Effects of Joint Capsule Tissue on Cartilage Degradation in an in Vitro Joint Injury Model

by

Stephanie Norris Lin

B.S. Mechanical Engineering
Massachusetts Institute of Technology, 2002

Submitted to the Department of Mechanical Engineering
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science in Mechanical Engineering

at the

Massachusetts Institute of Technology

September 2004

Signature of Author.....
Department of Mechanical Engineering
August 6, 2004

Certified by.....
Alan J. Grodzinsky
Professor of Mechanical Engineering
Thesis Supervisor

Accepted by.....
Ain Sonin
Chairman, Graduate Committee

EFFECTS OF JOINT CAPSULE TISSUE ON CARTILAGE DEGRADATION IN AN IN VITRO JOINT INJURY MODEL

by

STEPHANIE NORRIS LIN

Submitted to the Department of Mechanical Engineering
on August 6, 2004 in Partial Fulfillment of the
requirements for the Degree of Master of Science in
Mechanical Engineering

ABSTRACT

Osteoarthritis is a degenerative disease of the whole joint that affects an estimated 20.7 million Americans. Traumatic joint injury causes an increase in risk for the development of osteoarthritis. A previously developed model system involving co-culture of injured cartilage with joint capsule tissue or joint capsule conditioned medium was used to simulate the interaction of injured cartilage with other joint tissues. Experiments were carried out to characterize this model in greater detail and to quantify the release of GAG and changes in chondrocyte biosynthesis that occur as a result of co-culture. Model system parameters that were varied included the type of culture medium used, the duration of culture, and the size of the joint capsule tissue that was used. Experiments were also done to more carefully characterize properties of the joint capsule tissue such as GAG content, DNA content and variations in these properties with location. Attempts were also made to identify an unknown factor released by the joint capsule tissue that was potentially responsible for the observed decrease in chondrocyte biosynthesis as a result of co-culture. While this factor was not identified, convincing evidence suggested that it was not IL-1 or TNF-α. Systematic evaluation of this model system led to the conclusion that measurement of loss of total sGAG, alone, was not a definitive outcome measure, since it was difficult to distinguish the sGAG lost from cartilage from sGAG that was lost by the joint capsule tissue. The results suggested that more specific outcome measures such as gene expression of the cartilage or capsule and western analysis and biochemical analysis of matrix fragments lost to the medium would provide a more definitive interpretation of the interaction between joint capsule tissue and injured cartilage.

Thesis Supervisor: Alan Grodzinsky

Title: Professor of Mechanical Engineering

Table of Contents

Chapter 1: Background and Purpose

1.1 Background

It is estimated that osteoarthritis affects 20.7 million people in the United States with women and adults over 45 making up the majority of those affected.[19] Osteoarthritis is a degenerative joint disease whose hallmark is the loss of the joint's articular cartilage. In a healthy joint the ends of the bones are covered by cartilage. The whole joint is encapsulated by joint capsule tissue that is lined with the synovial membrane, which produces synovial fluid that helps act as a lubricant in the joint.[10] The joint capsule tissue itself is a dense fibrous connective tissue which is made up of parallel bundles of collagen and is populated with fibrocytes. The direction and arrangement of the collagen fibers depends on the loads that are applied to the tissue. Joint capsule tissue thickness can vary greatly depending on its location within a joint. Ligaments are considered local thickenings of the joint capsule tissue and so structurally they are the same. Joint capsule tissue is known to contain the small leucine rich proteoglycans decorin and fibromodulin.[13,24]

Figure 1 shows a sketch of a healthy joint.

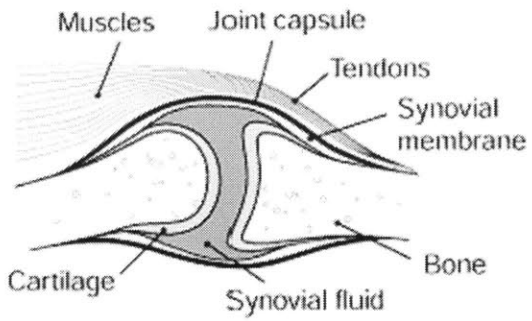

Figure 1: A healthy joint[10]

In a joint suffering from osteoarthritis, the cartilage no longer forms a smooth covering on the bone. Over time the cartilage becomes degraded and the bones can rub together. Osteophytes, small bone spurs, can also form on the bone and pieces of these can break off. This can cause joint pain and limited movement in those suffering from the disease.[10] Figure 2 shows a depiction of a joint suffering from osteoarthritis.

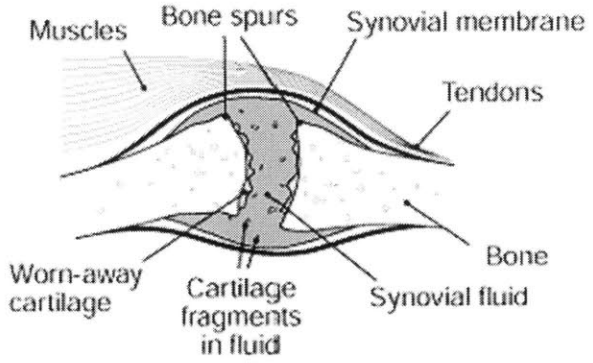

Figure 2: A joint suffering from osteoarthritis.[10]

Studies have shown that acute traumatic joint injury can cause an increase in risk for the development of osteoarthritis.[3,9] Because the pathways involved in this process are poorly understood, there has been an effort to study the process through in vitro models for acute traumatic joint injury.

Current in vitro models studying the effects of injury focus primarily on articular cartilage. These studies have shown that an acute injury to the joint can cause cartilage matrix damage[14,25] and chondrocyte cell death by apoptosis[2,16] They have also shown that chondrocyte biosynthesis may be decreased[11,29] and MMP-3 expression may increase[21].

While these in vitro models have been helpful in understanding how the cartilage alone responds to traumatic injury, they may be limited in that they don't take into account the response of other joint tissues to injury or the interaction of injured cartilage with those other joint tissues.

It is generally believed that osteoarthritis is a disease of the whole joint and not of just the cartilage.[20] In an effort to create a more complete in vitro model, recent studies in our lab have focused on co-culture of joint capsule tissue with injured cartilage.[22] The joint capsule tissue is found within the joint and contains the synovial membrane (Figure 1), which is responsible for the release of inflammatory agents in rheumatoid arthritis. Joint capsule tissue is removed during dissection of the joint and is punched to form disks that are either co-cultured with cartilage disks or used to condition medium by incubating a disk in a certain amount of medium for a given length of time. The studies done using

this co-culture system have shown a marked decrease in the biosynthetic activity of cartilage co-cultured with joint capsule tissue versus cartilage cultured alone and a synergistic increase in GAG loss from the cartilage after injury.[22]

1.2 Goal of experiments

The goal of this thesis is to follow up on these preliminary experiments done with the joint capsule tissue co-culture model by trying to identify the factor responsible for the decrease in biosynthesis or at least narrow down the list of possible suspects. The other goal of this thesis is to further characterize the joint capsule tissue co-culture model and conditioned medium model to better understand the parameters that regulate cartilage degradation. By doing this, it is hoped that consistent, reliable results will be able to be obtained from these model systems.

Chapter 2: Search for the Unknown Factor

2.1 Objective

As stated previously in the first chapter, when joint capsule tissue is co-cultured with cartilage or cartilage is cultured in joint capsule tissue conditioned medium there is a large decrease in chondrocyte biosynthesis levels. When injured cartilage is co-cultured with joint capsule tissue there also appears to be an increase in proteoglycan loss. The goal of the following experiments was to try and identify the factor responsible for the decrease in biosynthesis and the increase in proteoglycan loss. The initial studies performed with the co-culture system suggested that the unknown factor might be IL-1 because of the decrease in biosynthesis and the increased GAG loss seen in the system[7]. Dingle et al had also shown that IL-1 was released from minced porcine synovial cells and caused matrix degradation in cartilage[4]. It is also known that IL-1 is involved in rheumatoid arthritis[18]. Since the synovium is still attached to at least some of the joint capsule tissue samples, Il-1 appeared to be the most likely suspect. TNF-α was also a suspect because it is known to play a role in rheumatoid arthritis[18] and in previous experiments it had been shown to cause a decrease in chondrocyte biosynthesis levels as well as an increase in GAG loss with injury[21].

2.2 Eliminating IL-1α and TNF α

Because the previous experiments done by Patwari et al.[21,23] to show that IL-1 α and TNF- α were not responsible had been done using recombinant human cytokines and blockers in a bovine system, it was necessary to show that the recombinant blockers

would work against bovine cytokines in order to definitively show that those cytokines were not responsible for the decrease in biosynthesis and increase in proteoglycan loss.

2.2.1 Cross Reactivity of Etanercept with Bovine TNF- α

In order to make sure that Etanercept would block bovine TNF-α, as well as the recombinant human TNF- α , bovine TNF- α was provided by the USDA.

2.2.1.1 Methods

Cartilage was harvested from the femoropatellar groove of one to two week old bovine calves by using a drill press with a special bit to obtain nine millimeter diameter cylindrical cartilage on bone cores as described previously by Sah et al[27]. A microtome was then used to slice the cartilage into 1mm thick slices. These slices were then punched to obtain 3mm diameter by 1 mm thick cartilage disks.

The cartilage explants were allowed to equilibrate for three days in medium containing low-glucose Dulbeco's Modified Eagle Medium (Gibco, Grand Island, NY), 10% fetal bovine serum (HyClone, Logan, UT), and supplemented with 0.1mM non-essential amino acids, HEPES buffer and antibiotics and 0.4mM proline and ascorbate. The explants were incubated at 37° C and 5% CO_2.

After allowing the cartilage to equilibrate for three days, cartilage plugs were placed into one of four conditions with one plug from each slice going into each of the conditions:

1) Control, no TNF-α, no etanercept
2) 25 μg/ml etanercept
3) 100 ng/ml bovine TNF-α, no etanercept
4) 100 ng/ml bovine TNF-α and 25μg/ml etanercept

Each plug was incubated in 0.25 ml of medium for 6 days. On day 6 after intervention, the cartilage plugs were transferred to medium containing 10μCi/ml of ^{35}S-Sulfate and ^{3}H-Proline (proline results not shown). The disks were incubated in the labeled medium for 7 hours and then washed three times over 45 minutes in 0.4ml of PBS supplemented with 0.8mM sodium sulfate and 1mM proline to remove any unincorporated label. In the future this solution will be called R_x wash solution. Next the plugs were put in 1ml of Proteinase K and placed in a 60° water bath for two days to digest. After the disks were completely digested, radiolabel incorporation was measured using a scintillation counter. The counts per minute were converted to incorporation rate using the method previously described by Sah et al.[27]

2.2.1.2 Results:

Figure 1 shows sulfate incorporation in pmol/hr. The mean is plotted as well as the standard error. There were 8 samples per condition.

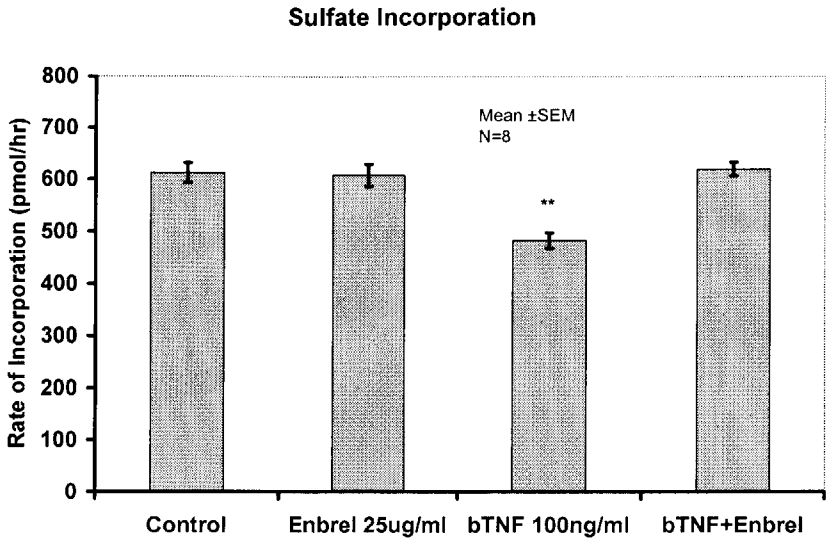

Figure 1: Rate of Sulfate incorporation for cartilage cultured in 10% FBS medium with and without 25 μg/ml etanercept and 100ng/ml of bovine TNF-α. Cartilage was allowed to equilibrate for three days after harvest before intervention and was incubated for 6 days in .25ml of medium prior to the radiolabel. '**' represents $p<0.0003$ by two sided student t test.

Figure 2 shows GAG loss after 6 days.

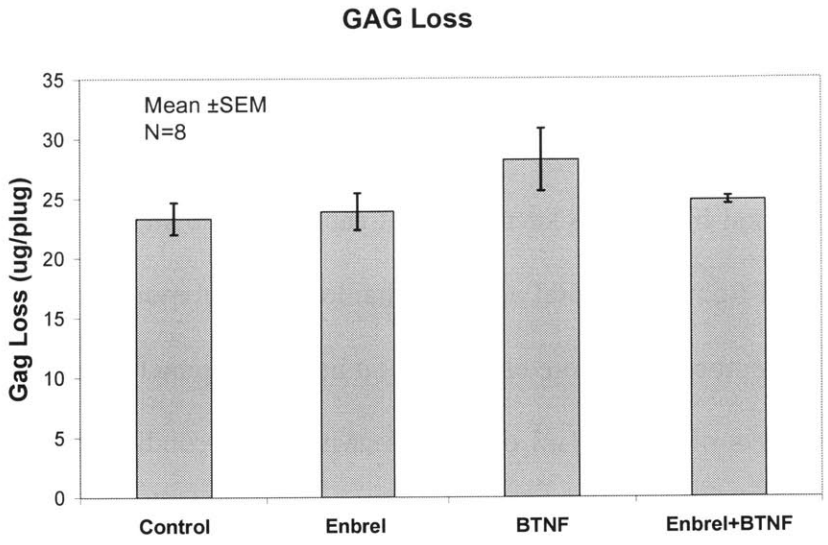

Figure 2: GAG Loss (μg/plug) for cartilage cultured in 10% FBS medium with and without 25 μg/ml etanercept and 100ng/ml of bovine TNF-α. Cartilage was allowed to equilibrate for three days after harvest before intervention and was incubated for 6 days in 0.25ml of medium prior to measuring GAG Loss.

From these graphs, it is evident that etanercept is successful at blocking the effect of bovine TNF-α thus showing that the results obtained by Fay et al.[7] are valid and that TNF-α is not responsible for the decrease in biosynthesis seen when cartilage is co-cultured with joint capsule tissue or cultured in joint capsule tissue conditioned medium.

2.2.2 Human Joint Capsule Tissue Conditioned Medium with IL-1ra and Sol-r

Because it was not possible to obtain bovine IL-1, joint capsule tissue was harvested from a human knee joint and used to condition medium. This medium conditioned with human joint capsule was then cultured with bovine cartilage to see if the same decrease in biosynthesis was seen and whether or not that decrease could be reversed by applying the recombinant human IL-1 blockers. This way it would be possible to definitively rule out IL-1 as the cause of the decrease in biosynthesis.

2.2.2.1 Methods

Joint capsule tissue was harvested from the knee joint of a human donor. The joint was dissected in the hood in order to keep the joint capsule tissue sterile. The joint capsule tissue was excised from the medial and lateral sides of the femoropatellar groove. The excised pieces of joint capsule were cleaned of fat tissue and punched to form 3mm disks. One 3 mm disk was placed in 1ml of medium which was conditioned for seven days. After seven days, joint capsule tissue cell viability was checked to ensure that the capsule was still alive. The conditioned medium was pooled and frozen for later use and then unthawed in a 37°C water bath. Medium was kept in the incubator along with the conditioned medium to act as a control. This medium was also frozen and unthawed like the conditioned medium.

Bovine cartilage was harvested in the usual manner and allowed to equilibrate for four days prior to intervention. The cartilage plugs were matched for location with the exception of the Il-1 and IL-1 + sol-r or IL-1 + IL-1ra treated plugs. Two separate experiments were run. One experiment using the IL-1 soluble receptor and another using the IL-1 receptor antagonist. For each experiment there were 6 conditions:

1) Control
2) 200 ng/ml IL-1ra or 5 μg/ml IL IL-1 solr
3) Human Joint Capsule Tissue conditioned medium
4) Human JCTCM + 200 ng/ml IL-1ra or 5 μg/ml IL-1solr
5) 2 ng/ml IL-1

6) 2 ng/ml IL-1 +200 ng/ml IL-1ra or 5 μg/ml IL-1solr

Cartilage plugs were incubated for four days under these conditions in 0.25ml of medium prior to being labeled with 10μCi/ml of ^{35}S-Sulfate and ^{3}H-Proline for 24 hours.

2.2.2.2 Results:

Figure 3 shows sulfate incorporation for the experiment done using 200 ng/ml IL-1ra.

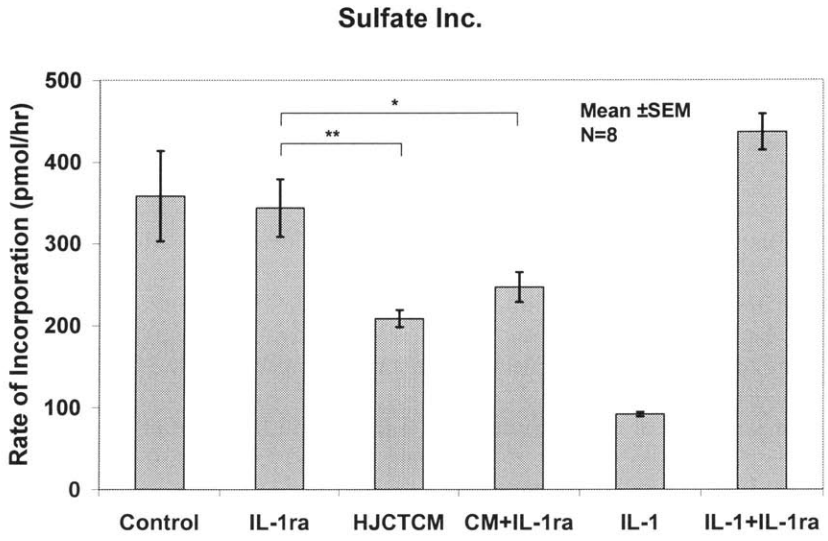

Figure 3: Rate of sulfate incorporation. Cartilage was allowed to equilibrate for four days before being placed in one of the above six conditions for four days. The concentration of IL-1ra was 200 ng/ml and the concentration of IL-1 was 2ng/ml. Human joint capsule tissue conditioned medium was conditioned by one 3mm piece of joint capsule tissue in 1ml of medium for 7 days. '**' represents $p<0.01$, '*' represents $p<0.02$ by a two sided student t test.

Figure 3 shows that IL-1ra is able to bring incorporation rates back to control levels for IL-1 treated cartilage but not for cartilage cultured in joint capsule tissue conditioned medium.

Figure 4 shows sulfate incorporation for cartilage treated with the IL-1 soluble receptor.

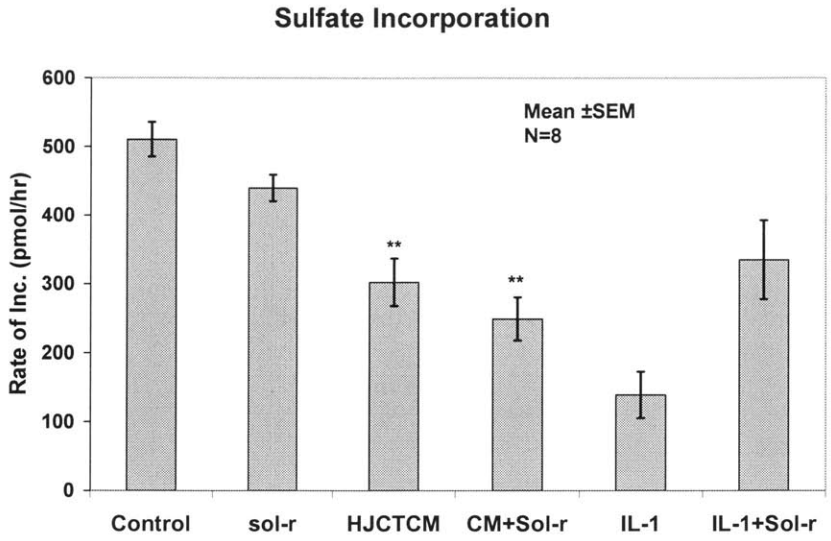

Figure 4: Rate of sulfate incorporation. Cartilage plugs were allowed to equilibrate for four days prior to being cultured in the above conditions for an additional 4 days. Joint capsule tissue conditioned medium was conditioned using one 3mm diameter piece of human joint capsule tissue in 1ml of medium for 7 days. IL-1 soluble receptor was used at a concentration of 5 μg/ml and IL-1 was at a concentration of 2ng/ml. '**' represents $p<0.01$ by a two sided student t test.

Figure 4 shows that the IL-1 soluble receptor also has no effect in reversing the decrease in biosynthesis for cartilage cultured in joint capsule tissue conditioned medium while it is able to bring biosynthesis levels back for IL-1 treated cartilage. In this case, the IL-1 concentration was twice as high as what is usually used for these experiments which probably accounts for the reason that biosynthesis levels never returned to control levels.

From Figures 3 and 4 it is possible to conclude that IL-1 is not responsible for the decrease in biosynthesis seen when cartilage is cultured in joint capsule tissue conditioned medium.

2.3 Confirming the factor is a protein

In order to verify that that the factor released by the joint capsule tissue is a protein and not an endotoxin, an experiment was done to test whether or not boiling joint capsule tissue conditioned medium would eliminate the decrease in biosynthesis seen in previous experiments. If the factor is a protein, boiling it should denature the protein and eliminate any activity that it might have. However, if it were an endotoxin, boiling would not have any effect.

2.3.1 Methods:

Cartilage was harvested as previously described. Joint capsule tissue was excised medial and lateral to the joint and punched to form 3mm disks. The cartilage plugs were allowed to equilibrate in 10 % FBS culture medium for three days prior to intervention. Two pieces of joint capsule tissue were used to condition 1 ml of medium. Medium was collected each day, pooled together and frozen.

Four milliliters of thawed joint capsule tissue conditioned medium and 10% FBS medium were boiled for 10 minutes in separate Erlenmeyer flasks on a hot plate. After 10 minutes, the flasks were placed on ice to cool the medium. Once the medium was cool, it was sterile filtered. Cartilage plugs were matched for location across the four conditions and were incubated in 0.5ml of medium for three days under one of the following four conditions. N=6 for all groups.

Conditions for this experiment:

1) Control
2) Conditioned Medium
3) Boiled Medium
4) Boiled Conditioned medium

After three days of culture, all groups were incubated for 18 hours in 0.5ml of medium containing 10μCi/ml of ^{35}S-sulfate and ^{3}H-Proline. After 18 hours the plugs were washed 3 times for 10-15 minutes each wash in Rx wash solution and then digested in Proteinase K. Once digested, proline and sulfate incorporation were measured using a scintillation counter.

2.3.2 Results:

Figure 5 shows sulfate incorporation for this experiment and Figure 6 shows proline incorporation.

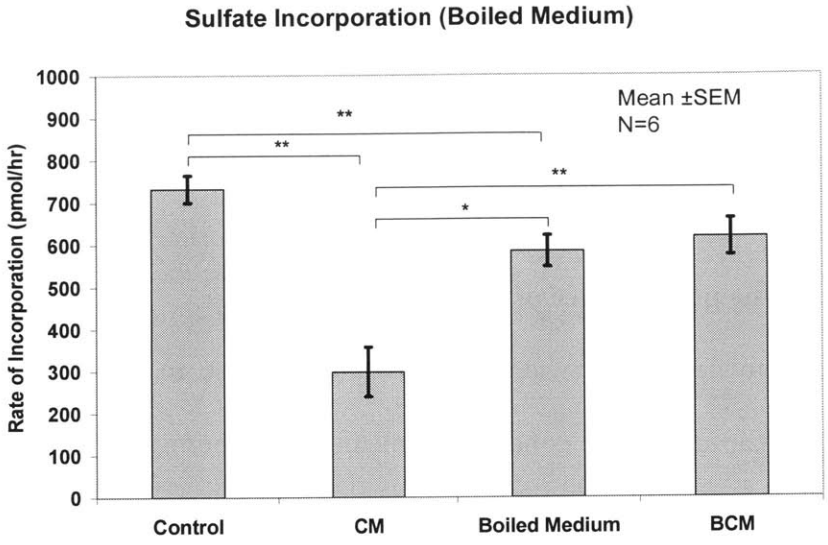

Figure 5: Rate of sulfate incorporation. Boiled medium and boiled conditioned medium were boiled for 10 minutes in Erlenmeyer flasks and then placed on ice to cool and sterile filtered. Cartilage was incubated for three days in 0.5ml of medium before beginning the radiolabel. '*' represents a p value of <0.02 and '**' represents a p value of <0.01 by a two sided student t-test.

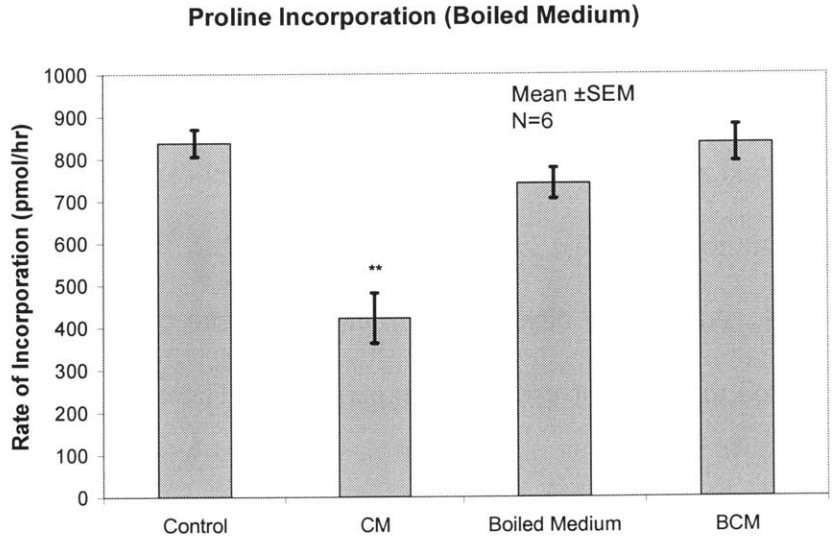

Figure 6: Rate of proline incorporation. Boiled medium and boiled conditioned medium were boiled for 10 minutes in Erlenmeyer flasks and then placed on ice to cool and sterile filtered. Cartilage was incubated for three days in 0.5ml of medium before beginning the radiolabel. '**' represents a p value of <0.01

From figures 5 and 6 we can see that boiling the conditioned medium was able to reverse the effect of the unknown factor. This would indicate that the unknown factor is some kind of protein.

2.4 Determining the permanence of the effect.

It was important to determine whether or not the decrease in biosynthesis seen after culturing in joint capsule tissue conditioned medium was permanent. If the effect was permanent even after the factor was removed, it would be important to make sure that the factor was never released. However, if the effects were reversible and biosynthesis levels could be brought back up to control levels, blocking it after it is released would be enough to prevent further damage.

2.4.1 Methods:

Cartilage was harvested in the usual manner. Joint capsule tissue was excised medial and lateral to the joint and punched to form 3mm disks. The joint capsule and cartilage plugs were allowed to equilibrate in 10% FBS culture medium. Two JCT disks were incubated in 1ml of medium. After three days, the joint capsule tissue conditioned medium was collected and pooled together for use in this experiment. There were four experimental groups:

1) Control (incubated for 3 days in normal 10%FBS medium)

2) JCTCM Control (incubated in conditioned medium for 3 days)

3) Recovery Control (Medium changed on day 3 and incubated an additional 5 days)

4) JC TCM Recovery (incubated in conditioned medium for 3 days, washed 3 times for 10-15 minutes in 10% FBS medium and then incubated for an additional 5 days).

Each plug was incubated in 0.5ml of medium. Groups 1 and 3 were incubated in freshly made 10% FBS medium while Group 2 and group 4 were incubated in the conditioned medium obtained from the joint capsule tissue. After three days of incubation, groups 1 and 2 were labeled with 10μCi/ml of ^{35}S-sulfate and ^{3}H-Proline for 18 hours. After 18 hours the plugs were washed 3 times for 10-15 minutes each wash in Rx wash. Each plug was then digested using pro K and radiolabel incorporation was measured using a scintillation counter. Groups 3 and 4 were washed 3 times in 10% FBS medium for 10-15 minutes a wash and then incubated an additional five days before being labeled and digested. N=6 for all groups and the plugs were matched for location across the four experimental groups.

2.4.2 Results:

Figure 7 shows sulfate incorporation for the recovery experiment.

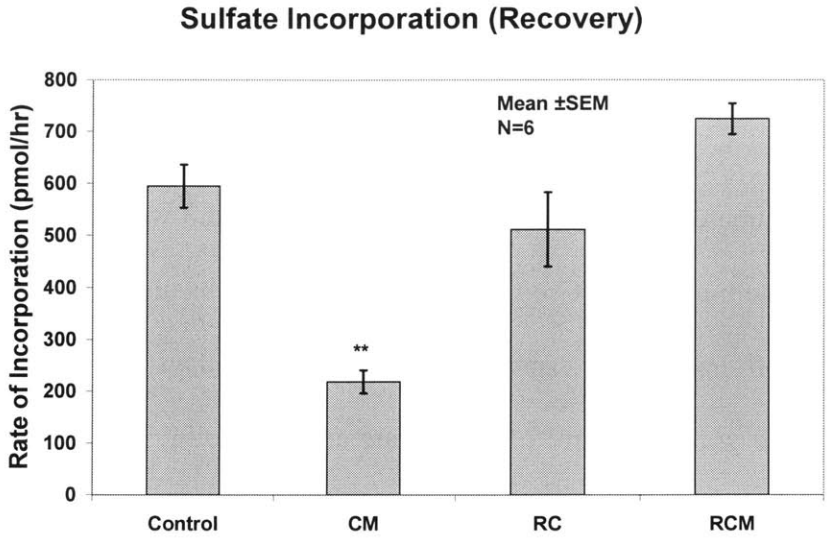

Figure 7: Rate of sulfate incorporation. Control and Conditioned medium cartilage were incubated for three days prior to being labeled with ^{35}S-sulfate and ^{3}H-proline. The recovery control had its medium changed on day three and was incubated an additional 5 days before being labeled with ^{35}S-sulfate and ^{3}H-proline. The recovery conditioned medium cartilage was washed three times for 10 minutes in 10% FBS medium before being placed in fresh medium that had not been conditioned by joint capsule tissue for 5 days. '**' represents a p value of <0.01 by a two sided student t test.

Figure 7 shows that the rate of sulfate incorporation for cartilage that has been cultured in conditioned medium and then cultured in fresh medium returns to control levels once the cartilage is no longer being cultured in conditioned medium. This indicates that the effect is not permanent and blocking it after it has been released should be sufficient to stop its effects.

2.5 Identifying the approximate size of the Factor

Because the unknown factor was not IL-1α or TNF-α, it was important to determine the approximate size in order to begin to gather more information in an effort to identify it.

Amicon centricon centrifuge filters were used to separate the medium components into three separate size ranges: greater than 3 KD, greater than 30 KD, and greater than 100KD.

2.5.1 Methods:

Joint capsule tissue conditioned medium from days 1-12 after harvest were pooled together to get a large amount of conditioned medium. One milliliter of this medium was then filtered through one of three sizes of Amicon centricon filters,YM-3, YM-30 or YM-100. The retentate from the 30 and 100 filters was washed three times with 250μl of DI water in order to desalt it. The retentate left on the YM-3 filter was washed once with 250μl of DI water and once with 100μl of DI water. Once most of the liquid had been filtered, the filtrate vial was removed and saved and the filter was flipped over and the retentate was reconstituted with 100μl of PBS. The reconstituted retentate was then added to 4.5ml of fresh 10% FBS medium. This solution was sterile filtered through a 0.2μm syringe filter and one milliliter of this filtered medium was put into 4 wells of a culture plate. This was repeated for each of the three different sizes. The control for this experiment was fresh medium which did not contain anything from the JCT conditioned medium. Cartilage disks harvested 7 days earlier were then transferred to the culture wells and cultured for three days. The cartilage disks were then labeled with medium containing 10 μCi/ml of ^{35}S-Sulfate and ^{3}H-proline. After 6 hours the disks were washed three times for 10-15 minutes each wash with Rx wash. The disks were then digested in 1ml of Proteinase K in a 60° water bath. Once digested, 100μl of the digested tissue and 2ml of ethyl alcohol were added to scintillation vials and a scintillation counter

was used to read the results. 250µl of the conditioned medium was digested with 100µl of Proteinase K and the GAG loss was measured.

2.5.2 Results:

Figure 8 shows sulfate incorporation for the size filtration experiment and figure 9 shows proline incorporation for the same experiment.

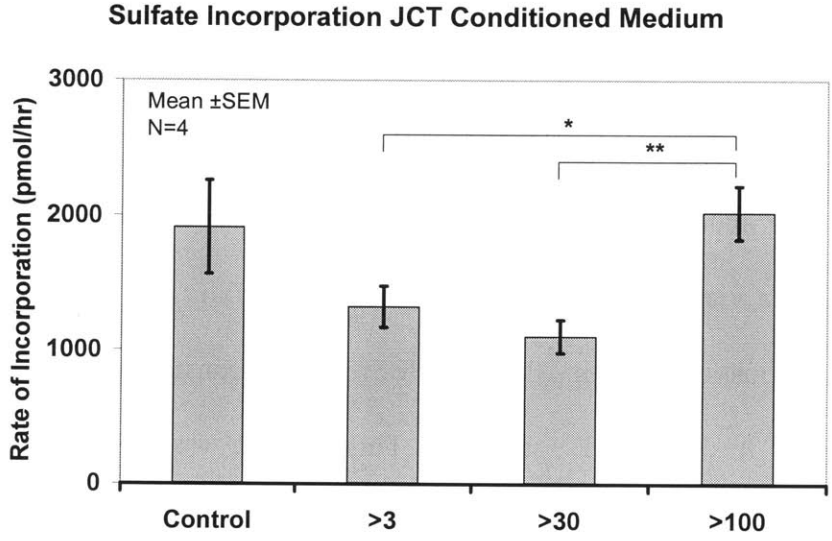

Figure 8: Rate of sulfate incorporation for cartilage plugs incubated for three days in control (10% FBS medium) and conditioned medium which has been fractionated using Amicon Centricon YM-3, YM-30 and YM-100 centrifuge filters. '*' represents $p<0.02$ '**' represents $p<0.01$ from a two sided student t test.

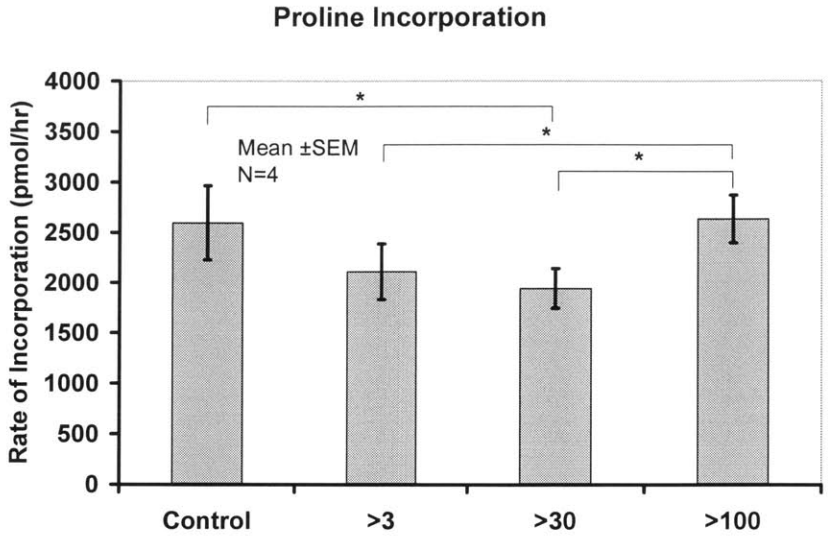

Figure 9: Rate of proline incorporation for cartilage plugs incubated for three days in control (10% FBS medium) and conditioned medium which has been fractionated using Amicon Centricon YM-3, YM-30 and YM-100 centrifuge filters. '*' represents $p<0.05$ from a two sided student t test.

Figures 8 and 9 show that radiolabel incorporation is significantly less for the >3KD and >30 KD samples than it is for the >100 KD samples which would indicate that the unknown factor is between 3-100 KD in size.

Figure 10 shows GAG loss for the same experiment.

