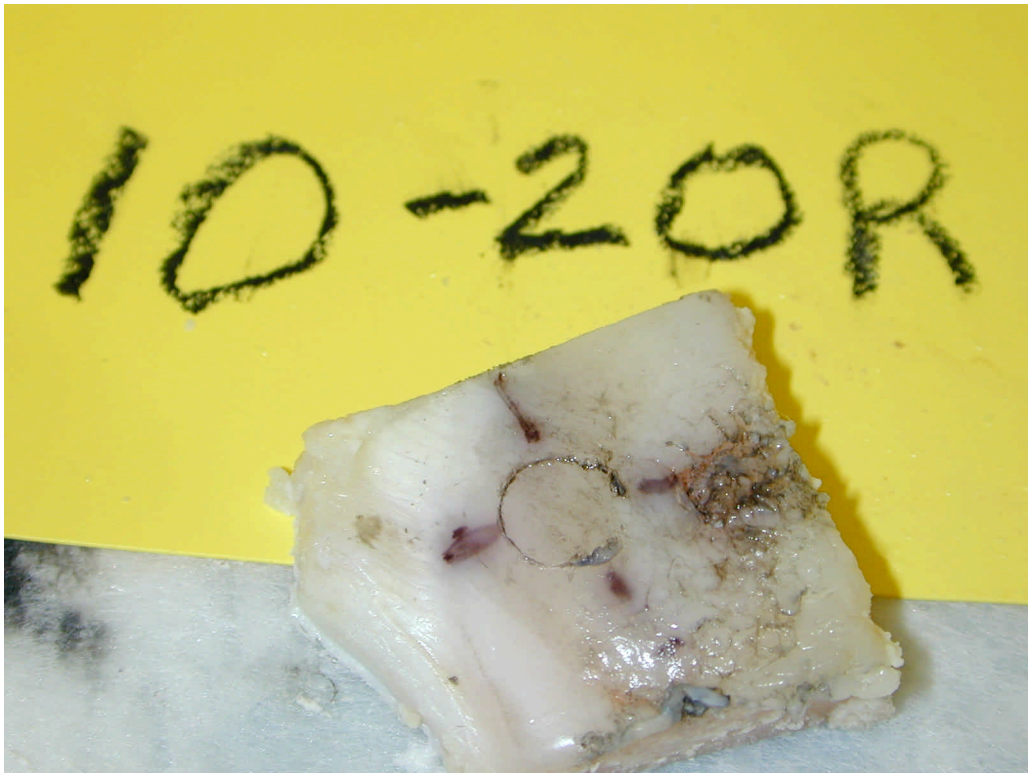
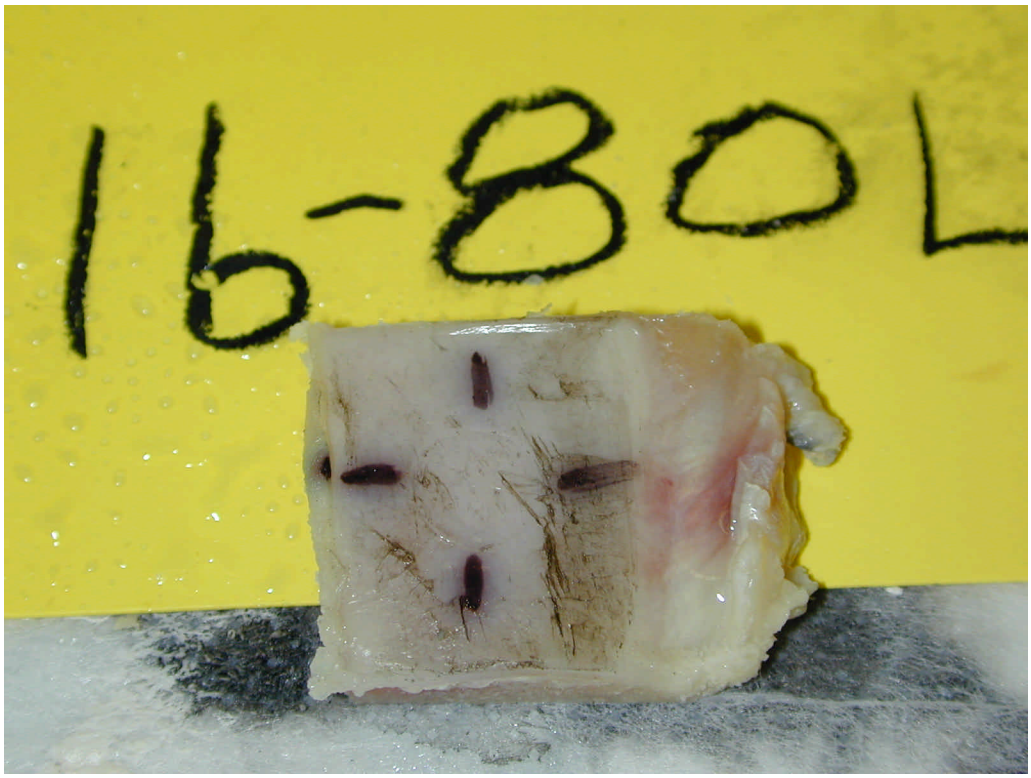

10-20R


16-80L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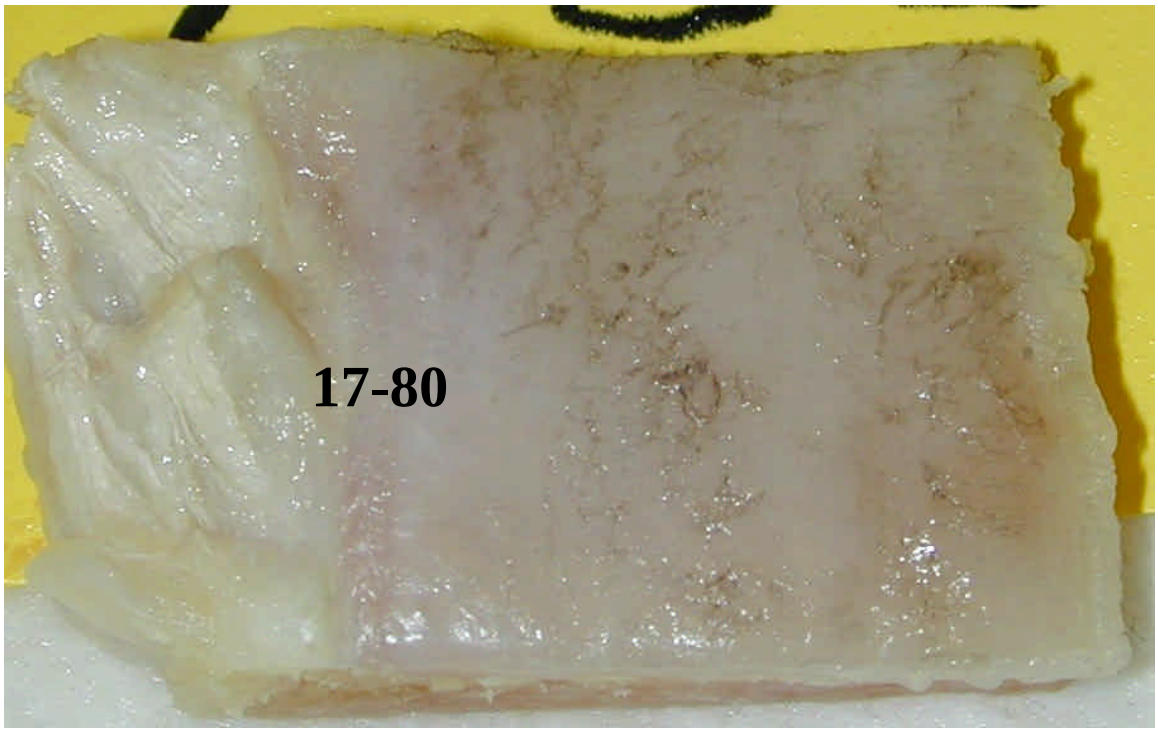
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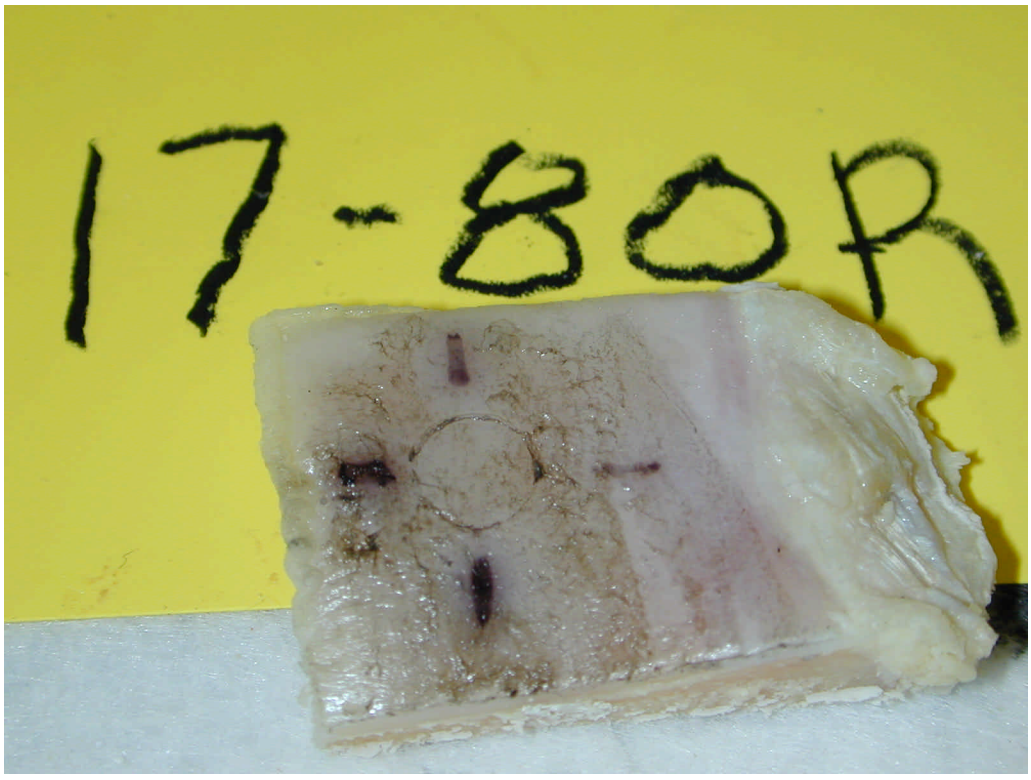
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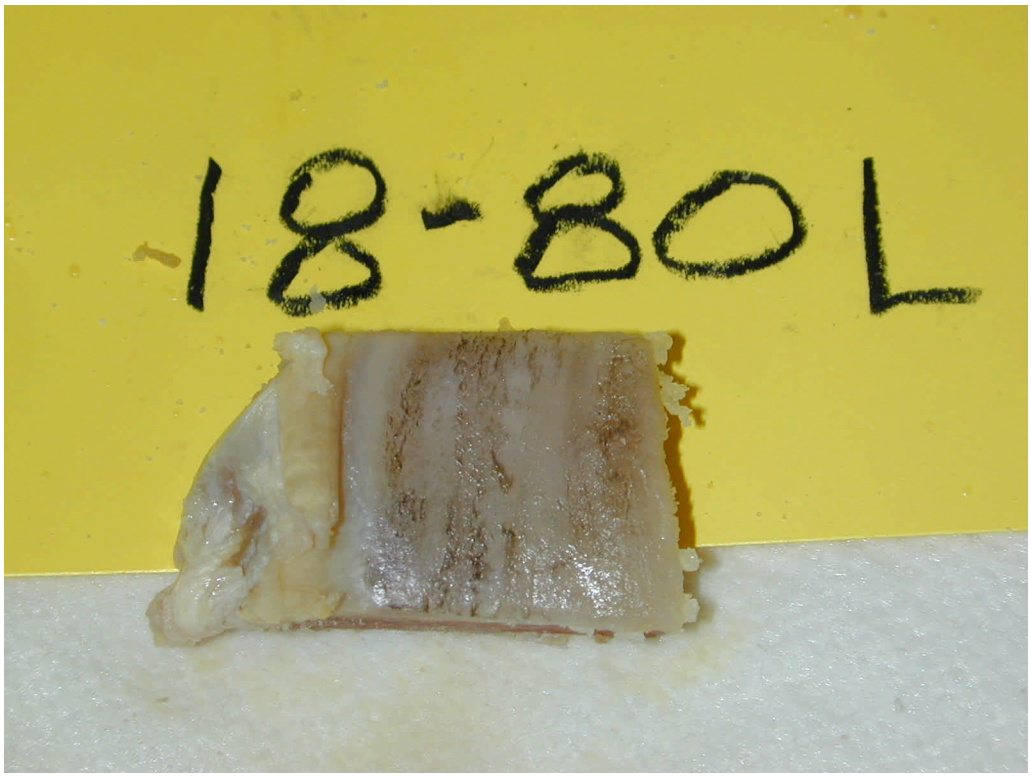
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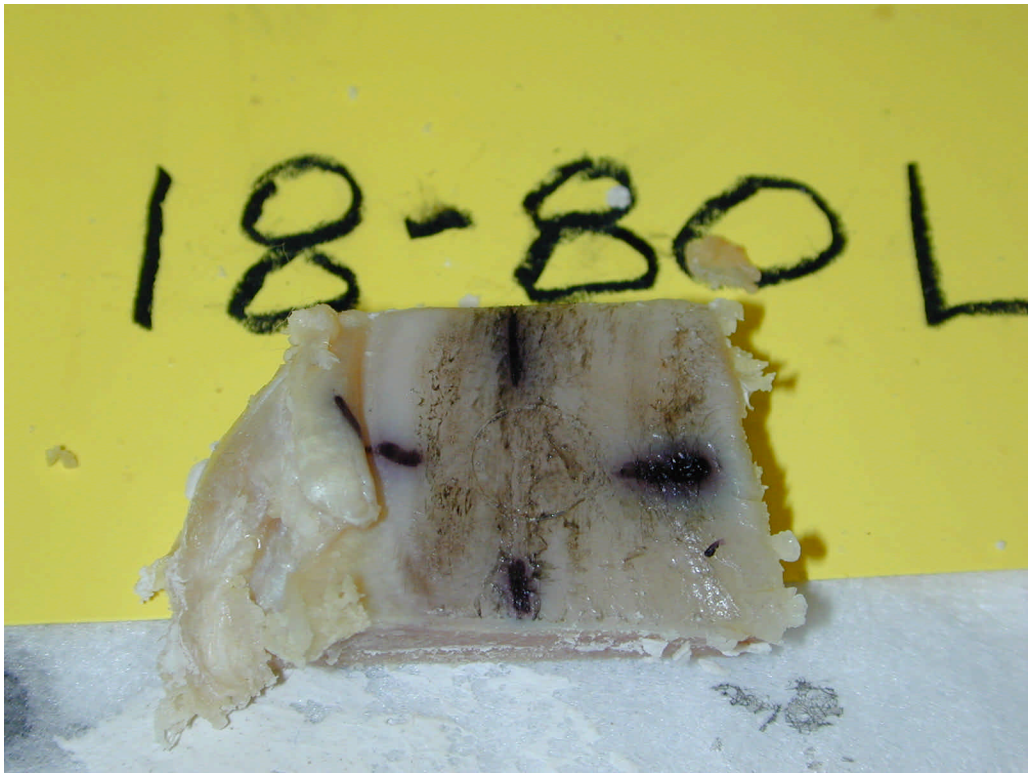
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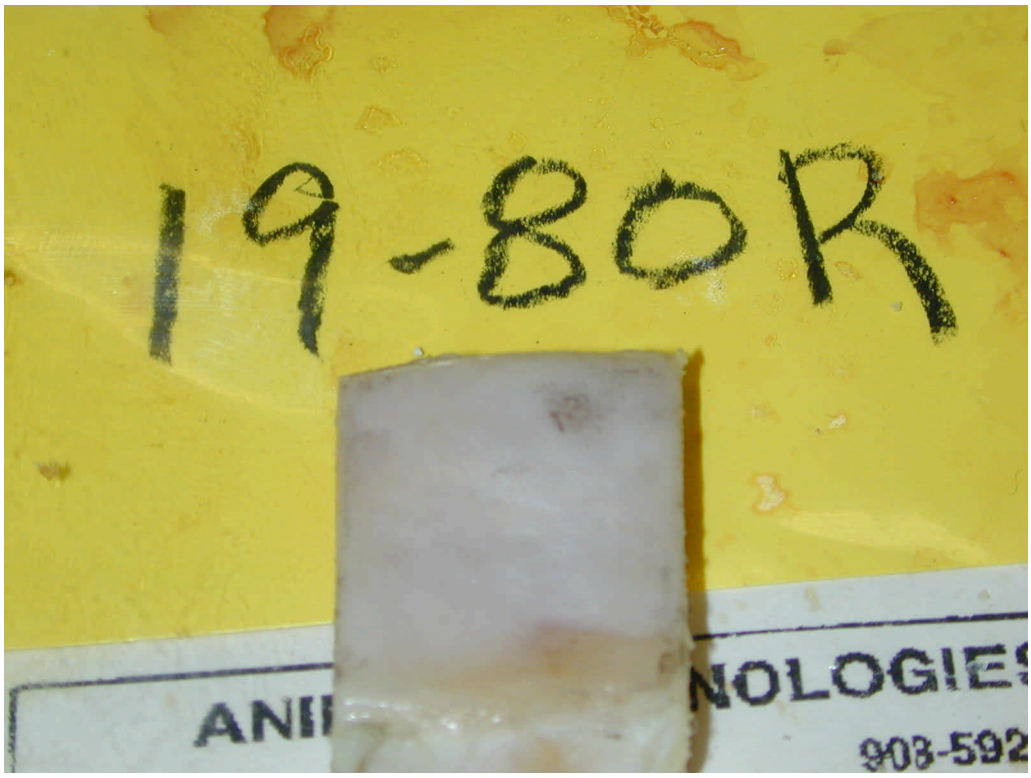
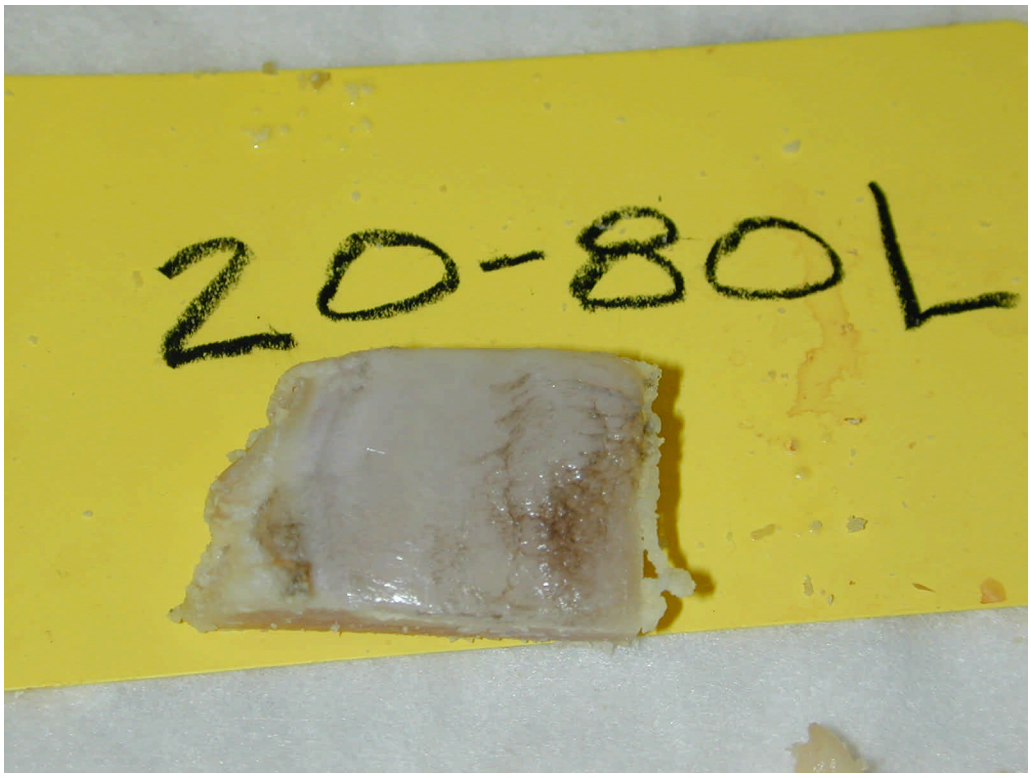