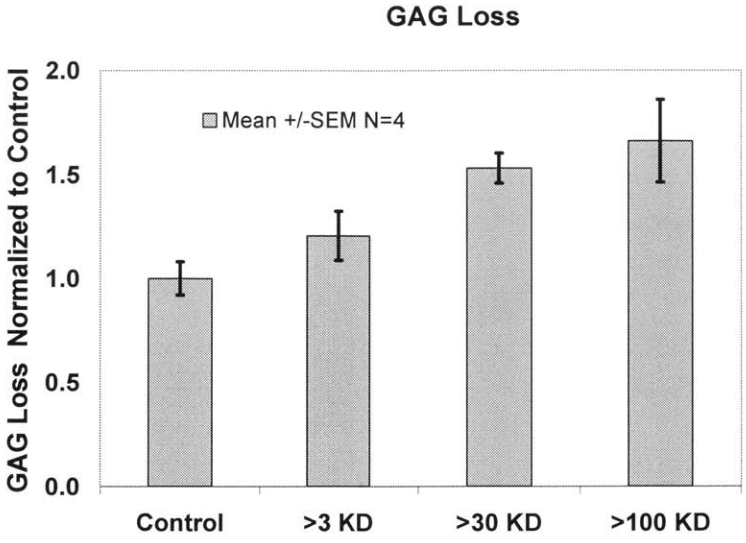

Figure 10: GAG loss normalized to control for cartilage plugs incubated for three days in control (10% FBS medium) and conditioned medium which has been fractionated using Amicon Centricon YM-3, YM-30 and YM-100 centrifuge filters.

In figure 10, it is unclear why the GAG loss appears to increase as the size of the centrifuge filter increases, perhaps this is because there are smaller proteins that are filtered out which help block GAG loss.

The same experiment was repeated but this time non conditioned medium and medium supplemented with 10ng/ml of IL-1 were also filtered through YM-3, YM-30 and YM-100 centrifuge filters. Because the cartilage plugs could not be matched for location for every condition, results were normalized to DNA.

Figure 11 shows sulfate incorporation for this experiment.

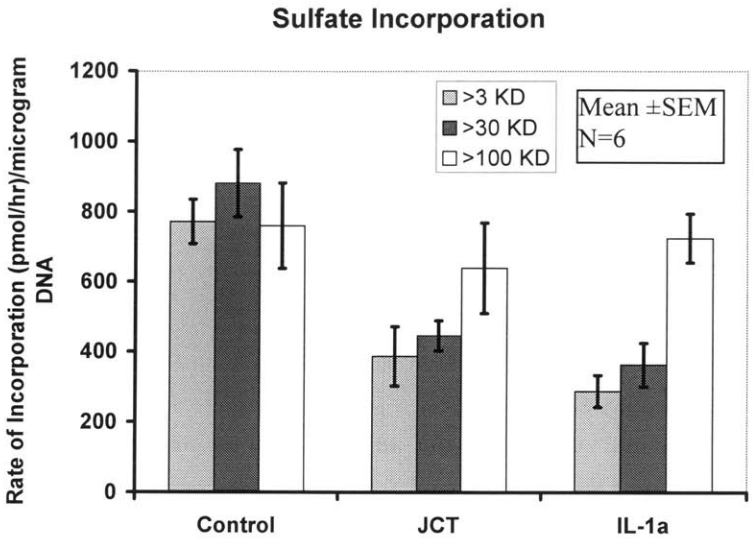

Figure 11: Rate of sulfate incorporation for cartilage plugs incubated for three days in fractionated 10% FBS medium, Joint capsule tissue conditioned medium and 10% FBS medium + 10ng/ml IL-1a. Results were normalized to DNA.

Figure 11 shows the same trends as figure 8. The filtered joint capsule tissue conditioned medium and the IL-1α treated medium have decreased radiolabel incorporation for the >3KD and >30KD fractions while incorporation levels are up for the >100KD sample. Because synthesis levels are down for the >30KD fraction for IL-1 treated medium and IL-1 is only about 20KD, it would indicate that the filter is either becoming saturated so smaller proteins cannot filter through or there is some interaction between molecules in the retentate that won't allow some other smaller molecules to filter through the filter. Filtering untreated 10% FBS medium appeared to have no effect on incorporation levels.

2.6 Isolating the unknown factor

After discovering the approximate size using centrifuge filters, an attempt was made to isolate the protein using a size filtration column. By identifying the column fraction which contained the protein, it might be possible to determine more information about it.

2.6.1 HPLC Separation

2.6.1.1 Method:

Joint capsule tissue was harvested in the usual manner and incubated in medium consisting of low glucose DMEM, ITS, PSA, Ascorbate, Proline, Non-essential amino acids and Hepes Buffer. This medium was then frozen and later unthawed and 4ml was removed. This 4 ml was filtered through an Amicon, Centricon YM-100 centrifuge filter, refrozen using liquid nitrogen and then lyophilized and reconstituted in 200ul of PBS. It was then injected into a Superose 12 column and fractionated into 1ml fractions. PBS was used as the running buffer. After the medium was fractionated, the ultraviolet absorbance at 280nm was measured to determine which fractions contained proteins.

2.6.1.2 Results:

Figure 12 shows the elution pattern of proteins that was observed by measuring UV absorbance at 280nm.

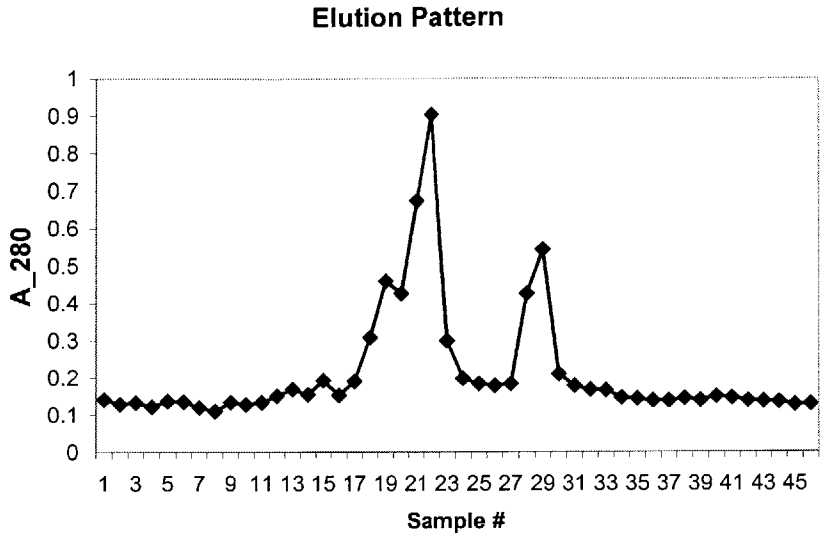

Figure 12: UV absorbance at 280nm of joint capsule tissue conditioned medium column fractions obtained from running concentrated conditioned medium through a Superose 12 column with PBS as the running buffer.

Figure 12 shows that there are peaks between fractions 18-23 and fractions 27-30. This indicates that these are the regions that contain proteins and will be the fractions that are used in later experiments to try and isolate the unknown factor. Because these experiments were done before IL-1α had been eliminated as a suspect, the same experiment was completed using ITS medium supplemented with 10ng/ml IL-1. Figure 13 shows the absorbance pattern for joint capsule tissue conditioned medium and IL-1 treated medium.

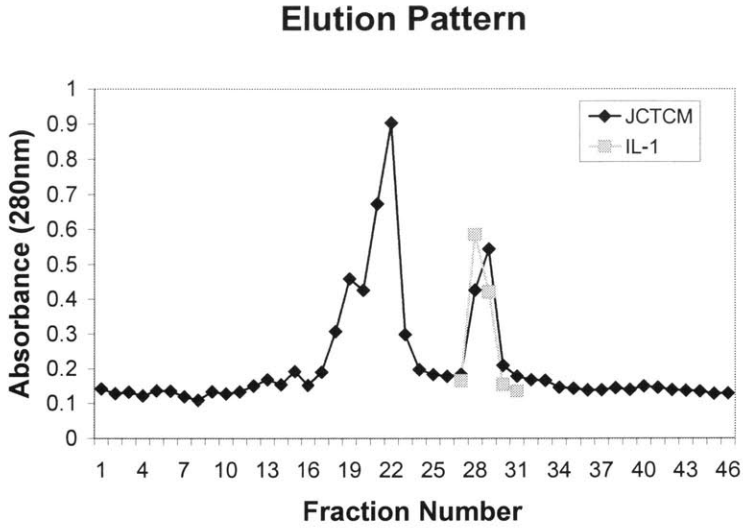

Figure 12: UV absorbance at 280nm for Superose 12 fractions of joint capsule tissue conditioned medium and IL-1 treated medium.

Figure 12 shows that the second peak seen in the plot lines up with the peak seen in the IL-1 treated medium. This means that the proteins in this peak are around 23 KD in size.

The fractions where the peaks were seen were split in half and half of the fraction was used to test the fraction's affect on chondrocyte biosynthesis and the other half was used to run an SDS PAGE Gel.

2.6.2 Biosynthesis levels of cartilage cultured with column fractions

2.6.2.1 Method:

In order to test the effects of each fraction on cartilage biosynthesis the samples were frozen, lyophilized and then reconstituted in 2.5 ml of 10% FBS medium and then sterile

filtered. Cartilage plugs were then cultured in 0.5ml of medium for three days and then labeled with 10μCi/ml of ^{35}S-sulfate and ^{3}H-proline.

2.6.2.2 Results:

Figure 13 shows sulfate incorporation normalized to DNA for this experiment.

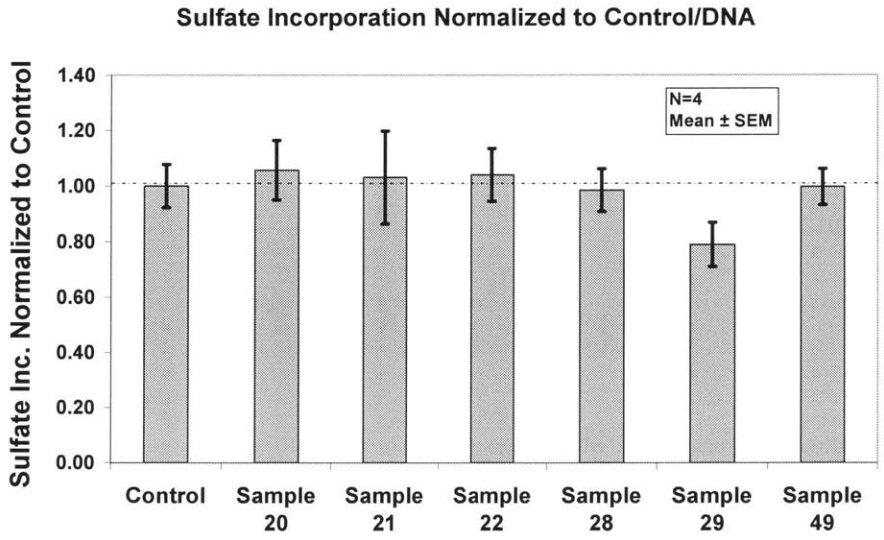

Figure 13: Sulfate incorporation normalized to control for cartilage plugs incubated in 10% FBS medium supplemented with 0.5ml of lyophilized column fractions. Column fractions were selected based on their UV absorbance at 280nm.

From Figure 13 it appears that Sample 29 is the most likely fraction to contain the unknown factor as it is the only fraction that appears to have any decrease in biosynthesis levels.

2.6.3 SDS PAGE Gel

2.6.3.1 Methods

Because it was determined that fraction 29 most likely contained the unknown factor, it was chosen to be run on an SDS page gel. It was run along with fraction 28 of the joint capsule tissue conditioned medium and fraction 29 of the medium supplemented with rhIL-1. The column fractions were frozen in liquid nitrogen, lyophilized and then reconstituted in 30 μl of de-ionized water. Thirty microliters of sample buffer was added to the reconstituted samples. These were boiled for 5 minutes and then spun down prior to loading. 20 μl was loaded into each lane and it was run at 175 V. It was then stained using a silver staining kit.

2.6.3.2 Results

Figure 14 show the gel after silver staining.

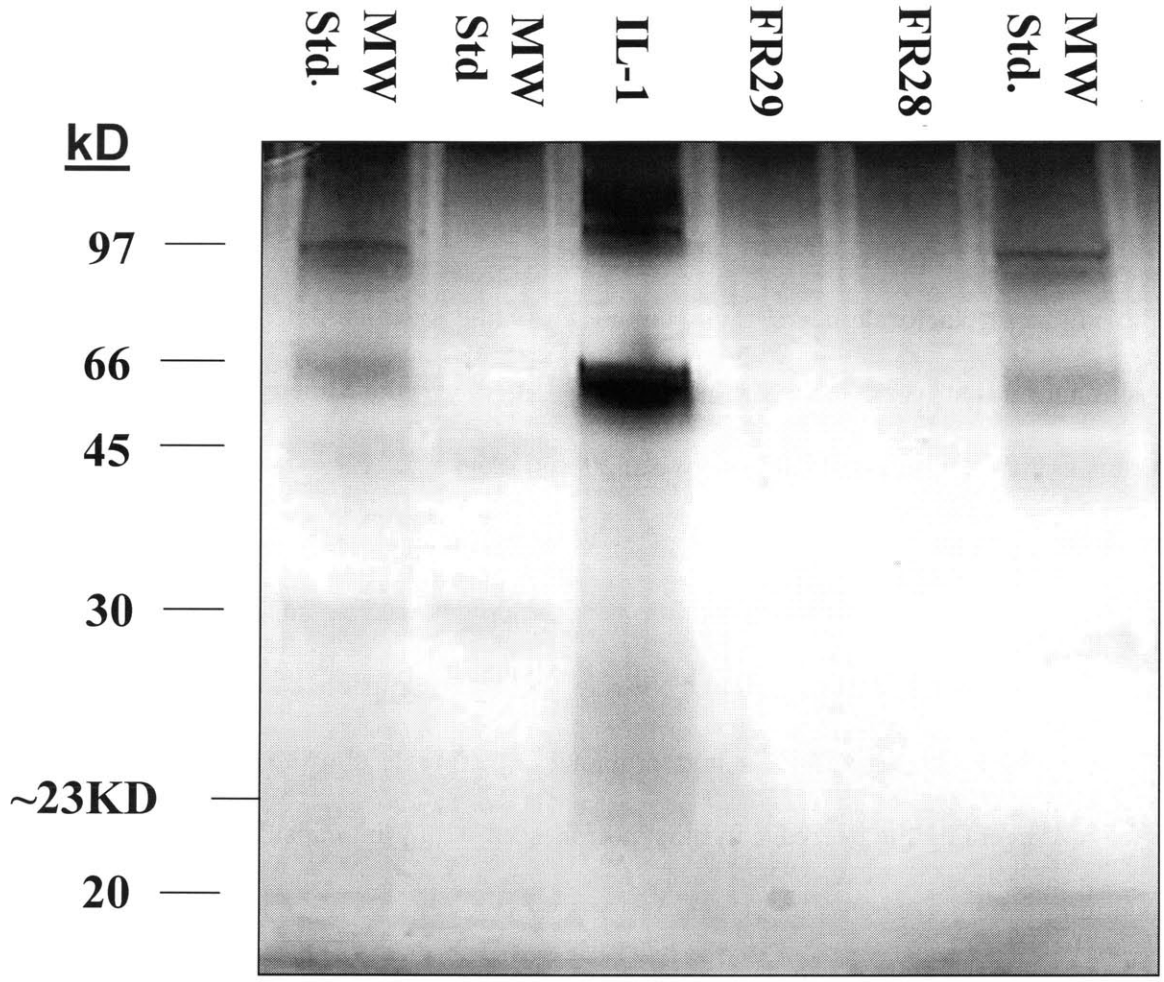

Figure 14: Silver stain of SDS page gel of column fractions identified to have the unknown factor in them as well as 1ng/ml of rh IL-1. The band at ~66KD is probably BSA and the band at 97KD is another additive to the IL-1 stock solution.

In the gel, one can see a faint line at ~23 KD in the IL-1 fraction, but no lines are visible anywhere in the joint capsule tissue conditioned medium fractions. This is probably because there is not a high enough concentration of the factor to show up by silver staining. In order for it to show up at it's appropriate MW, it is probably necessary to

concentrate the medium even more before running it through the column as well as potentially combining fractions from multiple column runs.

2.7 Discussion & Conclusions:

The unknown factor appears to be about the same size as Il-1α based on the size fractionation that was done with the centrifuge filters and the Superose 12 column. This means that the factor is probably between 20-30 KD in size. IL-1 itself can be eliminated as a suspect because of the experiments done using IL-1ra and the IL-1 soluble receptor with human joint capsule tissue conditioned medium showed no success at reversing the effects of the conditioned medium. TNF-α is also not responsible because etanercept was shown to block bovine TNF- α and previous experiments showed that it also had no effect on reversing the decrease in biosynthesis seen with joint capsule tissue conditioned medium.

These experiments also show that the joint capsule tissue is releasing something that results in a decrease in biosynthesis levels and that the decrease is not caused by nutrient depletion in the medium. If it were because of nutrient depletion, the size fractionation experiment should not have shown a decrease in biosynthesis levels as the retentate was added to fresh medium.

In the future, it may be useful to run more columns to separate out the unknown factor and then run those column fractions through a mass spectrometer to see if it is possible to identify what proteins are in the samples. By doing this, it may be possible to narrow

down the search to only a few suspects. Using mass spectrometry would probably also be a more efficient way of trying to identify the protein than trying to pick likely suspects and block their effect because it has a large database to draw on. Mass spectrometry should also be able to determine whether or not the factor has been previously identified.

The other possible future direction would be to look at other cytokines that are known to inhibit cartilage biosynthesis such as IL-6, oncostatin-M and leukemia inhibitory factor.[25] The drawback of this method is that unless one of these cytokines is responsible for the decrease in biosynthesis, there is no additional information about what the factor could be, only what it isn't. This does not significantly narrow down the field of suspects and is very time intensive for minimal returns.

Chapter 3: Joint Capsule Tissue Conditioned Medium Model

3.1 Background

While performing different experiments using the joint capsule tissue conditioned medium model it was observed that certain effects such as a synergistic increase in GAG loss with injury were seen in some experiments and not in others. It was suspected that this effect depended on the concentration of the unknown factor or the interaction of the joint capsule tissue and the injured cartilage. Up to this point there was no standardized protocol for the number of days to condition medium or any standardized number or size of punched pieces of joint capsule tissue per 1ml of medium. Therefore, the experiments in this chapter were directed at standardizing the protocol for the joint capsule tissue conditioned medium model.

3.2 Methods

Cartilage and joint capsule tissue were harvested as previously described in chapter 2. Medium was conditioned by putting one 3mm punched piece of joint capsule tissue in 1ml of either 10% FBS or ITS medium. These pieces were incubated in the medium for one, two, three or four days. Conditioned medium was supplemented with ascorbate just prior to use. At the same time, the cartilage was allowed to equilibrate for three days in 10% FBS medium. After three or four days of equilibrating the cartilage plugs were placed in one of four conditions with the cartilage plugs being matched for location across the four groups. Cartilage was injured by applying 50% strain at a strain rate of 1mm/sec. using a custom built incubator housed loading device as previously

described[8,21]. After intervention, cartilage plugs were incubated for four days. After four days, the cartilage plugs were radiolabeled with ^{35}S-Sulfate and ^{3}H-Proline for 24 hours after which they were washed, digested and counted as previously described[27]. On day four after intervention, the GAG lost to the medium was also measured by DMMB assay[6].

3.3 Number of days of conditioning necessary to obtain consistent, significant results

The goal of these experiments was to determine what difference it made conditioning medium for varying length of time. In this case, medium was conditioned for one day, two days or three days.

3.3.1 Methods

For these experiments, medium was conditioned by placing one 3mm punched piece of JCT in 1ml of 10% FBS medium for one, two or three days. After the appropriate number of days, the medium was pooled and supplemented with ascorbate. This medium was then used for two of the four groups. In this experiment each cartilage plug was incubated in 0.5ml of medium and n=6. The four groups for these experiments were:

1) Control
2) Injury
3) Conditioned Medium (one day, two days, or three days)
4) Conditioned Medium (one day, two days, or three days) + Injury

These experiments were started three days after harvest. Injury was to 50% strain at a rate of 1mm/sec using an incubator housed loading device.

A fourth experiment was also done which directly compared the effect of the number of days of conditioning on GAG Loss and radiolabel incorporation without injury. For this experiment N=5 and the four groups were as follows:

1) Control
2) JCTCM (conditioned for one day)
3) JCTCM (conditioned for two days)
4) JCTCM (conditioned for three days)

3.3.2 Results:

3.3.2.1 Medium conditioned for one day

Figure 1 shows GAG loss for injured and uninjured cartilage cultured in medium which had been conditioned for one day by one piece of joint capsule tissue per milliliter of medium.

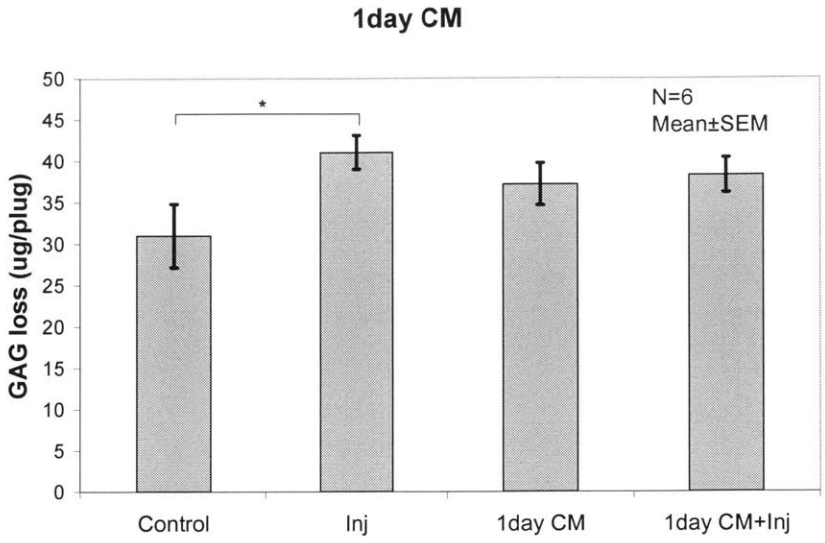

Figure 1: GAG loss (micrograms/plug) for uninjured and injured cartilage cultured in 10% FBS medium and 10% FBS medium which had been conditioned with one 3mm piece of joint capsule tissue for one day. '*' represents p value of <0.05 by two sided student t test.

The results of Figure 1 show that there is a slight increase in GAG loss in uninjured cartilage over control cartilage, however there is really no difference between the GAG loss of injured and uninjured cartilage that was cultured in the medium conditioned with joint capsule tissue for one day.

Figure 2 shows sulfate incorporation for the same experiment.

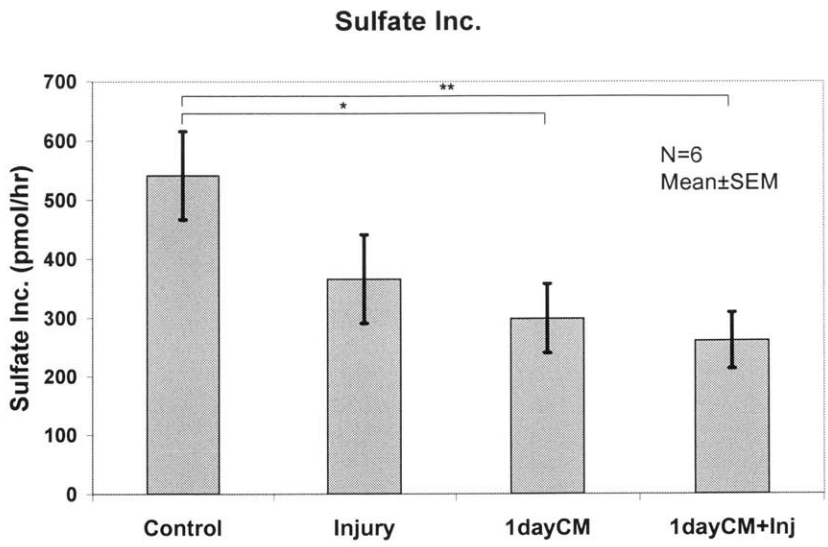

Figure 2: Sulfate Incorporation of injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for one day. '*' represents p value of < 0.05, '**' represents p value of < 0.02 by a two sided student t test.

The results of Figure 2 show that conditioning medium for one day with one 3mm piece of joint capsule tissue is not enough to get a significant decrease in sulfate incorporation when compared to the incorporation levels of injured cartilage. This means that a higher concentration of joint capsule tissue should be used to condition the medium or that the medium needs to be conditioned for longer.

3.3.2.2 Medium conditioned for two days

Figure 3 shows GAG loss from injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for two days.

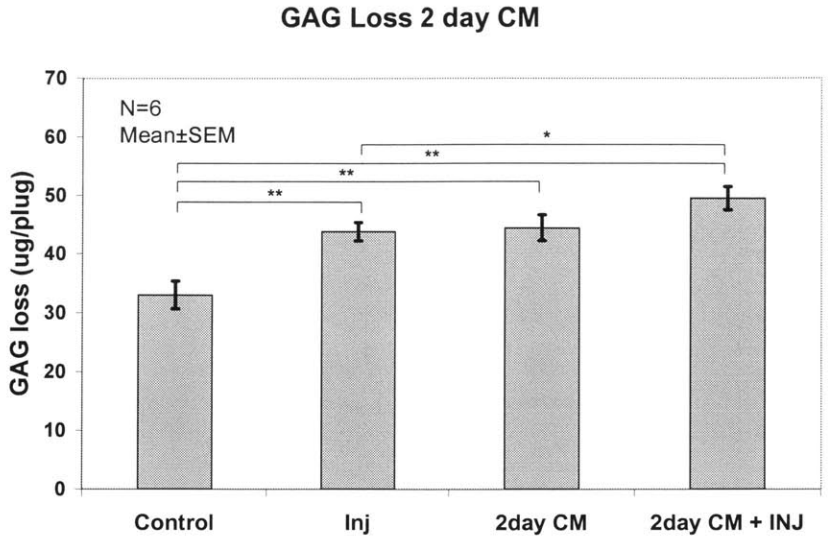

Figure 3: GAG loss of injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for two days. '*' represents p value of <0.05 and '**' represents p value of <0.006 by a two sided student t test.

Like the results in Figure 1, Figure 3 shows that although the GAG loss levels are higher for cartilage in conditioned medium, there is still not a significant increase in GAG loss with injury.

Figure 4 shows sulfate incorporation for injured and uninjured cartilage cultured in 10% FBS or 10% FBS medium conditioned for two days by one 3mm piece of JCT per milliliter of medium.

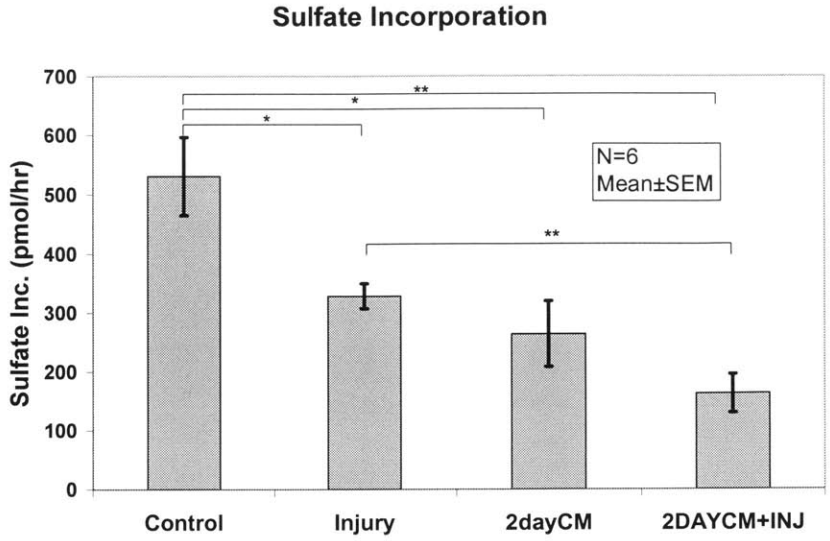

Figure 4: Sulfate incorporation of injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for two days. '*' represents $p<0.03$, '**' represents $p<0.003$ by a two sided student t test.

Similar to the results from Figure 2, Figure 4 shows that once again there is a significant decrease in radiolabel incorporation for injured and uninjured cartilage that is not cultured in conditioned medium and only a slight decrease in incorporation levels for injured and uninjured cartilage cultured in conditioned medium, this time however, the differences in radiolabel incorporation for injured and uninjured cartilage that is in unconditioned medium and conditioned medium are significant. This means that two days is probably the minimum amount of time needed for conditioning medium at this concentration of joint capsule tissue.

3.3.2.3 Medium conditioned for three days

Figure 5 shows GAG loss after four days for injured cartilage cultured in 10% FBS medium and 10% FBS medium conditioned with one 3mm piece of joint capsule tissue per milliliter for three days.

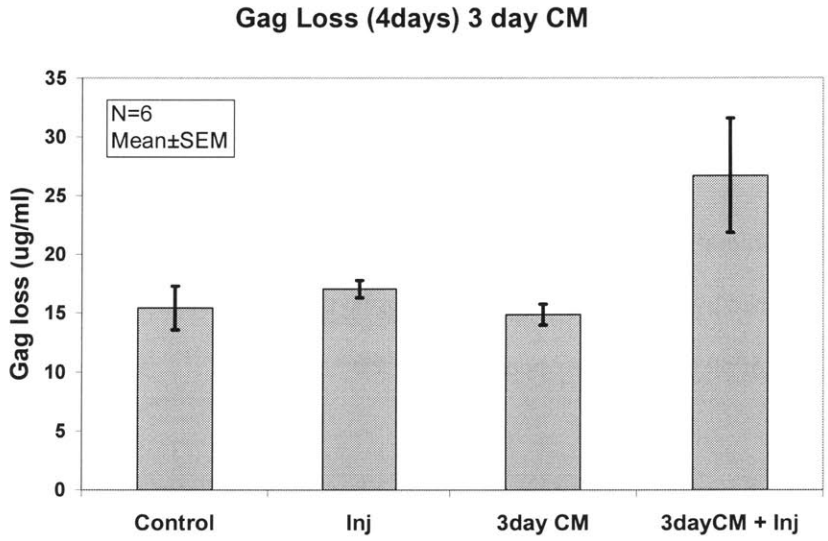

Figure 5: GAG loss of injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for three days.

The results in Figure 5 are different from the previous GAG loss results because this time there appears to be a large increase in GAG loss. However the results are not quite significant, $p=0.06$ by a two sided student t test, probably because the majority of the GAG loss appeared to come from only a couple of the cartilage plugs.

Figure 6 shows the sulfate incorporation for the same experiment.

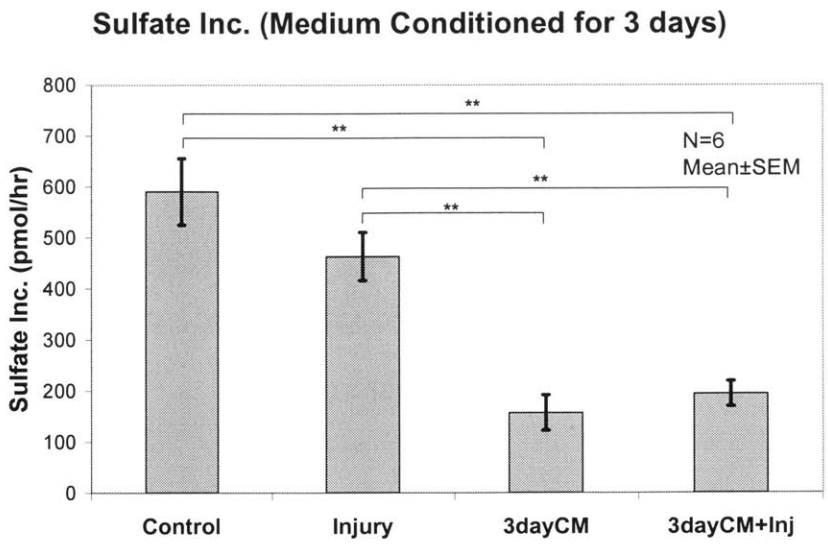

Figure 6: Sulfate incorporation of injured and uninjured cartilage cultured in 10% FBS medium or 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for three days. '**' represent p value of <0.002 by a two sided student t test.

After three days of conditioning the medium, there is finally a significant decrease in sulfate incorporation levels between injured cartilage in unconditioned medium and uninjured cartilage in conditioned medium. From figure 6, it appears that if one wants to be certain that the differences in radiolabel incorporation are significant, it is important to condition the medium for at least two days but preferably three days at a concentration of one 3mm punched piece per milliliter of medium.

3.3.2.4 Direct comparison of the number of days medium is conditioned.

Figure 7 is a comparison of GAG loss for uninjured cartilage cultured in unconditioned medium and conditioned medium that has been conditioned for one day, two days or three days by one 3mm punched piece of joint capsule tissue.

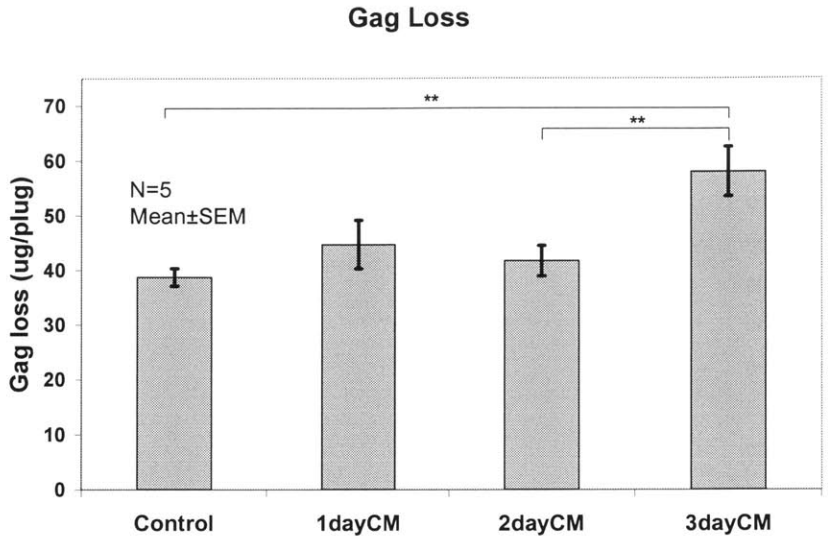

Figure 7: Comparison of GAG loss for cartilage cultured in 10% FBS medium and 10% FBS medium conditioned for one days, two days, three days. '**' represents p value of <0.02 by a two sided student t test.

Figure 7 shows that there doesn't appear to be any correlation between the number of days that the medium is conditioned and the amount of GAG loss. The three day conditioned medium is probably higher because it has more GAG from the joint capsule tissue than the 1 day and 2 day samples.

Figure 8 shows sulfate incorporation for the same experiment.

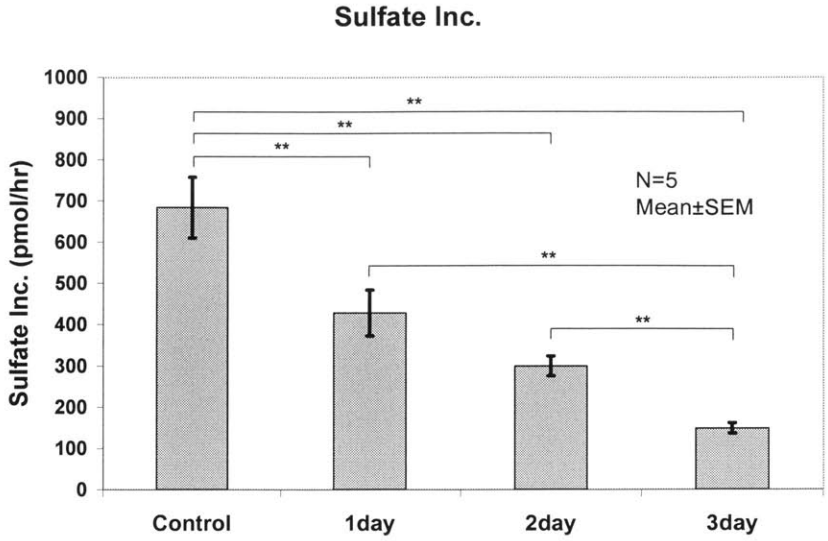

Figure 8: Comparison of Sulfate incorporation for cartilage cultured in 10% FBS medium and cartilage cultured in medium conditioned by joint capsule tissue for one, two or three days. '**' represents p value <0.02 by a two sided student t test.

Figure 8 shows that radiolabel incorporation appears to be linearly related to the number of days that the medium is conditioned. This agrees with the results from the earlier experiments and suggests that three days is probably a good number of days to condition the medium to ensure statistically significant results.

3.4 Which days after harvest can joint capsule tissue be used to condition medium?

Usually joint capsule tissue is obtained from the first three days after harvest. However, other times joint capsule tissue is used to condition medium 9-12 days after harvest. In these cases, there has always been a decrease in cartilage biosynthesis, results not shown, but GAG loss has not been looked at. Because there always appears to be a significant decrease in radiolabel incorporation, this experiment only looked at GAG loss.

3.4.1 Method

Joint capsule tissue was harvested in the usual manner and was punched to form 3mm disks. These disks were used to condition medium for a total of 12 days. Medium was pooled and collected every four days. Medium from the 8 days was stored in the incubator. Cartilage was harvested as previously described. A total of three experiments were done to look at GAG loss. The first looked at only uninjured cartilage, the second at injured cartilage and the third at the difference in GAG loss between uninjured and injured cartilage cultured in joint capsule tissue conditioned medium. 10% FBS medium was used for this experiment and cartilage was cultured in .5ml of medium after intervention. Cartilage was allowed to equilibrate for three days after intervention and then was cultured for an additional four days after intervention. After four days, GAG loss to the medium was measured.

Experiment 1: No injury

1) Control
2) Joint capsule tissue conditioned medium days 1-4 after harvest
3) Joint capsule tissue conditioned medium days 5-8 after harvest
4) Joint capsule tissue conditioned medium days 9-12 after harvest

Experiment 2: All plugs are injured

1) Injury
2) Joint capsule tissue conditioned medium days 1-4 after harvest + injury
3) Joint capsule tissue conditioned medium days 5-8 after harvest + injury
4) Joint capsule tissue conditioned medium days 9-12 after harvest + injury

Experiment 3:

1) Joint capsule tissue conditioned medium days 5-8 after harvest
2) Joint capsule tissue conditioned medium days 5-8 after harvest + injury
3) Joint capsule tissue conditioned medium days 9-12 after harvest
4) Joint capsule tissue conditioned medium days 9-12 after harvest + injury

Experiment 3 did not look at the GAG loss for the first four days after harvest with and without injury because several previous experiments have already done that. As in previous experiments plugs were matched for location across the four groups in each experiment.

3.4.2 Results

Figure 9 shows the results for experiment 1.

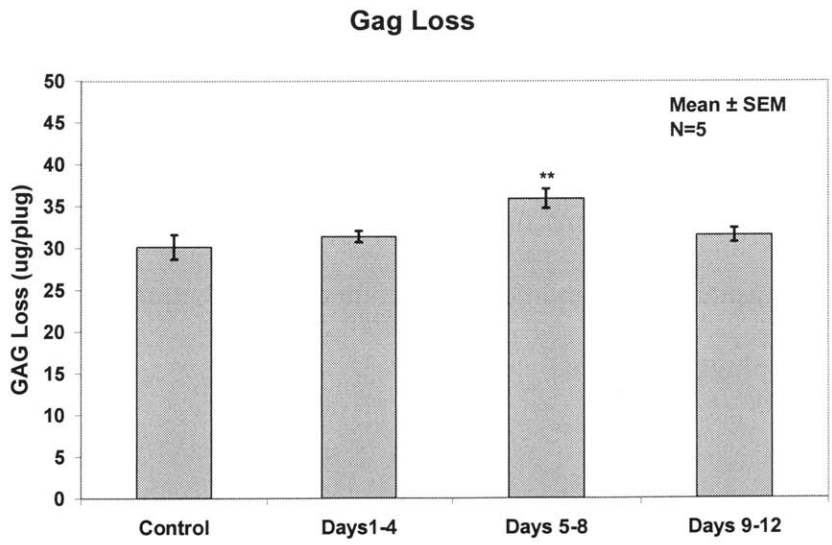

Figure 9: GAG loss after four days for uninjured cartilage cultured in 0.5ml of 10% FBS medium and 10% FBS medium conditioned by joint capsule tissue in the first four days after it was harvested, days 5-8 after it was harvested and days 9-12 after it was harvested. '**' indicates p value of <0.02 by a two sided student t test.

Figure 9 shows that the only group that had a significant increase in GAG loss was the group cultured in medium conditioned days 5-8 after harvest. This experiment would need to be repeated if one is concerned about the increase seen with the days 5-8 medium, otherwise it appears that it doesn't really matter which days after harvest you use for looking at GAG loss without injury.

Figure 10 shows the results for experiment 2.

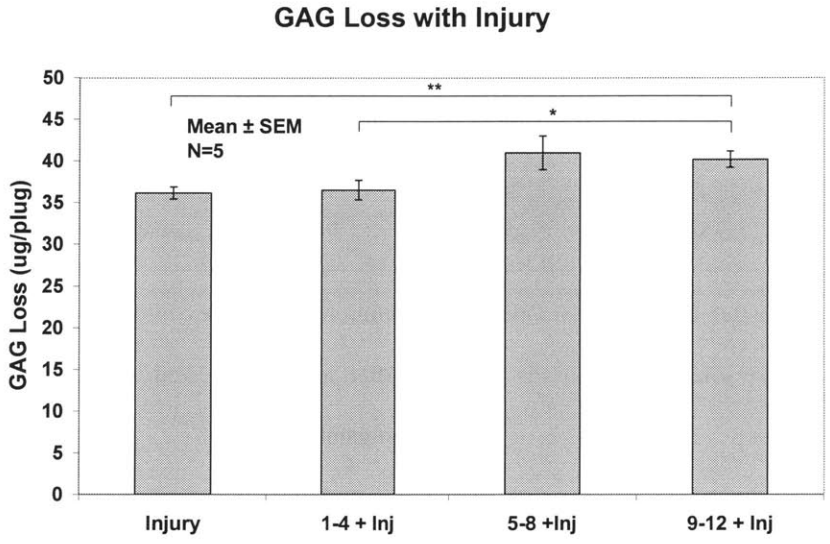

Figure 10: GAG loss after four days for injured cartilage cultured in 0.5ml of 10% FBS medium and 10% FBS medium conditioned by joint capsule tissue in the first four days after it was harvested, days 5-8 after it was harvested and days 9-12 after it was harvested. '*' indicates p value of <0.04, '**' indicates p value of <0.01 by a two sided student t test.

In figure 10, only days 9-12 is significantly higher than the injured cartilage in normal medium and the injured cartilage in days 1-4 of conditioned medium. This would seem

to indicate that like the results in figure 19, it is probably reasonable to assume that they are all roughly equivalent.

Figure 11 shows the results for experiment 3.

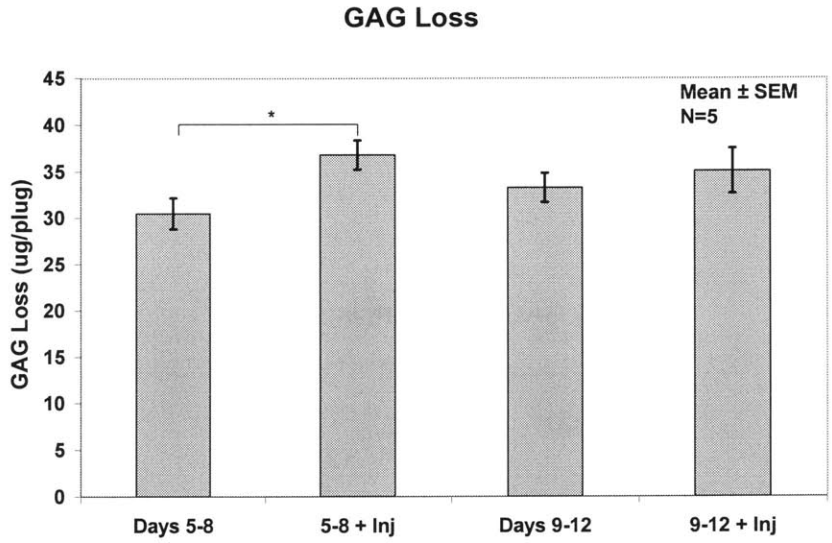

Figure 11: GAG loss after four days for injured and uninjured cartilage cultured in 0.5ml of 10% FBS medium conditioned by joint capsule tissue days 5-8 after it was harvested and days 9-12 after it was harvested. '*' indicates p value of <0.03 by a two sided student t test.

In figure 11, there is a significant increase in GAG loss when cartilage is injured with medium conditioned on days 5-8. However, this was not seen in previous experiments and is probably not a consistent result which would indicate that it probably does not matter which days after harvest are used to condition the medium. Also, combining the GAG loss results for uninjured cartilage in figure 11 with those in figure 19 shows that it doesn't really matter which days are used to condition medium because the overall GAG loss is pretty much the same.

3.5 Does human joint capsule tissue affect bovine cartilage?

Very few cytokine blockers have been made to work against bovine cytokines whereas there are a lot of available blockers for the human version. Because of this, it is very difficult to rule out possibilities for the unknown factor using a bovine system because there is always a question of cross species reactivity. Unfortunately, it is difficult to obtain human tissue and the tissue does not come on a regular schedule. This makes it difficult to use human tissue on a regular basis. However, if joint capsule tissue conditioned medium made using human joint capsule tissue were to have the same effect on bovine cartilage as bovine joint capsule tissue conditioned medium, it might be possible to make large quantities of conditioned medium using human joint capsule tissue and then use that conditioned medium to culture bovine cartilage. Human cytokine blockers could then be used without worrying about cross species interaction.

3.5.1 Methods

Human joint capsule tissue was harvested from the medial and lateral side of the femoropatellar groove, similar to the way bovine joint capsule tissue is harvested. The human joint capsule tissue was covered in a layer of fat which had to be cleaned off prior to culture in 10% FBS medium. Three millimeter punched pieces were cultured in 10% FBS medium for 3 days before the medium was collected and changed. One piece of joint capsule tissue was cultured per milliliter of medium. Left over 10% FBS medium was put in a 48 well plate in 1ml aliquots to serve as a control. This unconditioned medium was also left in the incubator for three days before being pooled and collected. Bovine cartilage was harvested in the usual manner and allowed to equilibrate for two

days prior to intervention. Two days after harvest some of the cartilage plugs were injured to 50% strain as previously described while others were left uninjured. Cartilage plugs were placed into one of four conditions:

1) Control
2) Injury
3) Human joint capsule tissue conditioned medium
4) Human joint capsule tissue conditioned medium + Injury

Cartilage plugs were incubated for three days in 0.5 ml of medium per plug prior to being radiolabeled for 24 hours with ^{35}S-Sulfate. Plugs were then washed and digested as previously described.

3.5.2 Results

Figure 12 shows sulfate incorporation for bovine cartilage cultured with human joint capsule tissue conditioned medium.

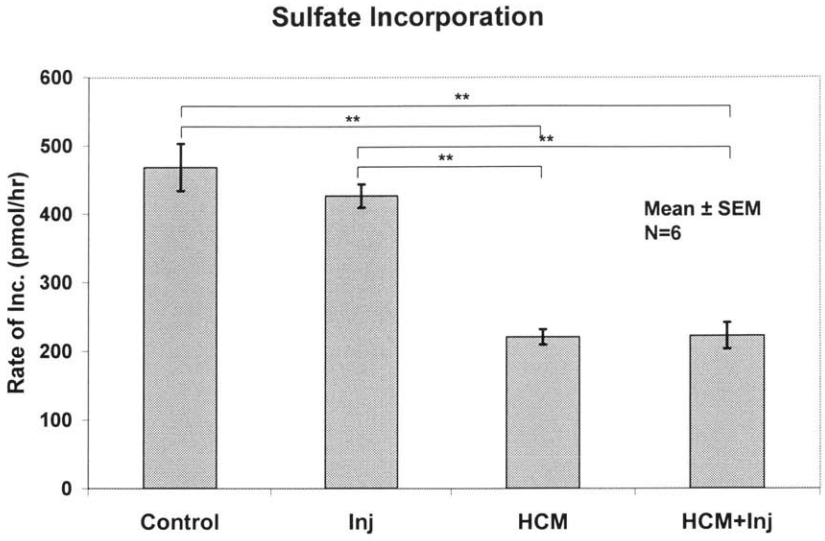

Figure 12: Sulfate incorporation for injured and uninjured bovine cartilage cultured in 10% FBS medium or 10% FBS medium conditioned with one 3mm piece of human joint capsule tissue per milliliter of medium for three days. Cartilage was cultured under these conditions for three days prior to being radiolabeled with ^{35}S-Sulfate for 24 hours. '**' represents p value of <0.0005 by a two sided student t test.

Figure 12 shows that there is still a significant decrease in radiolabel incorporation when bovine cartilage is cultured in medium conditioned by human joint capsule tissue instead of bovine joint capsule tissue. This would indicate that it should be possible to use medium conditioned by human joint capsule tissue on bovine cartilage in future experiments that make use of human blockers.

Figure 13 shows the result for GAG loss after three days.

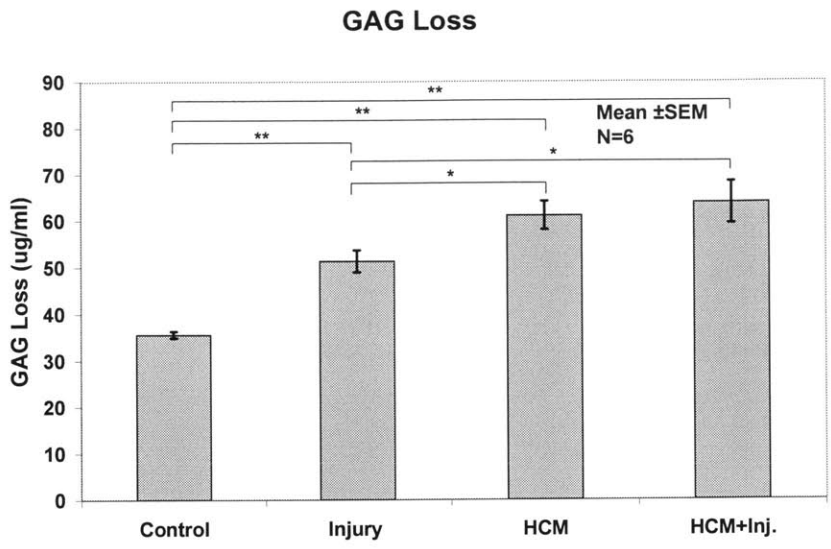

Figure 13: GAG loss for injured and uninjured bovine cartilage cultured in 10% FBS medium or 10% FBS medium conditioned with one 3mm piece of human joint capsule tissue per milliliter of medium for three days. Cartilage was cultured under these conditions for three days prior to being radiolabeled with ^{35}S-Sulfate for 24 hours. '*' represents p value of <0.05 '**' represents p value of <0.0002 by a two sided student t test.

Figure 13 shows that there is an increase in GAG loss when bovine joint capsule tissue is cultured with human joint capsule tissue, however, injury seems to have no effect on GAG loss.

3.6 Does dead joint capsule tissue have the same effect?

The next goal was to determine whether or not the same decrease in biosynthesis would be seen if dead joint capsule tissue was used to condition medium instead of live joint capsule. Because the tissue is dead, any decrease in biosynthesis would be attributed to something that was coming out of the tissue and not to nutrient depletion.

3.6.1 Methods

Joint capsule tissue was harvested as previously described and was punched using a 3mm dermal punch. These joint capsule tissue pieces were placed in a conical tube and frozen in the -80° C freezer for at least three days. The tissue was then thawed in a 37° C water bath. After being frozen for at least three days, one piece of joint capsule tissue was placed in 1ml of 10% FBS medium and incubated for three days. At the end of the three days, the medium was pooled together and supplemented with ascorbate. Cartilage was incubated in 0.5ml of medium for this experiment and N=6. The groups for this experiment were as follows:

1) Control
2) Injury
3) Co-Culture or Dead JCT Conditioned Medium
4) Co-Culture or Dead JCT Conditioned Medium + Injury

3.6.2 Results:

Figure 14 shows GAG loss from conditioned medium that was conditioned for three days by one 3mm piece of dead joint capsule tissue per milliliter of medium.

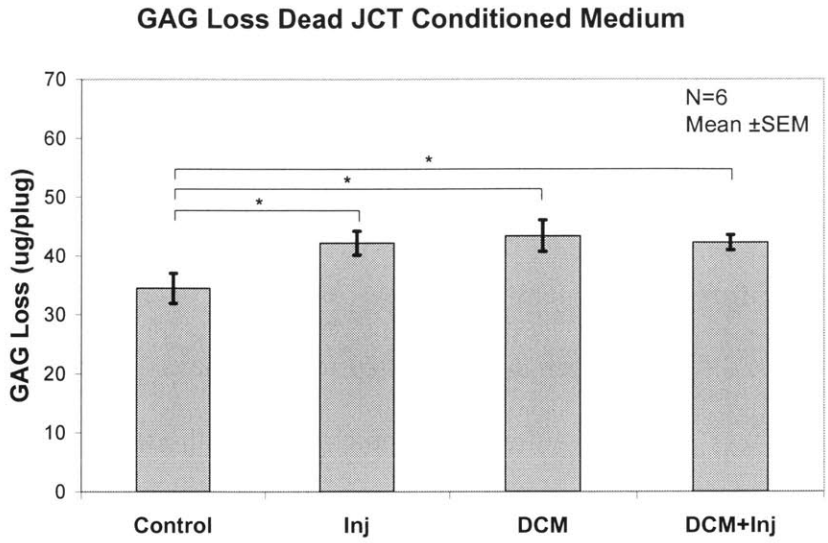

Figure 14: GAG loss for injured and uninjured cartilage cultured for four days in medium conditioned for three days with one 3mm piece of dead joint capsule tissue per one milliliter of 10% FBS medium. '*' represents p value of <0.05 by a two sided student t test.

Figure 14 shows there is no difference in GAG loss for injured and uninjured cartilage that was cultured in medium conditioned by dead joint capsule tissue. As will be discussed later in chapter 5 the increase in GAG loss seen between control cartilage and cartilage cultured in the dead joint capsule tissue conditioned medium is probably due to GAG loss by the joint capsule tissue.

Figure 15 shows sulfate incorporation for the same experiment.

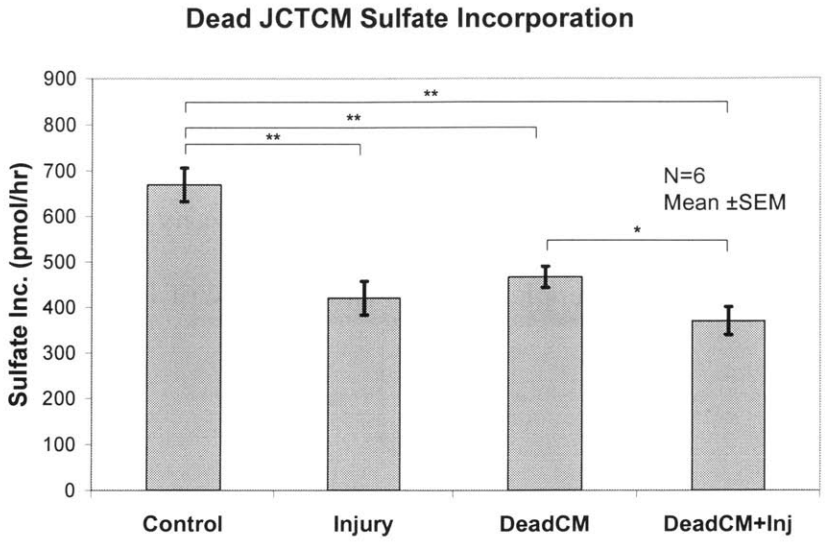

Figure 15: Sulfate incorporation for injured and uninjured cartilage cultured for four days in medium conditioned for three days with one 3mm piece of dead joint capsule tissue per one milliliter of 10% FBS medium. '*' represents p value of <0.05, '**' represents p value of <0.002 by a two sided student t test.

There appears to be a decrease in biosynthesis from culturing cartilage in medium conditioned by dead joint capsule tissue, however, there is no difference between incorporation levels for injured cartilage cultured in conditioned medium or non conditioned medium. This means that using dead joint capsule tissue to condition medium for an injury experiment may not be a good idea. There is also the danger that what is being released from the dead joint capsule tissue to cause the decrease in biosynthesis may not be the same as what is being released by the live joint capsule tissue because the cell membranes may have ruptured when the tissue was killed releasing other things into the medium.

3.7 ITS versus FBS medium

Because some experiments were done with 10% FBS medium and some experiments were done with ITS medium, it was important to see if the same trends in radiolabel incorporation and GAG loss were seen when there wasn't any serum. To do this, an experiment was done with ITS supplemented medium instead of 10% FBS medium.

3.7.1 Methods

3.7.1.1 ITS Conditioned Medium

ITS medium was conditioned for three days and the experiment was broken down into four groups like the previous experiments. The cartilage plugs were incubated in 0.5ml of medium for four days. N=6 for this experiment. The four groups for this experiment were:

1) Control
2) Injury
3) Conditioned Medium
4) Conditioned Medium + Injury

3.7.1.2 Comparison of FBS and ITS medium

In order to compare the absolute values of GAG loss and radiolabel incorporation, another experiment was done doing a direct comparison of ITS and 10% FBS medium conditioned by one 3mm piece of joint capsule tissue per milliliter of medium for three days. After intervention each plug was incubated in 0.5ml of medium for four days. In this case the four groups were:

1) ITSCM

2) ITSCM+Injury

3) FBSCM

4) FBSCM+Injury

3.7.2 Results:

3.7.2.1 ITS Conditioned Medium

Figure 16 shows GAG loss for injured and uninjured cartilage cultured in ITS medium or ITS medium conditioned by one 3mm piece of joint capsule tissue for three days.

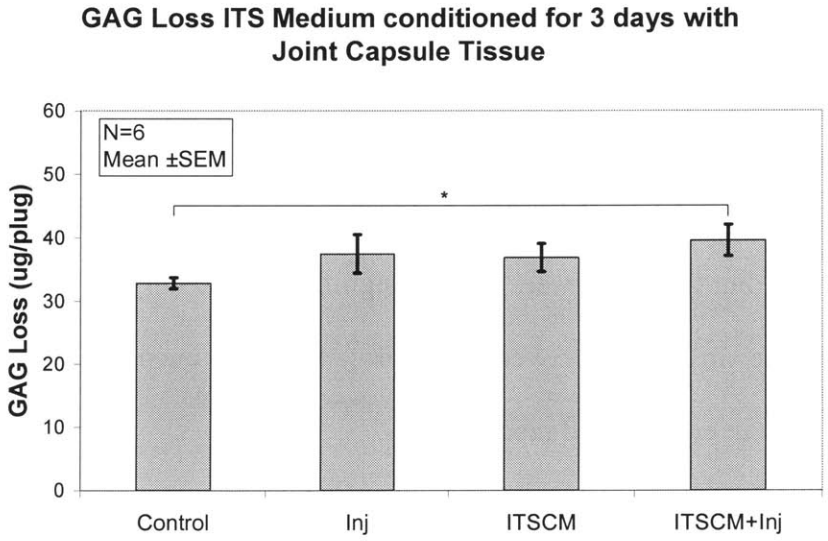

Figure 16: GAG loss for injured and uninjured cartilage cultured in ITS medium and ITS medium conditioned for three days with one 3mm punched piece of joint capsule per milliliter of medium. '*' represents p value of <0.05 by a two sided student t test.

Figure 16 shows that there appears to be no difference in GAG loss for conditioned medium with and without injury. Figure 17 shows radiolabel incorporation for the same experiment.

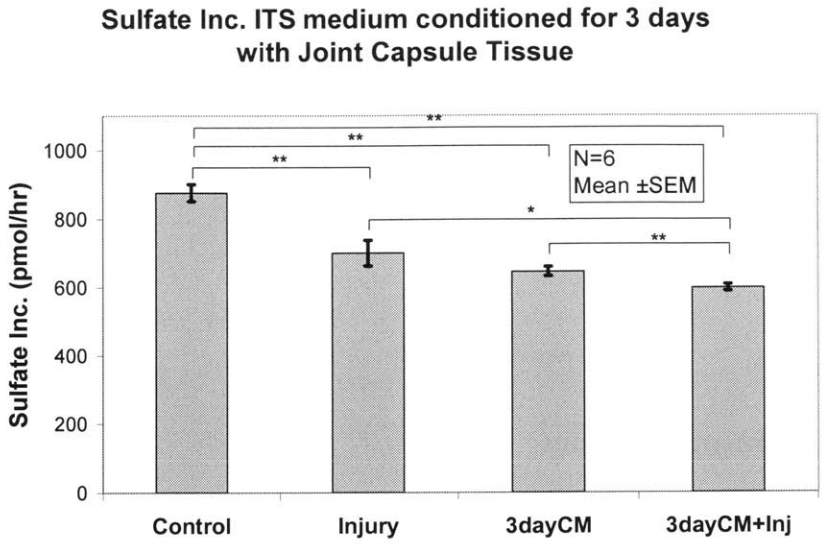

Figure 17: Sulfate incorporation for injured and uninjured cartilage cultured in ITS medium and ITS medium conditioned for three days with one 3mm punched piece of joint capsule per milliliter of medium. '*' represents $p<0.04$, '**' represents $p<0.001$ by a two sided student t test.

The differences in sulfate incorporation don't appear to be as large as those seen with 10% FBS medium but the differences are still significant. The only difference that wasn't significant was the difference between injured cartilage in unconditioned medium and uninjured cartilage in conditioned medium.

3.7.2.2 Direct Comparison of ITSCM and FBSCM

Figure 18 shows a comparison of GAG loss for injured and uninjured cartilage cultured in ITS joint capsule tissue conditioned medium and 10% FBS joint capsule tissue conditioned medium.

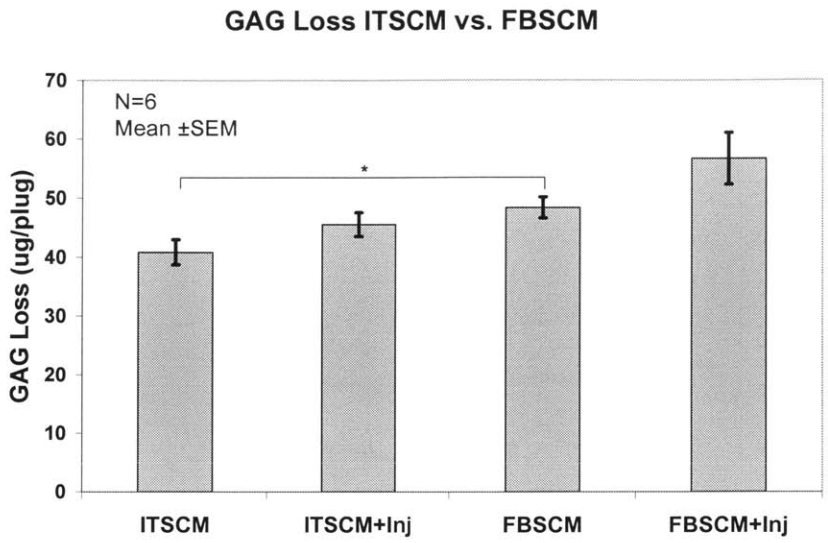

Figure 18: Comparison of GAG loss for injured and uninjured cartilage cultured for four days in ITS medium conditioned with joint capsule tissue for three days and 10% FBS medium conditioned with joint capsule tissue for three days. '*' represents $p<0.03$ by a two sided student t test.

There is higher initial GAG loss between ITS conditioned medium and 10% FBS conditioned medium. However the difference in GAG loss after injury is not significant between the two conditions.

Figure 19 shows a comparison of sulfate incorporation for ITS conditioned medium and 10% FBS conditioned medium.

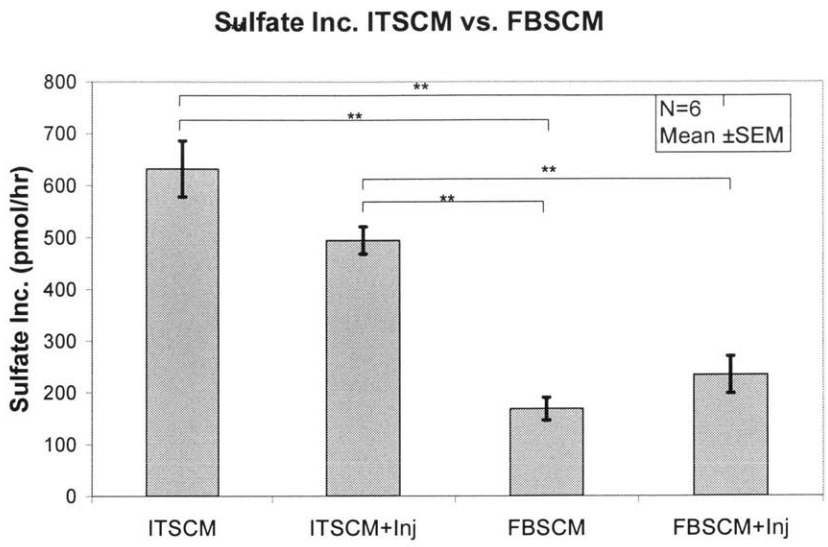

Figure 19: Comparison of sulfate incorporation for injured and uninjured cartilage cultured for four days in ITS medium conditioned with joint capsule tissue for three days and 10% FBS medium conditioned with joint capsule tissue for three days. '**' represents p value of <0.0002 by a two sided student t test.

As can be seen from Figure 19, there is significantly less sulfate incorporation for cartilage cultured in 10% FBS joint capsule tissue conditioned medium. Although cartilage cultured in ITS medium tends to have slightly higher incorporation levels than cartilage cultured in 10% FBS medium, this does not account for the dramatic difference seen in Figure 19. One possibility could be that there is something in the serum that interacts with either the joint capsule tissue to cause it to release more of the factor or it makes the cartilage more sensitive to the factor.

3.8 Effect of supplementing the medium with ascorbate vs. not supplementing the medium

In some experiments the joint capsule tissue conditioned medium had been supplemented with ascorbate and in other experiments it was not. Because of this it was important to do an experiment that looked at what differences supplementing vs. not supplementing the medium would cause. Because there has always been a significant decrease in radiolabel incorporation on GAG loss was measured.

3.8.1 Methods

Cartilage and joint capsule tissue was harvested in the usual manner. One 3mm piece of joint capsule tissue per milliliter of 10% FBS medium was used to condition medium for three days. Cartilage was allowed to equilibrate for three days prior to intervention and was cultured an additional three days after intervention in 0.5ml of medium before GAG loss was measured. The groups for this experiment were:

1) FBS (no ascorbate)
2) Joint capsule tissue conditioned medium (no ascorbate)
3) FBSS (ascorbate added)
4) Joint capsule tissue conditioned medium supplemented (ascorbate added)

Another experiment was done trying to compare the differences in 10% FBS medium and ITS medium when they were supplemented with ascorbate and when they were not. This experiment followed the same protocol as the first experiment except for in this case plugs were matched for location across the four groups without ascorbate in them.

1) FBS (no ascorbate)
2) FBSCM (no ascorbate)
3) ITS (no ascorbate)
4) ITSCM (no ascorbate)
5) FBSS (ascorbate)
6) FBSCMS (ascorbate)
7) ITSS (ascorbate)
8) ITSCM (ascorbate)

3.8.2 Results

Figure 19 shows GAG loss for supplemented and unsupplemented conditioned and unconditioned 10% FBS medium.

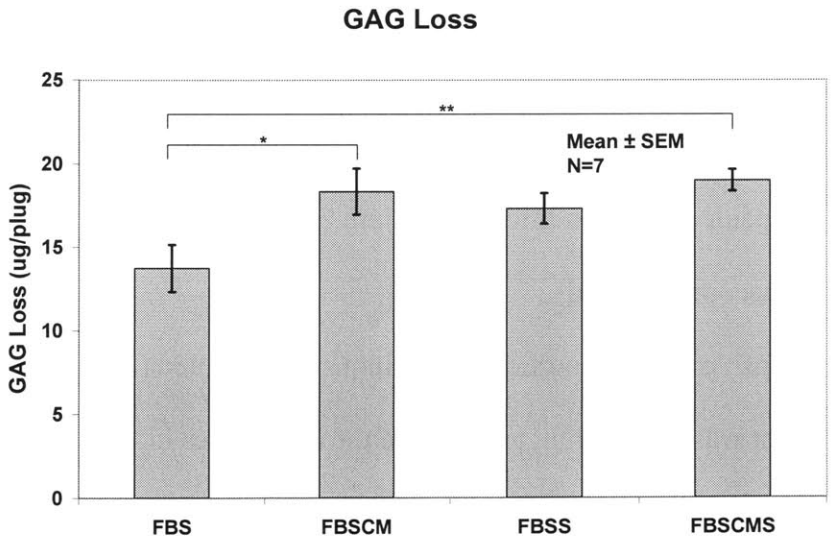

Figure 19: Comparison of GAG loss after three days for cartilage cultured in 10% FBS medium and joint capsule tissue conditioned medium that has and has not been supplemented with ascorbate. '*' represents p value of <0.04, '**' represents p value of <0.01 by a two sided student t test.

Figure 19 shows that supplementing the conditioned medium does not have an effect on the GAG loss measured. However, there is a large increase in GAG loss seen with unconditioned medium when it is supplemented with ascorbate.

Figure 20 shows the results for both ITS and FBS medium

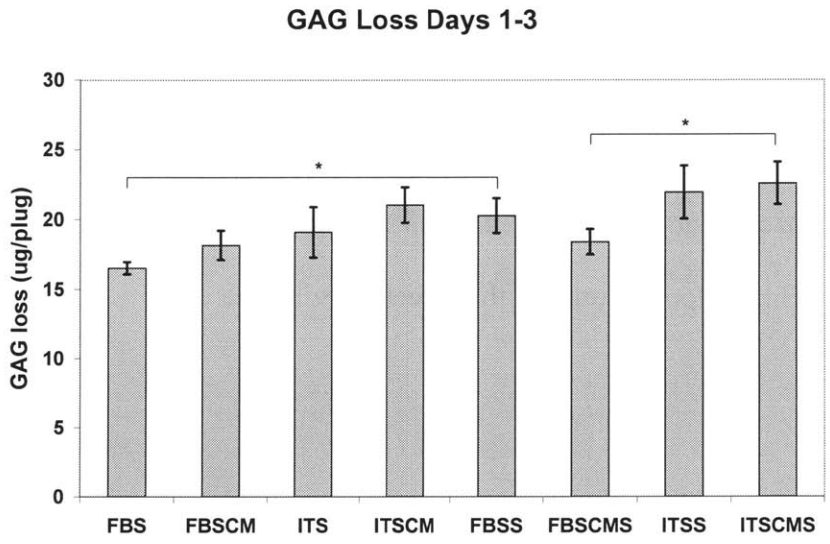

Figure 20: Comparison of GAG loss for 10% FBS and ITS Medium that has or has not been supplemented with ascorbate and 10% FBS medium and ITS medium conditioned by joint capsule tissue that has and has not been supplemented with ascorbate. '*' represents p value of <0.05 by a two sided student t test.

Once again there really is not any difference between the joint capsule tissue conditioned medium that has been supplemented with ascorbate and the medium that has not been supplemented. There is also still a large increase in GAG loss for cartilage cultured in 10% FBS medium that has been supplemented with ascorbate as opposed to medium that

has not been. ITS medium did not appear to be as sensitive to supplementation as the 10% FBS medium.

3.9 Effect of freezing joint capsule tissue conditioned medium

Another important thing to determine was whether or not freezing conditioned medium was a viable way of storing it. This was important for experiments that use human joint capsule tissue as it is very difficult to get and does not come on a regular schedule. If it is possible to store conditioned medium in the freezer, it would be possible to make up large quantities of conditioned medium, freeze it and then use it in later experiments. As many of the experiments done in chapter two which looked at biosynthesis levels had made use of previously frozen conditioned medium and still seen a large decrease in biosynthesis levels, this experiment looked only at the effect freezing the conditioned medium had on GAG loss.

3.9.1 Methods

Cartilage and joint capsule tissue were harvested as previously described. One 3mm piece of joint capsule tissue was used to condition 1ml of 10% FBS medium for four days. After four days of conditioning, some of the medium was quickly frozen in the minus 80° freezer and then unthawed in the incubator. The other part of the medium was stored in the incubator for later use. Cartilage was cultured for four days after intervention before GAG loss was measured. For these experiments all plugs were matched for location and N=6. The groups for the first experiment were as follows:

1) Control

2) Injury

3) Frozen conditioned medium

4) Frozen conditioned medium + Injury

The second experiment compared GAG loss of cartilage cultured in conditioned medium that had been frozen and conditioned medium from the same batch that had not been frozen. The groups for the second experiment were:

1) Joint capsule tissue conditioned medium (unfrozen)

2) Joint capsule tissue conditioned medium + injury (unfrozen)

3) Frozen joint capsule tissue conditioned medium

4) Frozen joint capsule tissue conditioned medium + injury.

3.9.2 Results

Figure 21 shows those results for GAG Loss.

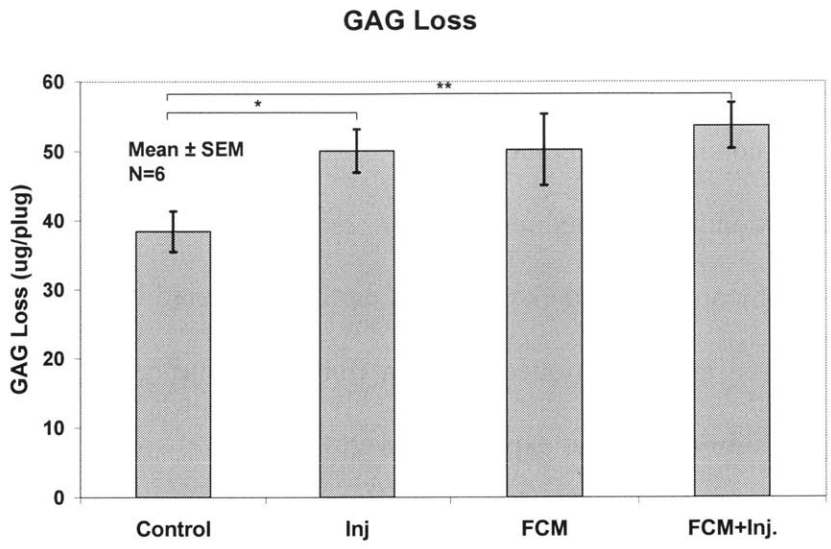

Figure 21: GAG Loss in injured and uninjured cartilage that was cultured in 10% FBS medium or 10% FBS medium which was conditioned for four days with one 3mm piece of joint capsule tissue/ml of medium, frozen, and thawed in a 37° water bath. '*' represents p value of <0.03, '**' represents p value of <0.007 by a two sided student t test.