19-80R


19-80R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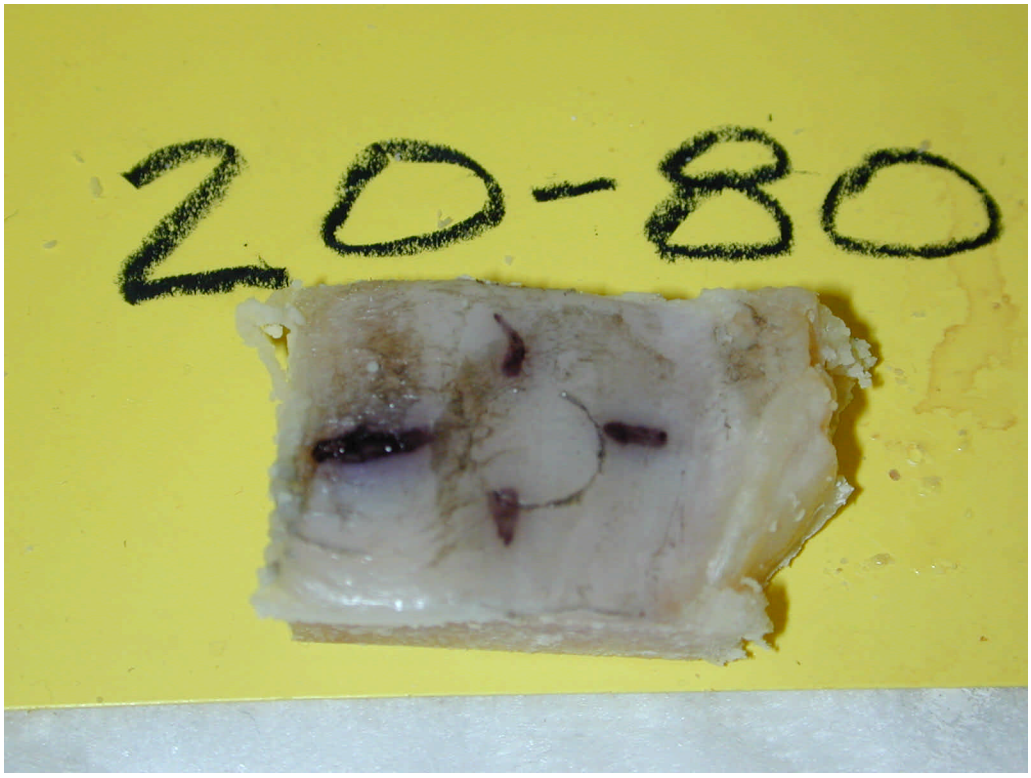
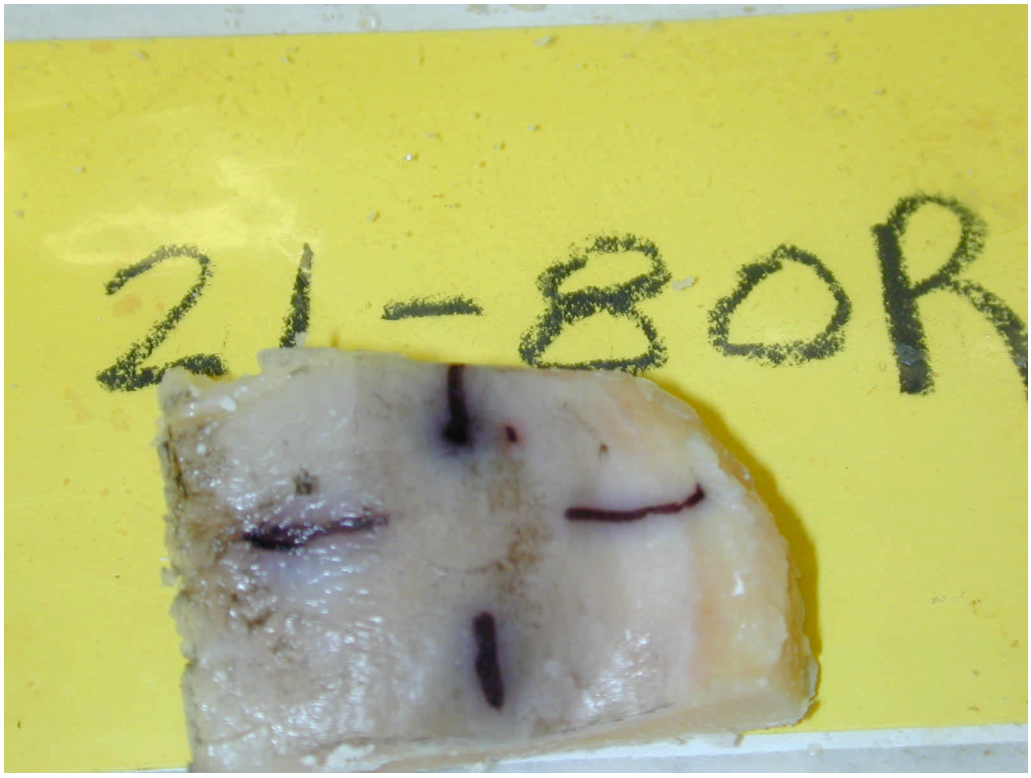
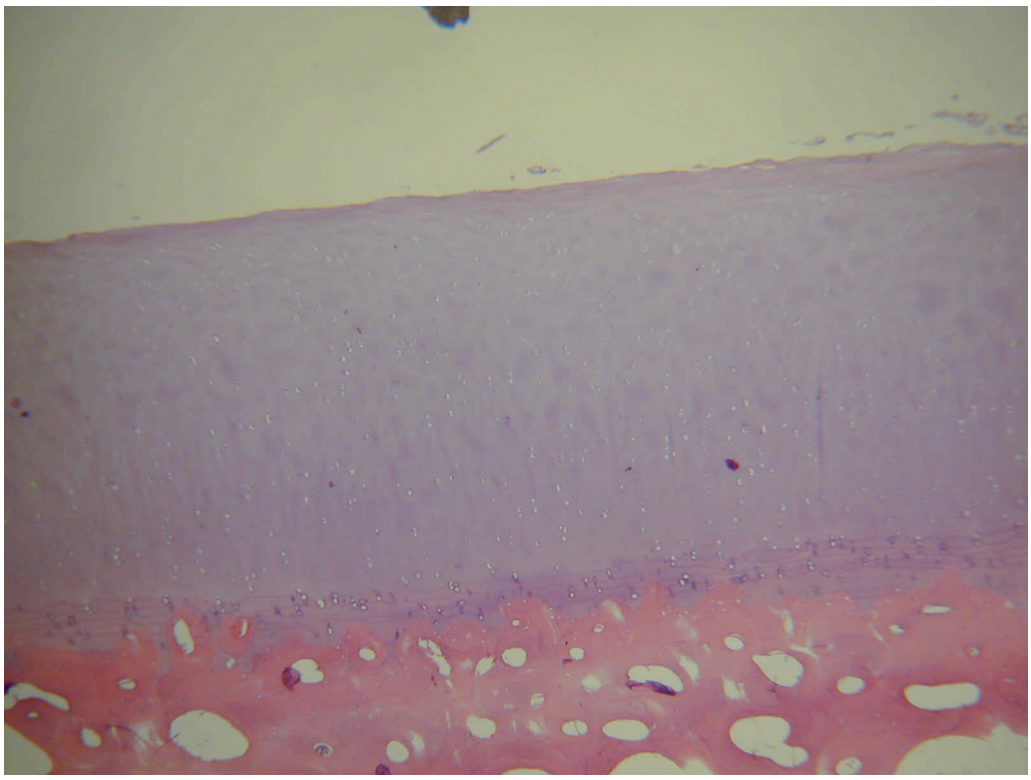

20-80


21-80R


21-80R

Appendix E: Histology Photographs



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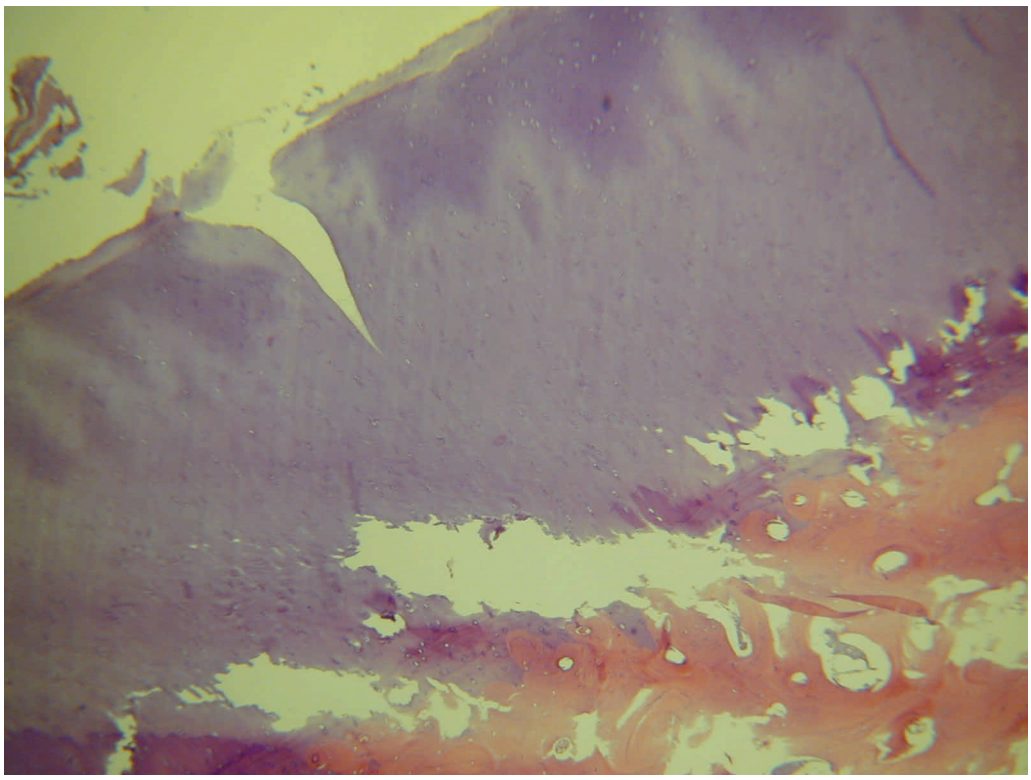

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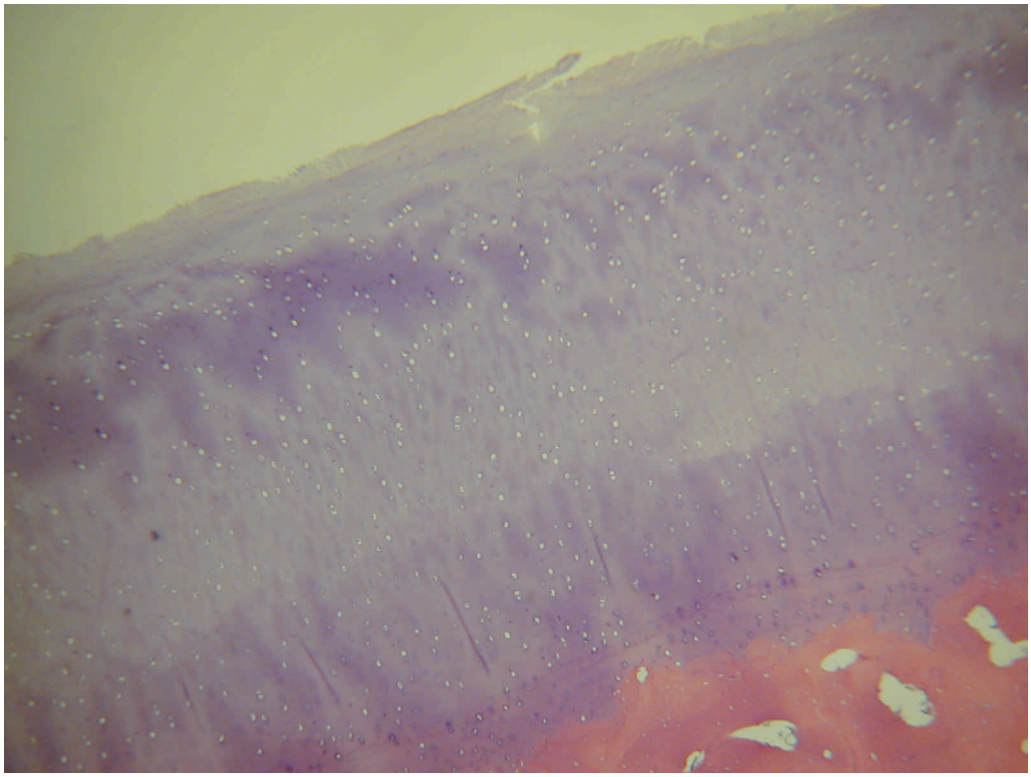

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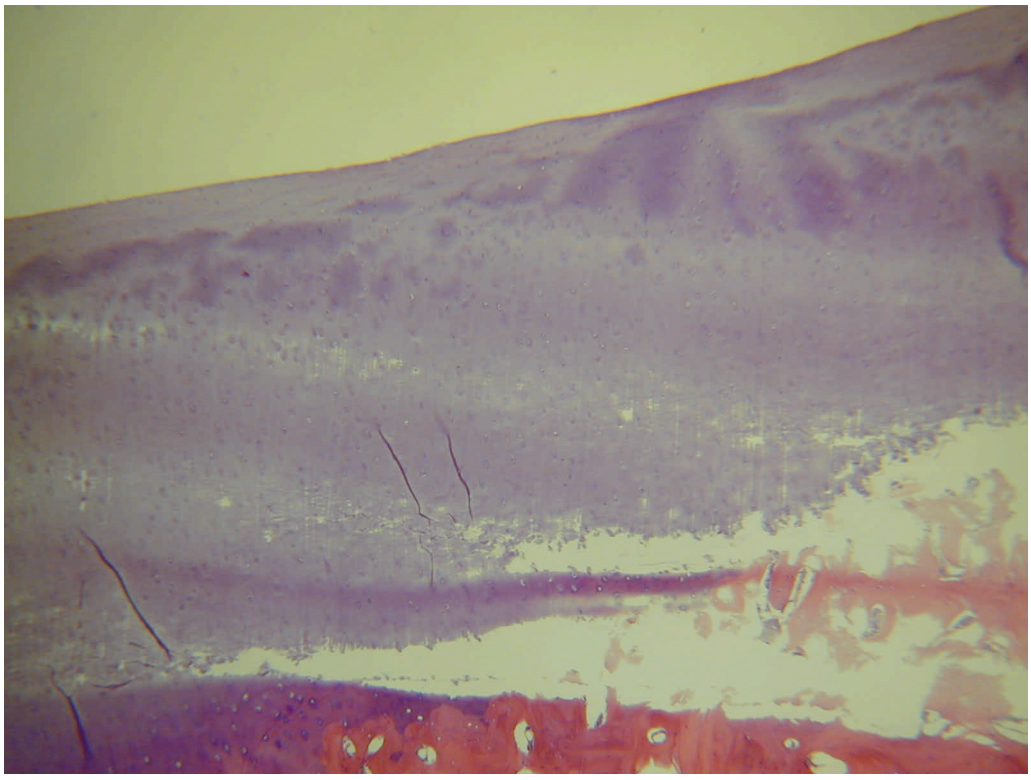

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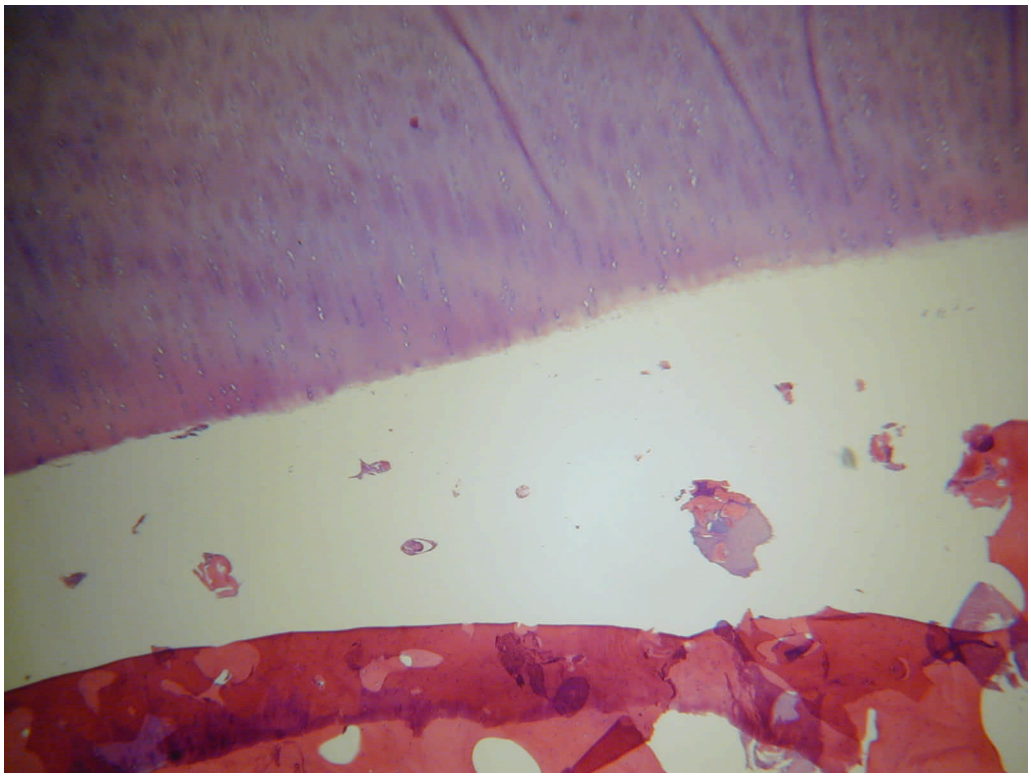
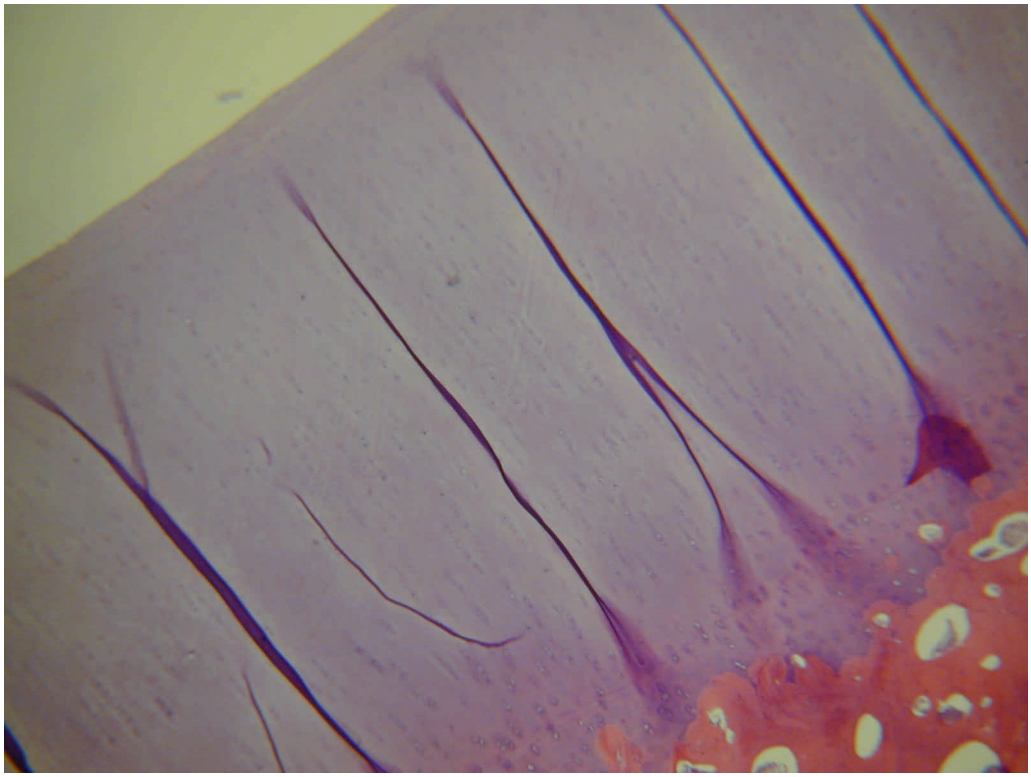


8-20



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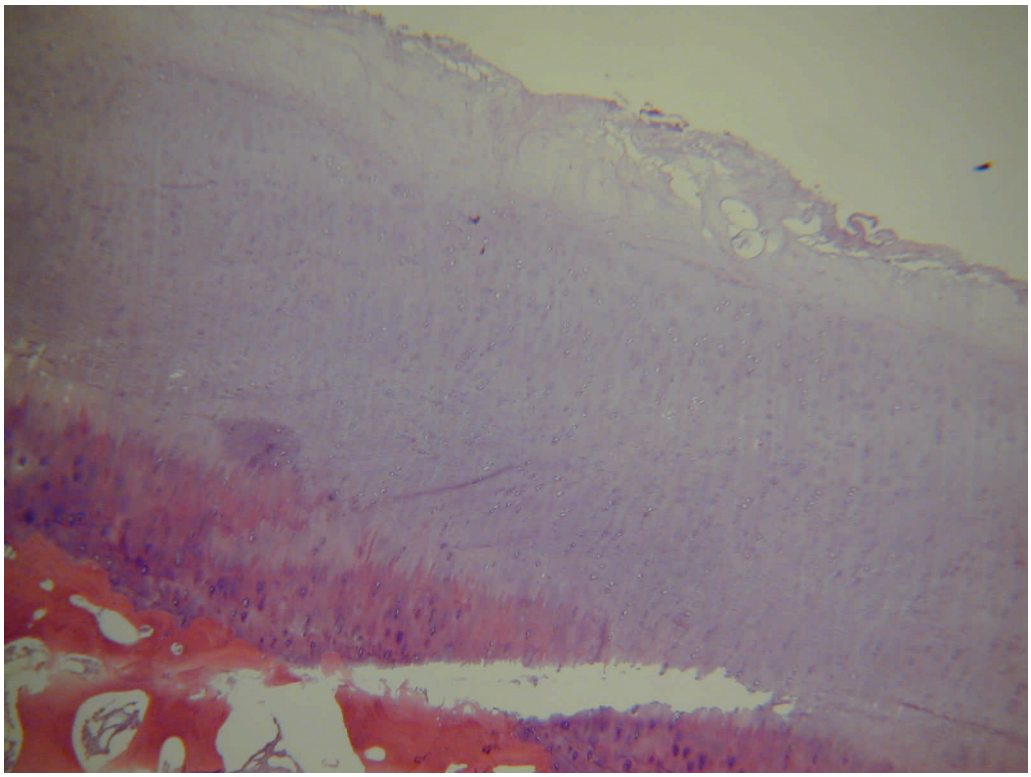

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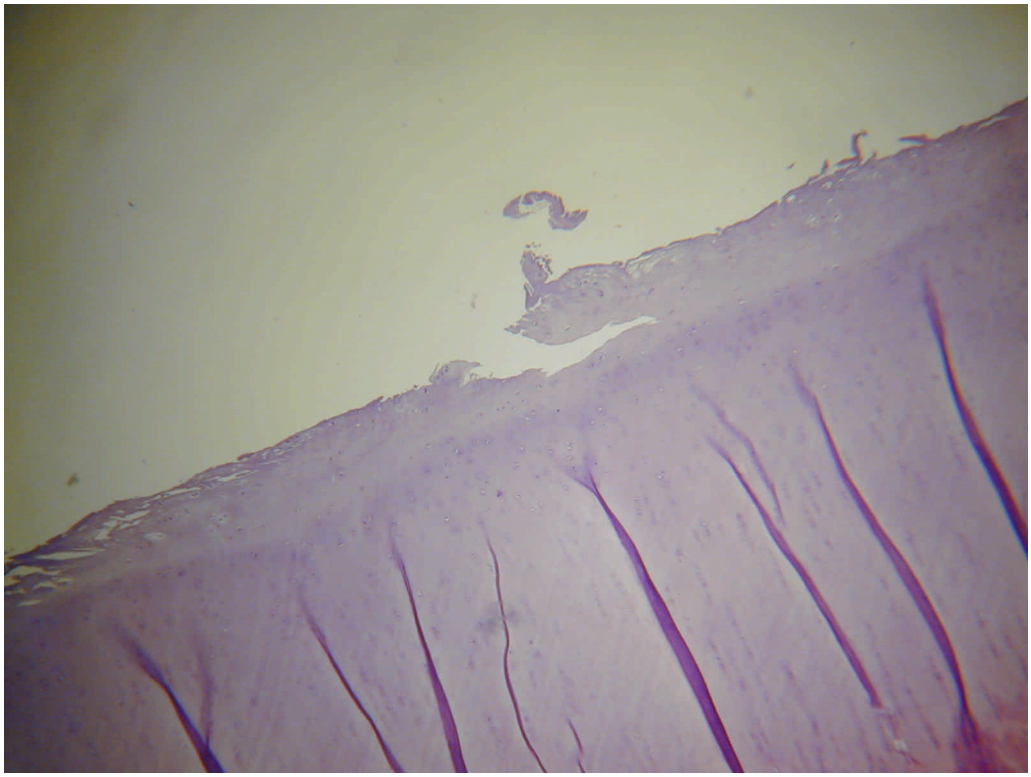

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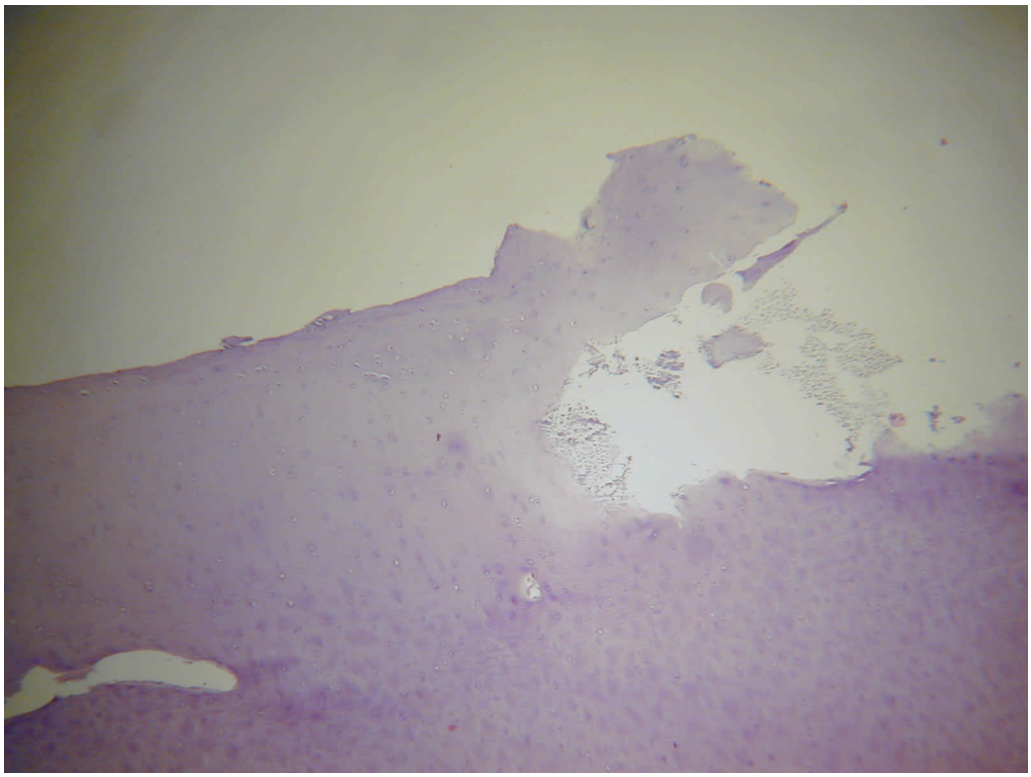

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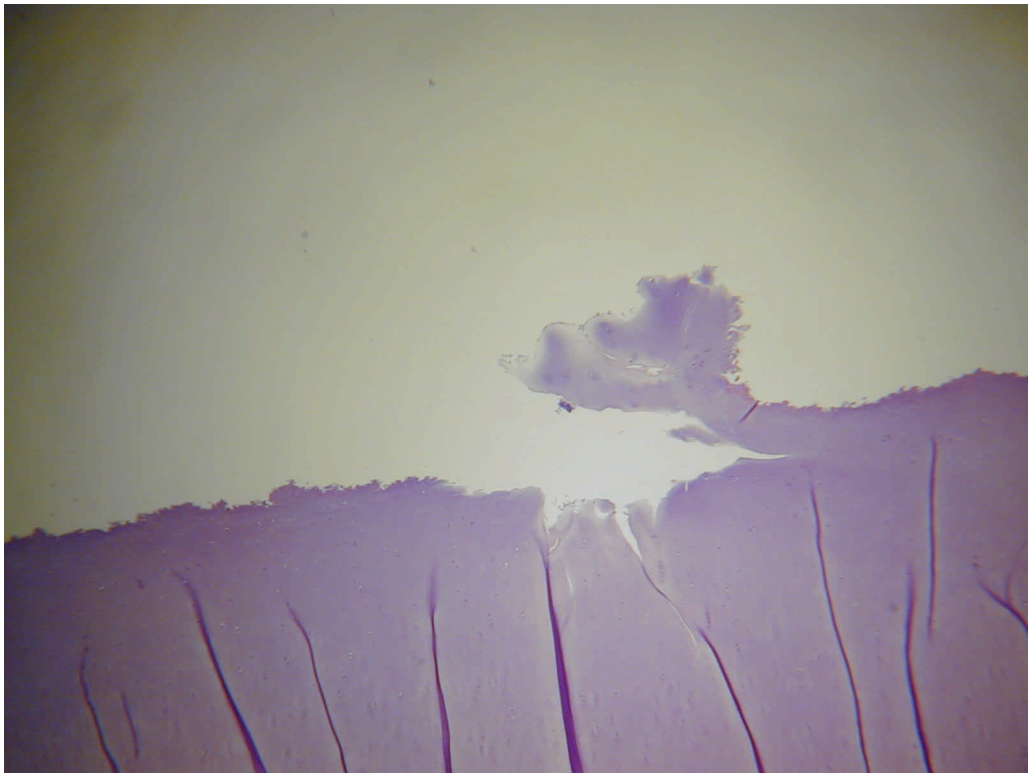

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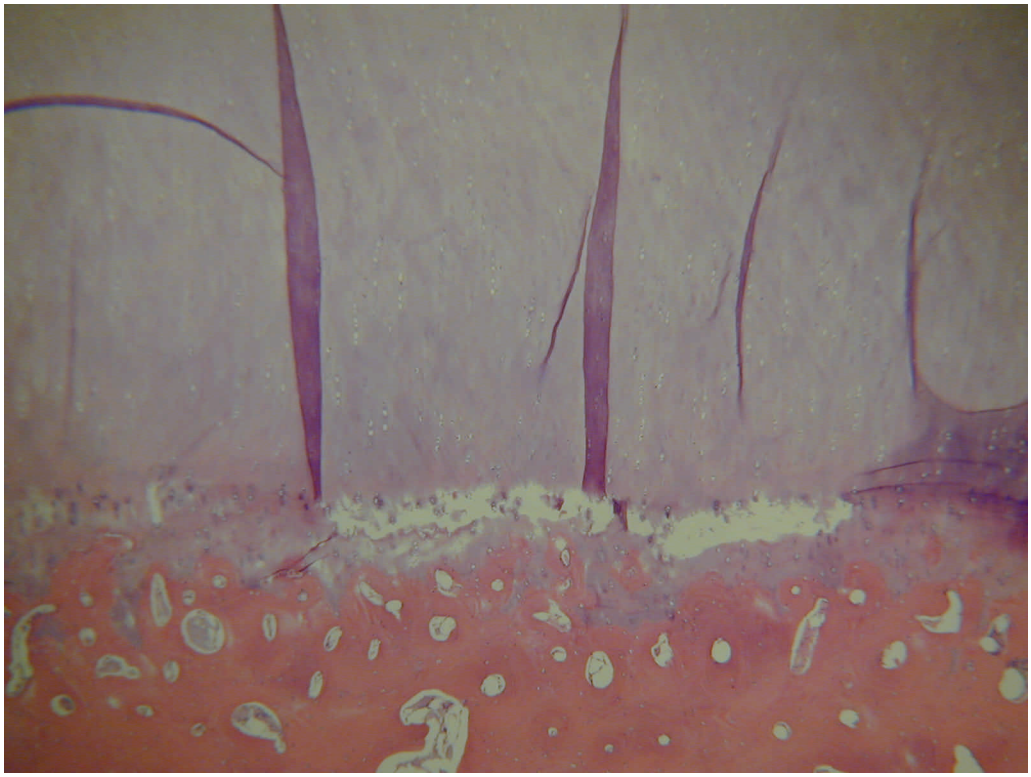

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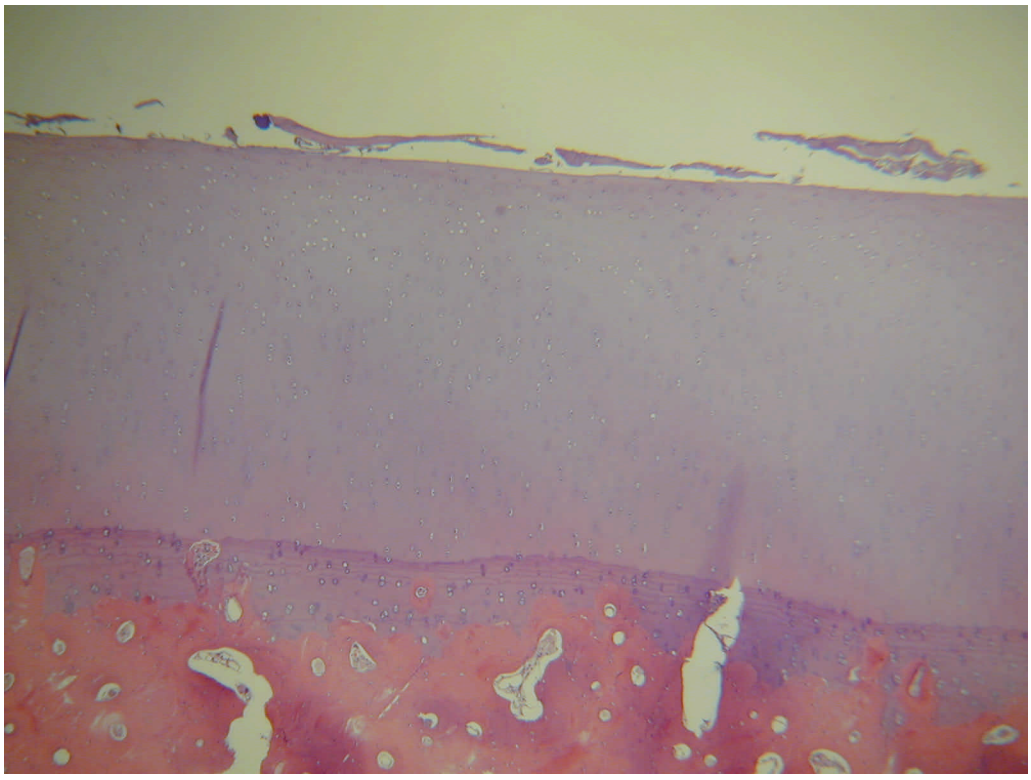

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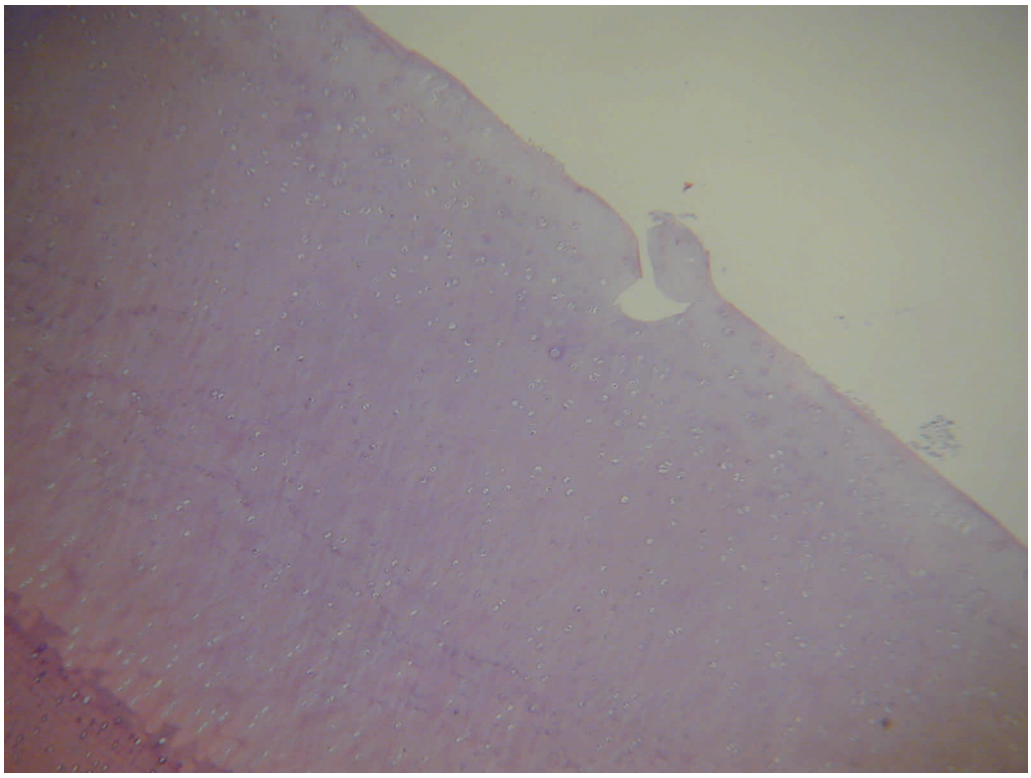

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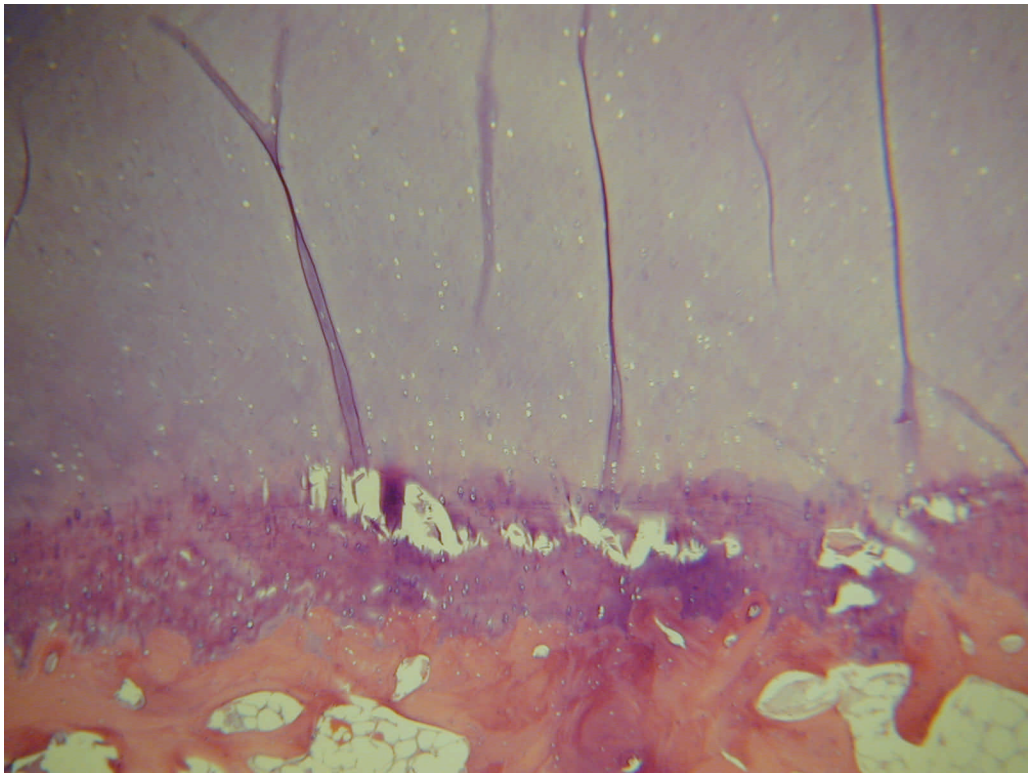

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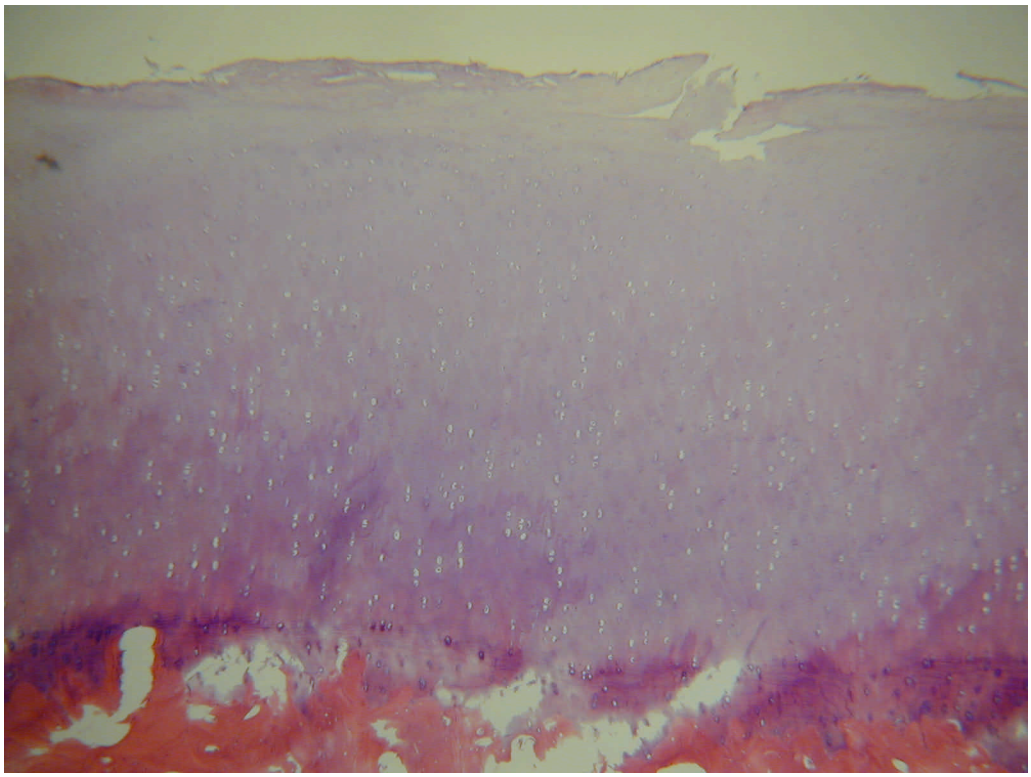