Figure 21 shows that there is basically no difference in the amount of GAG lost from injured and uninjured cartilage cultured in conditioned medium which had been previously frozen. This means that if one is only interested in looking at radiolabel incorporation, freezing the medium is still a viable option. However, previously frozen conditioned medium should probably not be used for injury experiments where GAG loss is of interest.

Figure 22 shows a comparison of GAG loss from joint capsule tissue conditioned medium that had been previously frozen and joint capsule tissue conditioned medium that had not been frozen.

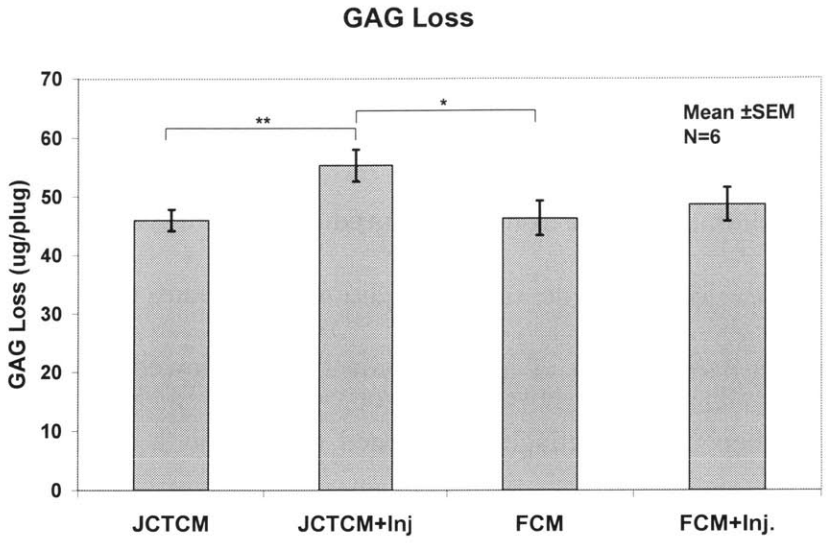

Figure 22: GAG Loss in injured and uninjured cartilage that was cultured in 10% FBS medium conditioned by conditioned for four days with one 3mm piece of joint capsule tissue/ml of medium, frozen, and thawed in a 37° water bath or stored in the incubator. '*' represents p value of <0.05, '**' represents p value of <0.02 by a two sided student t test.

Figure 22 shows that there is a significant increase in GAG loss with injury in the non frozen medium, whereas there was no increase in medium that had been previously frozen. Like figure 21 it indicates that if one is interested in looking at GAG loss it is probably wise not to freeze the medium. This could be because GAG loss is less sensitive to the unknown factor and when it is frozen and thawed, too much of it becomes denatured and there is not enough left to see an effect.

3.10 Using cartilage conditioned medium as a control

Because the joint capsule tissue is alive when medium is being conditioned it is eating up the nutrients that are in the medium, possibly making the medium nutrient deficient for the cartilage and being partly responsible for the decreased levels of biosynthesis. From

previous experiments, it is known that nutrient depletion is not responsible for all of the decrease seen. However, it is important to determine what portion of the decrease, if any, is caused by nutrient depletion. A preliminary experiment was done which compared unconditioned medium, cartilage conditioned medium, joint capsule tissue conditioned medium and frozen joint capsule tissue conditioned medium to make sure that all differences were still significant. This experiment was followed up by looking at the effect of injury when using cartilage conditioned medium as a control. Also, because joint capsule tissue is much more cellular than cartilage and is also thicker than the cartilage slices used, a higher concentration of cartilage per milliliter of medium was used to try and account for that difference. It is hoped that by using cartilage conditioned medium as a control, it might be possible to isolate the effect of the unknown factor and not the combination of nutrient depletion and the unknown factor.

3.10.1 Comparison of different types of conditioned medium

3.10.1.1 Methods

Cartilage and joint capsule tissue were harvested in the normal manner. One 3mm piece of joint capsule tissue or one 3mm piece of cartilage was used to condition 1ml of 10% FBS medium for three days. After three days of conditioning the cartilage conditioned medium and the joint capsule tissue conditioned medium were each pooled separately. Some medium was separated out from the joint capsule tissue conditioned medium and was frozen overnight in the -80° freezer. The experiment was begun four days after harvest and plugs were allowed to incubate in 0.5ml of medium under the following

conditions for an additional four days. All plugs were matched for location and N=8. The four groups for this experiment were:

1) Control (10% FBS medium)
2) Cartilage conditioned medium (3mm piece cartilage/1ml medium for 3 days)
3) JCTCM (3mm piece of JCT/1ml medium for 3 days)
4) Frozen JCTCM (3mm piece of JCT/1ml medium for 3 days and then frozen & unfrozen)

3.10.1.2 Results

Figure 23 shows sulfate incorporation for cartilage cultured in10% FBS medium and 10% FBS medium conditioned by cartilage or joint capsule tissue and joint capsule tissue conditioned medium that had been previously frozen.

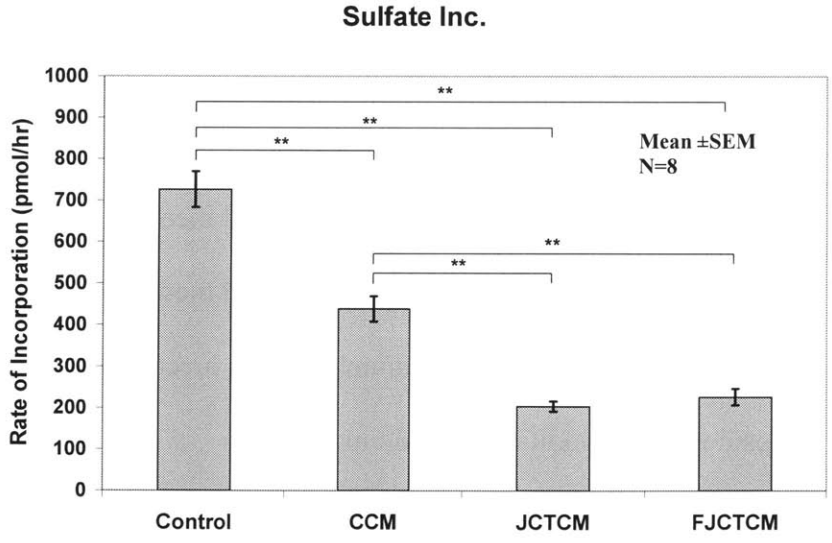

Figure 23: Sulfate incorporation for cartilage cultured for four days in 10% FBS medium, cartilage conditioned medium, joint capsule tissue conditioned medium, and joint capsule tissue conditioned medium which had been previously frozen. '**' represents $p<0.0001$ by a two sided student t test.

As can be seen in figure 23, it appears that freezing the joint capsule tissue has little effect on the rate of sulfate incorporation and there is still a significant drop in radiolabel incorporation when cartilage cultured in joint capsule tissue conditioned medium is compared to cartilage cultured in cartilage conditioned medium.

3.10.2 Cartilage conditioned medium follow up

3.10.2.1 Methods

Because cartilage is less cellular than the joint capsule tissue and is not as thick as many of the pieces of joint capsule tissue, cartilage conditioned medium was obtained by placing four 3mm punched pieces of cartilage in 1ml of 10% FBS medium for four days. Joint capsule tissue conditioned medium was obtained by culturing one 3mm piece of joint capsule tissue per milliliter of medium for four days. Injured and uninjured cartilage plugs were then cultured in either cartilage conditioned medium or joint capsule tissue conditioned medium and GAG loss and sulfate incorporation were measured after four days in culture. The groups for this experiment were:

1) Cartilage conditioned medium (4, 3mm plugs/1ml medium)
2) Cartilage conditioned medium (4, 3mm plugs/1ml medium) + Injury
3) Joint capsule tissue conditioned medium (1, 3mm piece/1ml medium)
4) Joint capsule tissue conditioned medium + Injury

3.10.2.2 Results

Figure 24 shows the results for GAG loss once the GAG loss from the cartilage used to condition the medium was accounted for.

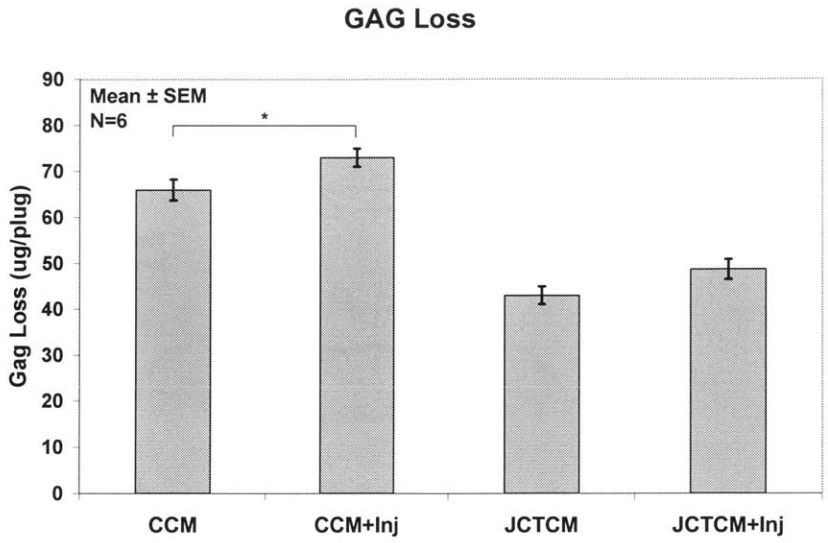

Figure 24: Comparison of GAG loss for injured and uninjured cartilage cultured for four days in 10% FBS medium that was either conditioned with cartilage (Four 3mm punched pieces/ml for 4 days) or joint capsule tissue (one 3mm punched piece/ml for 4 days). '*' represents p value <0.05 by a two sided student t test.

From figure 24 it appears that the same trends in GAG loss are seen for both cartilage conditioned medium and joint capsule tissue conditioned medium. The trends for the GAG loss with the cartilage conditioned medium appear to be the same as when it is cultured in unconditioned medium. GAG loss from the cartilage used to condition the medium is probably responsible for the high amount of GAG loss seen in the cartilage conditioned medium samples. In the future, medium samples of conditioned medium should be saved so that GAG already present in the medium at the start of the experiment can be subtracted out of the results.

Figure 25 shows sulfate incorporation for cartilage cultured in cartilage conditioned medium and joint capsule tissue conditioned medium.

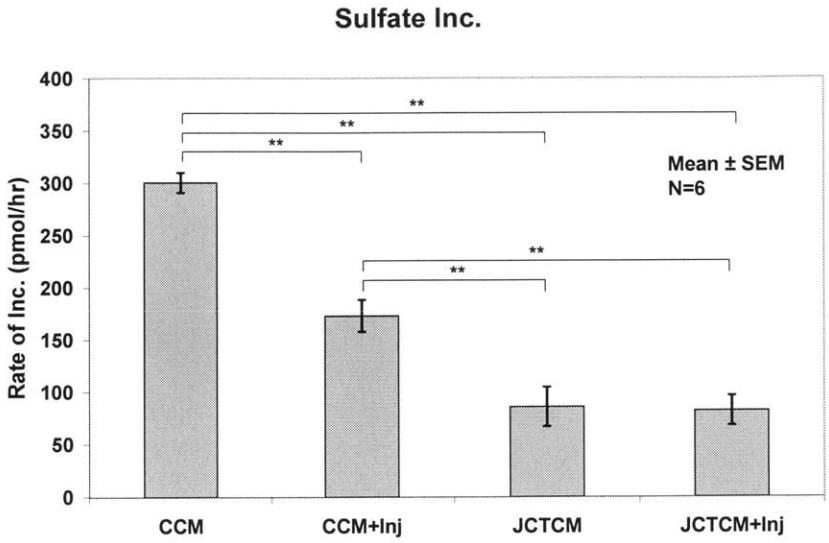

Figure 25: Rate of sulfate incorporation for injured and uninjured cartilage cultured for four days in 10% FBS medium that was either conditioned with cartilage (Four 3mm punched pieces/ml for 4 days) or joint capsule tissue (one 3mm punched piece/ml for 4 days). '**' represents $p<0.0002$ by a two sided student t test.

Figure 25 shows that cartilage cultured in joint capsule tissue conditioned medium still has significantly less sulfate incorporation than cartilage cultured in cartilage conditioned medium. Combining the results in Figure 25 with the results in Figure 23, this means that cartilage conditioned medium may be a better control when doing blocking experiments because it is probably not reasonable to expect that radiolabel incorporation levels will ever reach those of cartilage cultured in fresh 10% FBS medium because of nutrient depletion. For a comparison of radiolabel incorporation of cartilage cultured in fresh medium versus cartilage cultured in cartilage conditioned medium see Appendix A.

3.11 Discussion and Conclusions

While doing these experiments, it was discovered that joint capsule tissue conditioned medium contained a significant amount of GAG. This GAG was released by the joint capsule tissue itself and in some cases could account for as much as a third of the total GAG Loss measured. Unfortunately, medium samples were not saved as controls for these experiments, so this information should be kept in mind when interpreting all of the GAG loss results. In the future conditioned medium samples need to be saved for every experiment so that the GAG in the joint capsule tissue conditioned medium can be subtracted from the total GAG measured in each well at the end of the experiment so that the amount that is lost by the cartilage can be isolated. Chapter 5 will go into more detail about how much GAG is in the joint capsule tissue, how much is released and when it is released. In addition, data in Chapter 6 will provide more information about the structure of the joint capsule tissue itself.

In almost all of the joint capsule tissue conditioned medium experiments, there was more GAG seen in the conditioned medium without injury than was seen in the control wells. This difference is associated with the GAG that was lost by the joint capsule tissue when the medium was conditioned. The most surprising result is that for injured cartilage cultured in joint capsule tissue conditioned medium, very rarely is the GAG lost from this condition any higher than the GAG measured in the uninjured sample cultured in conditioned medium. On the other hand, there is a definite trend in almost all of the experiments for there to be an increase in GAG loss with injury when the cartilage is

cultured in unconditioned medium. This would seem to indicate that there might be something in the conditioned medium that is inhibiting GAG loss after injury.

It also appears from GAG loss data and other experiments that joint capsule tissue can be used to condition medium for up to two weeks without substantial change in the results seen. As an example several of the experiments in chapter two which looked at radiolabel incorporation made use of medium conditioned by joint capsule tissue that was almost two weeks old. Red/Green live dead assays were done on two week old joint capsule tissue and almost all of the tissue was still alive. (Unfortunately, no photograph was taken of that tissue.)

Because sulfate incorporation decreases linearly with the number of days that medium is conditioned, it indicates that the factor is probably released by diffusion and that the joint capsule tissue is depleting the nutrients in the medium at a constant rate which is another indicator that large amounts of cells are not dying.

If one is interested in making sure that there is a significant decrease in radiolabel incorporation for cartilage cultured in joint capsule tissue conditioned medium compared to control and injured cartilage in unconditioned medium, it is important to make sure that the medium is conditioned for at least two days, but preferably three at a concentration of one 3mm punched piece of joint capsule tissue per milliliter of medium. If three days is too long, increasing the number of pieces of joint capsule tissue per

milliliter of medium would probably give the same results as conditioning for longer periods of time with a lower concentration of joint capsule tissue to medium.

One exciting result was that culturing bovine cartilage in medium conditioned by human joint capsule tissue gave the same results as those seen when medium was conditioned by bovine joint capsule tissue. Because most cytokine blockers available are made to react with human cytokines, by using medium conditioned with human joint capsule tissue it will be possible to use these blockers on bovine cartilage, which is easier to get and more sensitive to change, to get definitive results. This will simplify future attempts that might try to identify the unknown factor.

While the human joint capsule tissue showed the same trends in GAG loss as bovine joint capsule tissue conditioned medium, it appeared that there was a lot more GAG loss in the uninjured cartilage. Unfortunately, none of the human joint capsule tissue conditioned medium that was used in the experiment included in this chapter was saved so there is no way to determine if the increase seen in GAG loss is because of GAG in the conditioned medium or because of some reaction with the cartilage. When human joint capsule tissue becomes available again, the amount of GAG that is lost to the medium should be measured as well as the total GAG found in the tissue.

Dead joint capsule tissue conditioned medium shows the same trends as the live joint capsule tissue conditioned medium. This is good because it shows that nutrient depletion is not the cause for the decrease in radiolabel incorporation. Unfortunately because it

was killed by freezing, the cell membranes could have ruptured releasing other factors into the medium that are different then what is released by live tissue.

Another positive result was that both ITS and 10% FBS medium showed the same trends for GAG loss and radiolabel incorporation. It is interesting to note though that there is a much more drastic decrease in radiolabel incorporation for cartilage cultured in 10% FBS medium. This means that one might want to condition ITS medium for longer or at a higher concentration in order to see more of a decrease. Another interesting thing to note was that supplementing either 10% FBS or ITS joint capsule tissue conditioned medium with ascorbate did not have any effect on GAG loss. The same was true for unconditioned ITS medium. Unconditioned 10% FBS medium was very sensitive to the addition of ascorbate as GAG loss increased significantly when it was added. 10% FBS medium seems to be much more sensitive to changes than ITS medium.

Freezing the conditioned medium appears to have little effect on radiolabel incorporation although it is unknown how it would react to multiple freeze/thaw cycles. This means that if one is only interested in looking at radiolabel incorporation freezing the medium should not be a problem. Freezing the conditioned medium might have an effect on GAG loss however more repeats would have to be done to determine whether or not that really is the case.

If one is interested in doing blocking experiments it appears that cartilage conditioned medium may serve as a better control because it can account for the decrease in

radiolabel incorporation that is caused by the depletion of nutrients. There is still a significant difference in radiolabel incorporation between cartilage conditioned medium and joint capsule tissue conditioned medium and because of nutrient depletion it seems more reasonable for a blocker to only return incorporation levels back to the cartilage conditioned medium levels. Four 3mm punched pieces of cartilage per milliliter of medium is probably a very safe concentration to condition medium with cartilage as it should compensate or possibly overcompensate for the differences in cellularity between the two tissues. As will be shown in chapter 5, cartilage has between 4-5μg of DNA for a 3mm by 1mm punched piece and joint capsule tissue can have between 5-25μg of DNA for a 3mm punched piece with the average being closer to 15. As with the joint capsule tissue since cartilage releases GAG to the medium, it is important to save a sample of the cartilage conditioned medium so that the GAG that was released during the conditioning process can be subtracted from the total amount measured at the end of the experiment.

The cartilage conditioned medium experiments as well as the dead conditioned medium experiments and the boiling experiments in chapter two show that there is definitely some factor released into the medium which is responsible for a good portion of the decrease in biosynthesis.

Overall the results in this chapter show that the conditioned medium model is fairly robust. It is important to make sure that the medium is conditioned for a long enough period of time to get a significant decrease in radiolabel incorporation and to save samples of the conditioned medium in order to subtract out the GAG lost by the joint

capsule tissue during the conditioning process from the total GAG measured at the end of the experiment.

Chapter 4: Joint Capsule Tissue Co-culture Model

4.1 Background and Purpose

Previously, some experiments were done using medium that had been conditioned by joint capsule tissue and some experiments had been done co-culturing joint capsule tissue and cartilage in the same well. It had been assumed that both models were equivalent since both resulted in a decrease in chondrocyte biosynthesis levels. Also, some previous experiments involved joint capsule tissue harvested with a 5mm dermal punch instead of a 3mm dermal punch. The experiments of this chapter were to determine whether or not conditioned medium yielded the same results as co-culturing joint capsule tissue with cartilage. In addition, experiments were performed to identify any differences seen when using 5mm versus 3mm diameter explants of joint capsule tissue.

4.2 General Methods

Cartilage and joint capsule tissue were harvested as previously described in chapter 2, however, some of the joint capsule tissue was punched using a 5mm dermal punch instead of a 3mm punch. These pieces were allowed to equilibrate for three to four days in 10% FBS medium prior to the beginning of the experiment. After equilibrating the cartilage plugs were placed into one of four conditions with the cartilage plugs being matched for location across the four groups. Cartilage was injured by applying 50% strain at a strain rate of 1mm/sec. using a custom built incubator housed loading device as previously described[8,21]. After intervention, cartilage plugs and joint capsule tissue were co-cultured for four days. After four days the cartilage plugs were labeled with ^{35}S-

Sulfate and ^{3}H-Proline for 24 hours after which they were washed, digested and counted as previously described[27]. On day four after intervention, the GAG lost to the medium was measured by DMMB assay[6].

4.3 Three millimeter co-culture and five millimeter co-culture models

The goal of these experiments was to look at the co-culture models and determine whether there was any difference in the trends of radiolabel incorporation and GAG loss when cartilage plugs are co-cultured with a 5mm punched piece of joint capsule tissue or a 3mm punched piece of joint capsule tissue.

4.3.1 Methods

Joint capsule tissue was harvested and punched using either a 3mm dermal punch or a 5mm dermal punch. For these two experiments N=7. Cartilage plugs were incubated in 1ml of 10% FBS medium for each of the four conditions. The four groups for each experiment were:

1) Control
2) Injury
3) 3mm or 5mm Co-culture
4) 3mm or 5mm Co-culture + Injury

4.3.2 Results

Figure 1 shows GAG loss for injured and uninjured cartilage co-cultured with a 3mm punched piece of joint capsule tissue.

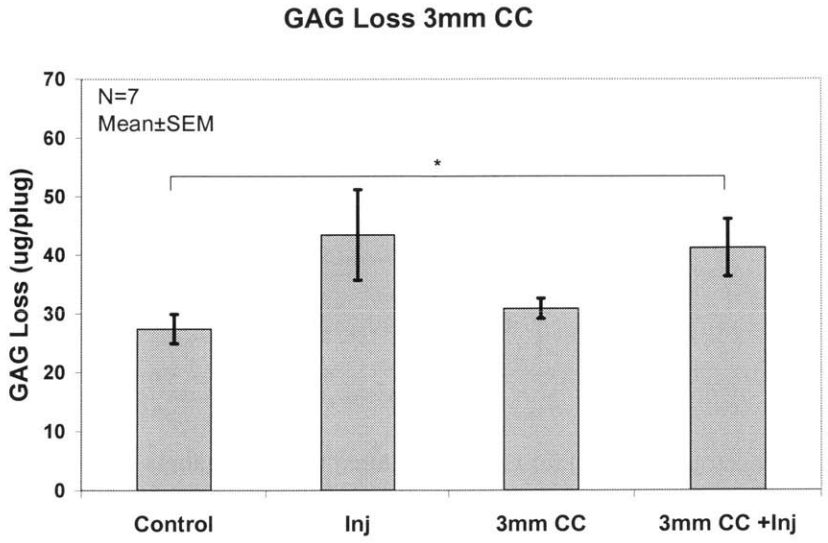

Figure 1: GAG Loss (micrograms/plug) for cartilage cultured with and without 3mm piece of joint capsule tissue and with and without injury. '*' indicates p value <0.05 by a two sided student t test.

From Figure 1, it appears that there is no difference in GAG loss for cartilage cultured with and without a 3mm piece of joint capsule tissue when directly comparing those left in free swell and those that were injured. The only significant difference was seen between the cartilage cultured alone and the cartilage that was injured and co-cultured with joint capsule tissue.

Figure 2 shows GAG loss after four days for injured and uninjured cartilage cultured with and without a 5mm punched piece of joint capsule tissue.

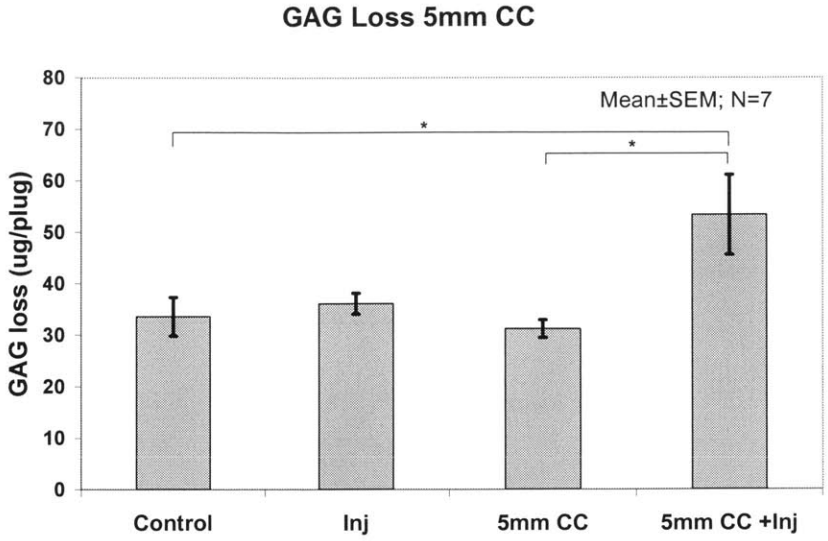

Figure 2: GAG Loss (micrograms/plug) for cartilage cultured with and without 5mm piece of joint capsule tissue and with and without injury. '*' represents $p<0.05$ by two sided student t test.

From figure 2 it appears that the combination of injury and co-culture with a 5mm punched piece of joint capsule tissue causes a large increase in GAG loss. Figure 3 shows sulfate incorporation for injured and uninjured cartilage cultured with and without a 3mm piece of joint capsule tissue for four days.

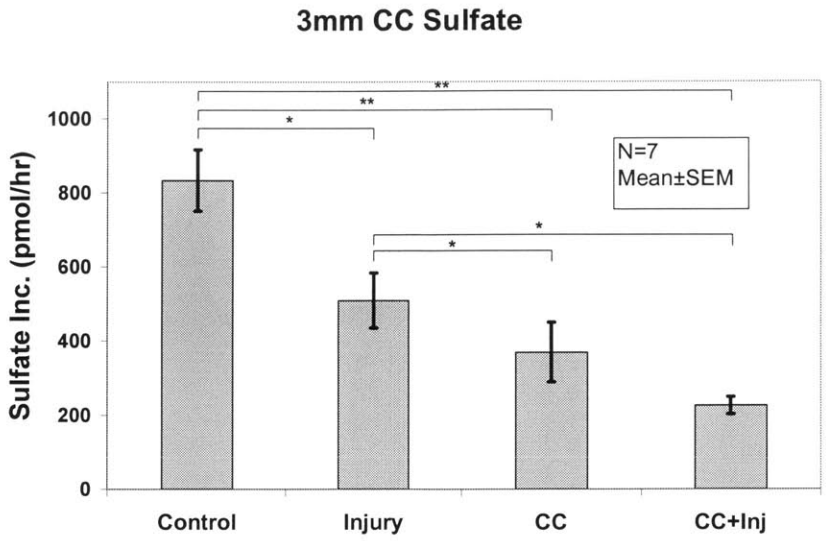

Figure 3: Sulfate Incorporation for cartilage cultured for four days with and without a 3 mm piece of joint capsule tissue and with and without injury. '*' represents $p<0.008$, '**' represents $p<0.0001$ by a two sided student t test.

Figure 3 shows the expected result that radiolabel incorporation decreases with both injury and co-culture. All results are significant except the difference between co-culture and co-culture plus injury.

Figure 4 shows sulfate incorporation for injured and uninjured cartilage cultured with and without a 5mm piece of joint capsule tissue.

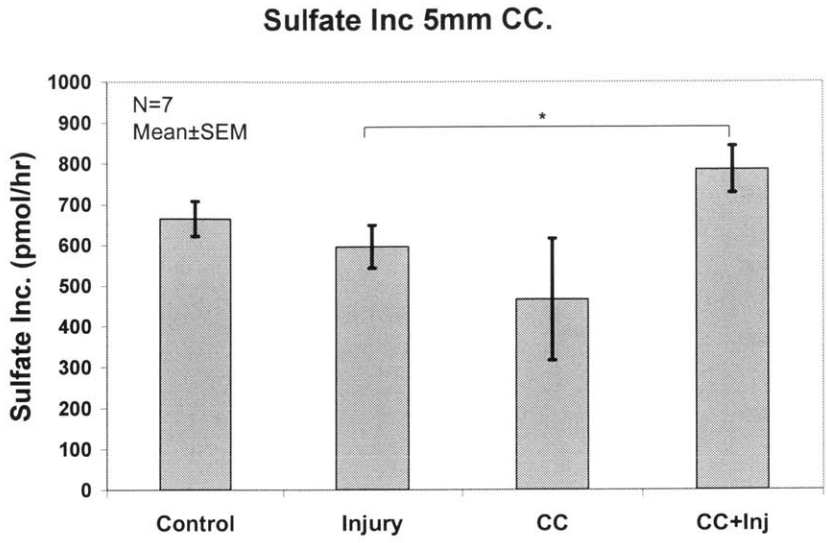

Figure 4: Sulfate Incorporation for cartilage cultured for four days with and without a 5 mm piece of joint capsule tissue and with and without injury. '*' indicates p value <0.05 by two sided student t test.

The results from Figure four are surprising because there is not a large decrease in sulfate incorporation with co-culture of the joint capsule tissue and the incorporation levels for co-culture plus injury are at the same level as control cartilage. While a slight increase in incorporation levels is often seen with co-culture and conditioned medium, it is usually still a lot less than the incorporation for control cartilage. More follow up experiments would have to be done to see how reproducible this result is before it can be taken too seriously. However, since the large increase in GAG loss is not seen in all co-culture experiments with a 5mm piece of joint capsule tissue, and sulfate incorporation is an indication of proteoglycan synthesis it is possible that either the injury or the joint capsule tissue triggered the chondrocytes to produce more GAG which was then lost into the medium.

4.4 Number of pieces of joint capsule tissue per well

All of the co-culture experiments that had been done in the past had co-cultured one piece of joint capsule tissue with one piece of cartilage. This experiment looks at whether or not there are different trends in GAG loss when two pieces of joint capsule tissue are co-cultured with one piece of cartilage.

4.4.1 Methods

Joint capsule tissue was harvested in the usual manner and was punched to form 5mm disks. Cartilage was also harvested in the usual manner. Both joint capsule tissue and cartilage were allowed to equilibrate for three days before intervention. After intervention, joint capsule tissue and cartilage were allowed to incubate an additional four days after which GAG loss was measured. Two experiments were done so as to compare the trends seen in GAG loss for co-culture with one piece of joint capsule tissue as opposed to two pieces of joint capsule tissue. By running two experiments with joint capsule from the same joints it was hoped to minimize differences in results that would be caused by variation in joint capsule tissue structure. In both experiments plugs were matched for location. One experiment had one 5mm punched piece of joint capsule tissue co-cultured with one piece of cartilage and the other experiment had two 5mm punched pieces of joint capsule tissue co-cultured with one piece of cartilage. The groups for the experiments were:

1) Control

2) Joint capsule tissue co-culture (one or two pieces)

3) Injury

4) Joint capsule tissue co-culture + injury (one or two pieces)

4.4.2 Results

Figure 5 shows the results for co-culture of one piece of joint capsule tissue with one piece of cartilage.

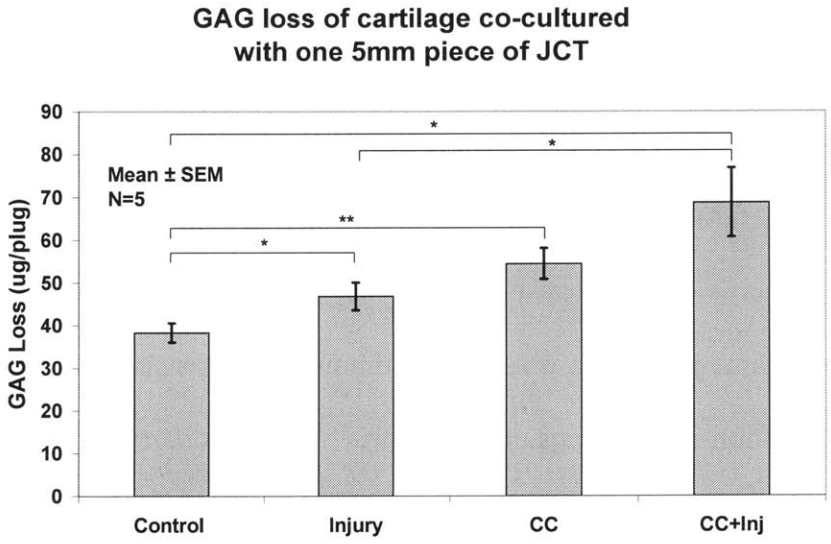

Figure 5: GAG loss for injured and uninjured cartilage co-cultured with one 5mm punched piece of joint capsule tissue for four days in 1ml of 10% FBS medium. '*' represents p value < 0.05, '**' represents p value of <0.007 by a two sided student t test.

These results are similar to the results seen in figure 2 except for there is much more GAG loss seen with co-culture than was seen in figure 2.

Figure 6 shows the results for co-culture of two pieces of joint capsule tissue with one piece of cartilage.

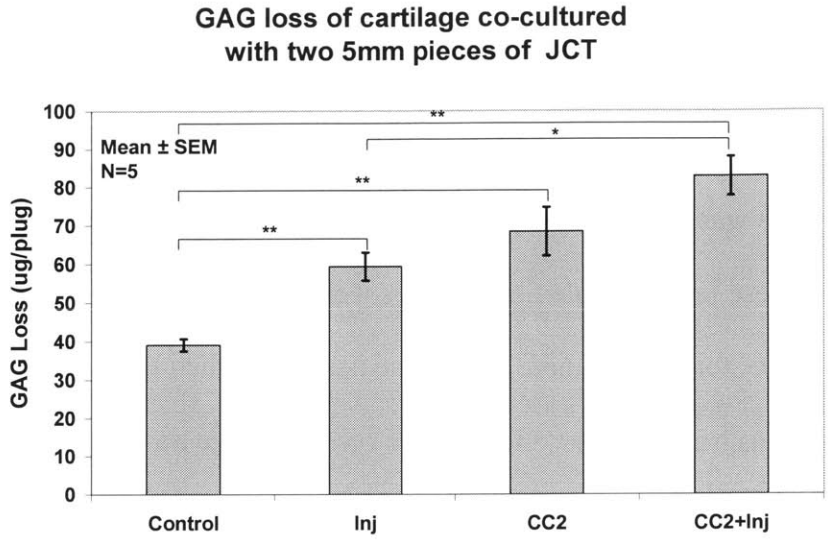

Figure 6: GAG loss of injured and uninjured cartilage co-cultured for four days in 1ml of 10% FBS medium with two 5mm punched pieces of joint capsule tissue. '*' represents p value of <0.02, '**' represent p value of <0.008 by a two sided student t test.

The results seen in figure 6 show the same trends as the results seen in figure 5. This would indicate that there is no need to increase the number of pieces of joint capsule tissue used in the co-culture experiment. The differences seen in these results as compared to the results seen in figure one and two could be due to differences in joint capsule tissue structure or variations from animal to animal.

4.5 Dead joint capsule tissue co-culture

The next goal was to determine whether or not the same patterns would be seen if dead joint capsule tissue was co-cultured with articular cartilage. If the same patterns were seen with dead joint capsule tissue, it would suggest that whatever is responsible for the

GAG loss and the decrease in biosynthesis is not released by a cellular response. It would also mean that it would be possible to harvest joint capsule tissue and then freeze it for use in later experiments.

4.5.1 Methods

Joint capsule tissue was harvested as previously described and was punched using a 3mm dermal punch. These joint capsule tissue pieces were placed in a conical tube and frozen in the -80° C freezer for at least three days. The tissue was then thawed in a 37° C water bath. Cartilage was harvested as and allowed to equilibrate as described above. The thawed pieces of joint capsule tissue were cultured in 1ml of medium along with articular cartilage. Cartilage plugs were incubated in 0.5ml of 10% FBS medium for this experiment. N=6. The groups for this experiment were:

1) Control

2) Injury

3) Dead joint capsule tissue co-culture

4) Dead joint capsule tissue co-culture + Injury

A follow up experiment was done to compare GAG loss of dead joint capsule tissue co-culture, live joint capsule tissue co-culture and joint capsule tissue conditioned medium. For this experiment cartilage plugs were incubated in 1ml of 10% FBS medium for 3 days after intervention. All plugs were matched for location and N=5.

1) Control

2) Live joint capsule tissue co-culture

3) Dead joint capsule tissue co-culture

4) Joint capsule tissue conditioned medium.

4.5.2 Results

Figure 7 shows GAG loss as a result of co-culturing injured and uninjured cartilage with a 3mm punched piece of dead joint capsule tissue.