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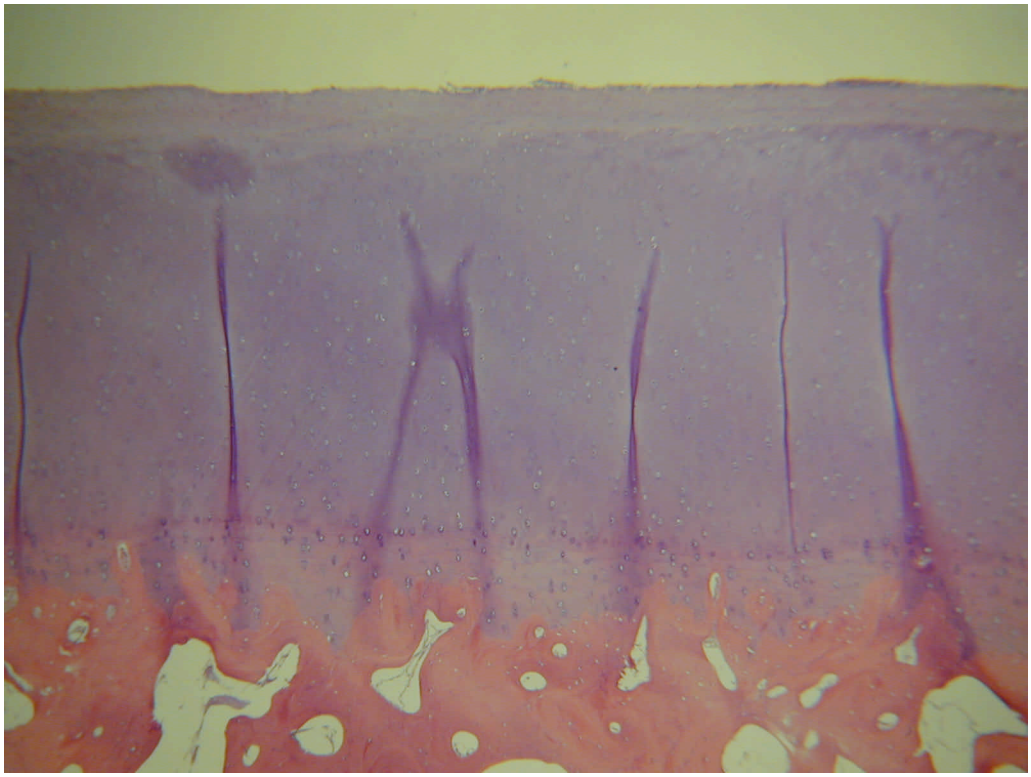

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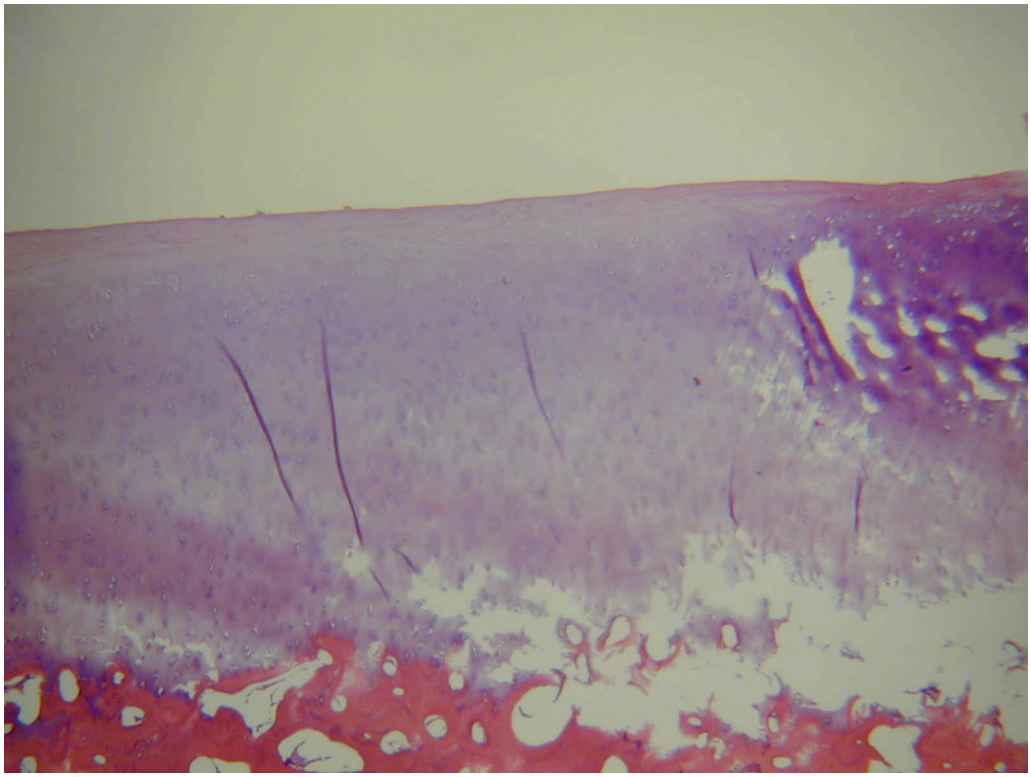

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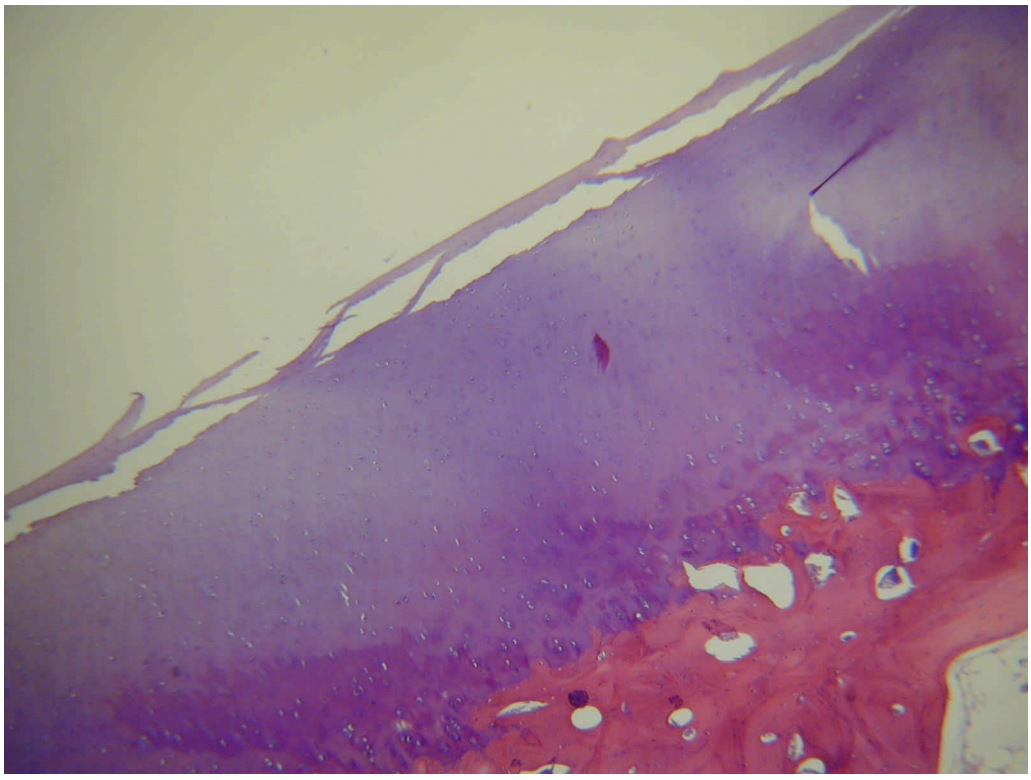

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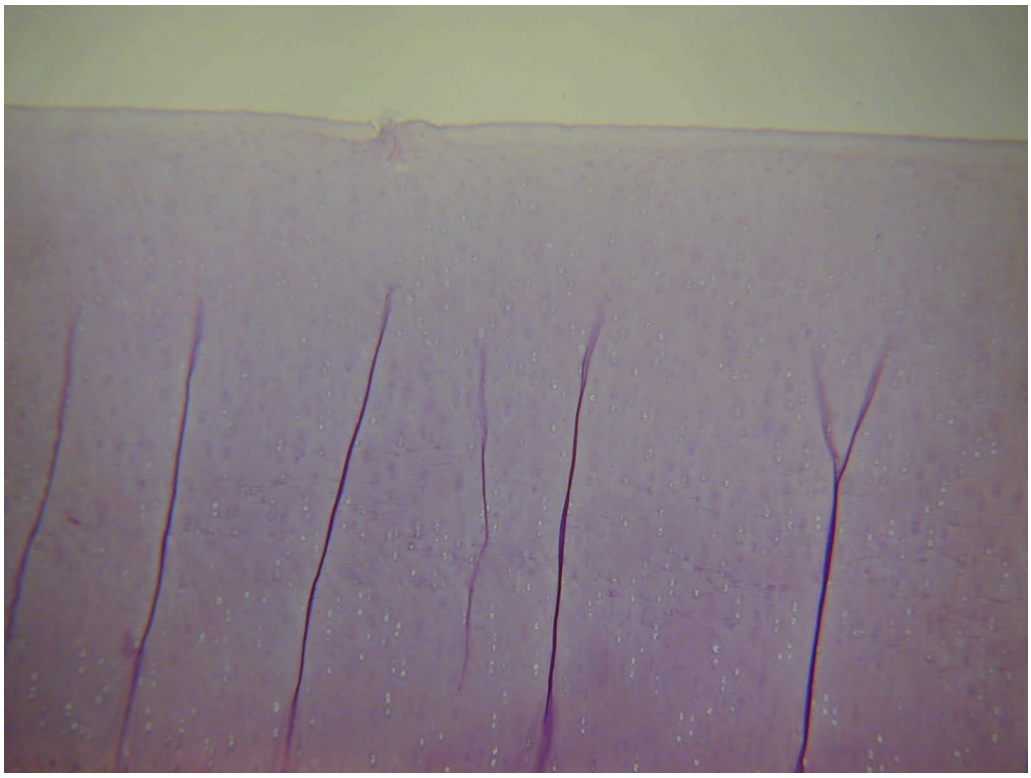

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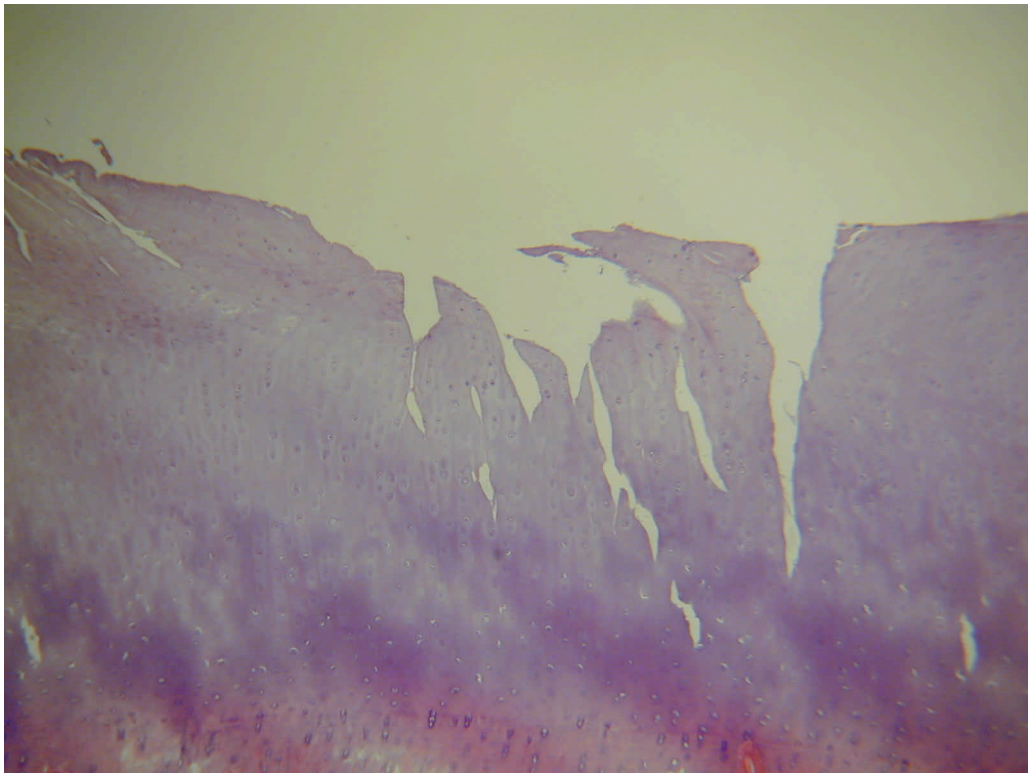

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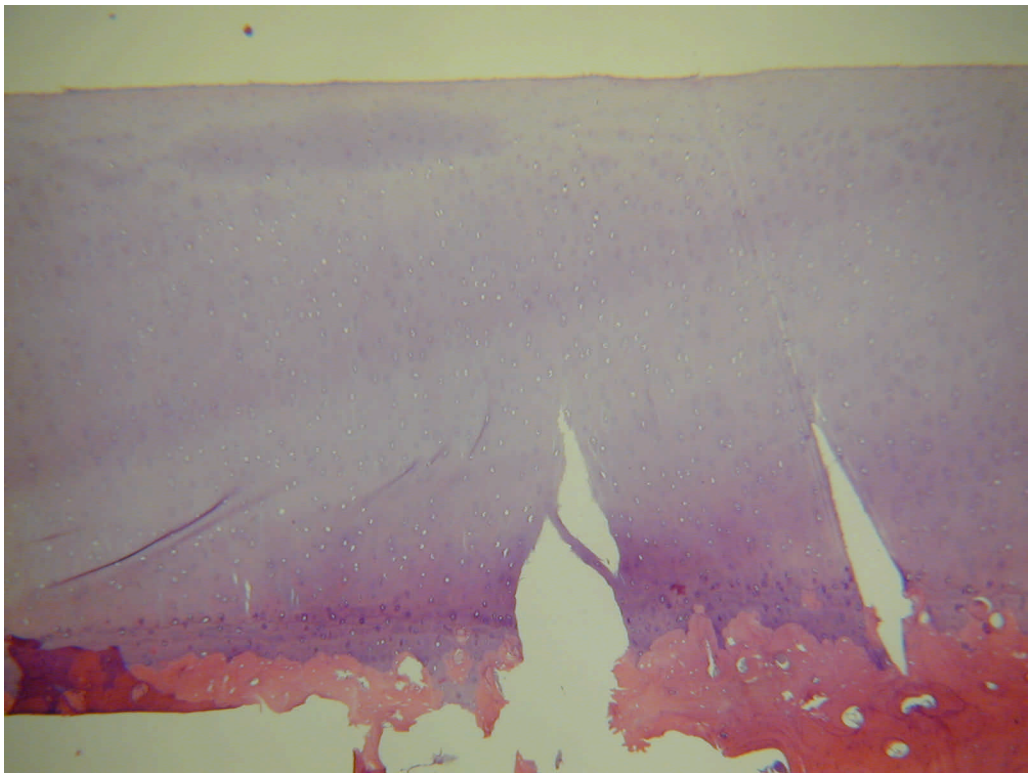

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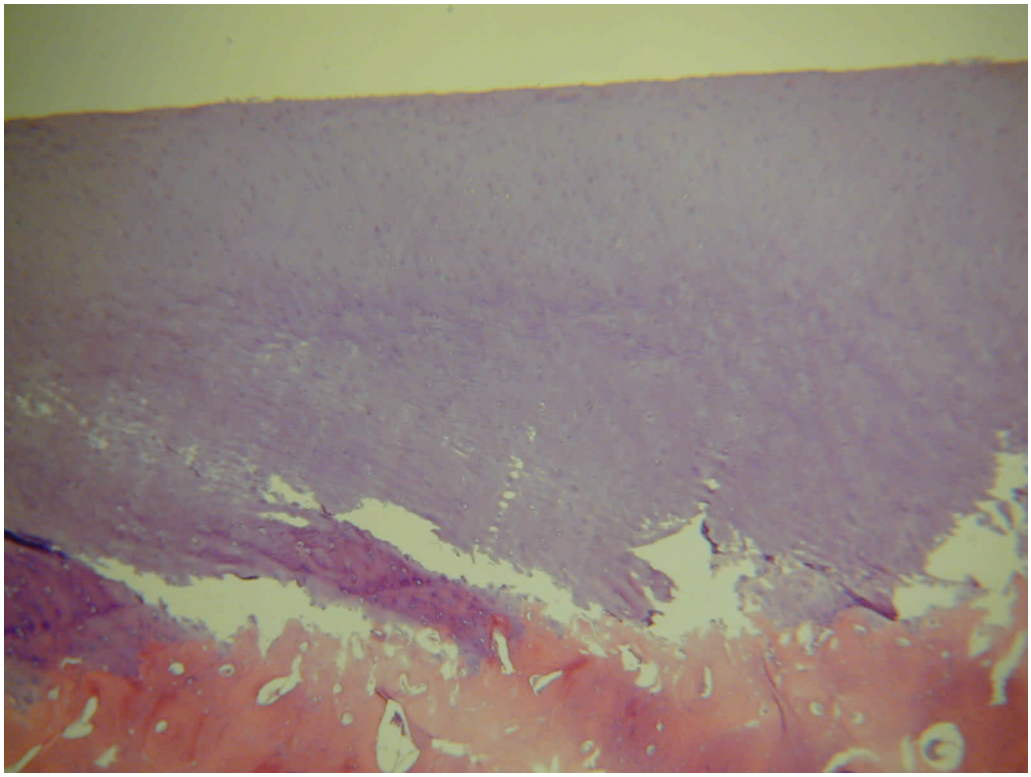
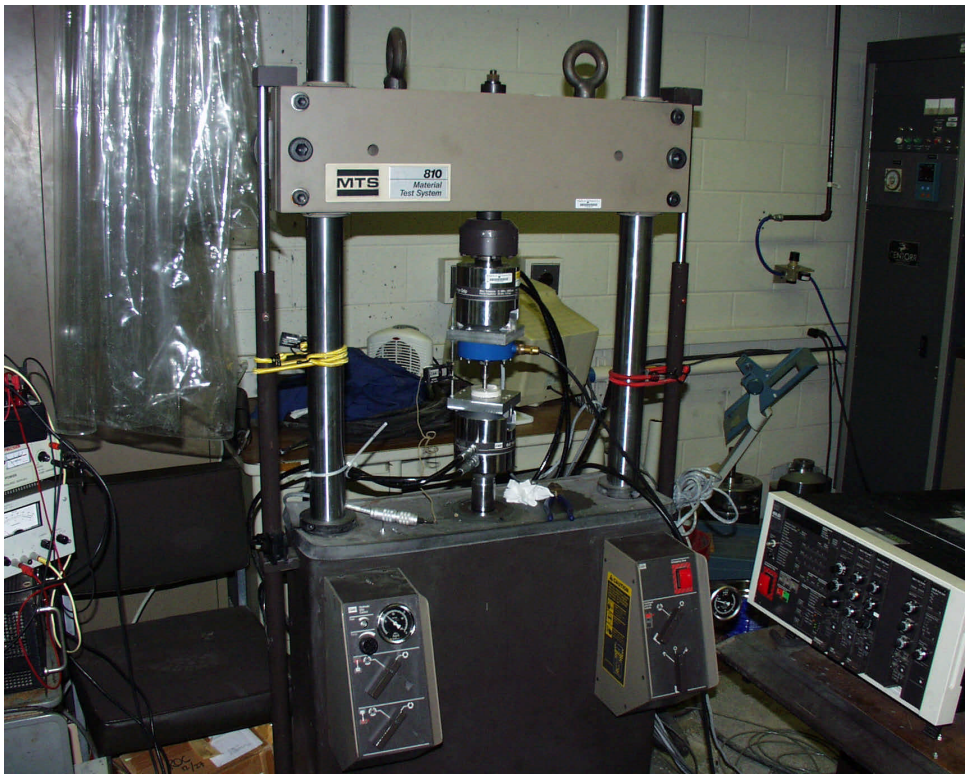
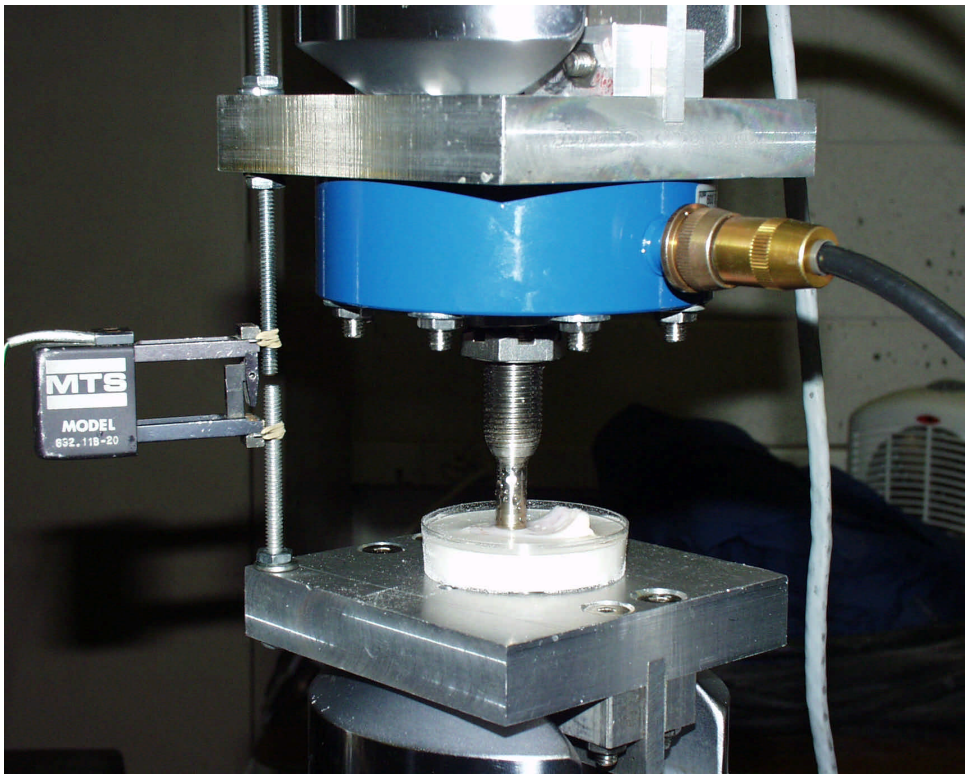
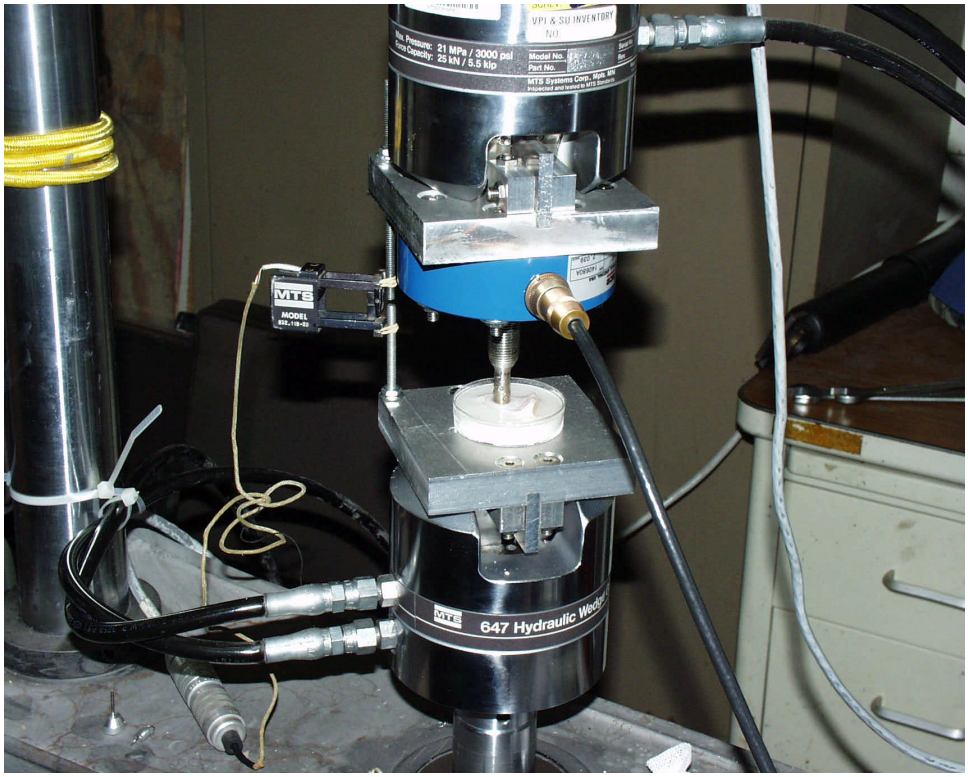


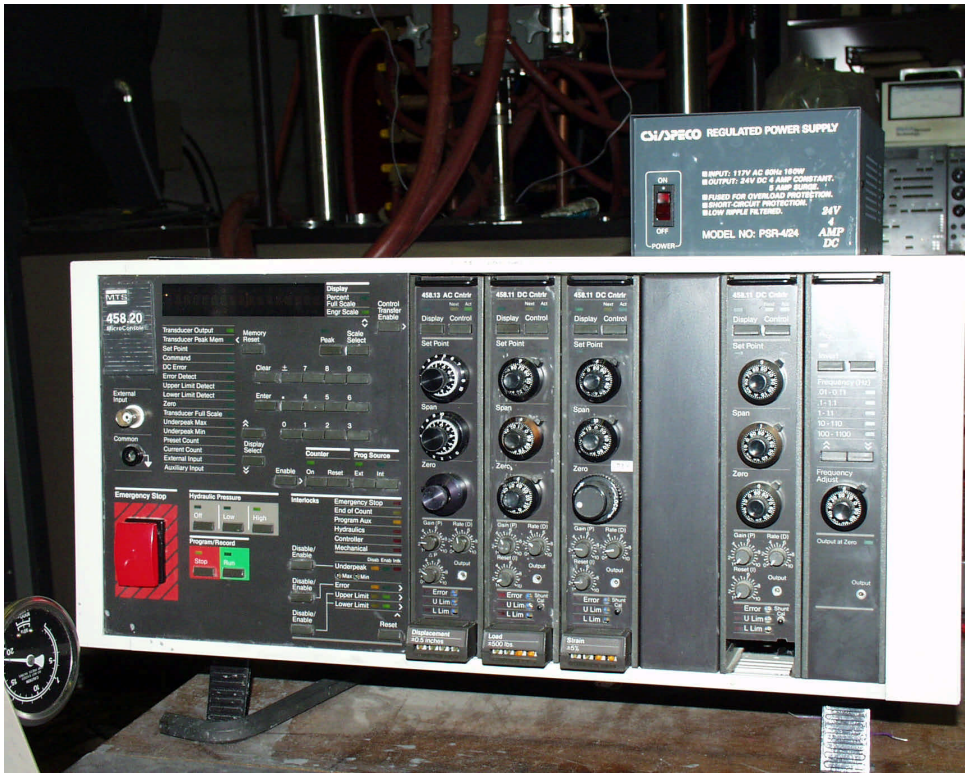
21F

Appendix F: Testing Apparatus Photographs






MTS
MODEL
647 Hydraulic Wedge


CIASPECO REGULATED POWER SUPPLY
MODEL NO: PSR-4/24
458.20
458.13 AC Cntlr
458.11 DC Cntlr


MTS
418.91
MicroProfiler™
Programmed
Direct
Remote
Mode Select
Display Output
Edit Program
Edit Block
Set Scale
Run Enable
Functn Select
Prog Readout
Run Prog
Hold
Resume Ret to 0
Output at 0
End
Program/Block
Segment
Rate
Ret Rate
Level
Time
#Repeats
Continuous
Block Retrieved
Ext Breakpoint
End Mark
Error Code
Display Select
Seg
Delete Seg
Insert Seg
Clear/ No
Undo
EEX
Enter/ Yes
Enab Dir Rate 1-9
DELL

Appendix G: Double Freeze Study

Circumstances may require that different body parts be tested at different times. Another possibility is that the same intact cadaver is used for various parts testing on separate occasions. This situation would subject the tissue to multiple freeze-thaw cycles.

The double freeze specimens were frozen at -20 C for 4 days, thawed for 24 hours, and refrozen for 5 days.

Economics and other circumstances often require use of the same cadaver for more than one test resulting in a part being subjected to two of these freeze-thaw cycles hence the double freeze protocol.

One would hypothesize that an alteration in stiffness due to a single freeze thaw cycle would also be realized, if not magnified by the double freeze thaw cycle. However, in the double freeze controls group, no such change was measured. Upon further examination of the double freeze control group, we recognized that two of the five plots appeared to be rather unconventional in shape and hysteresis size. These two fresh specimen F-D plots appear very different than the rest of the controls group as well as their contralateral limb counterparts. It is possible that these specimens were previously damaged prior to testing possibly altering the results.

- Results from these tests were not published.

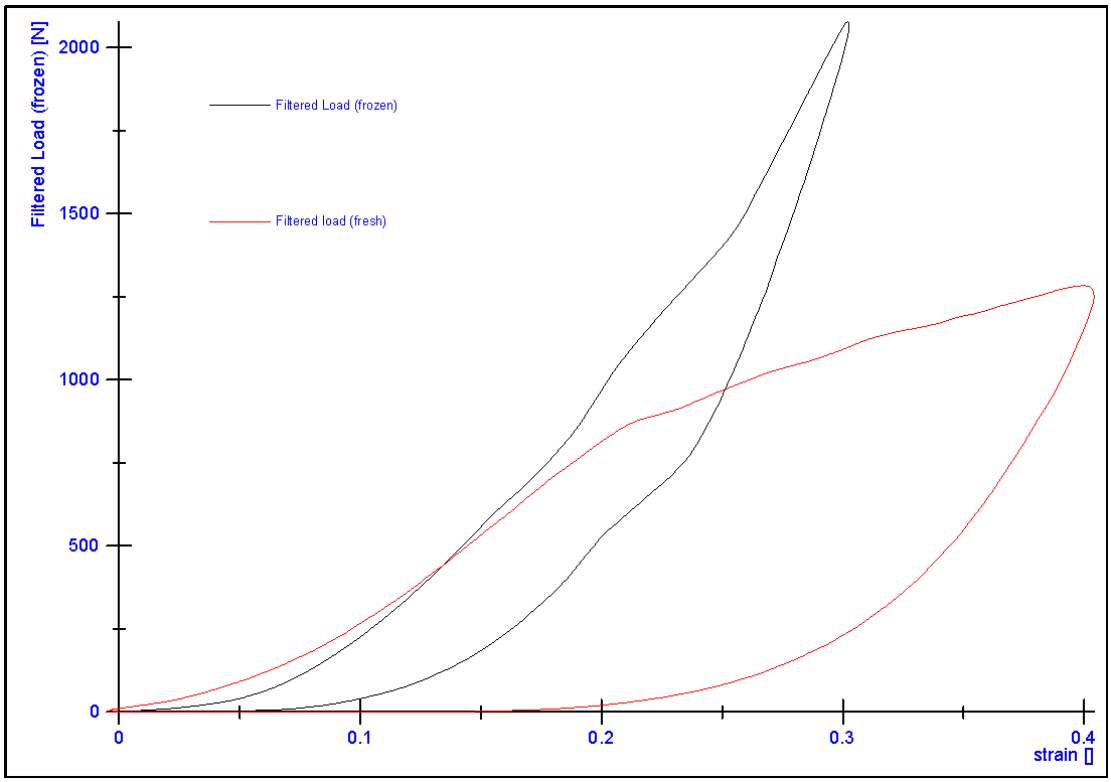
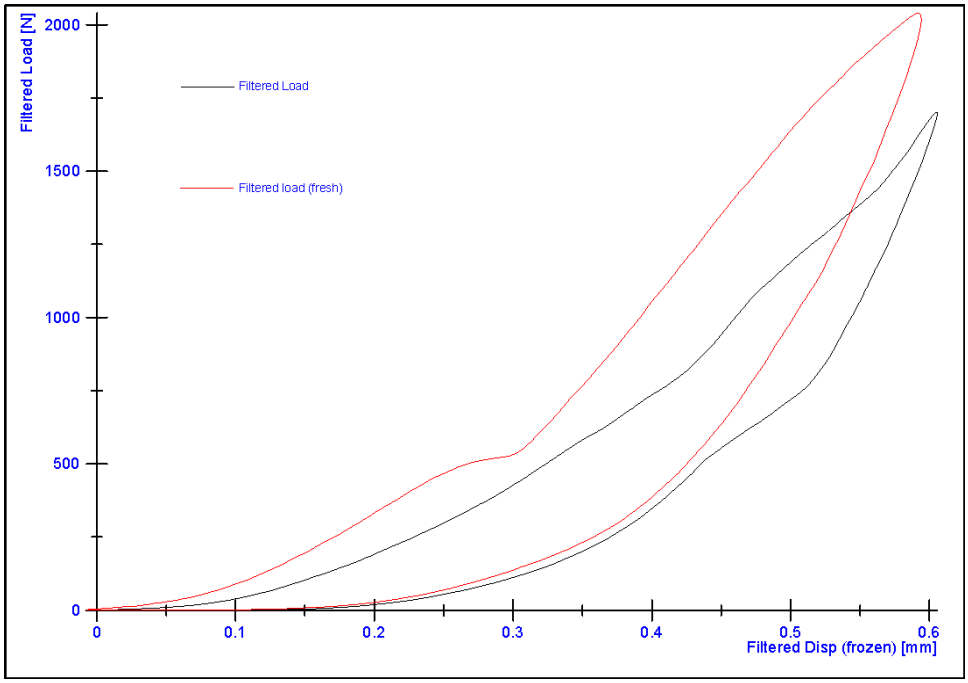
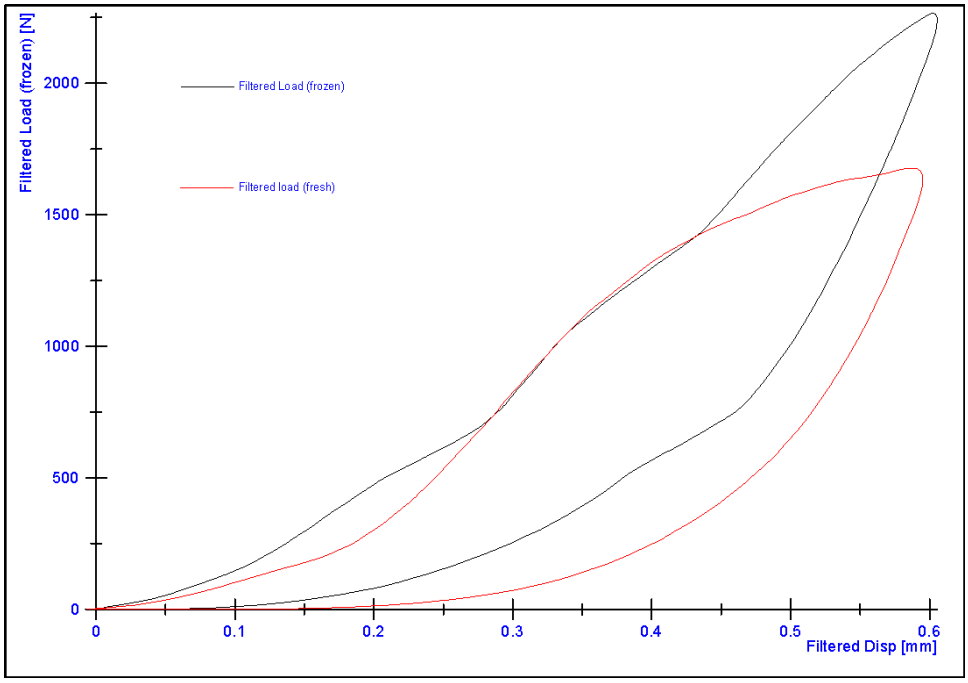


Figure G31: One of the two fresh tested specimens in the double fresh controls group exhibited unusual force displacement characteristics

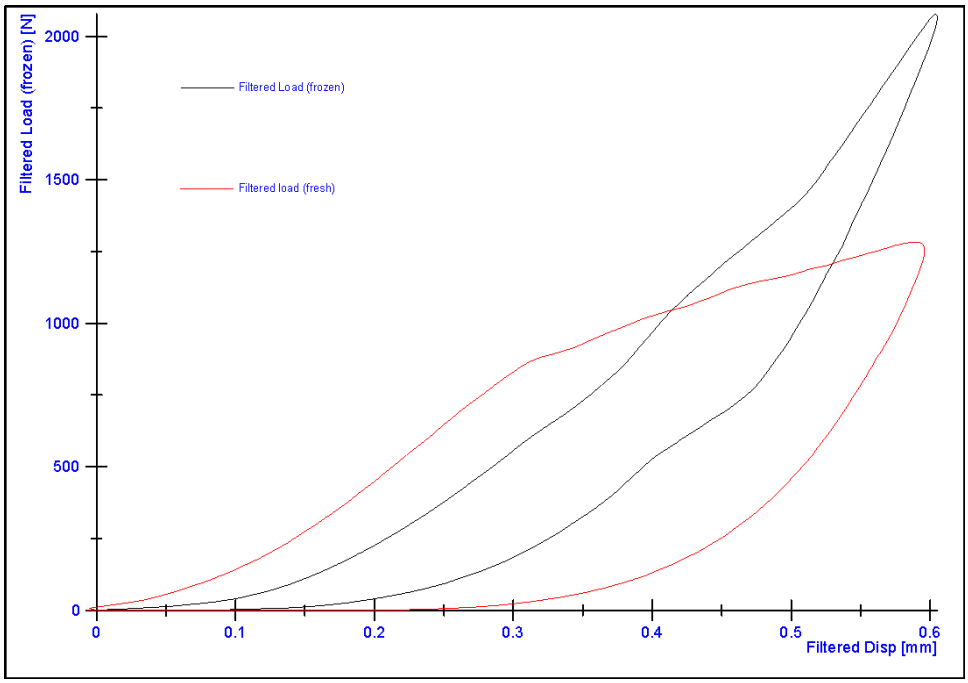
Force Displacement Data



Double Freeze (pair 11)



Double Freeze (pair 12)