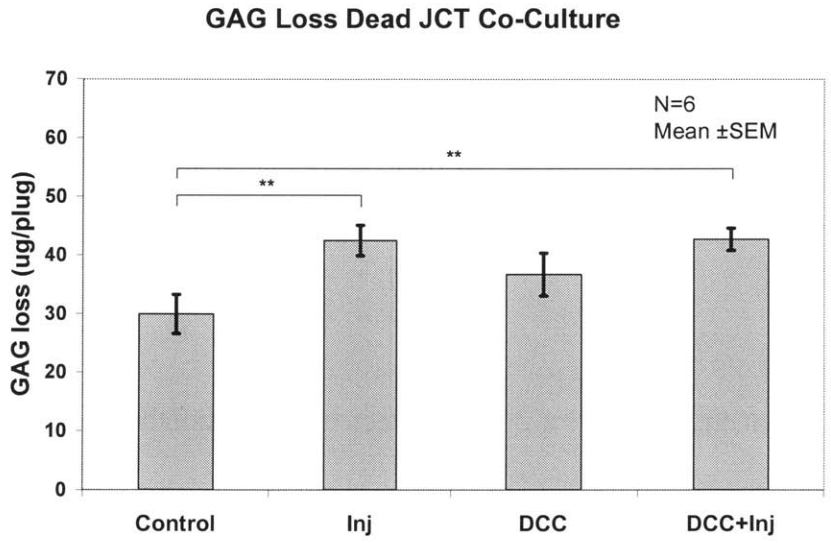

Figure 7: GAG loss for injured and uninjured cartilage cultured with and without a 3mm punched piece of dead joint capsule tissue for four days. '**' indicates p value <0.02 by a two sided student t test.

Once again, the GAG loss results are similar to those seen in the previous experiments, with no significant difference seen between injured and uninjured cartilage that was co-cultured with the dead joint capsule tissue.

Figure 8 shows sulfate incorporation for the same experiment.

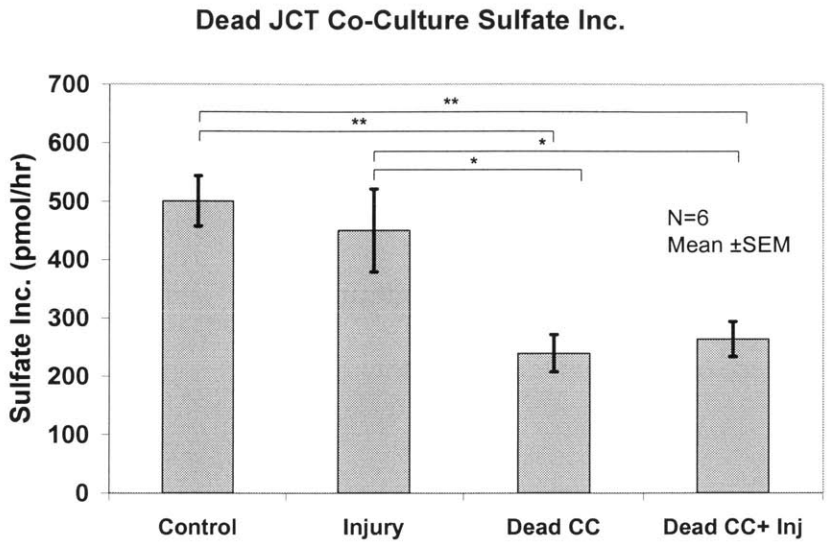

Figure 8: Sulfate incorporation for injured and uninjured cartilage cultured with and without a 3mm punched piece of dead joint capsule tissue for four days. Joint capsule tissue was killed by being frozen in a -80° C freezer for three days. '*' indicates p value of <0.05 and '**' indicates p value of 0.002 by a two sided student t test.

Figure 8 shows that there is still a dramatic decrease in radiolabel incorporation. This indicates that the tissue does not need to be alive to release the factor that causes the decrease in biosynthesis levels or because it was killed by being frozen, the cell membranes ruptured and are releasing other factors into the medium which are causing a decrease in biosynthesis.

Figure 9 shows GAG loss for cartilage cultured without joint capsule tissue, cartilage co-cultured with live joint capsule tissue or dead joint capsule tissue, and cartilage cultured in joint capsule tissue conditioned medium for three days.

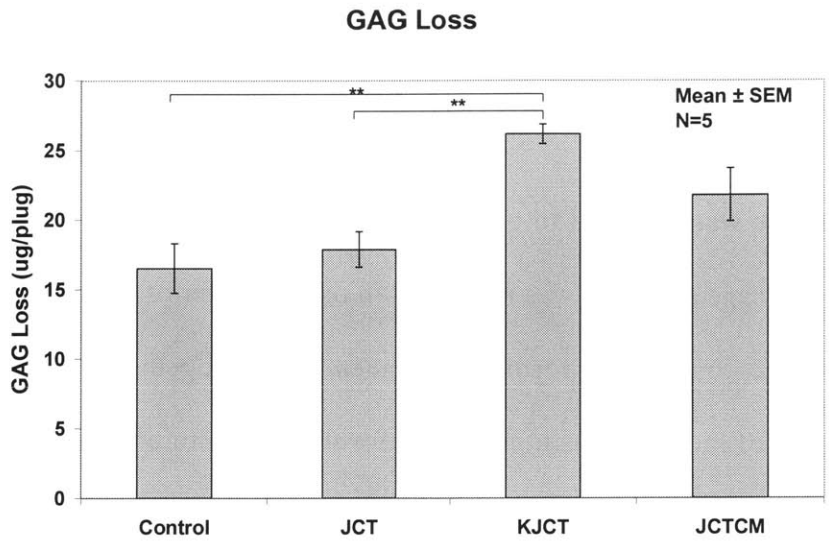

Figure 9: Direct comparison of GAG loss for cartilage cultured without joint capsule tissue, co-cultured with live joint capsule tissue or dead joint capsule tissue, and cartilage cultured in joint capsule tissue conditioned medium. Cartilage was allowed to equilibrate for three days before intervention and was cultured an additional three days after intervention. '**' represents a p value of <0.005 by a two sided student t test.

Figure 9 shows a significant increase in GAG loss in the dead joint capsule tissue co-culture and the joint capsule tissue conditioned medium. This result is different than the results in figure 7 and could be due to differences in the joint capsule tissue composition or the amount of damage that was done to the cell membranes of the joint capsule tissue when it was killed.

4.6 Direct comparison of joint capsule co-culture and conditioned medium

Because all of the previous experiments had been done with either the co-culture system or using conditioned medium, it was important to do an experiment that did a direct

comparison of the co-culture model and the conditioned medium model in order to determine which trends were the same and which were different.

4.6.1 Methods

Joint capsule tissue was punched to obtain 3mm punched pieces of joint capsule tissue. One piece of joint capsule tissue was incubated in one milliliter of medium for four days. Joint capsule tissue conditioned medium was then pooled together, supplemented with ascorbate and 1ml of medium was transferred to wells in a culture plate. Fresh 10% FBS medium was added to the other wells of the culture plate and the same pieces of joint capsule tissue which had been used to condition the medium were placed into those wells. There was one piece of joint capsule tissue per well. Cartilage was either left alone or injured to 50% strain as previously described. All plugs were matched for location across the four conditions. After four days of culture in either conditioned medium or with joint capsule tissue, cartilage plugs were radiolabeled ^{35}S-sulfate and GAG loss was measured. N= 6 for this experiment. The conditions for this experiment were:

1) Joint capsule tissue co-culture
2) Joint capsule tissue co-culture + injury
3) Joint capsule tissue conditioned medium
4) Joint capsule tissue conditioned medium + injury

4.6.2 Results

Figure 10 shows a comparison of GAG loss from cartilage cultured in conditioned medium and cartilage co-cultured with joint capsule tissue.

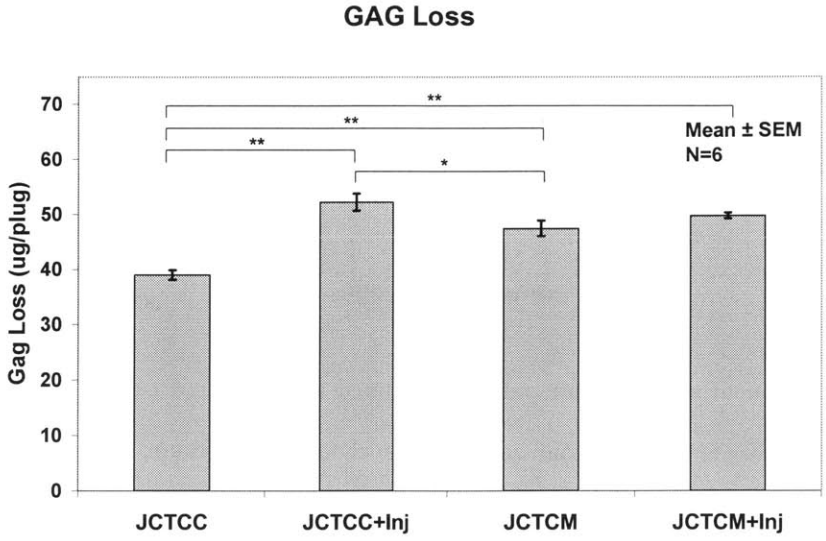

Figure 10: Comparison of GAG loss for injured and uninjured cartilage cultured with joint capsule tissue and cartilage cultured in joint capsule tissue conditioned medium. Cartilage cultured for four days under these conditions. '*' represents $p<0.05$, '**' represents $p<0.001$ by a two sided student t test.

Figure 10 shows that there is a significant increase in GAG loss when cartilage is injured in the co-culture system and no difference in the conditioned medium system. It is also interesting to note that the uninjured levels of GAG loss in the conditioned medium system are almost as high as the injured levels of GAG loss in the co-culture system. Figure 11 shows sulfate incorporation for the same experiment.

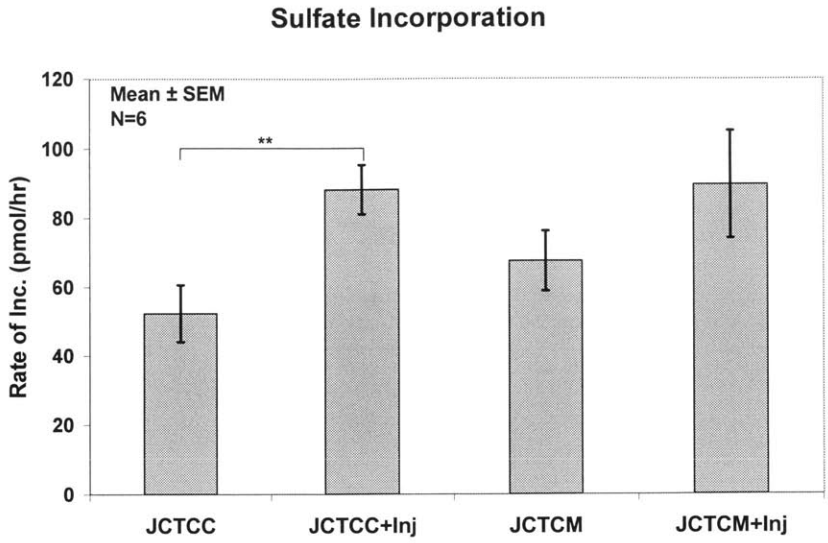

Figure 11: Comparison of sulfate incorporation for injured and uninjured cartilage cultured with joint capsule tissue and cartilage cultured in joint capsule tissue conditioned medium. Cartilage cultured for four days under these conditions. '**' indicates p value of <0.01 by a two sided student t test.

There is no significant difference in sulfate incorporation for conditioned medium and co-culture without injury and conditioned medium and co-culture with injury which means that if the only measurement one is interested in is radiolabel incorporation, it really doesn't make any difference whether one uses conditioned medium or co-culture to run the experiment. From figures 10 and 11 it appears that once again in the co-culture system a significant increase in GAG loss with injury is associated with an increase in sulfate incorporation.

4.7 Joint capsule tissue co-culture with joint capsule tissue cultured alone control

During one of the conditioned medium experiments it was discovered that the joint capsule tissue itself releases GAG into the medium and this amount is not always insignificant. In some cases it can account for as much as a third of the total GAG lost.

In the conditioned medium model, a sample of conditioned medium can be saved and the GAG in it can be measured so as to figure out how much GAG belongs to the cartilage and how much belongs to the joint capsule tissue. In the co-culture system, this becomes more difficult because both the cartilage and the joint capsule tissue are cultured together in the same well and there is no way to account for how much GAG comes from the joint capsule tissue and how much GAG comes from the cartilage. The goal of this experiment was to try using a piece of joint capsule tissue cultured alone as a control for the GAG lost by the joint capsule tissue.

4.7.1 Methods

Cartilage and joint capsule tissue were harvested in the usual manner. Joint capsule tissue was punched to form 5mm pieces. Both cartilage and joint capsule tissue were matched for location across the different conditions. They were both allowed to equilibrate for three days prior to intervention. After intervention they were incubated in 1ml of 10% FBS medium for three days at which time the medium was changed and GAG loss was measured. They were then allowed to incubate for an additional 3 days after which GAG loss was measured a second time. The groups for this experiment were:

1) Cartilage alone
2) Joint capsule tissue alone
3) Joint capsule tissue + cartilage
4) Injured cartilage alone
5) Injured cartilage + joint capsule tissue

4.7.2 Results

Figure 12 shows GAG loss for the first three days after intervention.

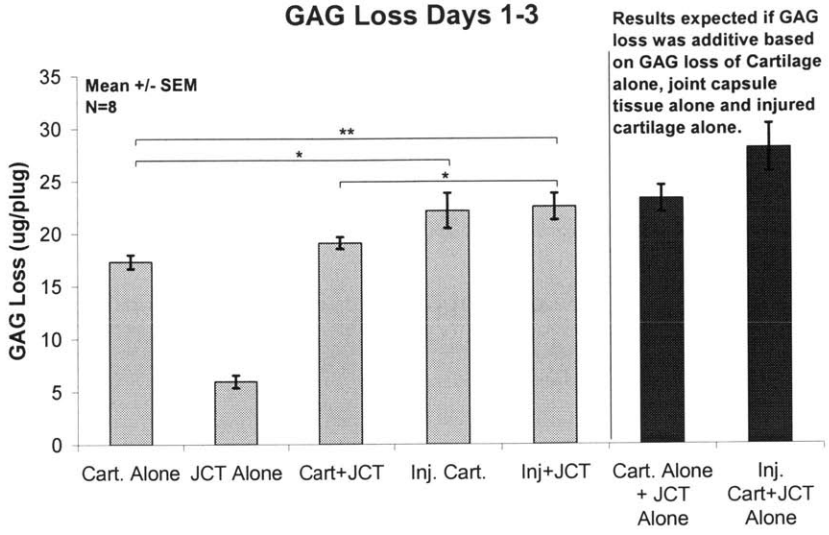

Figure 12: GAG loss for injured and uninjured cartilage cultured with and without a 5mm punched piece of joint capsule tissue and joint capsule tissue cultured alone for days 1-3 after intervention. The two bars on right are the mathematical sum of the experimental results on the left for cartilage cultured alone + joint capsule tissue cultured alone, and injured cartilage cultured alone + joint capsule tissue cultured alone. '*' represents p value of <0.04, '**' represents p value of <0.004.

For the first three days after intervention, it appears that there must be some interaction between the joint capsule tissue and the cartilage that causes inhibition of GAG loss in one or both of the tissues. If there were no interaction at all, we would expect the cartilage plus joint capsule tissue group and the injury plus joint capsule tissue group to be higher as can be seen on the right. Instead, they appear to be at about the same level as the cartilage that is cultured alone ~1.5 to 2 μg higher.

Figure 13 shows GAG loss for days 4-6 after intervention.

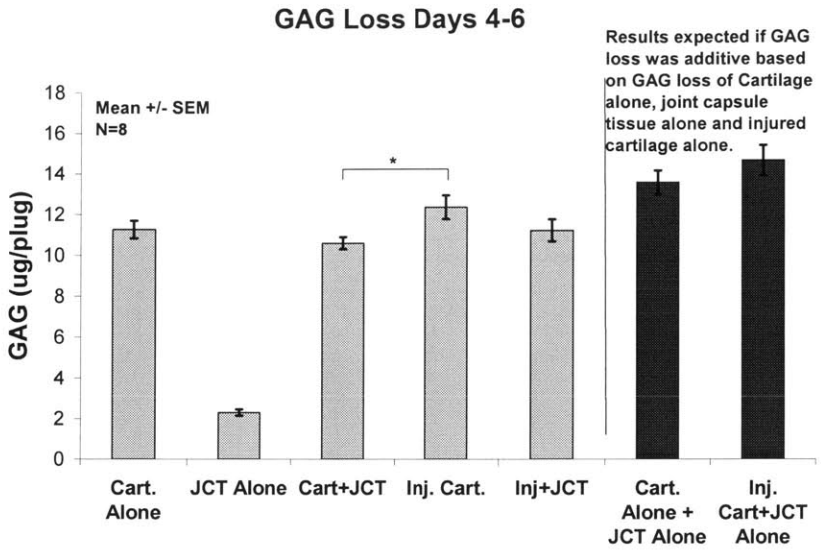

Figure 13: GAG loss for injured and uninjured cartilage cultured with and without a 5mm punched piece of joint capsule tissue and joint capsule tissue cultured alone for days 4-6 after intervention. The two bars on right are the mathematical sum of the experimental results on the left for cartilage cultured alone + joint capsule tissue cultured alone, and injured cartilage cultured alone + joint capsule tissue cultured alone. '*' represents p value of <0.03.

Figure 13 shows the same trends as in figure 12 except for this time it appears that the wells that have cartilage co-cultured with joint capsule tissue actually has less GAG loss than the cartilage cultured alone. While the GAG loss is practically equivalent, it is surprising to see that it is slightly less by about 1 μg in figure 13 whereas it had been slightly higher by about 1.5-2 μg in figure 12. Granted these differences are really too small to mean something without more repeats of the experiment, however, if they hold up in multiple repeats, it would mean that the joint capsule tissue and the cartilage interact in a way that inhibits GAG loss.

Figure 14 shows the combined GAG loss for days 1-3 and 4-6 after intervention.

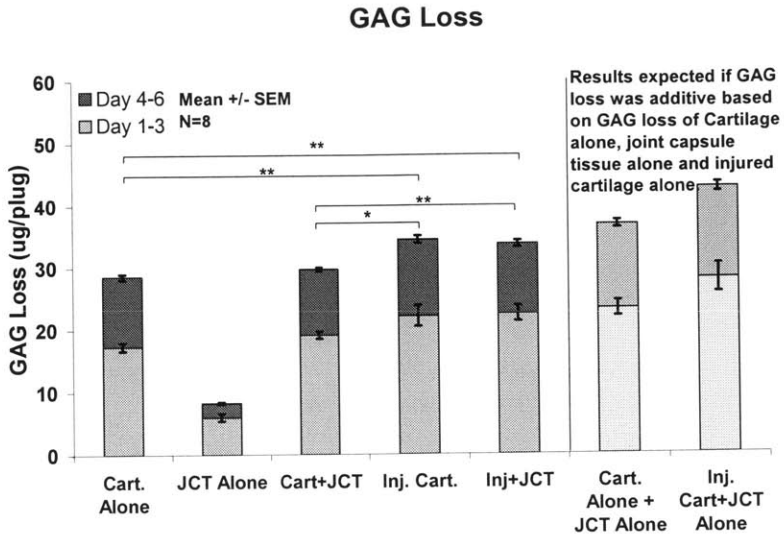

Figure 14: GAG loss for injured and uninjured cartilage cultured with and without a 5mm punched piece of joint capsule tissue and joint capsule tissue cultured alone for days 1-6 after intervention. The two bars on right are the mathematical sum of the experimental results on the left for cartilage cultured alone + joint capsule tissue cultured alone, and injured cartilage cultured alone + joint capsule tissue cultured alone. The lighter bars on the bottom are the results for days 1-3 and the darker bars on top are the results for days 4-6. '*' represents p value of <0.02, '**' represents p value of <0.003.

Figure 14 shows that the cumulative GAG loss for cartilage cultured alone and the co-culture system ends up being the same over six days. This is surprising since the joint capsule tissue cultured alone lost on average about 8 μg of GAG in those six days. If the two tissues were not interacting with each other to inhibit GAG loss in some way, the co-culture system should have had significantly higher GAG loss than the cartilage cultured alone.

4.8 Discussion and Conclusions

The results for section 4.3 do not definitively say whether or not one should use a 3mm punched piece of joint capsule tissue or a 5mm punched piece of joint capsule tissue. It appears that depending on what outcome measures one is interested in, either one may be preferable. If one is interested in trying to obtain more GAG loss after injury, the 5mm co-culture system is preferable however the results using the 5mm co-culture system are more variable from experiment to experiment although there are only a limited number of outcomes which are seen. On the other hand, using a 3mm punched piece of joint capsule tissue gives much more consistent results although they rarely include the large increase in GAG loss with injury. The 3mm punched pieces probably yield more consistent results because they are more tightly grouped as far as where they were harvested in the joint and therefore are more consistent in structure and thickness giving more consistent results with tighter error bars. Often, all of the joint capsule tissue harvested is used when using the 5mm punch whereas only a small portion of the joint capsule tissue is used when using the 3mm punch.

Another good piece of information is that adding an additional piece of joint capsule tissue to the well does not affect the results and so there is no need to use a larger piece of tissue than the 5mm punched piece.

The co-culturing dead joint capsule tissue should not be considered a viable alternative to using live tissue because its results looked more similar to the results of the conditioned

medium model. Also, there is no way to be certain that the decreases seen in radiolabel incorporation are not due to other factors released from damaged cell walls as a result of freezing the tissue.

It appears that the majority of the GAG loss is seen during the first three days after harvest and there is no reason to go out to four days or longer. From the last experiment it looks like the same amount of GAG loss was seen no matter what the conditions were for days 4-6 so there is no need go longer than three days although going longer did not affect the trends seen.

Experiment 4.7 showed that it was good to have joint capsule tissue cultured alone as a control for GAG loss. If radiolabel incorporation is done on joint capsule tissue conditioned medium in the future, it will also serve as a useful control for that. By having the joint capsule tissue as a control it showed that there must be some kind of tissue-tissue interaction going on. A follow up to experiment 4.7 might want to look at GAG loss days 7-9 after intervention as it would be interesting to see if the amount of GAG loss in the co-culture wells continued to decrease and if that decrease would become statistically significant. If it did, it would show that there is some inhibitory response due to the interaction of the two tissues.

The results in this chapter also confirm that there are some differences in the results seen with the co-culture system as opposed to the conditioned medium system. The most striking difference is the difference in the amount of GAG measured in the medium. In

the co-culture system, the GAG loss tends to be the same whether or not joint capsule tissue is present in the medium. The exception appears to be when something triggers an increase in sulfate incorporation and there is a large increase in GAG loss to the medium. In the co-culture system a significant increase in GAG loss is often seen with injury over uninjured cartilage co-cultured with joint capsule tissue. Although there is rarely any difference statistically between the two different injured groups, there is usually no difference statistically for the GAG loss measured injured and uninjured cartilage cultured in conditioned medium. These results as well as the results presented in section 4.7 indicate that there is some interaction between the joint capsule tissue and the cartilage that is affecting GAG loss. An interesting follow up to this might be to do co-culture experiment using cyclohexamide to block the cellular response of both tissues and compare the results to the previous co-culture and conditioned medium experiments.

Another result that should be investigated further is what is responsible for the increase in sulfate incorporation after injury. While this increase was not always statistically significant, the trend was seen in many of the co-culture experiments and a few of the conditioned medium experiments. It would be interesting to see if it had something to do with the location either tissue was harvested from or the amount of damage received from the injury.

In future experiments involving the co-culture system and radiolabel incorporation, it would be a good idea to radiolabel joint capsule tissue as well as cartilage to get an idea of what is happening with regards to biosynthesis and proteoglycan synthesis in the joint

capsule tissue itself. It would be interesting to see if these levels also fall or increase depending on the conditions.

Total GAG for the cartilage should also be measured to determine if all of the extra GAG that is being made is being released into the medium or is staying in the tissue.

Because it appears that there is some kind of interaction between the two tissues, the joint capsule tissue co-culture model and the joint capsule tissue conditioned medium model should be treated as two different models and should not be assumed to be the same. The differences in the two models may be unimportant for some experiments yet important in others. The model used should be picked based on what outcome measures are going to be used and what the experiment is trying to test.

Chapter 5: Joint Capsule Tissue Characterization

5.1 Background

It had been previously assumed that all GAG loss found in joint capsule tissue conditioned medium experiments and joint capsule tissue co-culture experiments came from the cartilage. However, while doing the experiments documented in chapter 3 it was found that the joint capsule tissue conditioned medium contained a significant amount of GAG before any cartilage had been cultured in it. This meant that the joint capsule tissue contributed to the GAG that was measured. It was also observed while harvesting the joint capsule tissue, the medial side capsule tissue was much smaller and much more elastic than the joint capsule tissue on the lateral side. The lateral side capsule tissue was about twice as large and, structurally, it resembled ligament tissue as well as the more elastic membranous joint capsule tissue found on the medial side of the joint. This meant that the lateral side was much easier to punch than the medial side and, therefore, was used more often in experiments. This chapter examines the mechanism of GAG loss from the joint capsule tissue, total GAG of the tissue as well as the correlation of GAG loss, total GAG, total DNA, location, and size to the wet weight of the tissue.

5.2 GAG loss measured in conditioned medium

While doing the experiments found in chapter three, it was discovered that joint capsule tissue itself loses a significant amount of GAG into the medium. This section looks at the amount of GAG that was measured in a variety of conditioned medium samples that had

been saved. Unfortunately, there is no way to match the samples to the experiment they were used with.

5.2.1 Methods

Left over joint capsule tissue conditioned medium that had been conditioned for varying numbers of days and by different sizes of joint capsule tissue had been left in the incubator for use with future experiments. Three aliquots were taken from each of these conditioned medium samples and the GAG in the medium was measured by DMMB assay.

5.2.2 Results

Figure 1 shows the GAG that was measured in the various conditioned medium samples as well as how long each sample was conditioned, unless otherwise stated the concentration was one 3mm piece of joint capsule tissue per milliliter of 10% FBS medium.

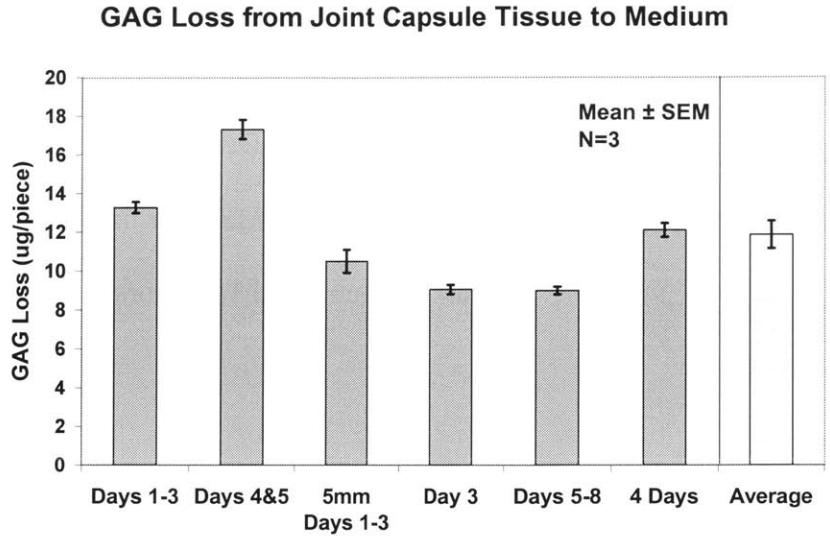

Figure 1: GAG measured in left over medium samples that had been stored in the incubator. Three aliquots were taken from each medium sample and GAG was measured for those aliquots. The average GAG loss is an average of the GAG measured in all of the different conditioned medium samples.

Figure 1 shows that there is a substantial amount of GAG that is lost to the medium by the joint capsule tissue. The values range from 9 μg per piece of joint capsule tissue to as much as 17 μg with the average being around 12 μg. In most of the conditioned medium and co-culture experiments, the GAG loss for the control cartilage was about 30 μg and uninjured cartilage cultured in joint capsule tissue conditioned medium was about 40 μg. This means that the joint capsule tissue could be responsible for a quarter to a third of the GAG that is being measured in each well. As was stated in chapter three, this means that samples of conditioned medium need to be saved so that the GAG lost by the joint capsule tissue can be subtracted from the GAG lost by the joint capsule tissue.

5.3 GAG loss vs. Wet Weight

In order to have a better idea of how much GAG was being lost into the medium and if there was any correlation between that amount and the size, location and wet weight of the joint capsule tissue an experiment was done that recorded that information as well as measured GAG loss after 24 hours, 48 hours, 72 hours and 144 hours.

5.3.1 Methods

Joint capsule tissue was harvested in the usual manner from three separate joints. This time the medial side and the lateral side were kept separated and six 3mm punched pieces were taken from the medial side and the lateral side. An additional six 5mm punches were taken from the lateral side. Wet weight of all joint capsule tissue pieces was measured. Each piece of joint capsule tissue was then placed in .5ml of 10% FBS medium for culture. After 24 hours, 48 hours, 72 hours and 144 hours the medium was changed and the GAG loss was measured.

5.3.2 Results

The results presented in this section are presented as an average of 6 samples from each of three joints. The data was also analyzed for each joint individually and while the values varied some, the trends were all the same and the differences were small. Appendix B contains the plots which break the data down by individual joints as well as additional GAG loss vs. wet weight plots for each of the days.

5.3.2.1 GAG loss vs. Location and Punch Size

Figure 2 shows GAG loss for the first 24 hours, 24-48 hours, 48-72 hours and 72-144 hours as well as the total GAG loss based on the location of harvest and punch size.

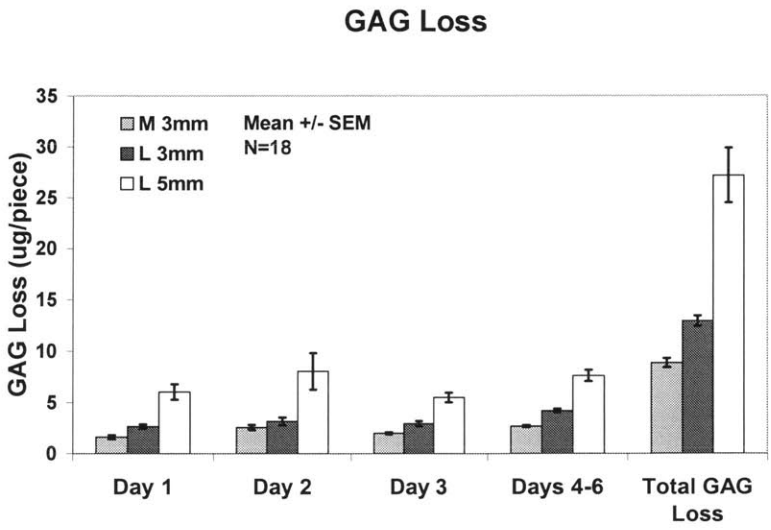

Figure 2: GAG loss as a function of time and location. All results $p<0.02$ by a two sided student t test except for the difference between the M 3mm and L 3mm for the Day 2 time point.

Figure 2 shows that there is a significant difference in GAG loss at each time point for the different locations and punch sizes. The only non significant difference was for the day 2 time point when there wasn't a significant difference between the GAG loss for the Medial 3mm piece and the Lateral 3mm piece.

Figure 3 also shows GAG loss as a function of location and time.

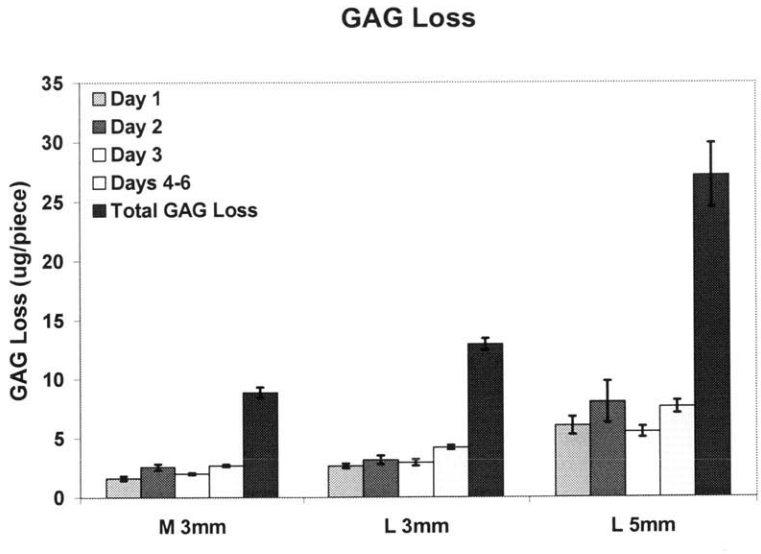

Figure 3: GAG loss as a function of time and location.

Figure 3 shows that GAG loss is pretty much constant for the first three days after harvest and then appears to taper off for the last three days with the cumulative GAG loss being similar to that seen for the first three days. Because GAG loss appears to be fairly constant, this would be consistent with the GAG being lost by diffusion as opposed to some other mechanism which might result in a spike in GAG loss for one of the days.

5.3.2.2 GAG loss vs. Wet Weight

Figure 4 shows total GAG loss over the 6 days as a function of the tissues wet weight. It has been fit with a best fit line.

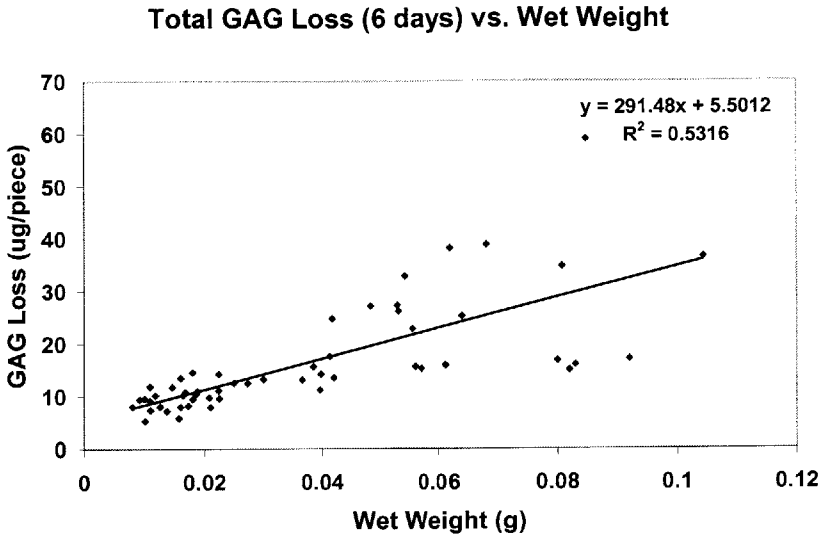

Figure 4: GAG loss over six days as a function of wet weight. Data fit by a linear regression. It is important to note that the line does not go through zero and can not be taken as an accurate predictor of GAG loss for tissue weighting less than 8mg.

Figure 4 shows that the total GAG loss increases linearly as the wet weight of the tissue increases. It is important to note that the best fit line does not go through zero as the best fit line that went through zero underestimated the GAG loss of the lower weight pieces of tissue. This plot indicates that in general it is the tissue weight that determines how much GAG is lost to the medium. It also shows that as the wet weight of the tissue increases, so does the variability in the amount of GAG that is lost. Using smaller pieces of tissue will decrease this variability and yield more consistent GAG loss results.

5.3.2.3 Wet Weight vs. Location and Punch Size

Figure 5 shows joint capsule tissue wet weight based on location of harvest and punch size.

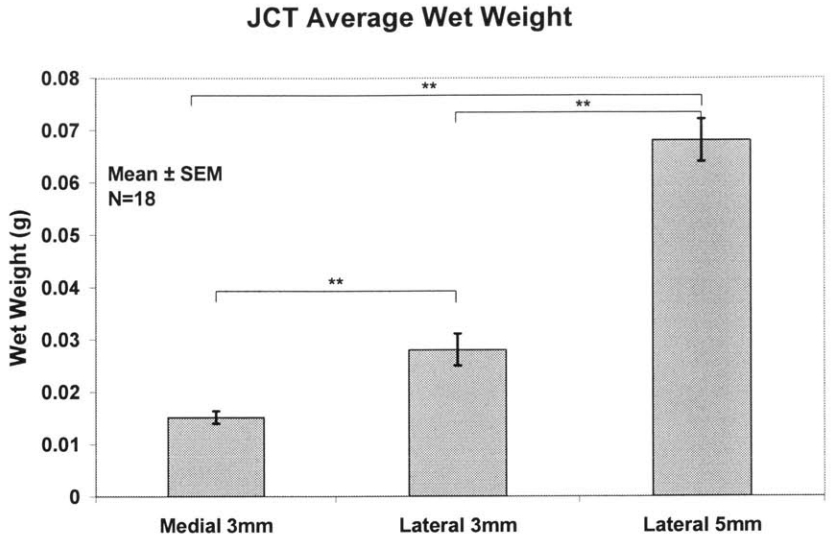

Figure 5: Joint capsule tissue average wet weight based on location and punch size. '**' represents p value of <0.0007.

Figure 5 shows that the medial side has the lowest wet weight and the lateral side has the largest wet weight. This is most likely due to the differences in structure between the two sides. The 5mm punch is about 2.5 times as heavy as the 3mm punch for the lateral side which is what is expected based on the difference in volume of the pieces.