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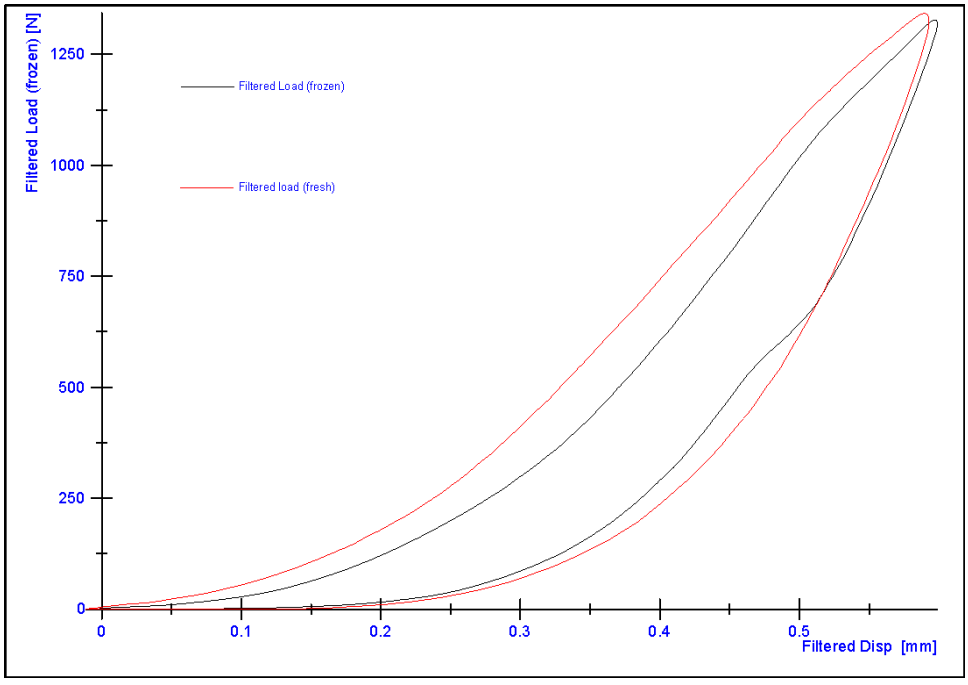
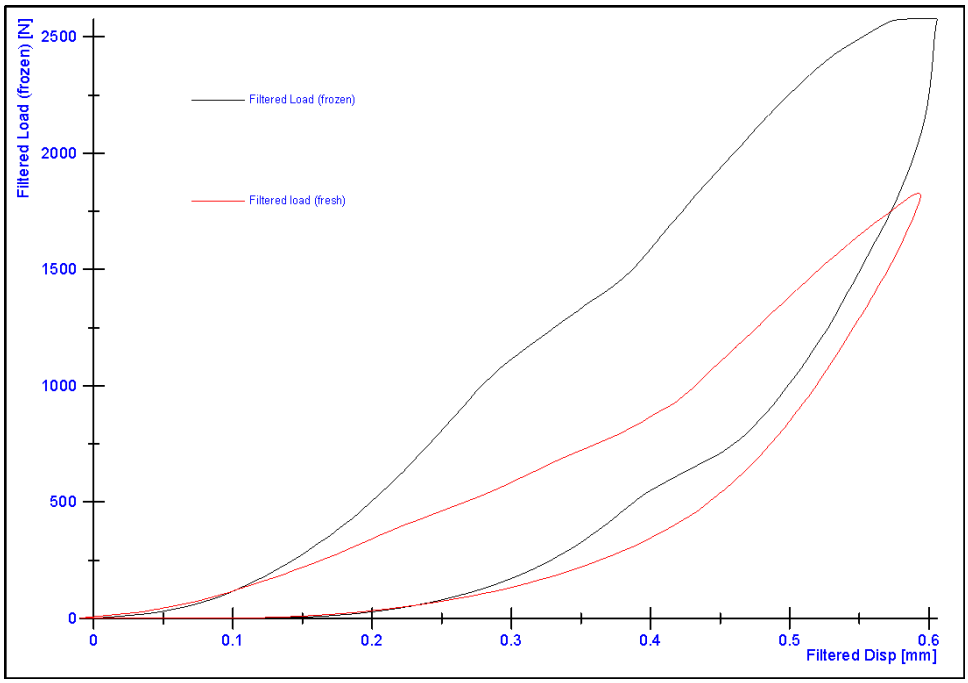
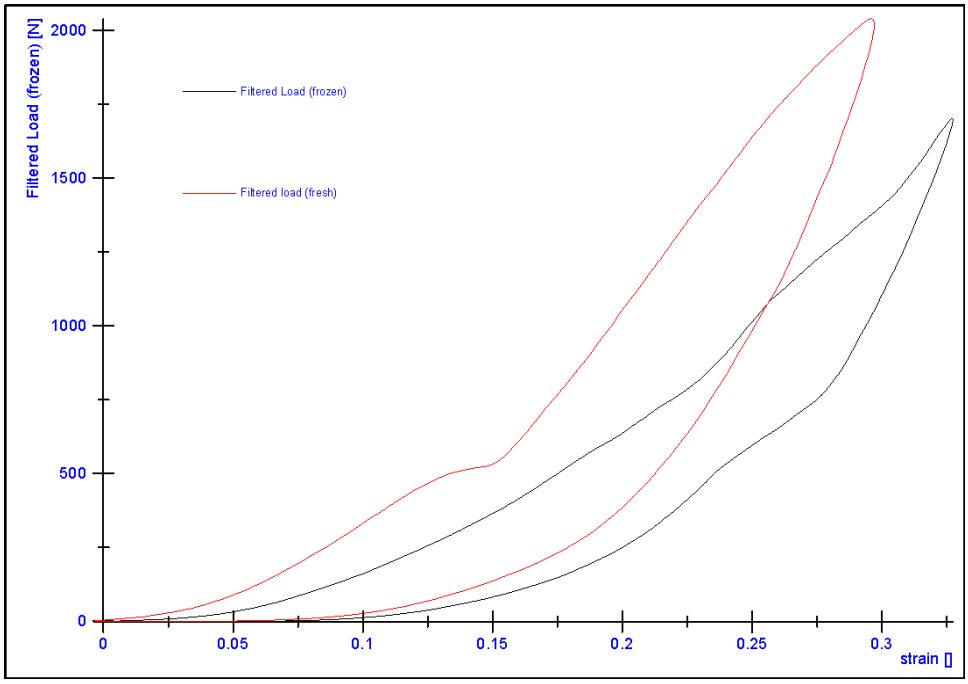


Double Freeze (pair 14)



Double Freeze (pair 15)

Stress Strain Plots



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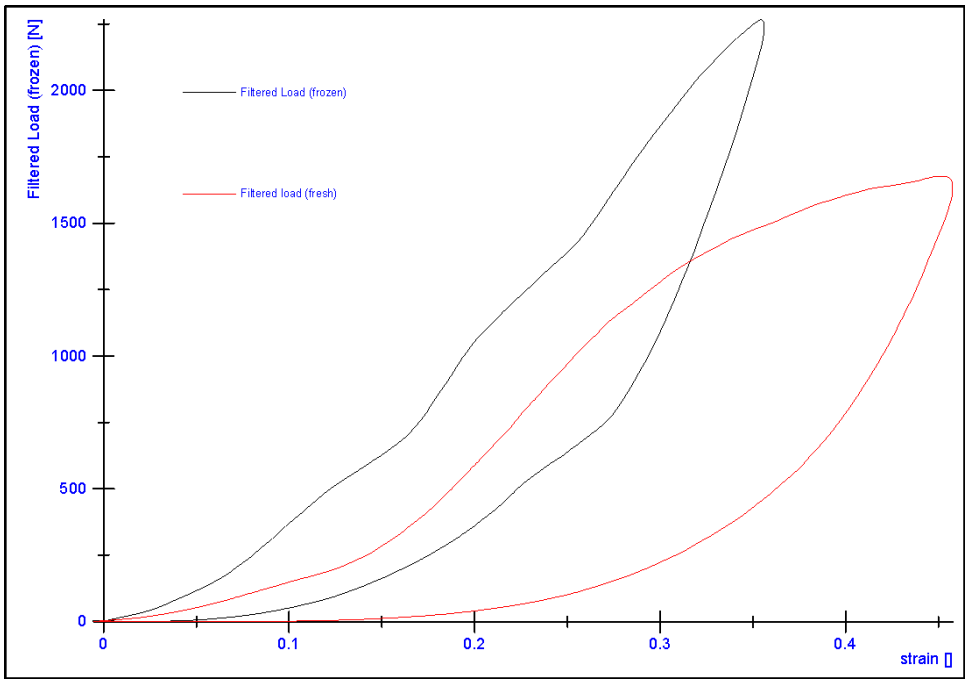

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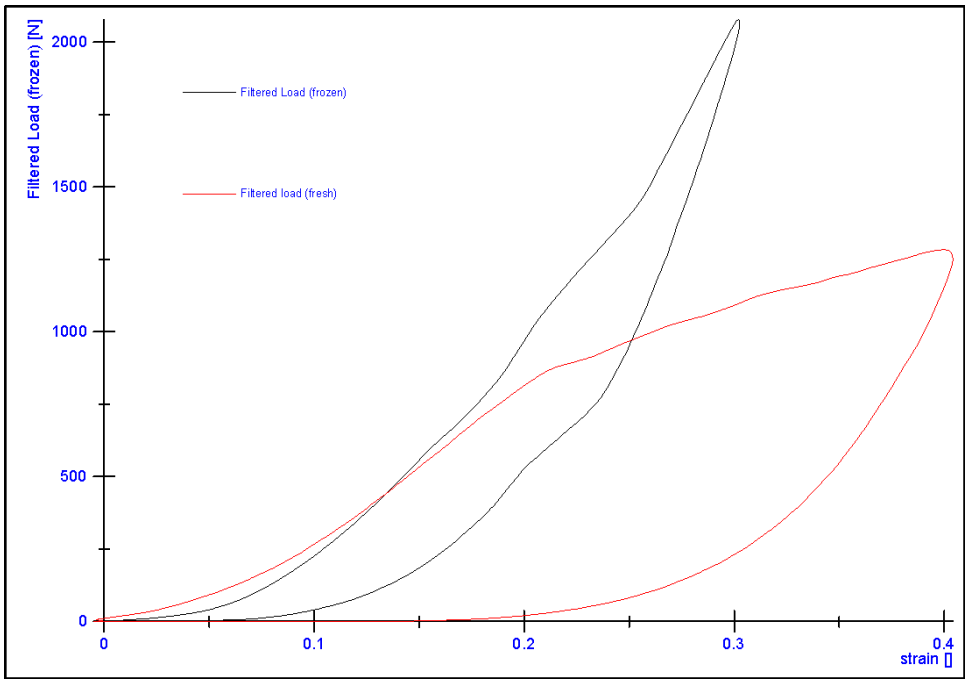

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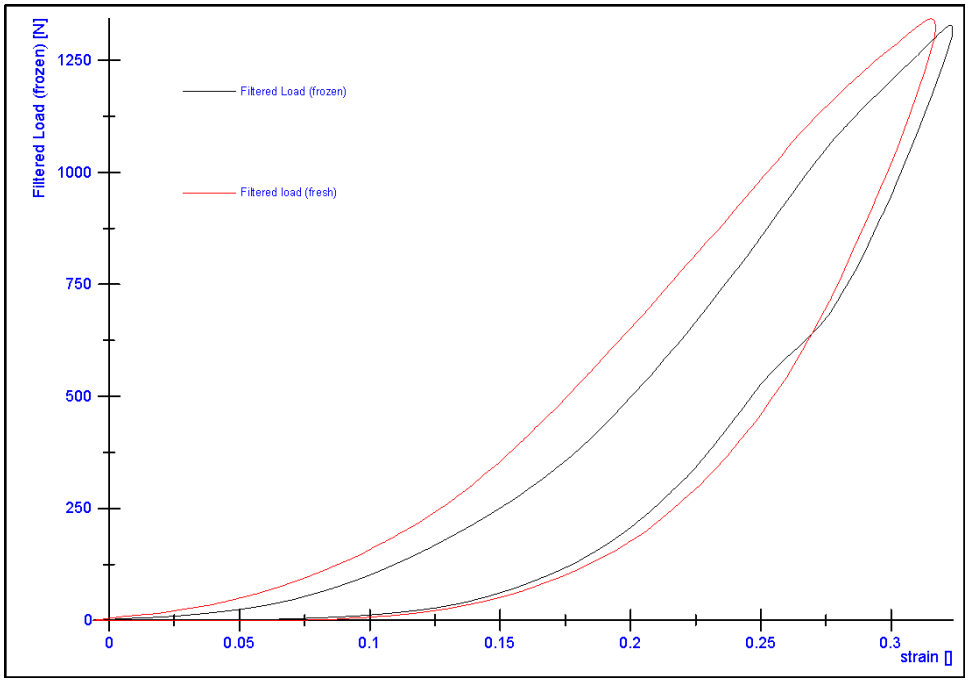

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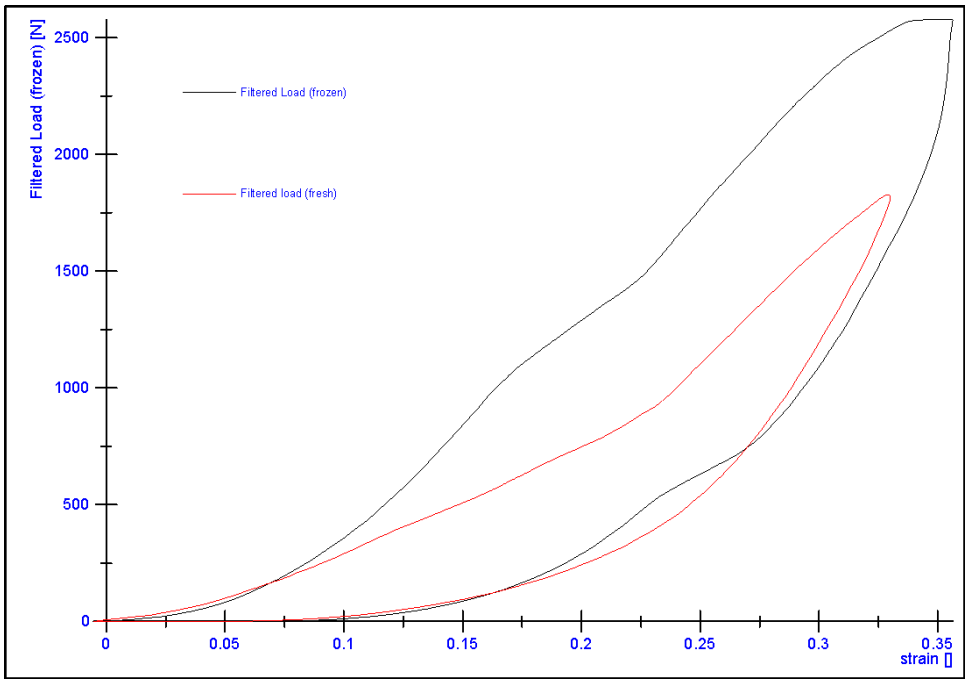

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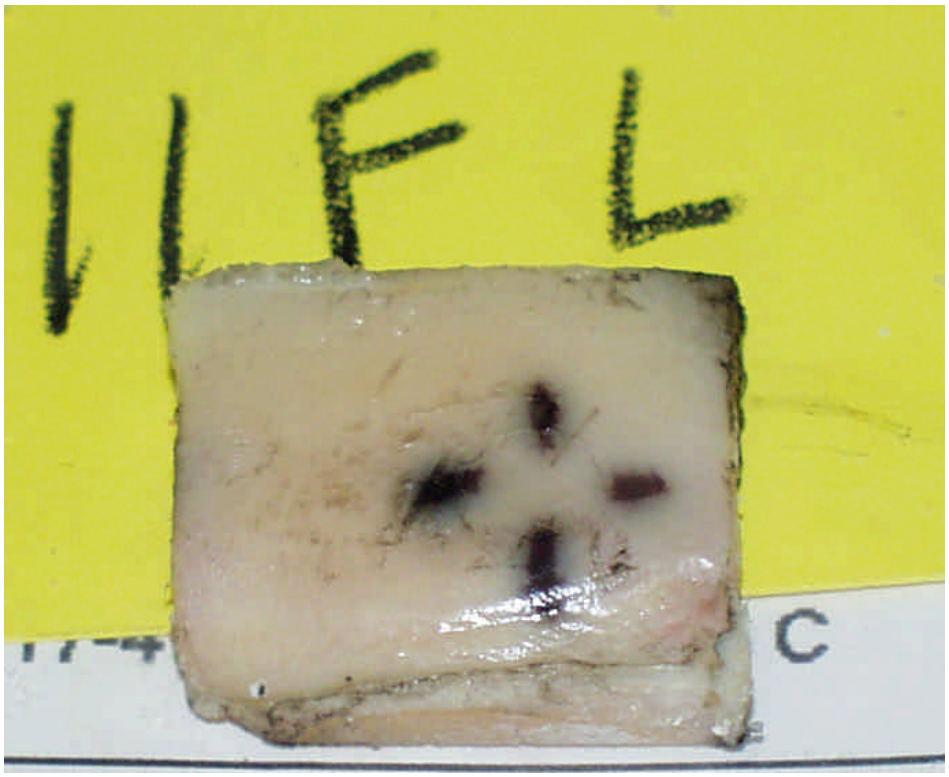
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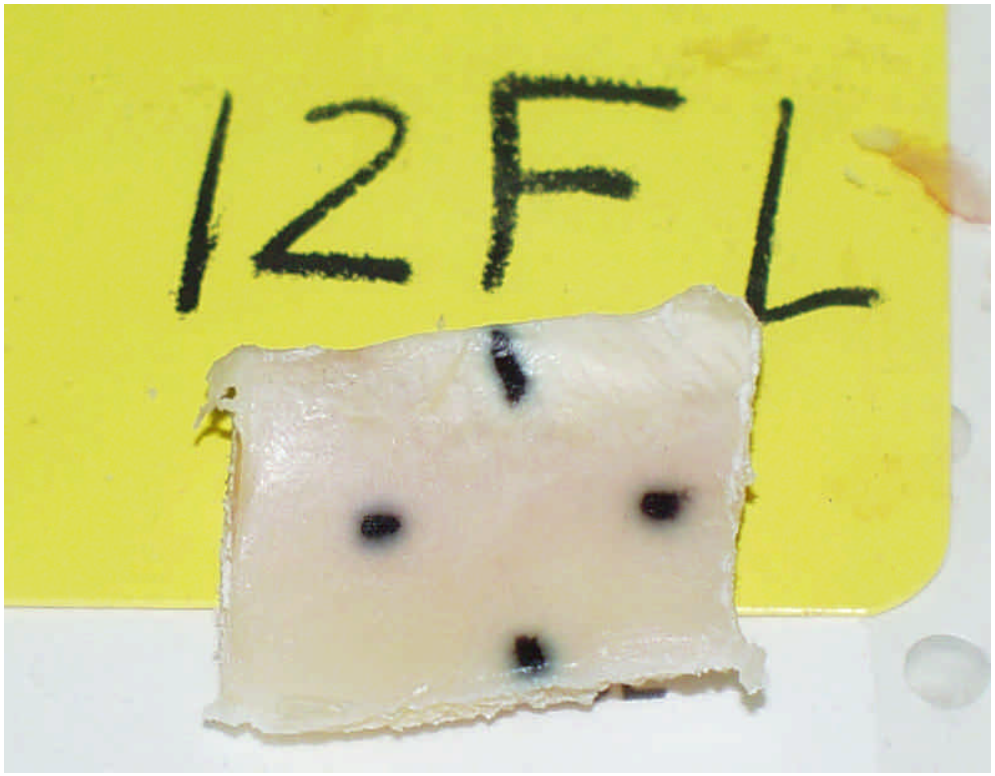
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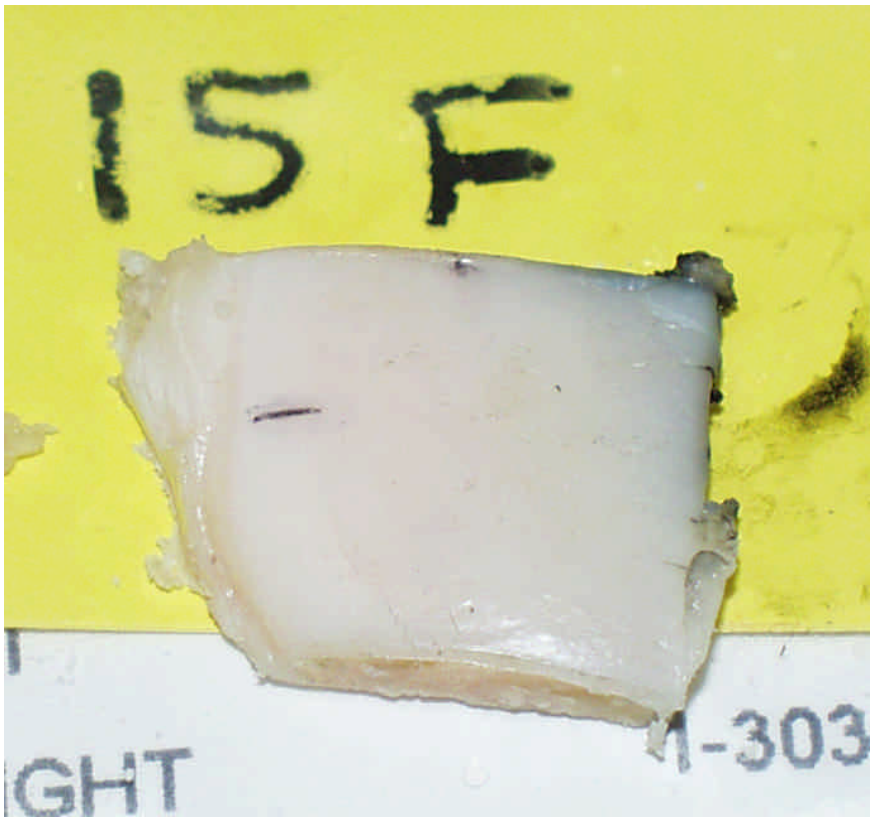
13F
1 RIGHT
TECHNOLOGIES,


14F
RIGHT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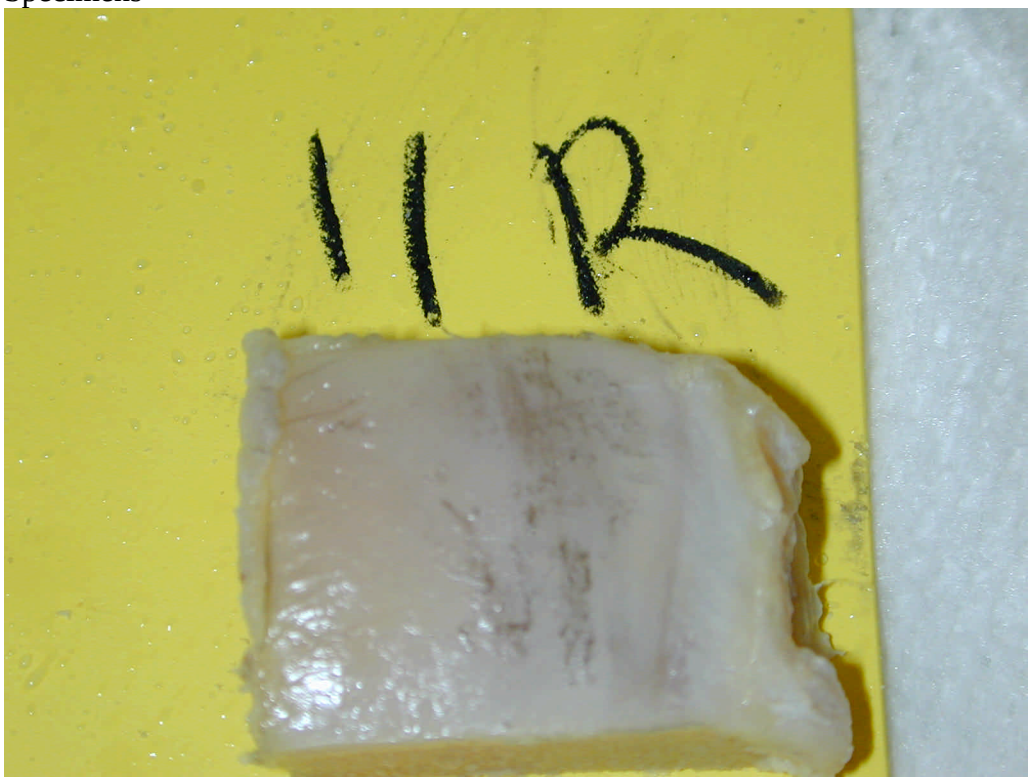
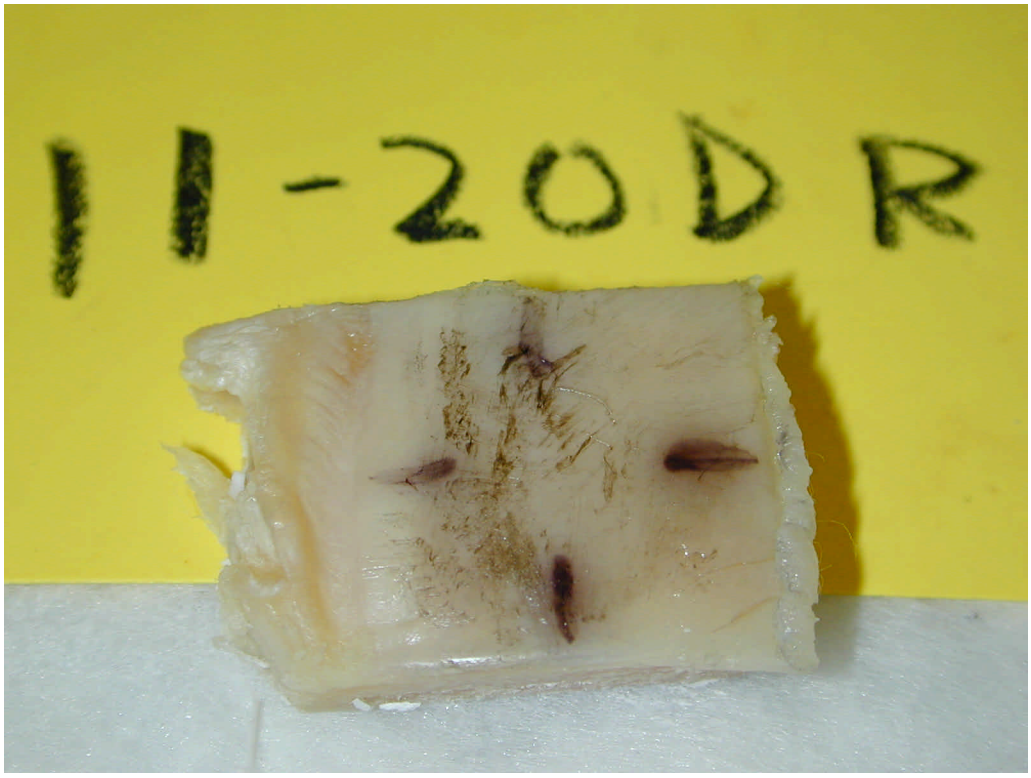
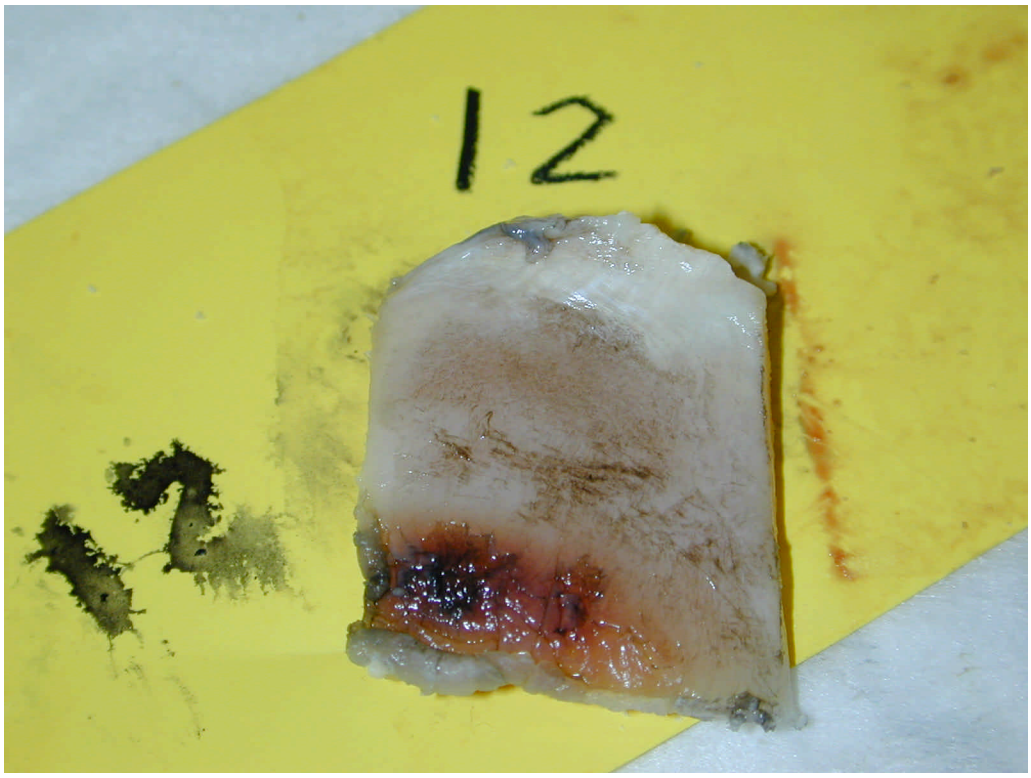
-303

Frozen
Specimens



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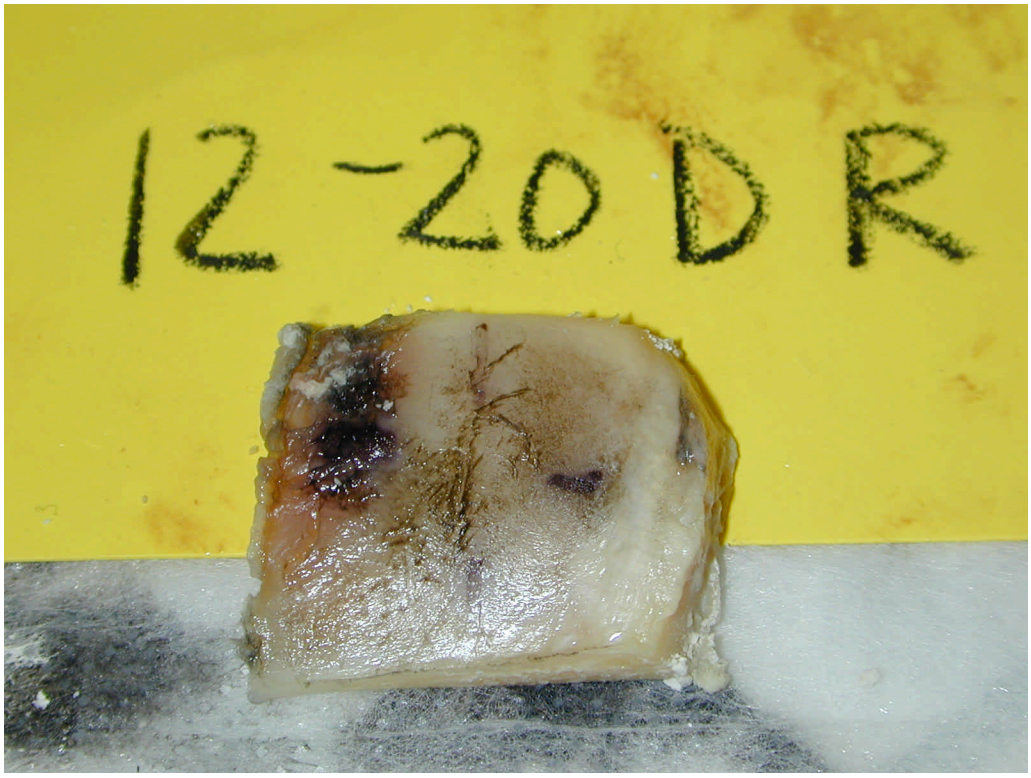
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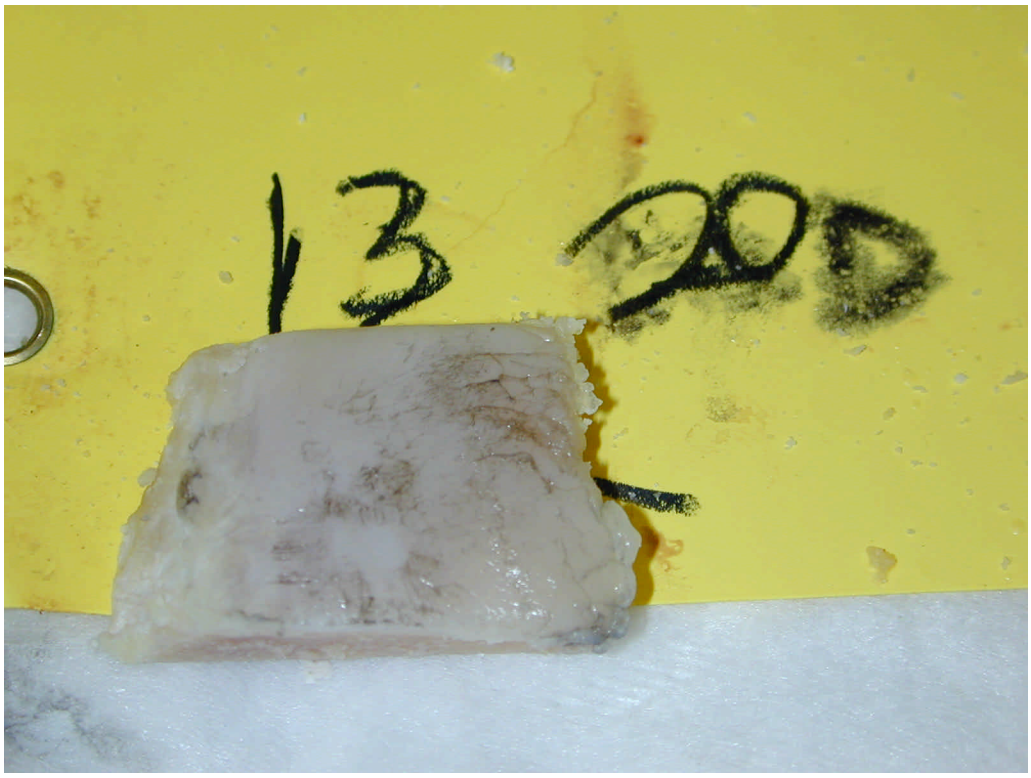
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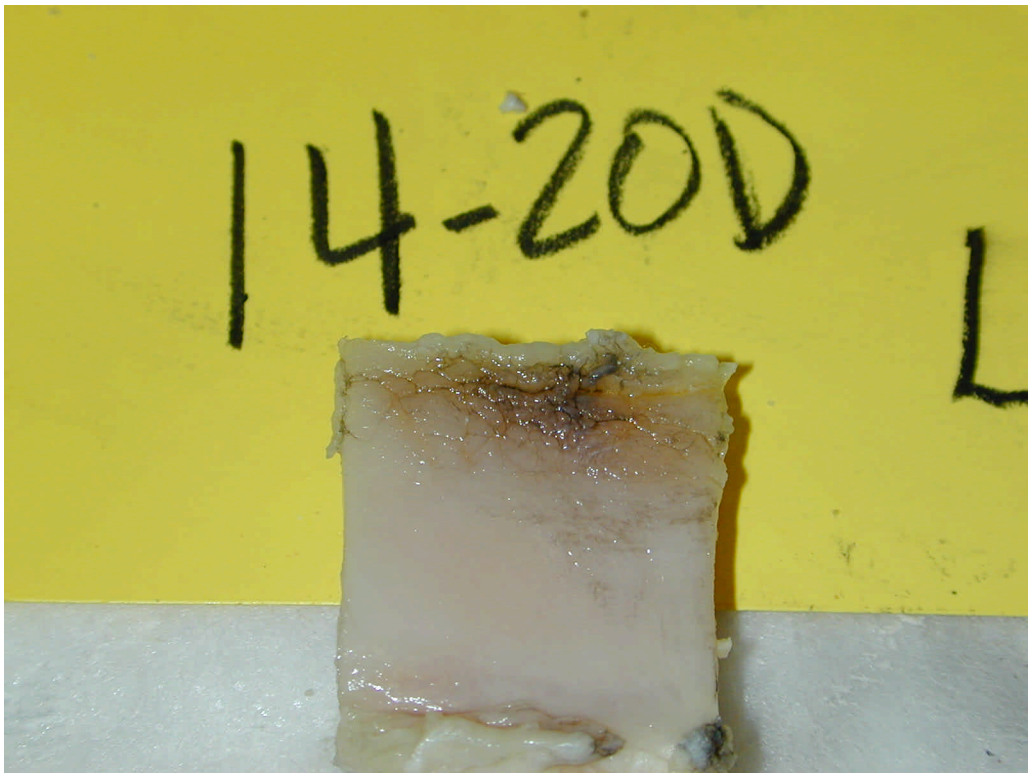
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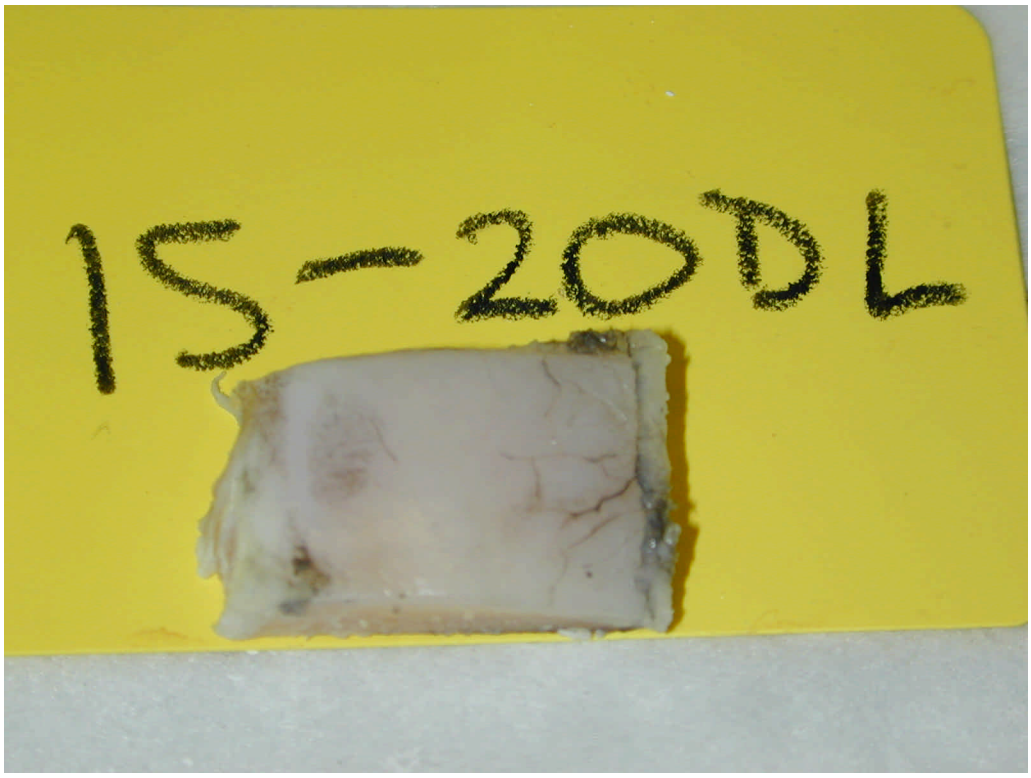
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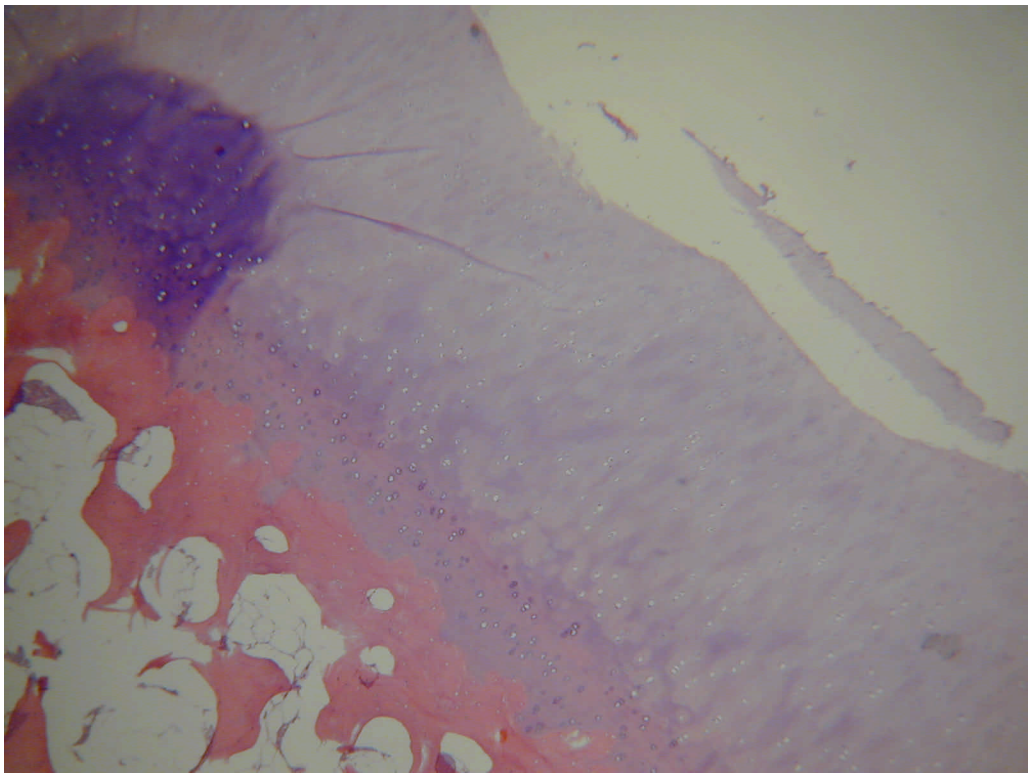

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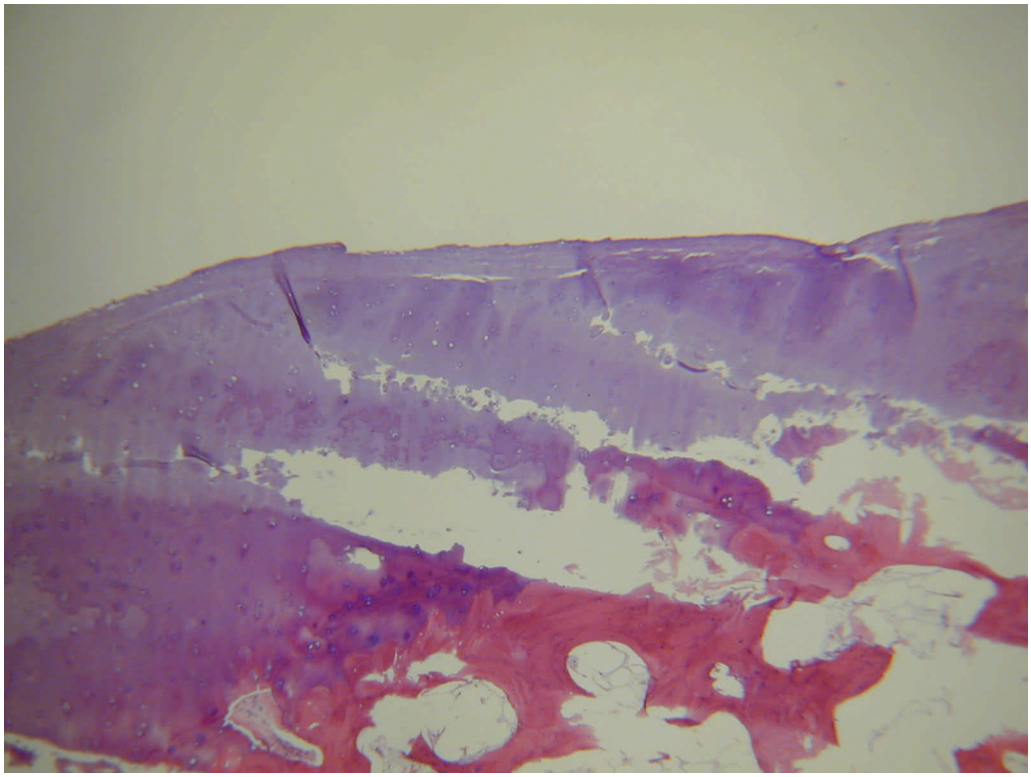