5.4 Total GAG, DNA and wet weight of joint capsule tissue and cartilage

Since little was known about how much total GAG was in joint capsule tissue as well as how that compared to cartilage, an experiment was done to look at the relative wet weights and amounts of GAG and DNA in joint capsule tissue and cartilage. At the same time it was possible to look at what effect location and size had on these parameters for joint capsule tissue.

5.4.1 Methods

Joint capsule tissue and cartilage were harvested in the usual manner from two joints. Cartilage was sliced to form 1mm thick slices and punched with a 3mm dermal punch. The location of each slice was recorded as well as its thickness and wet weight. Cartilage plugs were digested in 1ml of proteinase K for two days in a 60° C water bath. Joint capsule tissue location was also recorded. Four 3mm punches were taken from the medial side of each joint. Four 3mm punches and four 5 mm punches were taken from the lateral side of each joint. Wet weight was measured and the joint capsule tissue was digested in proteinase K at a concentration that was 10 X the concentration used for the cartilage. After the joint capsule tissue and the cartilage were fully digested, total GAG and total DNA were measured. See Appendix D for more detail on how to perform GAG assays and DNA assays.

5.4.2 Results

The data in this section came from combining the results from two different cows. Twelve samples were taken from each cow. The joint capsule tissue results are presented for each individual joint. The cartilage results are presented as an average of the two joints as there was very little variation between the two joints and the number of samples from the lateral side was two small to do any kind of statistical comparison.

5.4.2.1 Joint Capsule Tissue Results

Figure 6 shows joint capsule tissue total GAG vs. total DNA.

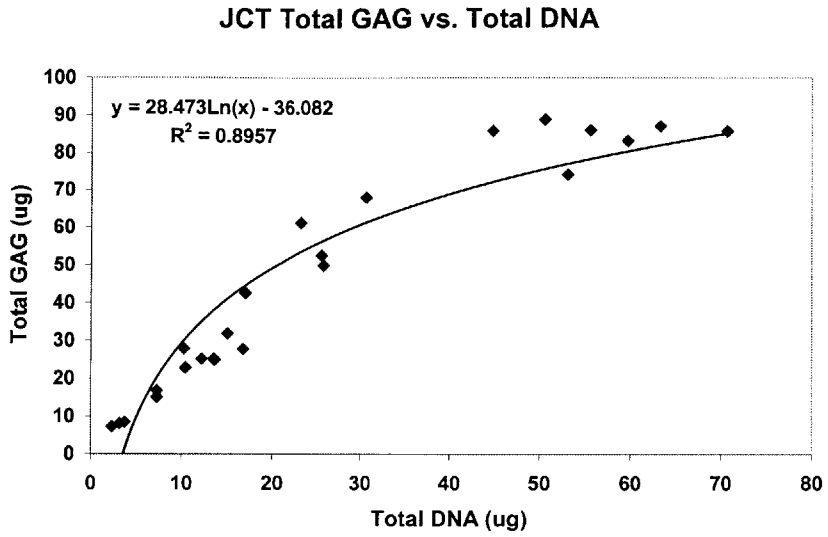

Figure 6: Joint capsule tissue total GAG plotted as a function of total DNA. Results are fit with a logarithmic approximation.

Figure 6 shows that although a logarithmic approximation gave the best fit for all of the data, the total GAG appears to vary linearly for lower amounts of total DNA and then remain constant for higher values. The total GAG appears to top off at around 90μg.

Figure 7 shows joint capsule tissue total GAG as a function of wet weight.

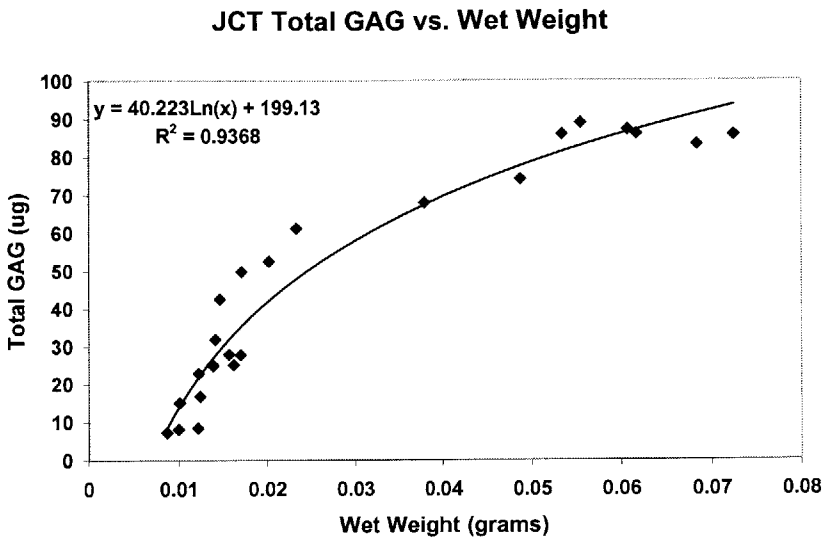

Figure 7: Joint capsule tissue total GAG as a function of wet weight fit with a logarithmic best fit approximation.

Figure 7 shows that the total GAG appears to vary logarithmically with the wet weight of the tissue and once again top off at around 90μg for the highest wet weight samples.

Figure 8 shows joint capsule tissue total DNA as a function of the wet weight.

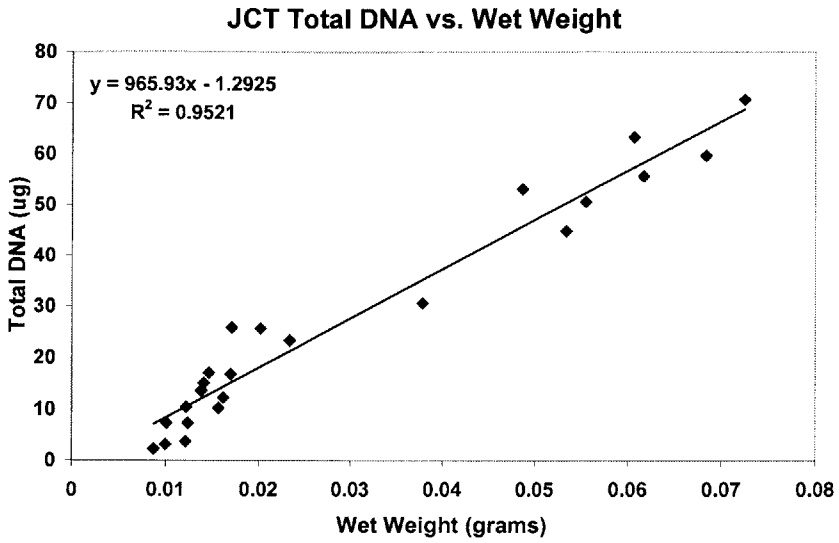

Figure 8: Joint capsule total DNA as a function of wet weight. Data is fit with the best fit linear approximation.

Figure 8 shows that the total DNA varies linearly with the wet weight of the sample.

Figure 9 shows joint capsule tissue average total DNA by location and size.

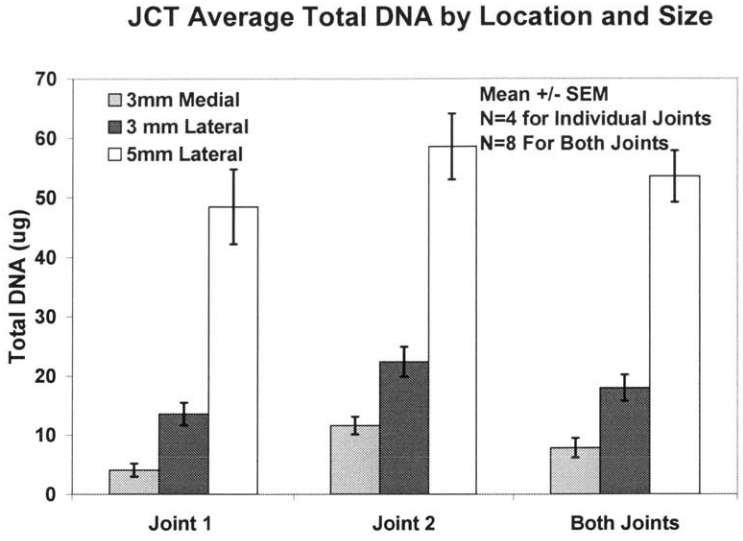

Figure 9: Joint capsule tissue total DNA by location and size. All differences are significant $p<0.05$ by a two sided student t test except for the difference in Total DNA for the 5mm lateral samples for joints 1 and joints 2.

Figure 9 shows that there is a significant increase in total DNA on the lateral side of the joint as compared to the medial side of the joint with the lateral side having about twice the amount of DNA as the medial. It also shows that there can be significant differences in the total DNA from joint to joint so a one size fits all approximation for every joint may not be possible.

Figure 10 shows joint capsule tissue total GAG by location and size.

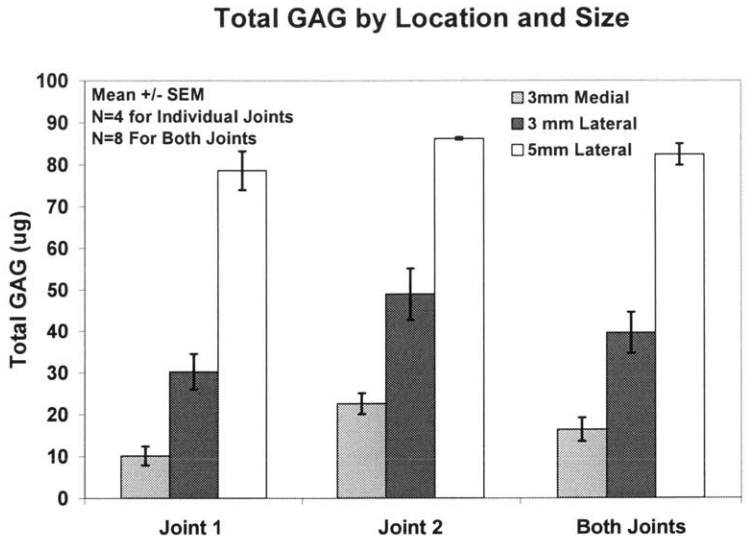

Figure 10: Joint capsule tissue total GAG as a function of location and size. All differences within individual joints are significant as well as the difference in total GAG for the 3mm Medial samples from joint 1 to joint 2. $p<0.01$ by a two sided student t test.

Figure 10 shows that there are even more significant differences in total GAG based on where the tissue is harvested from. The lateral side has as much as three times the amount of GAG that the medial side has and these values can vary greatly between joints, ~2x for the medial side.

Figure 11 shows wet weight by location and size.

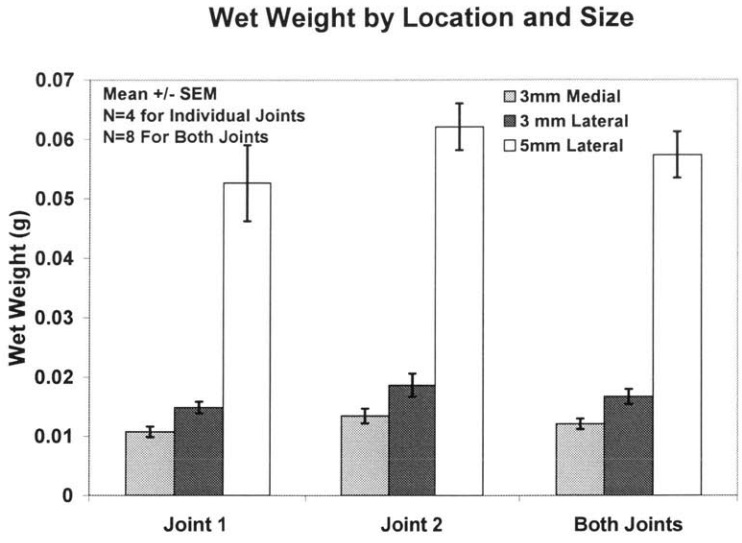

Figure 11: Wet weight as a function of location and size. There are no significant differences from joint to joint.

Figure 11 shows that the wet weight of the tissue appears to remain fairly constant from joint to joint and that the variation from the medial side of the joint to the lateral side of the joint is much smaller than for total GAG and total DNA.

Table 1 shows average wet weight, total GAG and total DNA by size and location for the two joints.

Table 1: Average wet weight, total GAG and total DNA by size and location

	Medial 3mm	**Lateral 3mm**	**Lateral 5mm**
Wet Weight	12.2 mg	16.8 mg	57.3 mg
Total GAG	16.4 μg	39.6 μg	82.4 μg
Total DNA	7.9 μg	18 μg	53.5 μg

5.4.2.2 Cartilage Results

Figure 12 shows cartilage total GAG as a function of total DNA.

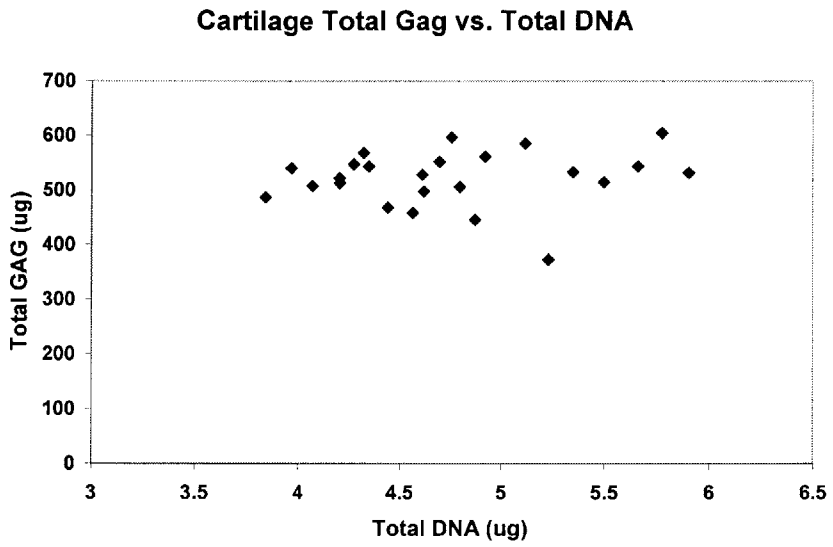

Figure 12: Cartilage total GAG as a function of total DNA

Figure 12 shows that unlike the joint capsule tissue, total GAG in the tissue appears to remain constant at around 500μg despite variations in the amount of total DNA.

Figure 13 shows cartilage total GAG as a function of its wet weight.

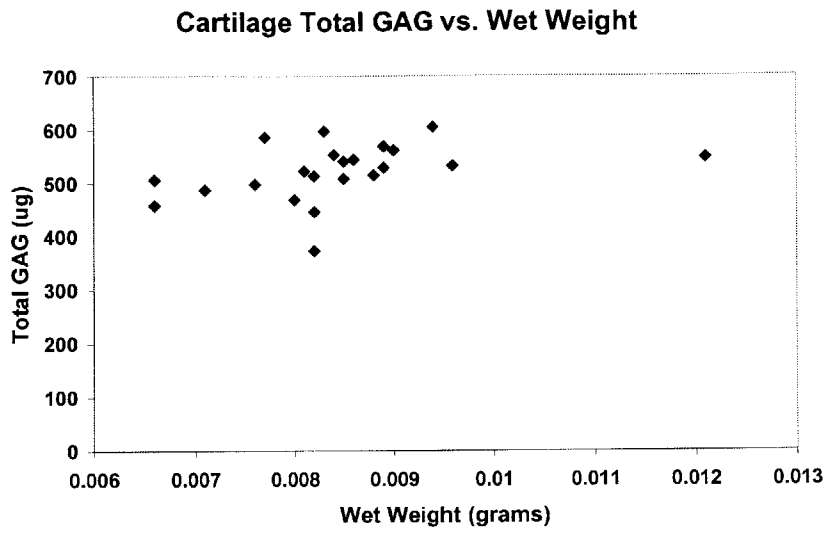

Figure 13: Cartilage total GAG as a function of wet weight

Similar to the results for figure 12, it appears from figure 13 that the wet weight of the tissue does not affect the amount of total GAG.

Figure 14 shows total DNA vs. wet weight.

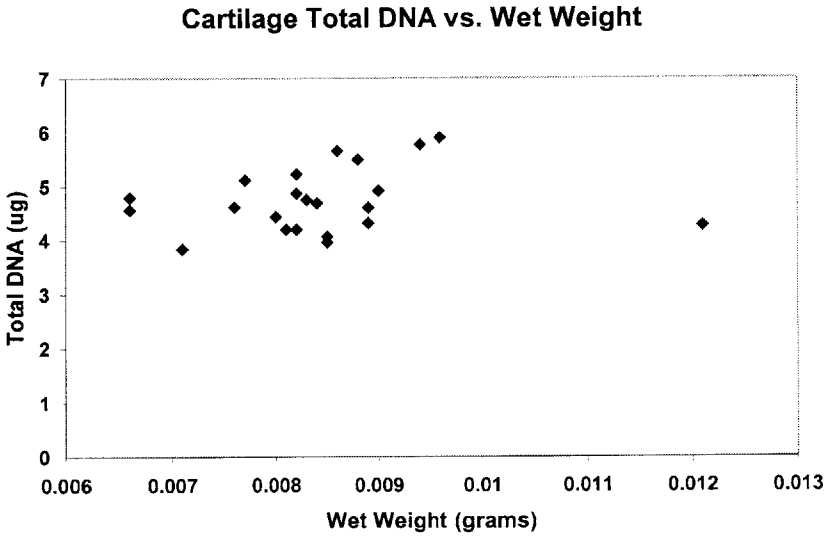

Figure 14: Cartilage total DNA vs. Wet Weight

Similar to the results in figures 12 and 13, figure 14 shows that the amount of total DNA does not appear to depend on the wet weight of the tissue.

Figure 15 shows cartilage wet weight normalized to the volume of the tissue.

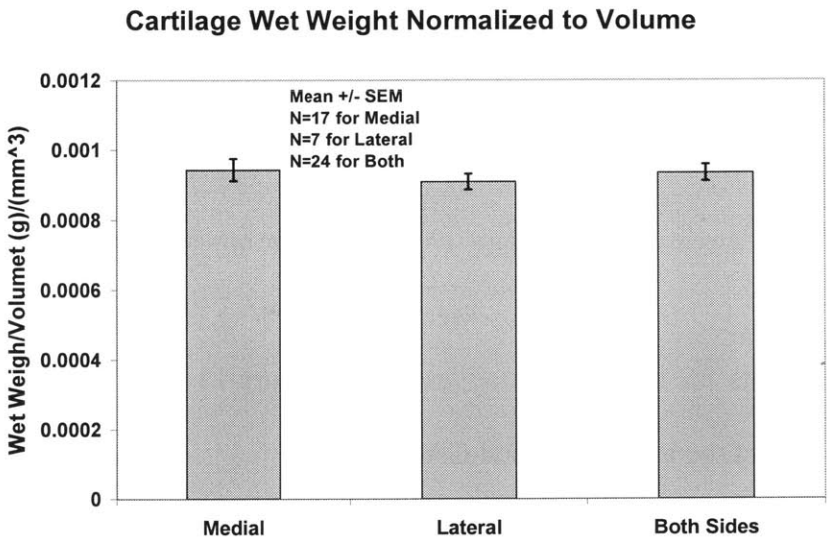

Figure 15: Cartilage wet weight normalized to volume. Results are presented as the average of data collected from two joints.

Figure 15 shows that the average wet weight remains constant from the medial to the lateral side of the joint.

Figure 16 shows cartilage total GAG normalized to volume for the medial and lateral side of the joint.

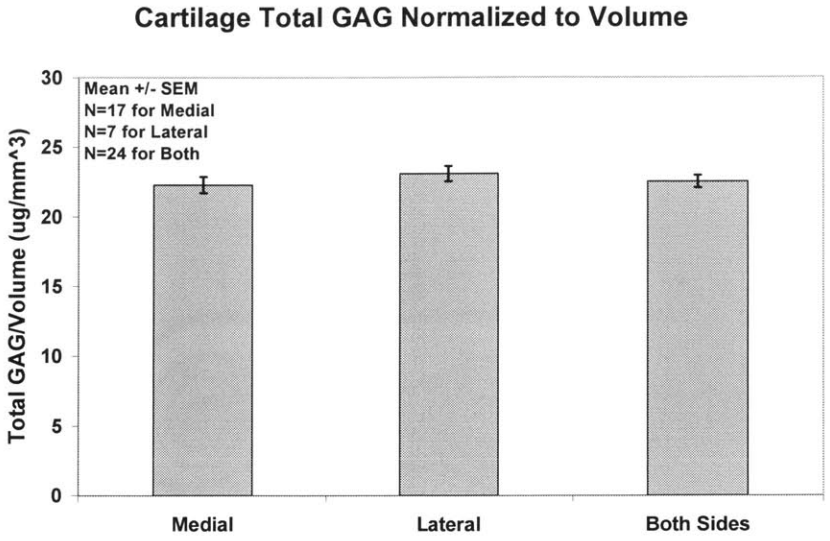

Figure 16: Cartilage total GAG for the medial and lateral side of the joint normalized to volume. Results are presented as the average of data taken from two joints.

Figure 16 shows that total GAG does not appear to vary from the medial side of the joint to the lateral side of the joint.

Figure 17 shows cartilage total DNA normalized to volume.

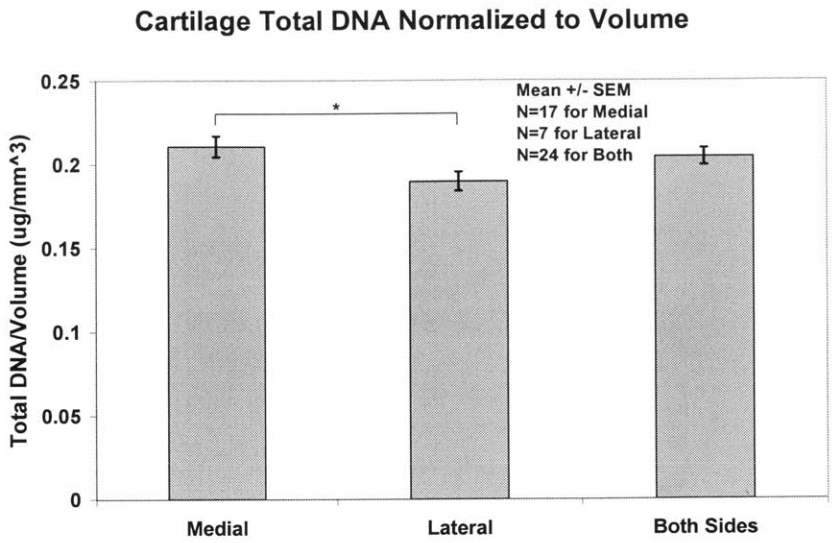

Figure 17: Cartilage total DNA for the medial and lateral side of the joint normalized to volume. Results are presented as the average of data taken from two joints. '*' represents p value of <0.05.

Figure 17 shows that the lateral side appears to have less total DNA/volume than the medial side of the joint.

Table 2 shows the average wet weight, total DNA and total GAG for the cartilage plugs.

Table 2: Average wet weight, total DNA, and Total GAG

Wet Weight	8.4 mg
Total DNA	4.8 μg
Total GAG	530 μg

5.4.2.3 Comparison of Results

Table 3 shows the data from tables 1 and 2 combined.

Table 3: Average wet weight, total DNA, and Total GAG

	Cartilage	Medial 3mm	Lateral 3mm	Lateral 5mm
Wet Weight	8.4 mg	12.2 mg	16.8 mg	57.3 mg
Total DNA	4.8 μg	7.9 μg	18 μg	53.5 μg
Total GAG	530 μg	16.4 μg	39.6 μg	82.4 μg

Table 4 shows the average values for the joint capsule tissue divided by the average values of the joint capsule tissue.

Table 4: Average JCT values/Average Cartilage Values

	Medial 3mm	Lateral 3mm	Lateral 5mm
Wet Weight	1.5	2	6.8
Total DNA	1.6	3.75	11.1
Total GAG	0.03	0.07	.16

Tables 3 and 4 show that joint capsule tissue has higher wet weight and total DNA but a lot less total GAG than the cartilage.

5.5 Superose 6 column fractionation

Because joint capsule tissue was known to contain small leucine rich proteoglycans such as decorin and fibromodulin,[13] it was thought that it might be possible to separate out the GAG that was lost by the joint capsule tissue from the GAG that was lost by the cartilage.

If this was not possible running samples through the superose 6 column would at least provide information about the relative size of the proteoglycans.

5.5.1 Methods

Medium was conditioned by joint capsule tissue and cartilage as previously described in chapter 3. This conditioned medium was frozen, lyophilized and reconstituted in 2ml of ammonium acetate running buffer. The reconstituted samples were sterile filtered through a 0.2μm syringe filter. 250 μl of the filtered samples were then run individually on a Superose 6 column at a rate of 0.5ml/min. Fractions were collected every minute and GAG was measured by DMMB assay. Cartilage and joint capsule tissue extracts were prepared by freezing the tissue (3mm punched pieces) in the minus 80° C freezer and then were crushed to create a fine powder using the pulverizer used for preparing the PCR samples. Liquid nitrogen was used to keep the pulverizer and the samples cool during this process. An equal wet weight of each tissue was powdered. The proteoglycans were extracted using a guanidine extraction with protease inhibitors, after the extraction was complete, they were ethanol precipitated at -20° C overnight, spun for 20min, and dried. See appendix D for more details. The dried precipitate was separated into two samples. The samples that were to be run down the Superose 6 column were reconstituted in 250μl of ammonium acetate running buffer and run individually down the column. Fractions were collected at the same rate as for the conditioned medium samples. 200 μg of pure aggregan was run down the column to calibrate the column and to determine which fraction would contain it.

5.5.2 Results

Figure 18 shows the results of fractionating tissue extracts, conditioned medium and aggrecan using a Superose 6 column.

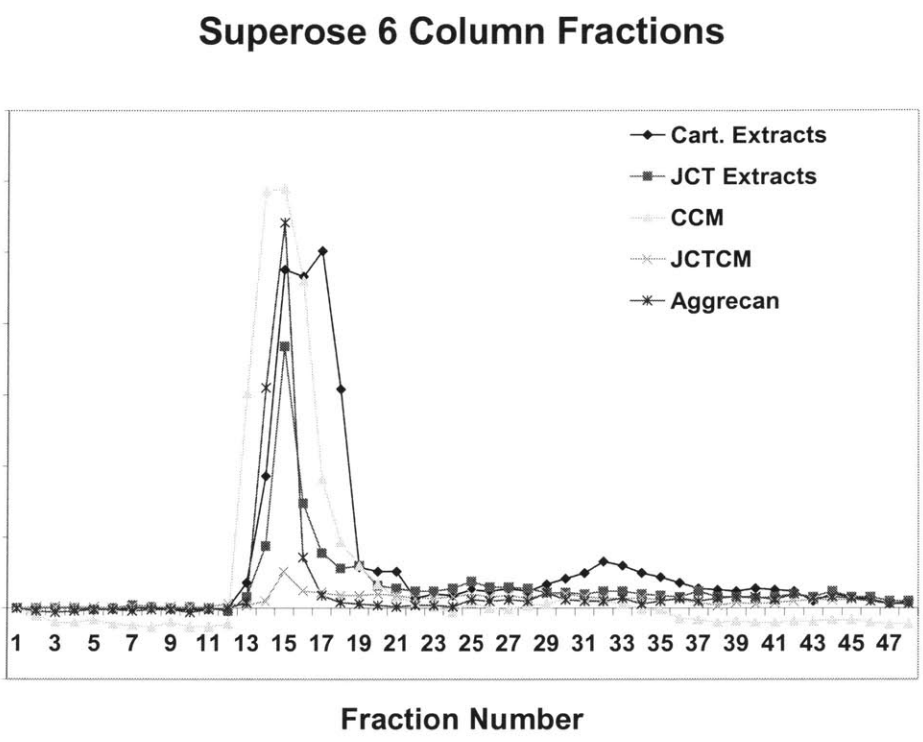

Figure18: GAG content of Superose 6 column fractions for cartilage and joint capsule tissue extracts and conditioned medium samples as well as pure aggrecan. Y axis has no meaning as all measurements were taken at different concentrations.

Figure 18 shows that all of the fractions peaked at fraction 15 including the aggrecan fractions. This would indicate that Aggrecan or another large proteoglycan is being released from the joint capsule tissue. Another interesting thing is that there is a wide peak for the cartilage extract that extends from fraction 29-37. Appendix B contains a plot in which this peak is more visible. Figure 18 also shows that it will be impossible

separate the GAG released by the joint capsule tissue from the GAG released by the cartilage using any kind of size filtration.

5.6 Western Analysis

Because all of the peaks for the joint capsule tissue samples run down the Superose 6 column had a peak where aggrecan came out, it was important to determine if it was aggrecan that was being released or another large proteoglycan that was about the same size. One definitive way to determine whether or not aggrecan was being released was to run a western blot and probe with the anti-G1 antibody as it ought to pick up any aggrecan fragments that might be in the tissue.

5.6.1 Methods

Tissue extracts were prepared using the same method as for the Superose 6 column. Once the ethanol precipitated samples had dried they were digested with protease-free chondroitinase ABC for two hours and keratanase II and endo-ß-galactosidase for four more hours. These samples were then dried and resuspended in sample buffer and run on a 4-15% gradient Tris-HCL gel from BioRad. The proteins were then transferred to a nitrocellulose membrane and probed with an anti-G1 antibody.

5.6.2 Results

Figure 19 shows the results of the Western Blot with the anti-G1 antibody.

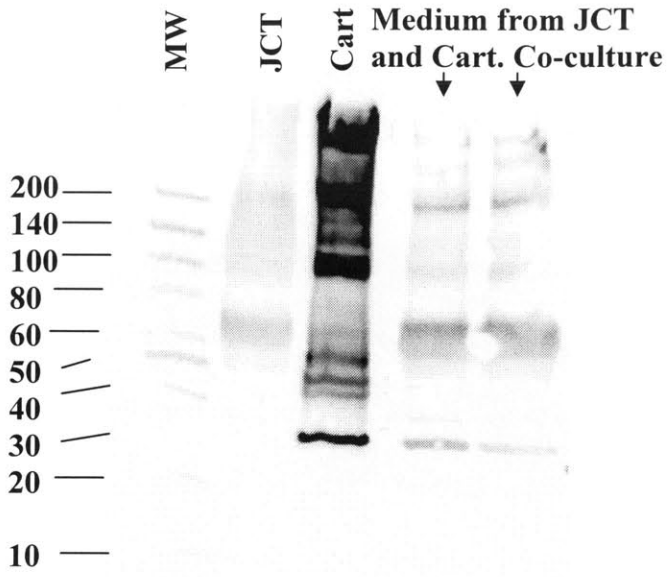

Figure 19: Western blot with anti-G1 antibody. First lane is standards, the second lane is joint capsule tissue extracts, the third lane is cartilage extracts and the last 2 lanes are medium samples obtained from a joint capsule tissue and cartilage co-culture experiment done by Jenny Lee.

Figure 19 shows that there are two faint bands in the joint capsule tissue extract lane at 200 KD, and between 60-80 KD. These two bands match up well with two of the bands seen in the lanes that are medium from a JCT and cartilage co-culture experiment done by Jenny Lee. These two bands indicate that there are aggrecan fragments in the tissue extract which means that the peak seen in the superose 6 column fractions is most likely aggrecan. The western should be repeated using a higher concentration of tissue in order to get verify the result seen in figure 19.

5.7 Discussion and Conclusions

It was surprising to discover that the average amount of GAG loss to the conditioned medium samples that were saved was 12 μg/plug. This is not an insignificant amount of

GAG that can be neglected as it can account for as much as a third of the total GAG loss that is measured in an experiment.

It was not surprising that the medial side loss less GAG than the lateral side because it was thinner than the lateral side and lacked the structural integrity of the lateral side. The structure of the joint capsule tissue on the lateral side looked more like a flattened ligament, whereas the medial side bore more resemblance to a thick membrane.

GAG loss was pretty much constant for the first three days after harvest and then decreased for the last three days. This indicates that the GAG is probably lost through diffusion and there is no need to worry about a spike in GAG loss on a certain day when collecting conditioned medium samples. GAG loss also appeared to be linearly proportional to wet weight which would indicate that it may be possible to guess the amount of GAG that a given piece of joint capsule tissue will lose based on its wet weight.

There appears to be an upper limit of ~90 μg for the amount of total GAG found in the pieces of joint capsule tissue. Because of this upper limit, a logarithmic equation provided the best fit for the relationship of total GAG to DNA and wet weight. However, it appeared that for lower amounts of DNA and to a lesser extent wet weight, that there was a linear relationship.

Because total GAG of joint capsule tissue appeared to vary from side to side and joint to joint, a one size fit all approximation will probably not work. Instead, it is probably preferable to develop some kind of control as was discussed in chapters three and four.

While total GAG in joint capsule tissue appeared to vary as a function of DNA and wet weight, the total GAG in cartilage appeared to remain constant for varying wet weights and amounts of total DNA. It is important to note though that the variations in wet weight for cartilage were very small compared to the variations seen for joint capsule tissue as it was only punched with a 3mm punch. It is possible that there might be some kind of relationship over a larger range of wet weights or that it might solely be dependent on the size punch that is being used.

Total GAG for cartilage as well as wet weight appeared to be constant from the medial to the lateral side of the femoropatellar groove. It was surprising though that the lateral side appeared to have less DNA than the medial side. It is unknown why this might be and more experiments would have to be done to make sure that this result holds up over more joints with a larger N.

Overall the results of sections 5.2, 5.3 and 5.4 showed that the GAG loss and amounts of GAG in the joint capsule tissue are not as unpredictable as they first appeared because there were definite relationships between wet weight, total GAG and total DNA. While the results were not totally unpredictable, they reiterated the need for keeping controls to

account for joint capsule tissue GAG loss in future conditioned medium and co-culture experiments.

The results that were surprising were the results of the Superose 6 column and the western blot. As was stated earlier, it was expected that the joint capsule tissue conditioned medium and the joint capsule tissue extracts would have peaks that corresponded to lower molecular weight proteoglycans such as decorin and fibromodulin since it was known to contain those proteoglycans. Instead, the peaks for both samples lined up perfectly with the peak for pure aggrecan, the cartilage tissue extract and cartilage conditioned medium. As a follow up to that, the western was run with the G1 antibody in hopes of determining whether or not aggrecan was present in the joint capsule tissue. Although the results of the gel are very faint, they indicate that there is some aggrecan present in the joint capsule tissue. Another western should be done with a higher concentration of joint capsule tissue extract to make sure that the bands get darker and see if any other bands appear. It might also be good to repeat the gel and use other antibodies to look for other aggrecan fragments.

After reviewing the literature, it appears that it is not unreasonable for joint capsule tissue to contain aggrecan although this has not been shown before.[12] More experiments would need to be done to show that aggrecan really is present in the tissue.

Joint capsule tissue is a broad term that applies to all of the tissue that surrounds the joint cavity. As stated in chapter 1, ligaments are just local thickenings of the joint capsule

tissue and some even include tendons as part of the capsule, especially the patellar tendon and the quadriceps tendon.[24] This does not seem unreasonable as ligaments have a very similar structure to tendon except for ligaments have fewer collagen fibers, higher percentage of proteoglycan matrix and more elastin.[17]

Compressively loaded tendons have been shown to become fibrocartilaginous and contain aggrecan.[5,28,30,31,32] However, this process does not appear to begin until after birth.[5] The bovine deep flexor tendon is an example of a tendon which has been shown to contain aggrecan.[31] A version of aggrecan that is missing the G1 domain has also been shown to be present in areas of adult tendon which are in tension.[32] Also, ligament was shown to contain a large unknown proteoglycan capable of forming aggregates with hyaluronan and decorin like aggrecan can.[1] In addition to this, joint capsule has been shown to become fibrocartilaginous where it attaches to the bone and in regions where it articulates with the joint.[24] Joint capsule tissue in the proximal interphalangeal joint has also been shown to contain chondroitin 6 sulfate which is associated with aggrecan.[15] In some animals such as the mouse, rat, dog and rabbit, the capsule forms a structure called the suprapatella which is a thick fibrocartilage that has been shown to contain aggrecan.[24,28] This suprapatella, which is not present in cows or humans, is located in approximately the same location as the joint capsule tissue used in the experiments presented in this chapter and earlier chapters. Combining all of this information together, it does not seem unreasonable for there to be some aggrecan in the joint capsule tissue as it is present in similar locations of the capsule in other animals. Also, since tendon, ligament and capsule appear to be difficult to separate structurally and often combine

together in the joint to form the capsule, the fact that aggrecan appears in tendon in tension and compression indicates that it could also be present in the joint capsule even though it is not necessarily compressively loaded. Finally, since joint capsule tissue structure has been shown to vary drastically from joint to joint and from location to location within a joint, it does not seem unreasonable for there to be areas of joint capsule tissue which contain aggrecan and areas that do not.

Chapter 6

GAG Loss from Free Swelling and Injured Cartilage by Location and Depth

6.1 Background and Purpose

The purpose of experiments in this chapter is to characterize the variations in GAG loss from free swelling and injured cartilage with location and depth in the tissue. Cartilage was harvested from the femoropatellar groove of 1-2 week old bovine calves, cored, and then sliced to form 1mm thick slices. Currently the number of slices taken and how deep those slices are taken from is partly a function of the number of samples needed to perform a particular experiment or set of experiments. If location and depth do affect the amount of GAG loss seen after injury or the change in GAG loss with injury, it might indicate that more care needs to be taken in choosing the location and depth of the cartilage samples harvested for use in experiments or more care may need to be taken in selecting which slices to assign to a particular experimental groups in order to ensure consistent results across a set of experiments.