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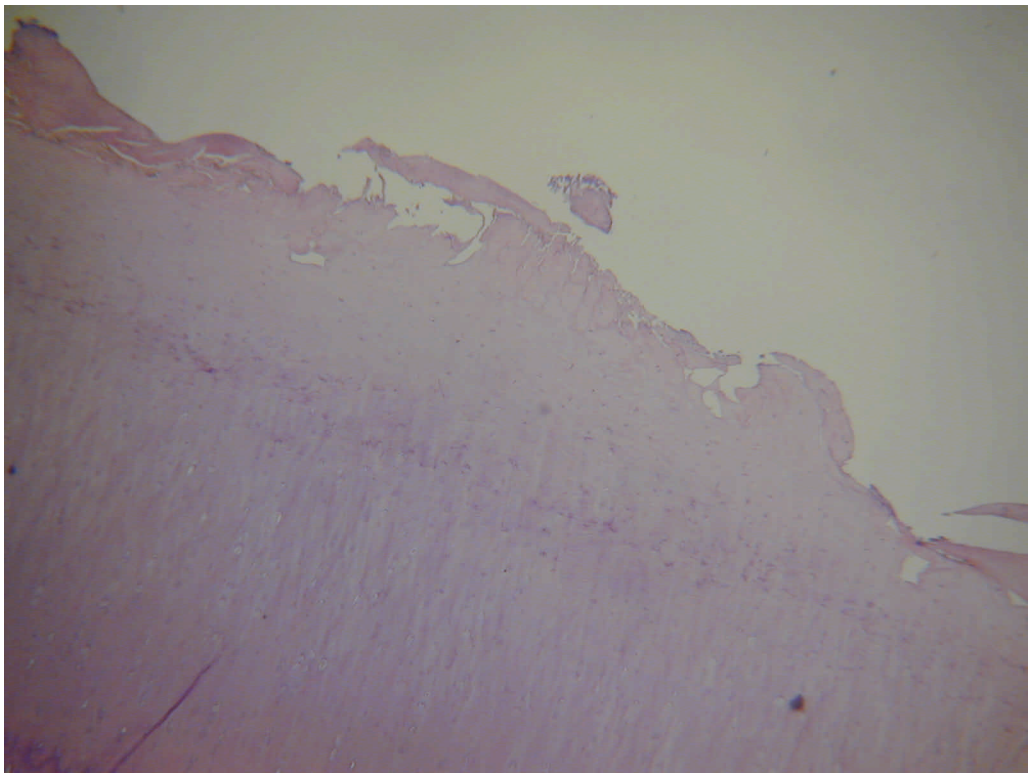

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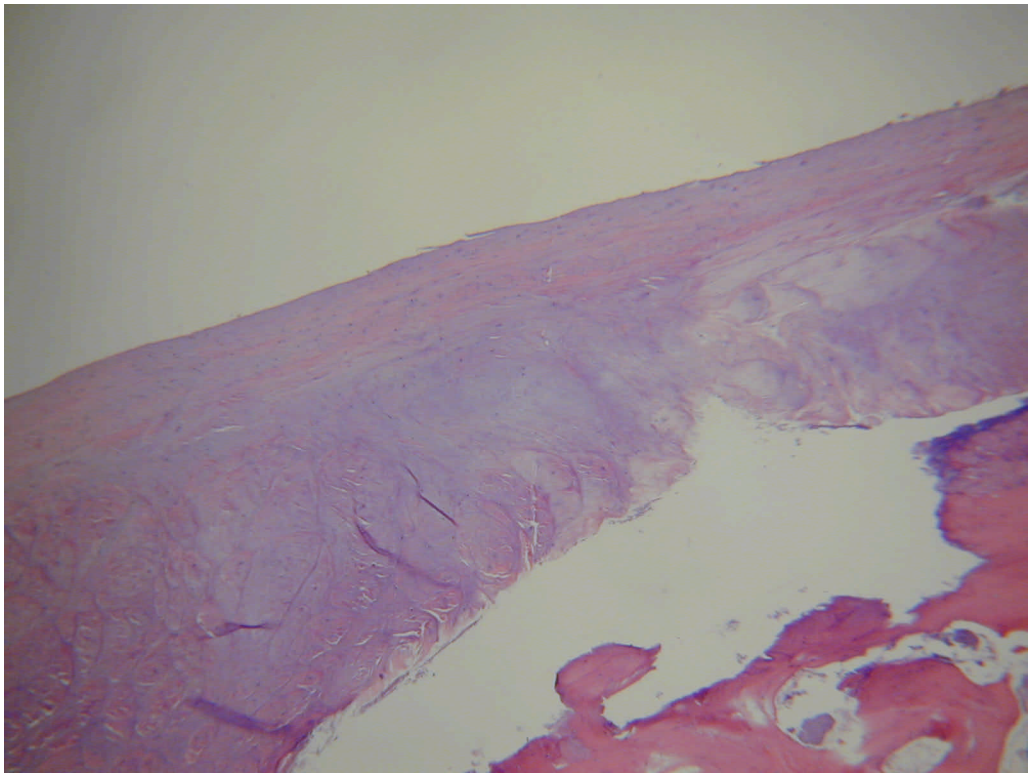

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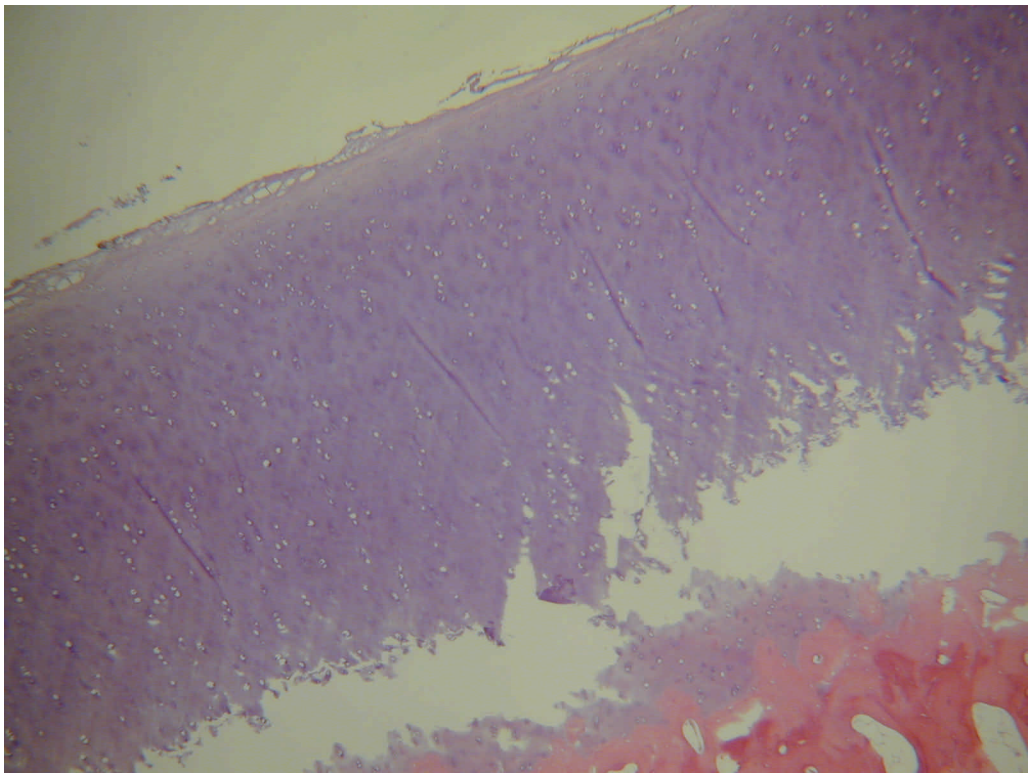

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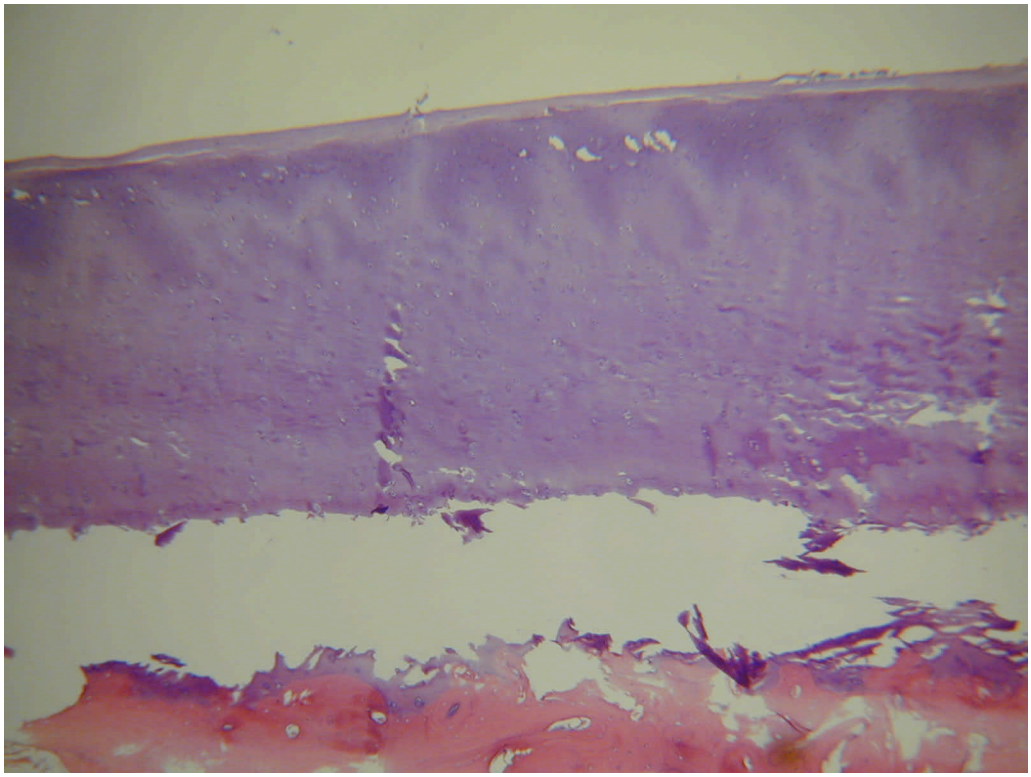

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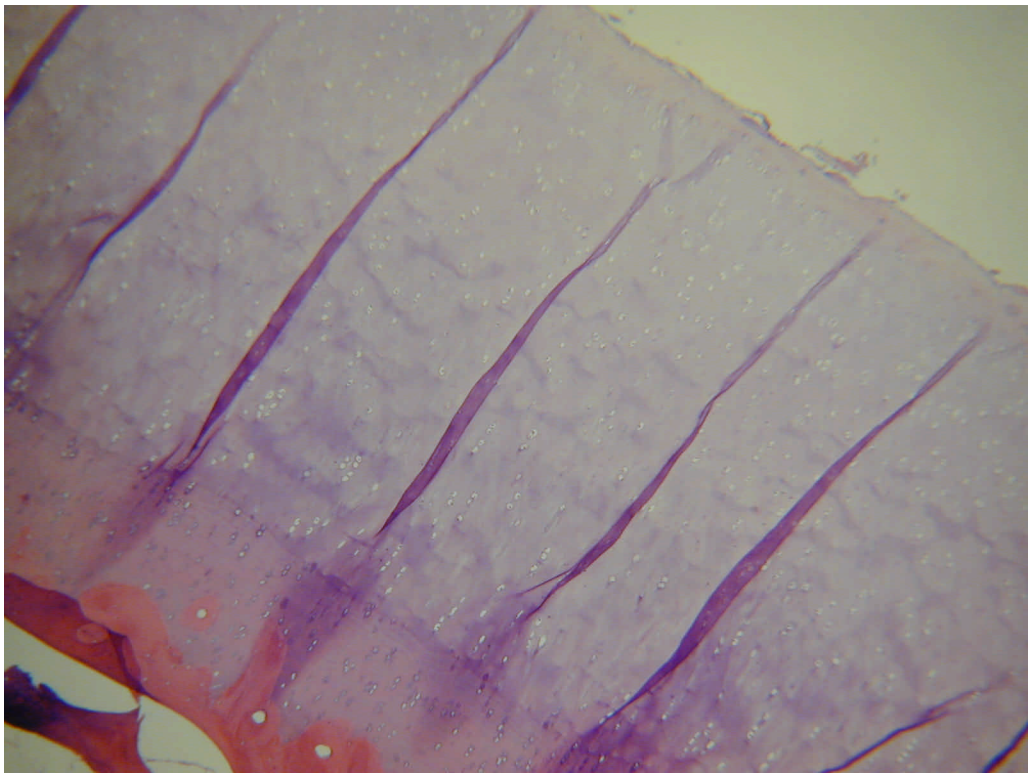

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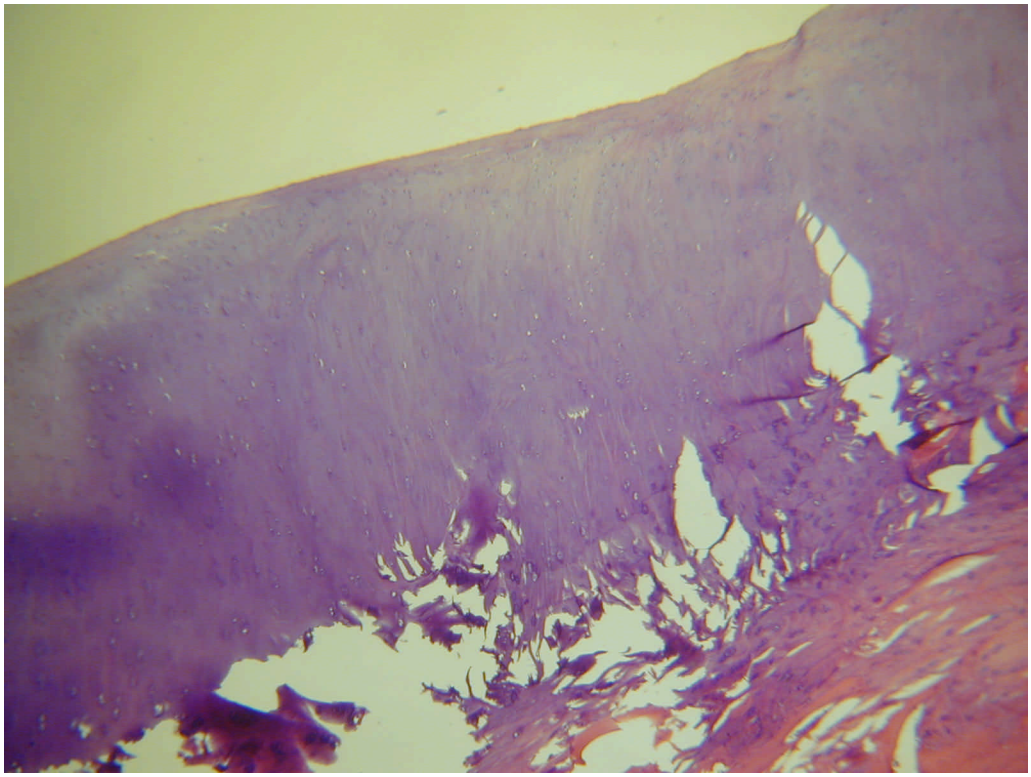

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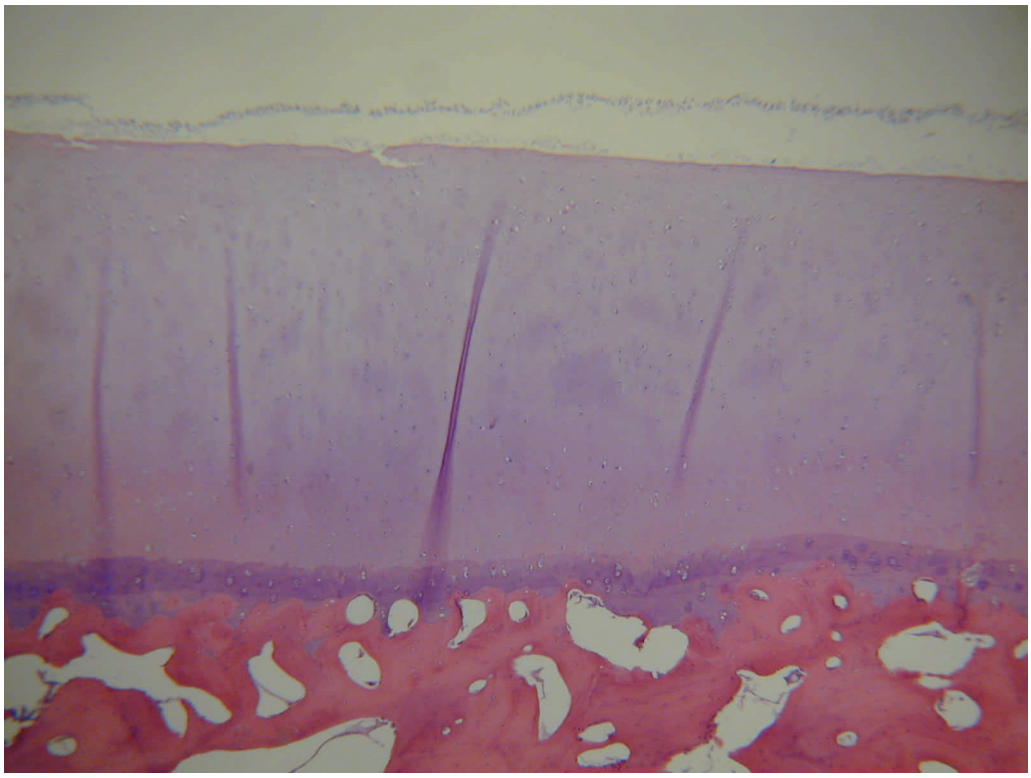

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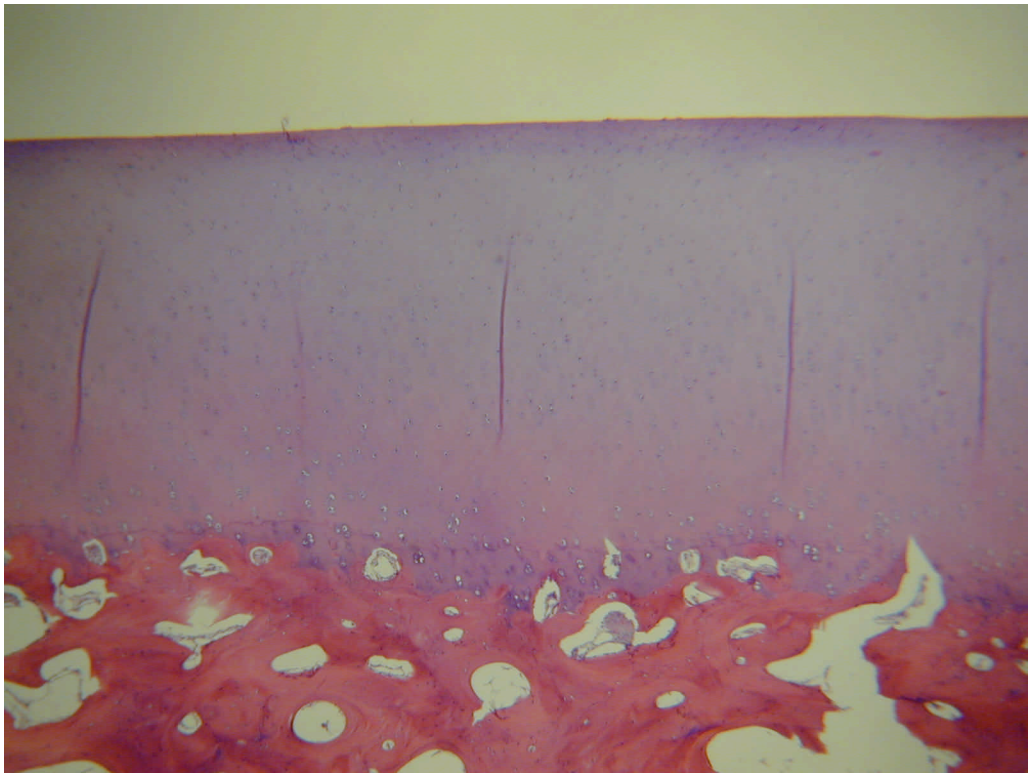

15F

Vita

David Sebastian Tordonato was born on January 14, 1980. He is the son of Candace and Sebastian Tordonato and brother of Katherine Tordonato Sorenson. He graduated from Robinson High School in Fairfax, Virginia in 1998. He went on to study mechanical engineering at Virginia Tech and received a Bachelor of Science degree in 2002. At Virginia Tech, was accepted into the 5 year B.S./M.S. program and chose to remain at there for his Masters of Science while specializing in biomechanics. David is fond of the outdoors and enjoys many outdoor different activities such as hiking, mountain biking, snowboarding, and water sports. At this point, his plans for the future are still uncertain but he has aspirations of attending Medical School and becoming a doctor.

For further information please write his permanent address:

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Clifton VA, 20124