6.2 Methods

Cartilage was harvested as previously described and was allowed to equilibrate for three days prior to intervention. One plug from each slice was injured to 50% strain as previously described and one plug was placed in free swell conditions. Plugs were cultured in 10% FBS medium for four days. After four days GAG loss was measured. GAG loss was then correlated to the side of the joint the tissue was harvested on, the

depth and location on each side of the joint as well as the shape of the plug after injury. Cartilage explants used in this analysis came from a total of 7 joints.

6.3 Results

For interpreting results by location, L denotes the lateral side of the joint and M denotes the medial side of the joint. M1 denotes the core that was furthest away from the chondyle with M4, M5, or M6 being the core closest to the chondyle depending on the size of the joint. The same is true for the lateral side. Top slice indicates the first slice taken from each core and the 4^{th} slice is the last slice that was taken indicating that it was closer to the bone.

6.3.1 Free Swell

Figure 1 shows GAG loss of free swelling cartilage based on whether the tissue was harvested on the lateral or medial side of the joint.

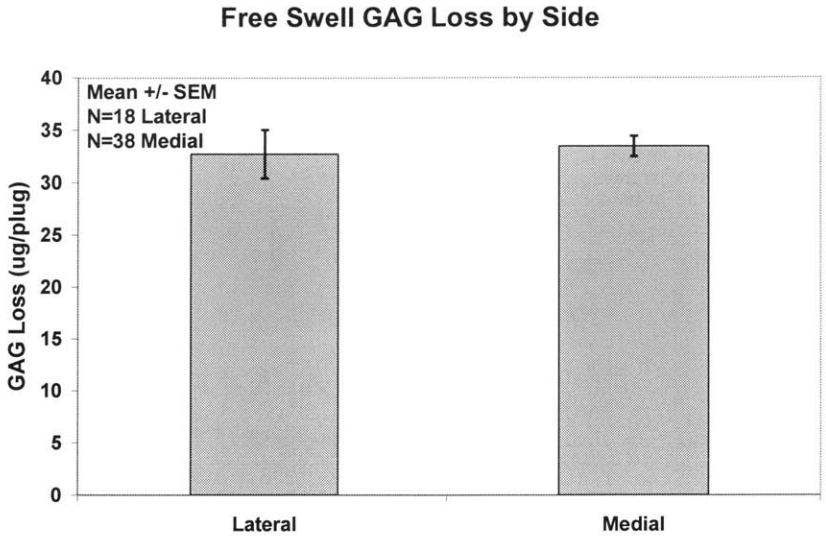

Figure 1: GAG loss for free swelling cartilage harvested from either the medial or the lateral side of the joint and cultured in 10% FBS medium for four days. Cartilage was allowed to equilibrate for three days prior to intervention.

From figure 1 it appears that there is no difference in the GAG lost to the medium if the cartilage is harvested from the lateral or medial side of the femoropatellar groove.

Figure 2 shows free swelling cartilage GAG loss separated by medial and lateral side as well as whether the plug was from the first, second, third or fourth slice. The first slice is the slice closest to the surface.

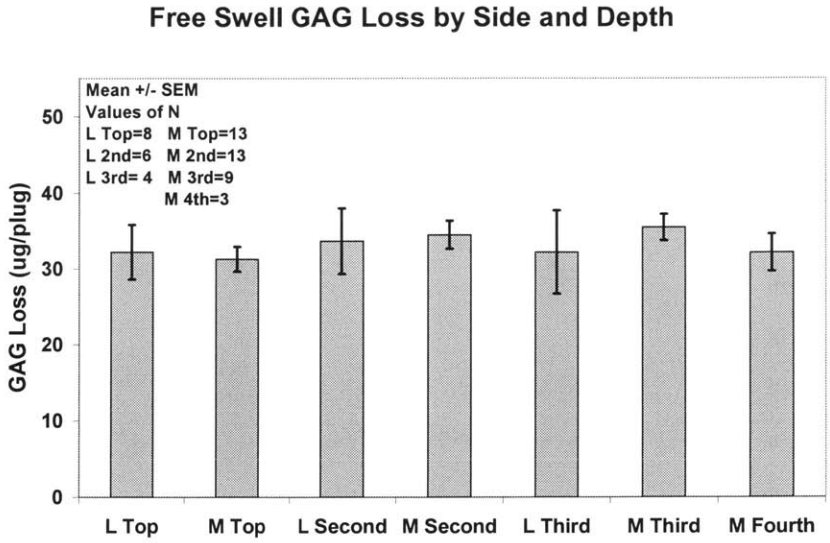

Figure 2: GAG loss of free swelling cartilage after four days of culture in 10% FBS medium. Results are separated by the side of the joint the cartilage was harvested on and whether the cartilage came from the first, second, third or fourth slice.

From figure 2 it also appears that there is no difference in the GAG loss based on the side or depth of the slice taken. It is important to note for this graph and subsequent graphs that include depth information that due to variations in cartilage thickness, it is possible for a second slice to be closer to the bone than a fourth slice.

Figure 3 shows the same data except for separated only by depth.

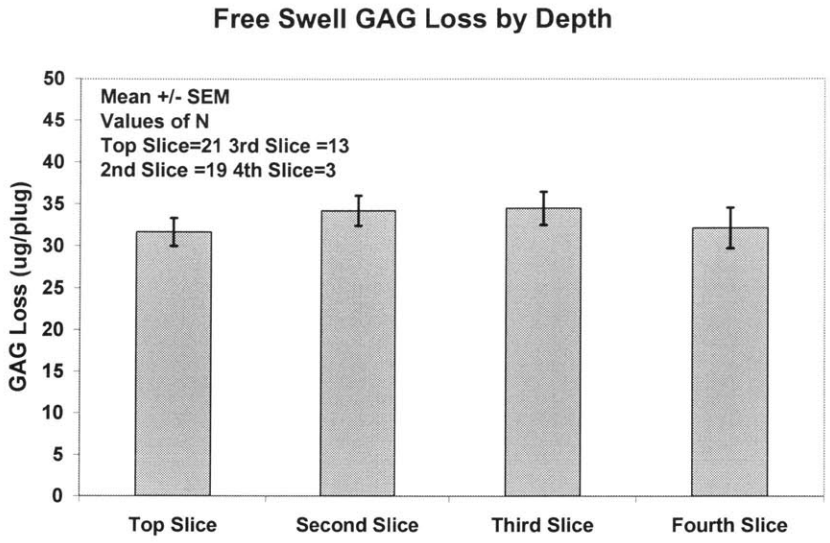

Figure 3: GAG loss by depth of free swelling cartilage after four days of culture in 10% FBS medium. Top slice indicates the top most slice taken, with the second, third and fourth slices getting progressively closer to the bone.

Once again, it appears that there is no difference in free swelling GAG loss based on depth.

Figure 4 shows GAG loss based on the surface location from which it was harvested. M and L denote medial and lateral sides and the numbers indicate distance away from the femur. A one denotes a plug from a core that was taken at the bottom of the femoropatellar groove and closest to the femur and a four indicates a core that was taken on the upper portion of the groove or the section closest to the chondyle.

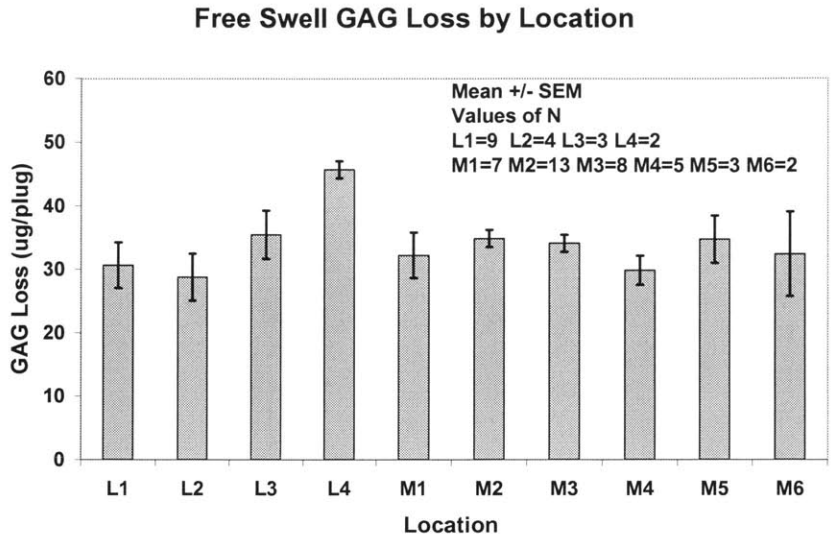

Figure 4: GAG loss of free swell cartilage after based on location on the joint. L1 indicates the lateral side furthest from the chondyle and closest to the femur whereas L4 indicates the lateral side and position closest to the chondyle. The same is true for the medial side. Because of differences in joint size, many times only three cores were taken from the lateral side and four cores were taken from the medial side.

Figure 4 shows that there does not appear to be any difference in GAG loss based on the location of harvest. Although L4 is significantly higher than the other bars, there are only two samples and so it can not be taken as an indication of what would happen if there were more samples.

6.3.2 Injury

Figure 5 shows GAG loss by side for injured cartilage.

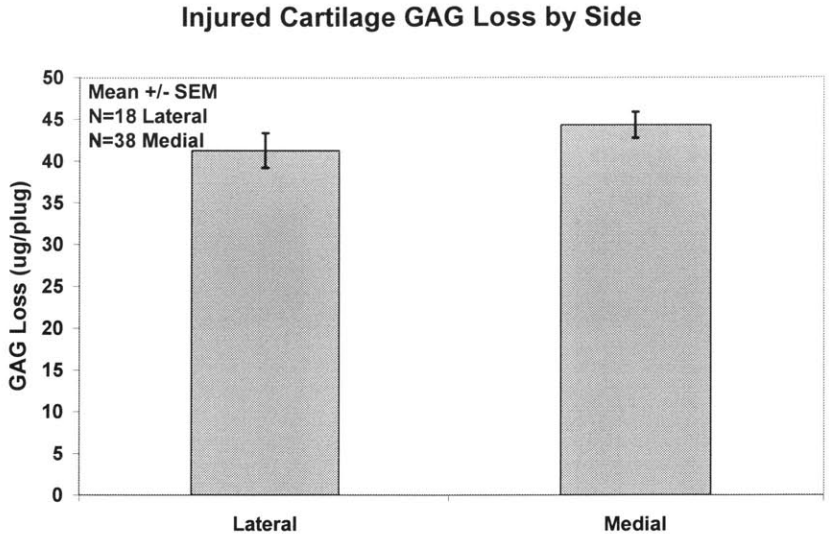

Figure 5: GAG loss for injured cartilage harvested from either the medial or the lateral side of the joint and cultured in 10% FBS medium for four days. Cartilage was allowed to equilibrate for three days prior to intervention.

The results of figure 5 mirror the results from the free swelling cartilage and show no difference in GAG loss based on the side of the joint the tissue was harvested from.

Figure 6 breaks down the information further and looks at GAG loss by side and depth of injured cartilage.

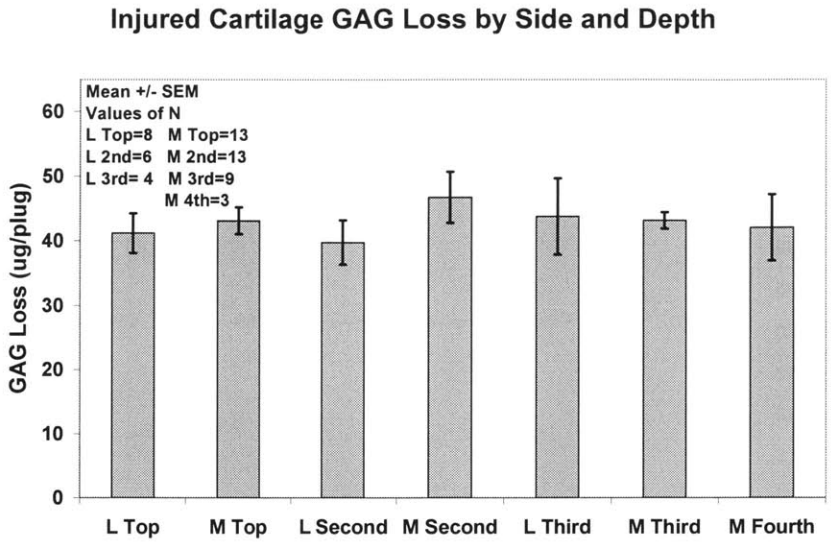

Figure 6: GAG loss of injured cartilage after four days of culture in 10% FBS medium. Results are separated by the side of the joint the cartilage was harvested on and whether the cartilage came from the first, second, third or fourth slice.

The results of Figure 6 also mirror the results seen for free swelling cartilage and indicate that side and depth don't affect the GAG loss seen.

Figure 7 shows GAG loss by depth for injured cartilage.

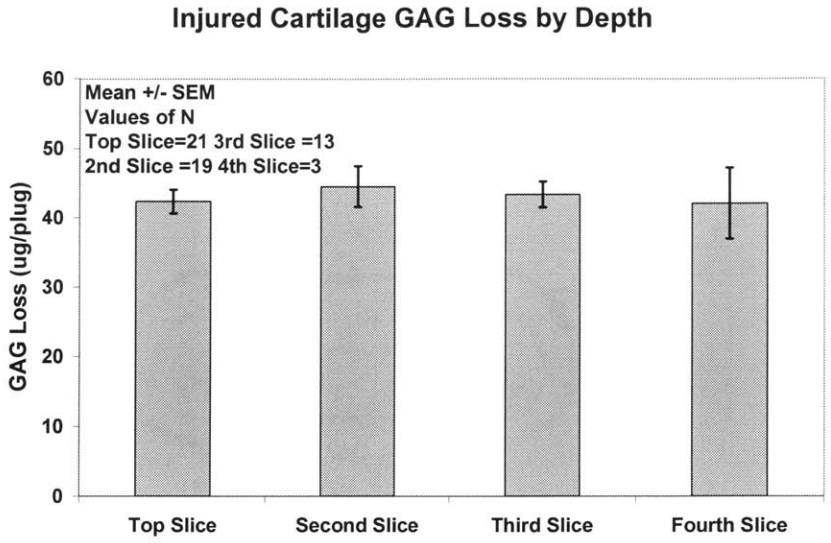

Figure 7: GAG loss by depth of injured cartilage after four days of culture in 10% FBS medium. Top slice indicates the top most slice taken, with the second, third and fourth slices getting progressively closer to the bone.

Once again, depth does not seem to affect the GAG lost to the medium.

Figure 8 shows GAG loss of injured cartilage by location.

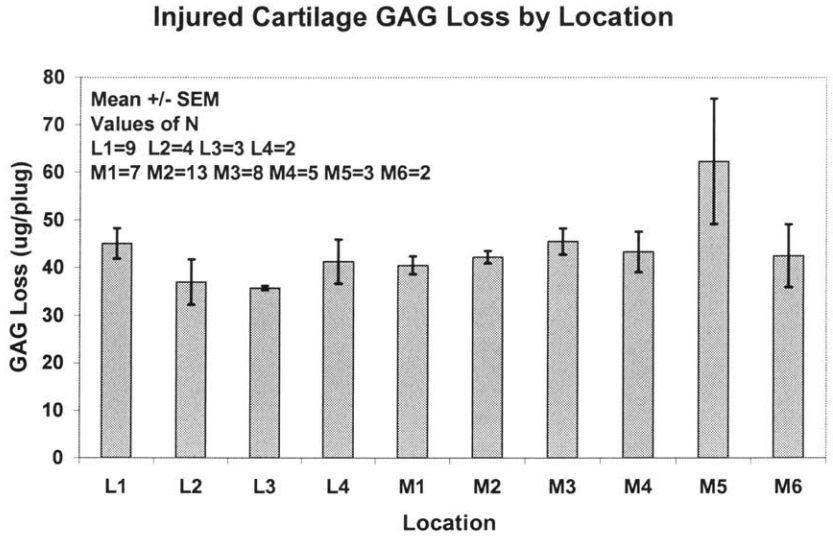

Figure 8: GAG loss of injured cartilage after based on location on the joint. L1 indicates the lateral side furthest from the chondyle and closest to the femur whereas L4 indicates the lateral side and position closest to the chondyle. The same is true for the medial side. Because of differences in joint size, many times only three cores were taken from the lateral side and four cores were taken from the medial side.

Once again there appears to be no difference in GAG loss based on the location of harvest.

Figure 9 shows GAG loss based on the shape of the plug after injury. Elliptical denotes plugs that were deformed after injury and round denotes plugs that retained their shape.

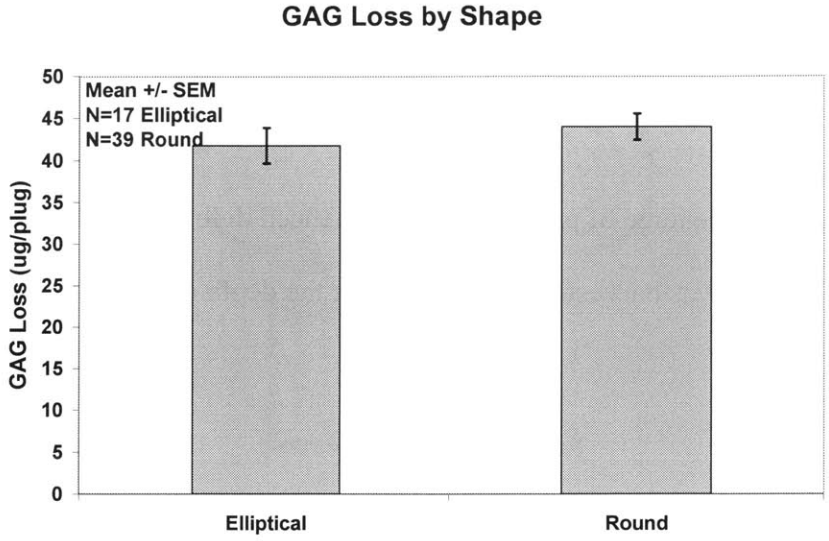

Figure 9: GAG loss of injured cartilage explants after four days of culture in 10% FBS medium based on their shape after injurious compression. Elliptical denotes deformation of the cartilage plugs, whereas round denotes that the plugs kept their original shape.

Figure 9 shows that there is no difference in the amount of GAG lost by plugs that were deformed during injury and those that weren't although at 50% strain, the majority of the plugs retained their round shape.

Table 1 shows the percentage of plugs which experienced deformation based on the side of the joint they were harvested from.

Table 1: Percent of plugs which experienced deformation by side of harvest.

Side	% Elliptical
Lateral	44.44
Medial	23.7

Table one shows that the lateral side had a higher incidence of deformed plugs than the medial side.

Table 2 shows the percentage of plugs which experienced deformation broken down by both the side the plug was harvested from as well as the depth of the slice the plug was taken from.

Table 2: Percept of plugs which experienced deformation by side and depth of harvest.

Side and Depth	% Elliptical
L Top	25
L 2nd	50
L 3rd	75
M Top	15.4
M 2nd	15.4
M 3rd	33.3
M 4th	66.7

Table 2 shows that both the medial and the lateral side follow the same trend of an increasing percentage of deformed plugs as the depth of the slice from which the plugs were taken increases.

Table 3 shows that same information presented only by depth.

Table 3: Percent of plugs which experienced deformation by depth

Depth	% Elliptical
Top Slice	19
Second Slice	26.3
Third Slice	46.1
Fourth Slice	66.7

Table three shows that there is a clear trend of increasing deformation as the depth of the slice increases.

6.3.3 Difference between Injury and Free Swell

Figure 10 shows the difference in GAG loss of injured and free swelling cartilage based on the side of the joint from which the cartilage was harvested.

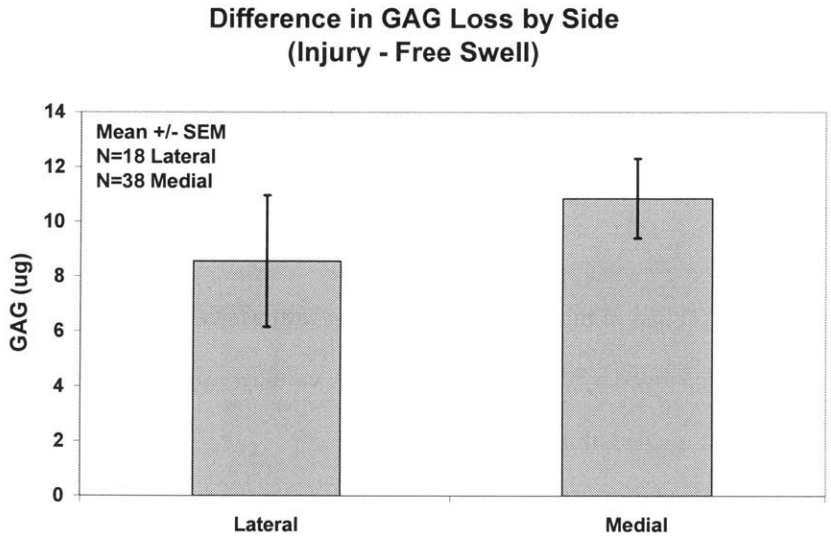

Figure 10: Difference in GAG loss for injured and free swelling cartilage harvested from either the medial or the lateral side of the joint and cultured in 10% FBS medium for four days. Cartilage was allowed to equilibrate for three days prior to intervention.

Figure 10 shows that there is no significant difference in the change in GAG loss seen between injured and uninjured plugs that were harvested on either the medial or the lateral side.

Figure 11 shows the difference in GAG loss between injury and free swelling cartilage based on the side of harvest as well as the depth.

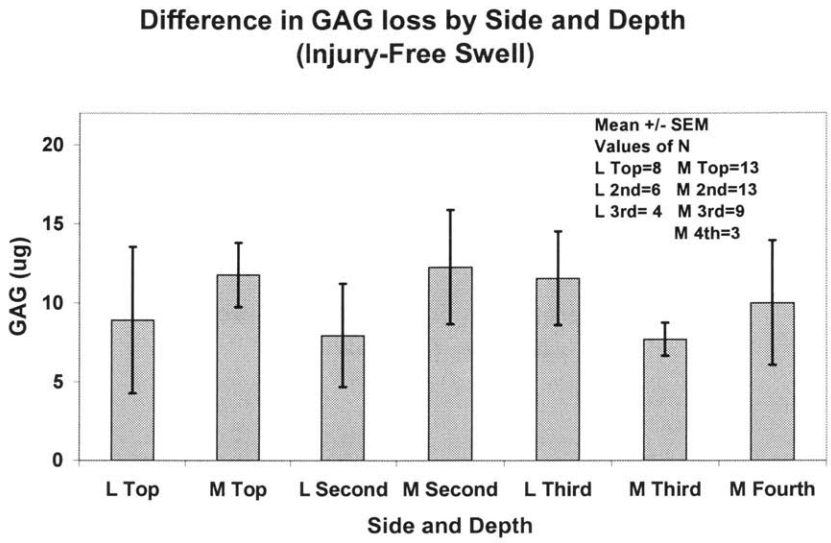

Figure 11: Difference in GAG loss of injured and free swelling cartilage after four days of culture in 10% FBS medium. Results are separated by the side of the joint the cartilage was harvested on and whether the cartilage came from the first, second, third or fourth slice.

From Figure 11 it appears that side and depth do not affect the difference in GAG loss seen between injury and free swelling cartilage.

Figure 12 shows the difference in GAG loss based on the depth of the slice from which the cartilage was harvested.

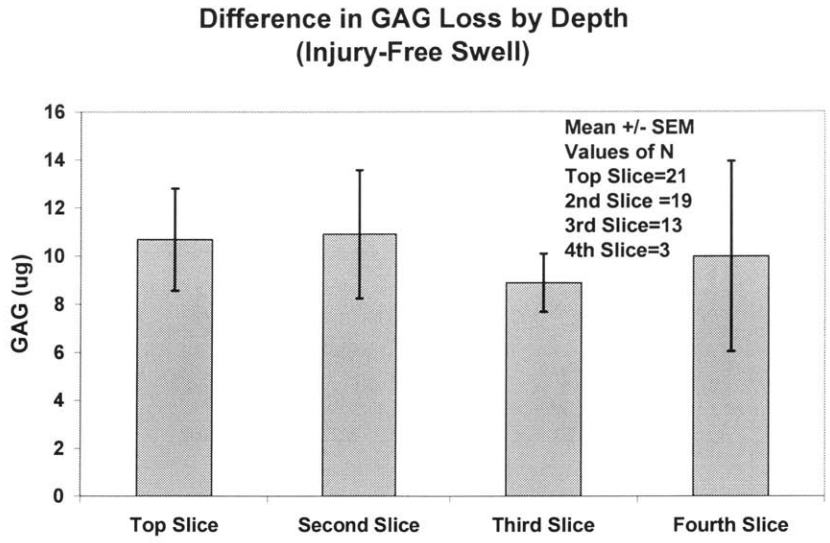

Figure 12: Difference in GAG loss by depth of injured and free swelling cartilage after four days of culture in 10% FBS medium. Top slice indicates the top most slice taken, with the second, third and fourth slices getting progressively closer to the bone.

Figure 12 also shows that depth does not appear to affect the difference in GAG loss seen between injured and uninjured cartilage.

Figure 13 shows the difference in GAG loss for injured and uninjured plugs based on location.

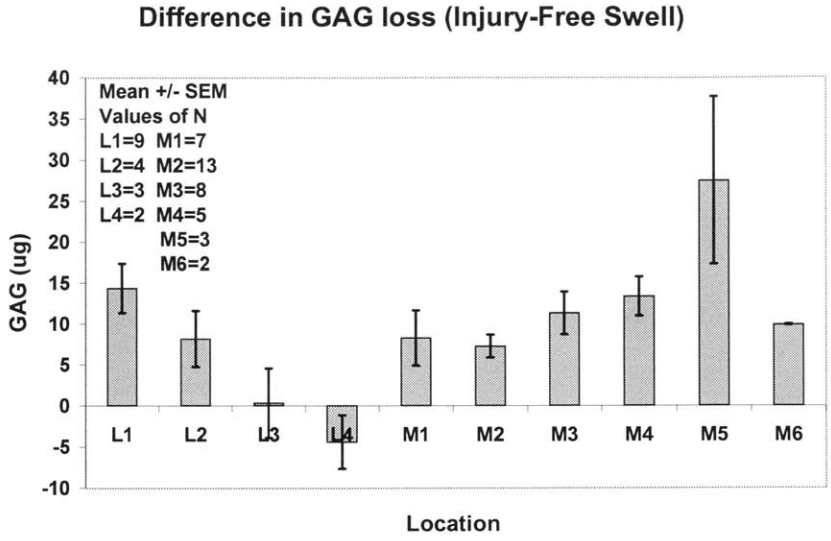

Figure 13: Difference in GAG loss of injured and free swelling cartilage after based on location on the joint. L1 indicates the lateral side furthest from the chondyle and closest to the femur whereas L4 indicates the lateral side and position closest to the chondyle. The same is true for the medial side. Because of differences in joint size, many times only three cores were taken from the lateral side and four cores were taken from the medial side.

From Figure 13 it appears that location may have an effect on the difference in GAG loss measured for free swell and injured cartilage although the trends appear to be reversed for the lateral and medial side. Unfortunately there were very small N for many of the locations so more experiments would have to be done to see if these same trends hold up for a larger number of samples.

6.4 Discussion and Conclusions

Based on the results presented in this chapter, it appears that GAG loss does not seem to vary much for free swelling and injured cartilage based on the side of the joint that the cartilage is harvested from, the location with respect to the chondyles and the depth of the

tissue from the surface of the joint. This is good news because it means that across an experiment, one does not need to make sure that all of the plugs come from a certain depth or a certain location to get consistent GAG loss results.

The results for cartilage deformation were surprising because in the absence of outside factors, increased deformation after injury did not result in an increase in GAG loss. This was good news because it indicated that having an abnormally high number of deformed plugs in an experiment should not change the results and should not be cause for concern. It was also interesting that the lateral side was more susceptible to deformation and that the occurrence of deformation increased as the depth increased. This probably has to do with the structure of the cartilage in the deeper zones. Also, the lateral side is probably more susceptible because it is not as thick as the cartilage on the medial side and so it doesn't take as many slices to get to the middle and deep zones of the cartilage.

Just because the results indicated that location, depth and deformation did not affect GAG loss does not mean that they do not have an affect on other outcome measures such as gene expression. Further experiments looking at this would have to be done to rule this possibility out. Also, future experiments would need to be done to ensure that the increased deformation of the lateral side and the deeper zones does not affect GAG loss when the cartilage is injured and cultured in the presence of outside factors such as cytokines. The increased deformation may make it easier for these factors to diffuse into the matrix causing increased GAG loss that isn't seen in tissue that does not have the same amount of deformation.

It would also be a good idea to do more follow up experiments that look at the difference in GAG loss between injury and free swell based on location as the number of samples in figure 13 was too small to draw any conclusions. This would just involve keeping track of location and GAG loss for free swelling and injured cartilage in future injury experiments that are done and could be done in conjunction with other studies.

Chapter 7: Conclusions and Recommendations

An important conclusion that can be drawn from the data presented in Chapters 2-5 is that the DMMB GAG assay is not a good outcome measure to use for joint capsule tissue co-culture and joint capsule tissue conditioned medium experiments. In the joint capsule tissue co-culture model, it is difficult to separate the contribution of the joint capsule tissue to the total GAG loss since there is currently no way to determine what GAG came from the cartilage and what GAG came from the joint capsule tissue. With both models, the trends seen can be inconsistent. In one experiment there may be a large increase in GAG loss while in another there may be no difference. Better outcome measures may be looking at gene expression or doing western analysis on the medium.

One problem with the conditioned medium model is nutrient depletion. While it appears that using cartilage conditioned medium as a control might account for this, it might be preferable to fractionate the conditioned medium using centrifuge filters. For example medium could first be filtered through a large centrifuge filter to remove any excess pieces of tissue in the medium as joint capsule tissue conditioned medium has a tendency to clog filters when it is syringe filtered. Next, the medium could be filtered using a very small filter. Once the medium has been filtered through a smaller filter, the molecules stuck on the filter could be reconstituted in fresh medium. This way, the unknown factor should still be present in the medium, but the effects of nutrient depletion should be negated. This also ought to provide a way to run a more controlled experiment.

Because joint capsule can be digested by using a higher concentration of proteinase K, 10X the concentration used for cartilage, future radiolabel experiments for the co-culture system should also label the joint capsule tissue to see what effect co-culture has on it. The combined results of joint capsule tissue radiolabel incorporation and chondrocyte radiolabel incorporation might yield some interesting and possibly unexpected results.

By adding new outcome measures to the co-culture and conditioned medium experiment as well as by using human joint capsule tissue instead of bovine tissue, it may be possible to learn additional information about the effects of the unidentified factor or factors which might aid in their identification.

Chapter 5 raised the concern about differences in the outcome of experiments performed using joint capsule tissue from the medial side versus the lateral side. As stated previously, most experiments made use of the lateral side. However, it is possible due to structural differences in the two tissues that the two sides might give different results given the same experimental setup. A control experiment should be done to determine if this should be a concern or not.

One way to help keep track of this would be to keep more detailed notes about the appearance of the joint capsule tissue and the location of harvest. It is also important to note that in many of the joints, there appeared to be some inflammation of the synovium which might also have an effect on the results of conditioned medium and co-culture

system experiments. This should also be incorporated into the protocol for all future experiments.

Follow up experiments should also be done to determine whether or not aggrecan or other large proteoglycans are present in the joint capsule tissue that is used for the conditioned medium and co-culture experiments and if it is present, what form these are in. The identification of specific large proteoglycan species in joint capsule tissue would be an interesting discovery.

Finally, experiments should be done to look at whether or not the increased deformation seen after injury in cartilage harvested further from the surface of the joint has an effect on GAG loss when cartilage is cultured in the presence of outside factors. If there is a difference in the results seen for deformed versus undeformed cartilage, it would be important to make sure that there was a consistent level of deformation across all of the injured samples.

References

1. Campbell MA, Winter AD, Ilic MZ, Handly CJ: Catabolism and loss of proteoglycans from cultures of bovine collateral ligament. Arch. Biochem Biophys. 328:1:64-72, 1996.

2. D'Lima DD, Hashimoto S, Chen PC, et al: Impact of mechanical trauma on matrix and cells. Clin Orthop 391S:S90-S99, 2001.

3. Davis MA, Ettinger WH, Neuhaus JM, et al: The association of knee injury and obesity with unilateral and bilateral osteoarthritis of the knee. Am J Epidemiol 130:278-288, 1989.

4. Dingle JT, Saklatvala J, Hembry R, Tyler J, Fell HB, Jubb R, 1979. A cartilage catabolic factor from synovium. Biochem J 184, 177-180.

5. Evanko SP, Vogel KG. Proteoglycan synthesis in fetal tendon is differentially regulated by cyclic compression in vitro. Arch. Biochem. Biophys. 307(1) 153-164.

6. Farndale RS, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta 1986; 883: 173-177.

7. Fay J., Patwari P., Grozinsky A.,, "Cartilage degradation after injurious mechanical compression in the presence of joint capsule tissue or exogenous interleukin-1," Trans 48. Orthop Res Soc, Dallas, TX, Feb 10-13, 27:64, 2002.

8. Frank EH, Jin M, Loening AM, Levenston ME, Grodzinsky AJ, 2000. A versatile shear and compression apparatus for mechanical stimulation of tissue culture explants. J Biomech 33, 1523-1527.

9. Gelber AC, Hochberg MC, Mead LA, et al: Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. Ann Intern Med 133:321-328, 2000.

10. "Handout on Health: Osteoarthritis" National Institute of Arthritis and Musculoskeletal and Skin Diseases. May 2002 <http://www.niams.nih.gov/hi/topics/arthritis/oahandout.htm>

11. Jeffrey JE, Thomson LA, Aspden RM: Matrix loss and synthesis following a single impact load on articular cartilage in vitro. Biochim Biophys Acta 1334: 223-232, 1997.

12. Kleftogiannis F, Handley CJ, Campbell MA: Charicterization of extracellular matrix macromolecules from bovine synovial capsule. J Orthop Res. 12(3):365-374, 1994.

13. Koopman WJ, Arthritis and allied conditions 14th ed. Vol. 1. Lippincott Williams & Wilkins, Philadelphia, 2001.

14. Kurz B, Jin M, Patwari P, et al: Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orhop Res. In press.

15. Lewis AR, Ralphs JR, Kneafsy B, Benjamin M.: Distribution of collagens and glycosaminoglycans in the joint capsule of the proximal interphalangeal joint of the human finger. Anat. Rec. 250:281-291, 1998.

16. Loening AM, James IE, Levenston ME, et al: Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 381:205-212, 2000.

17. "Ligament and Tendon Structure and Function." Univ. of Michigan. August 8, 2004 <http://www.engin.umich.edu/class/bme456/ligten/ligten.htm>

18. Meyer O., Role of TNF-α and cytokines in the physiopathology of rheumatoid arthritis. Therapeutic perspectives. Bull Acad Natl Med. 2003; 187(5);935-54;discussion954-5.

19. "Osteoarthritis" Arthritis Foundation. May 2002 <http://www.arthritis.org/conditions/DiseaseCenter/oa.asp>.

20. Osteoarthritis: New Insights, Part 1: The disease and its Risk Factors. Ann. Intern Med. 133: 635-646, 2000.

21. Patwari P., Cook M.N., DiMicco M.A., et al., Proteoglycan degradation after injurious compression of bovine and human articular cartilage in vitro: Interaction with exogenous cytokines, Arthritis and Rheum, 2003 May; 48(5):1292-301.

22. Patwari P, Fay J, Cook MN, et al: In vitro models for investigation of the effects of acute mechanical injury on cartilage. Clinical Orthopaedics and Related Research 391S: S61-S71, 2001.

23. Patwari P, Norris SA, Kumar S, Lark MW, Grodzinsky AJ, "Inhibition of bovine cartilage biosynthesis by coincubation of joint capsule tissue is mediated by an interleukin-1 independent singalling pathway," Trans 49th Orthop Res Soc, New Orleans, LA, Feb 1-5, 28:158, 2003.

24. Ralphs JR, Benjamin M: The joint capsule: structure, composition, ageing and disease. J. Anat., 1994 June; 184(3): 503-509.

25. Repo RU, Finlay JB: Survival of articular cartilage after controlled impact. J Bone Joint Surg 59A: 1068-1076, 1977.

26. Rowan A.D., Koshy P.J., shingleton W.D., et al., 2001. Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen Breakdown. Arthritis Rheum 44, 1620-1632

27. Sah RL, Kim YJ, Doong JY et al: Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res 7(5): 619-36, 1989.

28. Tischer T, Milz S, Shieker M, Benjamin M. An immunohistochemical study of the rabbit suprapatella, a sesamoid fibrocartilage in the quadriceps tendon containing aggrecan. J Histochem Cytochem. 2002 July; 50(7), 955-960.

29. Torzilli PA, Grigiene R, Borrelli J, et al: Effect of impact load on articular cartilage: Cell metabolism and viability, and matrix water content. J Biomech Eng 121:433-441, 1999.

30. Vogel KG. What happens when tendons bend and twist? Proteoglycans. J Musculoskel Neuron Interact 2004; 4(2):202-203.

31. Vogel KG, Koob TJ: Structural specialization in tendons under compression. Int Rev Cytol 115:267-293, 1989.

32. Vogel KG, Sandy JD, Pogany G, Robbins JR: Aggrecan in bovine tendon. Matrix Biol. 1994:14: 171-179.

Appendix A: Miscellaneous other experiments

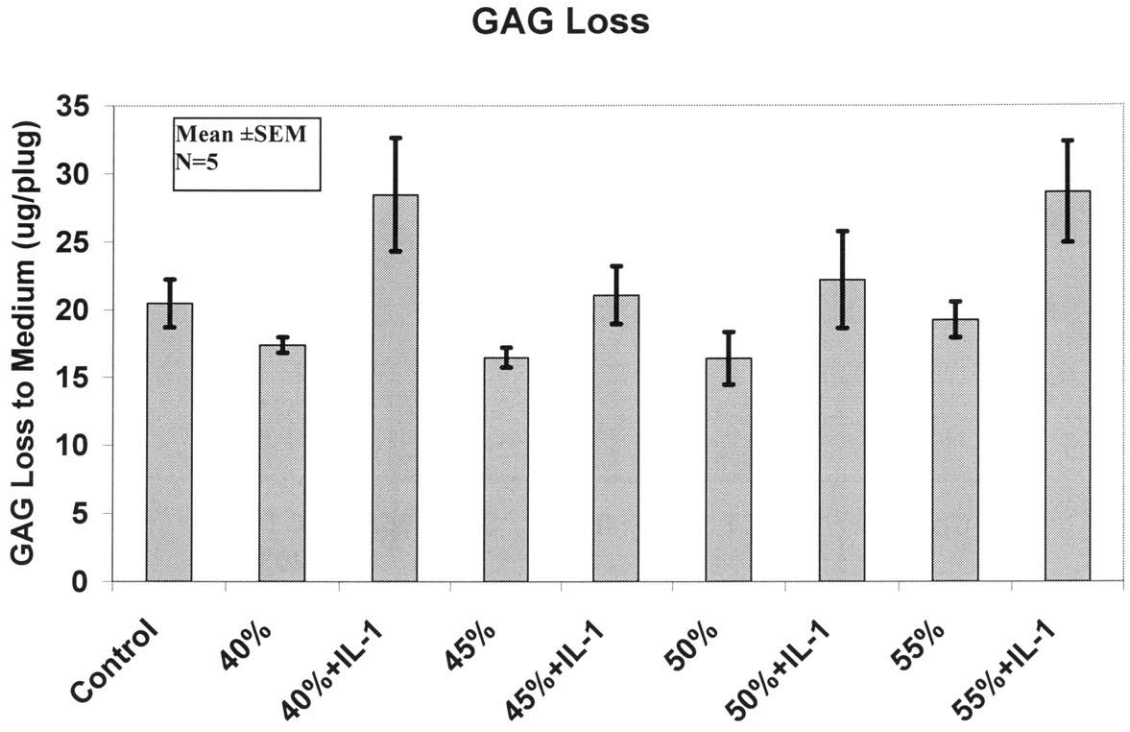

Figure 1: GAG loss (μg/plug) for cartilage injured at 40%, 45%, 50% and 55% and cultured in .5ml of ITS medium or ITS medium treated with 1ng/ml of IL-1. Cartilage plugs were allowed to equilibrate for two days prior to intervention and were allowed to culture for three days before GAG loss was measured.

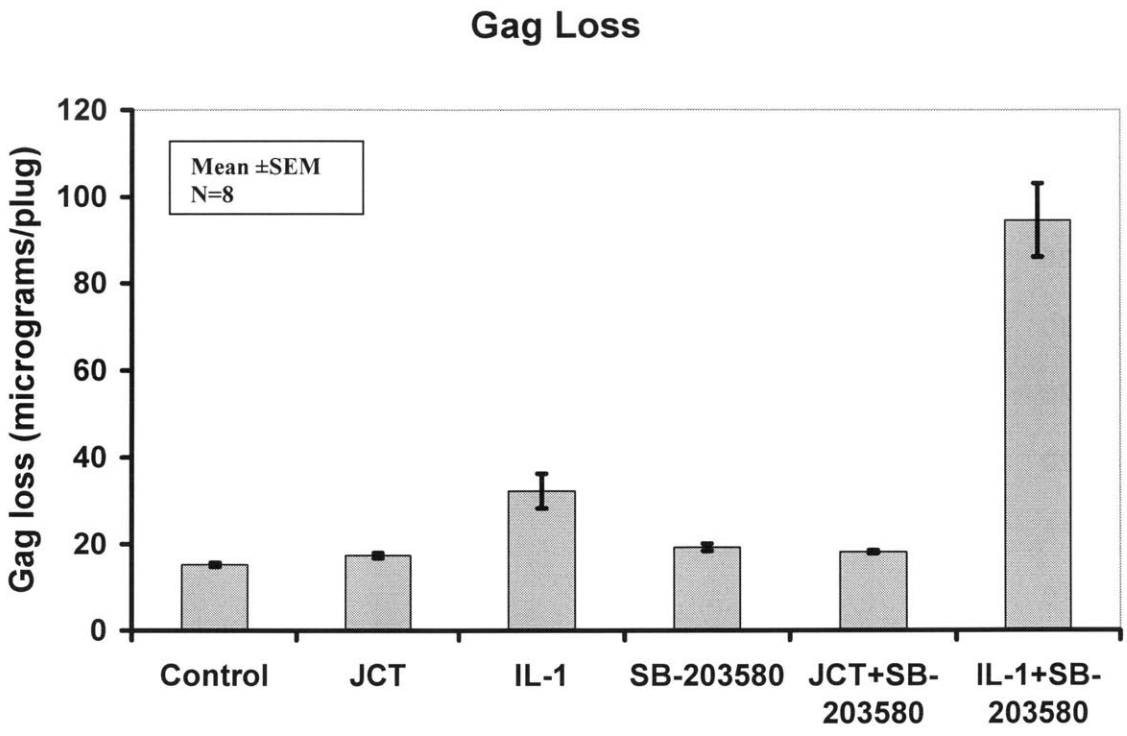

Figure 2: GAG loss (μg/plug) for cartilage cultured in 10% FBS medium cultured under one of the following six conditions. 1) 10% FBS medium 2) Joint capsule tissue conditioned medium 3) Medium treated with 1ng/ml of IL-1 4) Medium treated with 10μM p38 inhibitor 5) Joint capsule tissue conditioned medium + p38 inhibitor and 6) IL-1 + p38 inhibitor. Cartilage was allowed to equilibrate for two days prior to intervention and was cultured under these conditions for three days prior to measurement of GAG loss.

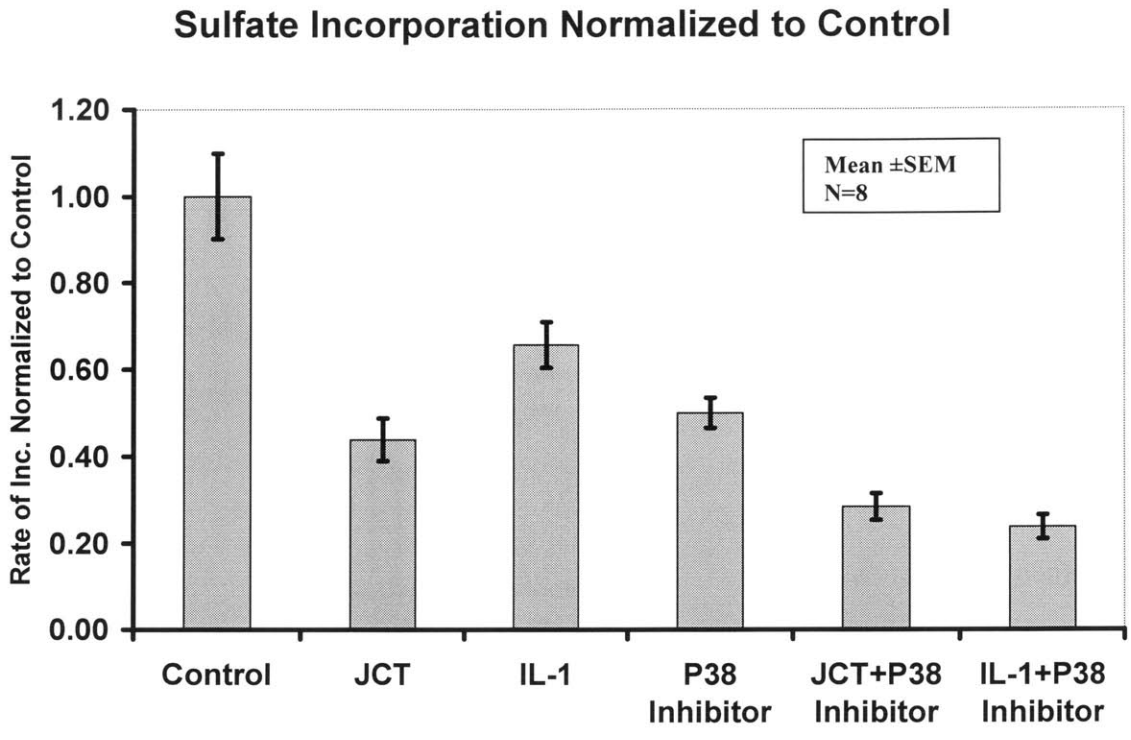

Figure 3: Sulfate incorporation for cartilage cultured in 10% FBS medium cultured under one of the following six conditions. 1) 10% FBS medium 2) Joint capsule tissue conditioned medium 3) Medium treated with 1ng/ml of IL-1 4) Medium treated with 10μM p38 inhibitor 5) Joint capsule tissue conditioned medium + p38 inhibitor and 6) IL-1 + p38 inhibitor. Cartilage was allowed to equilibrate for two days prior to intervention and was cultured under these conditions for three days prior to being radiolabeled.

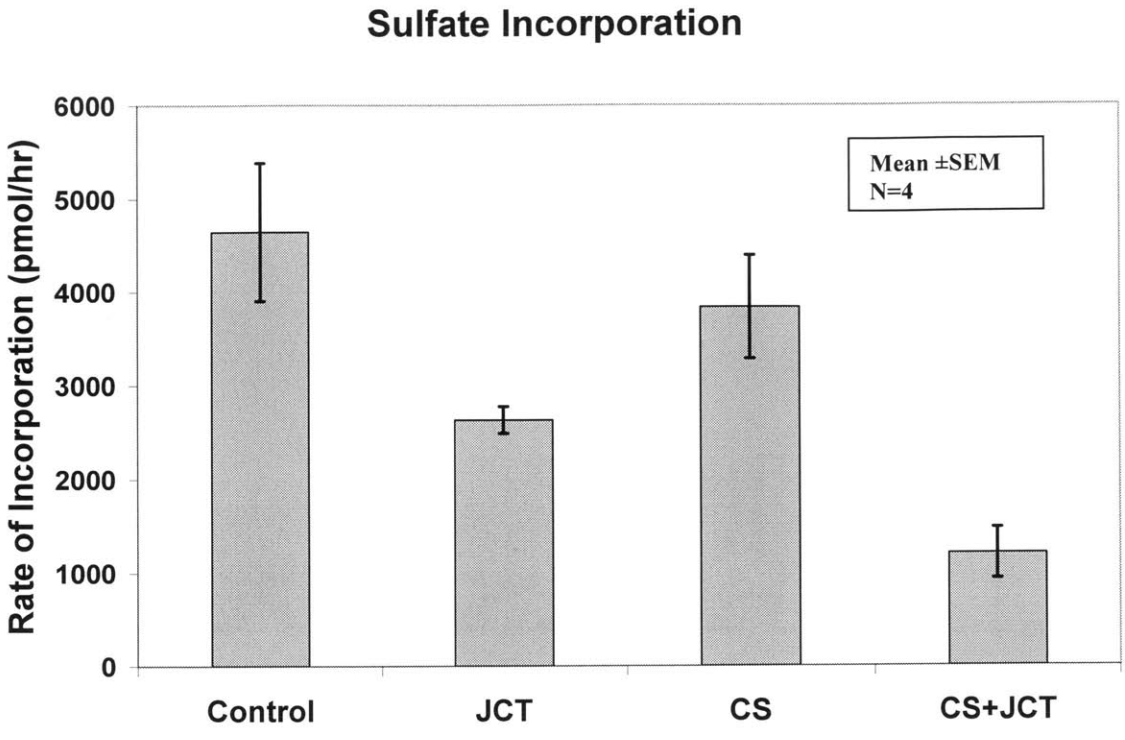

Figure 4: Sulfate incorporation for cartilage cultured under one of the following four conditions 1) Control, 10% FBS medium, 2) Joint capsule tissue co-culture 3) Medium treated with 200μg/ml of chondroitin sulfate and 4) Joint capsule tissue co-culture plus 200μg/ml of chondroitin sulfate. Cartilage plugs were allowed to equilibrate three days before intervention and then were cultured for three days .5ml of medium before being radiolabeled.

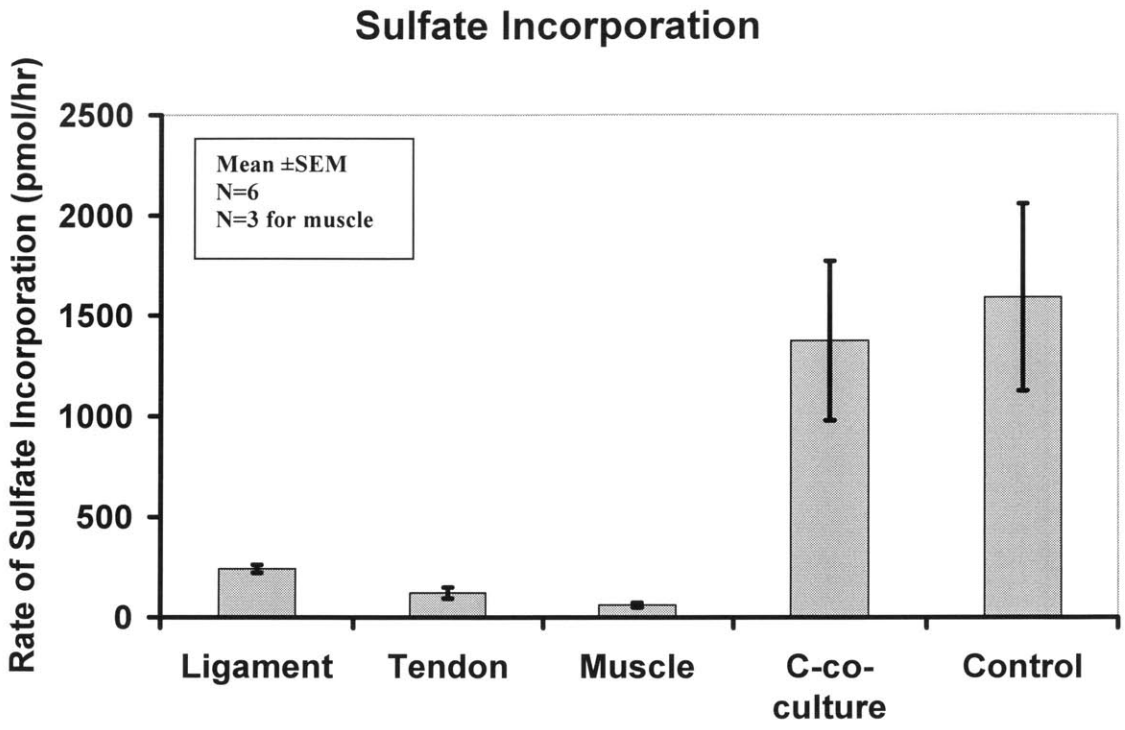

Figure 5: Sulfate incorporation for different joint tissues co-culture experiment. Cartilage was allowed to equilibrate for three days and then cultured for three days in 10% FBS medium under one of the following conditions before being radiolabeled: 1) Ligament co-culture 2) Tendon Co-culture 3) Muscle Co-culture 4) Co-culture and 5) 10% FBS medium. None of the pieces of tissue were the same size due to difficulty cutting after harvest.

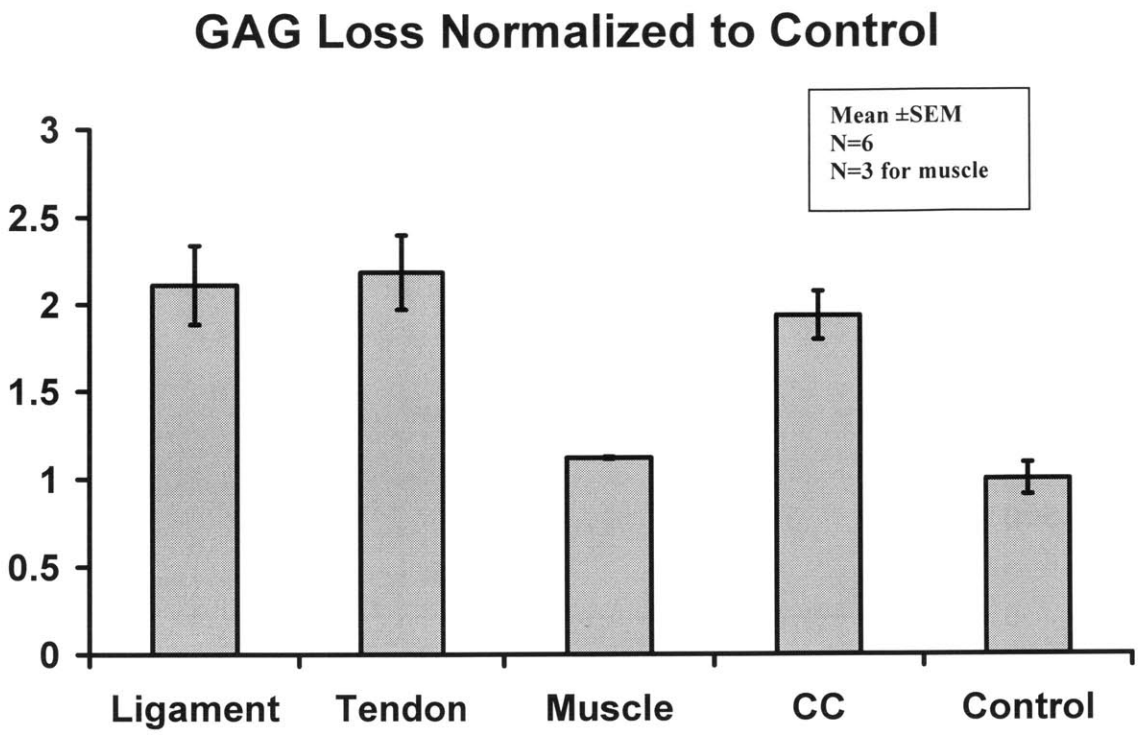

Figure 6: Normalized GAG loss for different joint tissues co-culture experiment. Cartilage was allowed to equilibrate for three days and then cultured for three days in 10% FBS medium under one of the following conditions before GAG loss was measured: 1) Ligament co-culture 2) Tendon Co-culture 3) Muscle Co-culture 4) Co-culture and 5) 10% FBS medium. None of the pieces of tissue were the same size due to difficulty cutting after harvest.

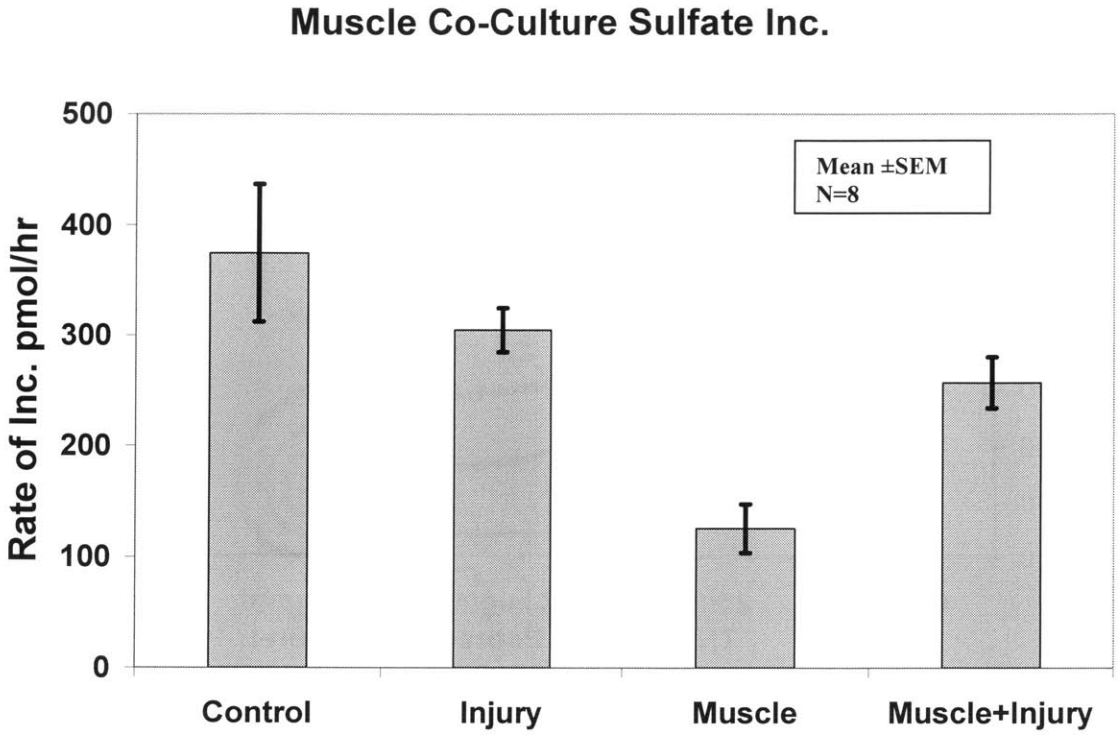

Figure 7: Sulfate incorporation for muscle co-culture experiment. Muscle was sliced into 2mm thick sheets and punched using a 3mm dermal punch. Both cartilage and muscle explants were allowed to equilibrate for three days prior to being co-cultured together in 1ml of 10% FBS medium for three days before being radiolabeled.

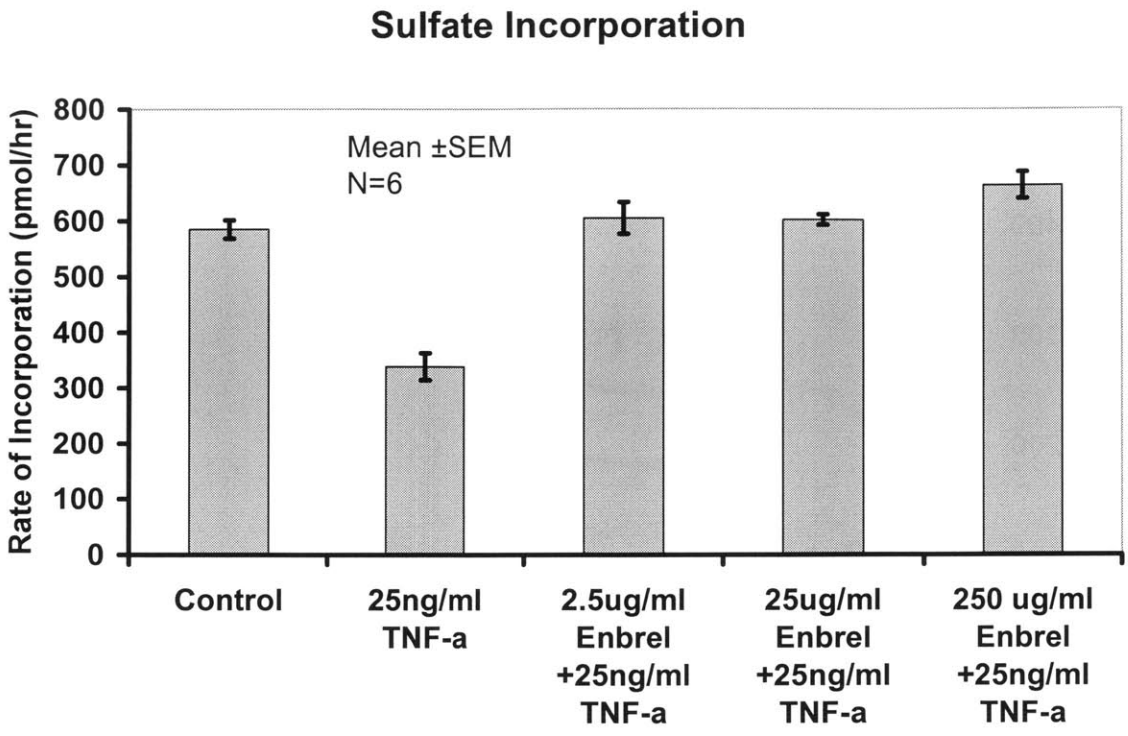

Figure 8: Sulfate incorporation for Etanercept dose response with 25ng/ml of TNF-α. Cartilage was allowed to equilibrate for three days prior to intervention. Cartilage was then cultured in .25ml of medium for an additional three days under one of the conditions listed above before being radiolabeled.

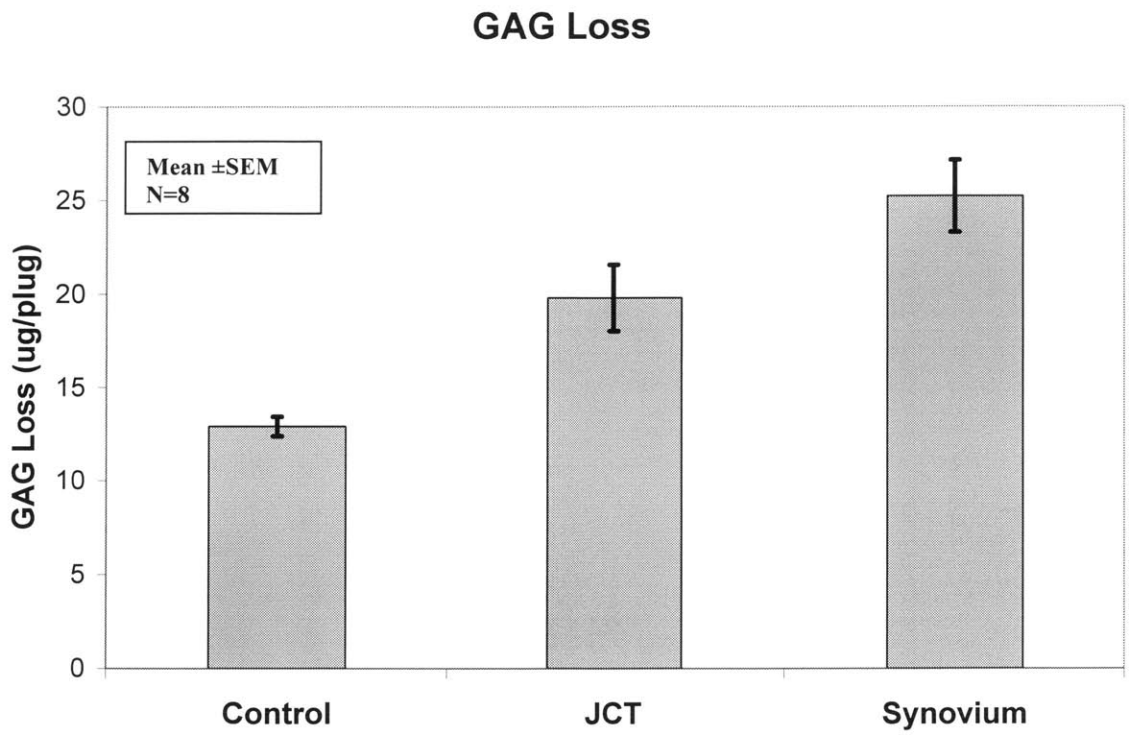

Figure 9: GAG loss (μg/plug) for cartilage co-cultured under one of the following three conditions for three days: 1) control, 10% FBS medium; 2) Joint capsule tissue co-culture; 3) Synovium co-culture. All cartilage plugs were allowed to equilibrate for three days prior to intervention.

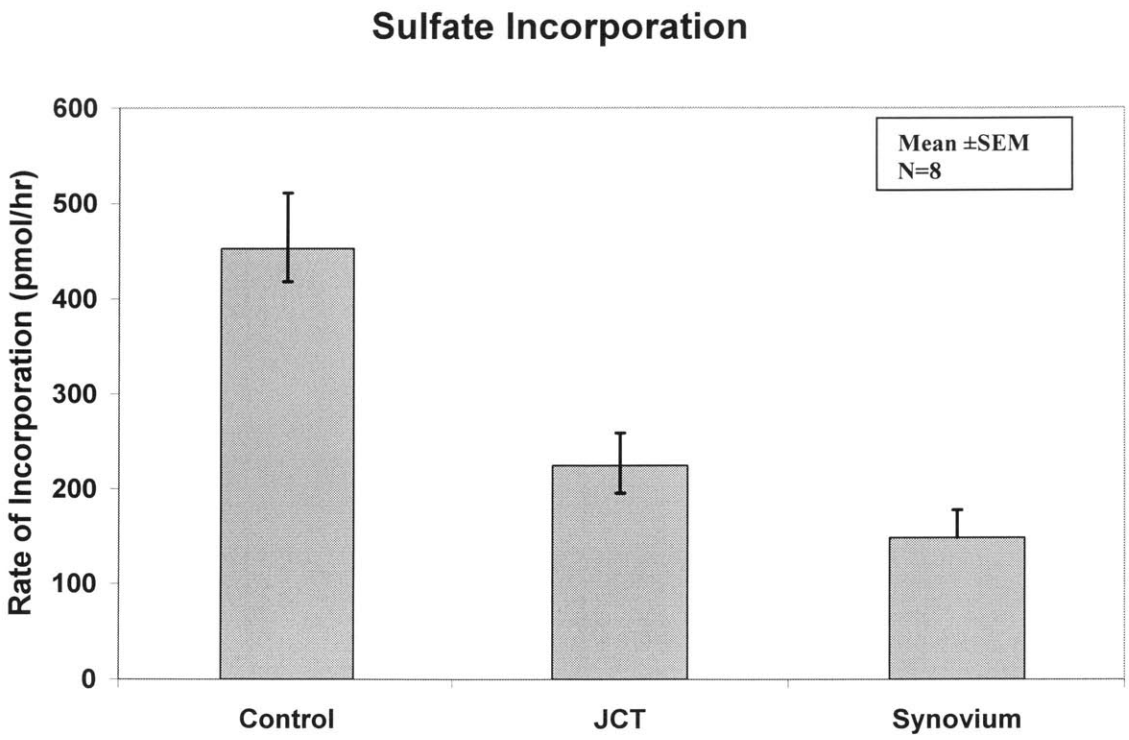

Figure 10: Sulfate Incorporation for cartilage co-cultured under one of the following three conditions for three days: 1) control, 10% FBS medium; 2) Joint capsule tissue co-culture; 3) Synovium co-culture. All cartilage plugs were allowed to equilibrate for three days prior to intervention.

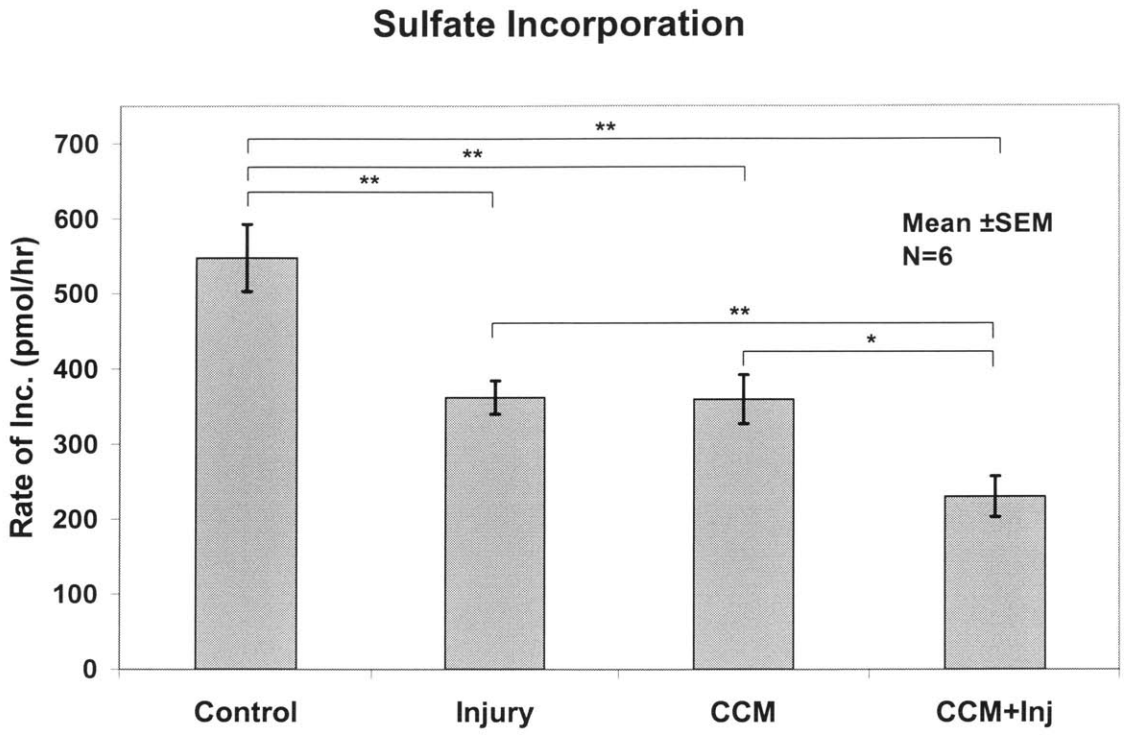

Figure 11: Sulfate Incorporation for injured and uninjured cartilage cultured in 10% FBS medium and 10% FBS medium conditioned by one 3mm piece of cartilage per milliliter of medium for three days. Cartilage was allowed to equilibrate for three days prior to intervention. Injury was to 50% strain at a strain rate of 1mm/sec. Cartilage plugs were cultured for three days after intervention before being radiolabeled. '*' represents p value of <.02, '**' represents p value of <.004 by a two sided student t test.

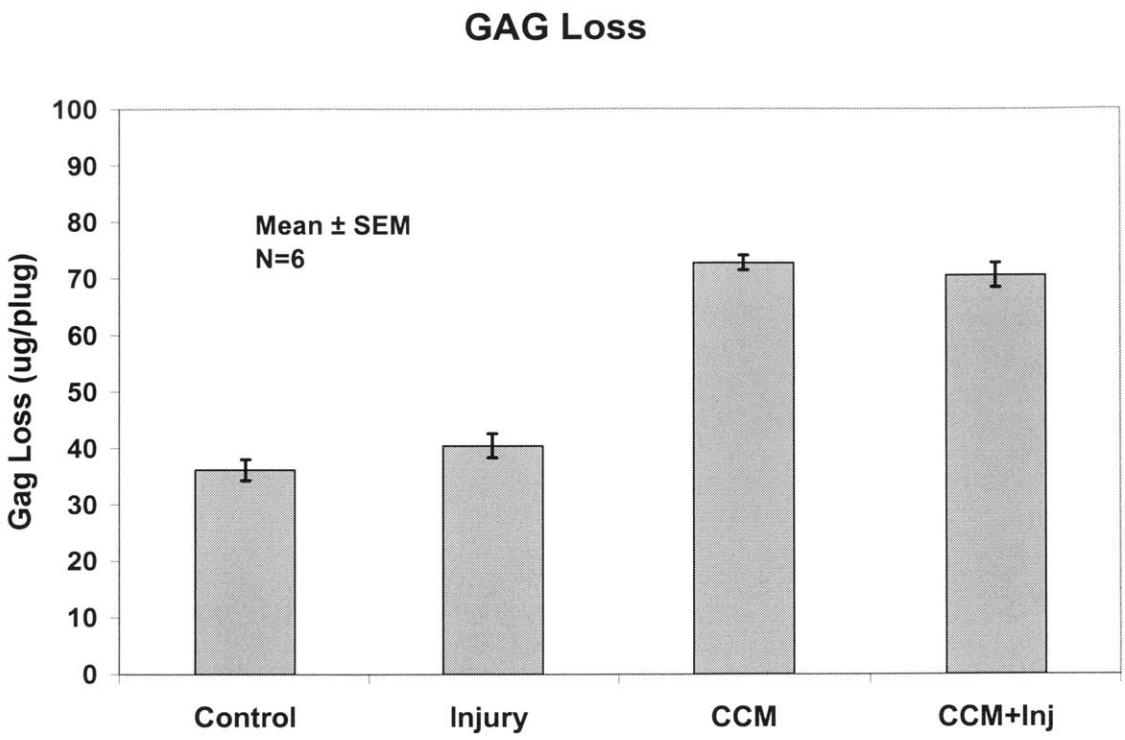

Figure 12: GAG Loss for injured and uninjured cartilage cultured in 10% FBS medium and 10% FBS medium conditioned by one 3mm piece of cartilage per milliliter of medium for three days. Cartilage was allowed to equilibrate for three days prior to intervention. Injury was to 50% strain at a strain rate of 1mm/sec. Cartilage plugs were cultured for three days before GAG loss was measured. These results do not account for GAG loss to the medium during the conditioning process.

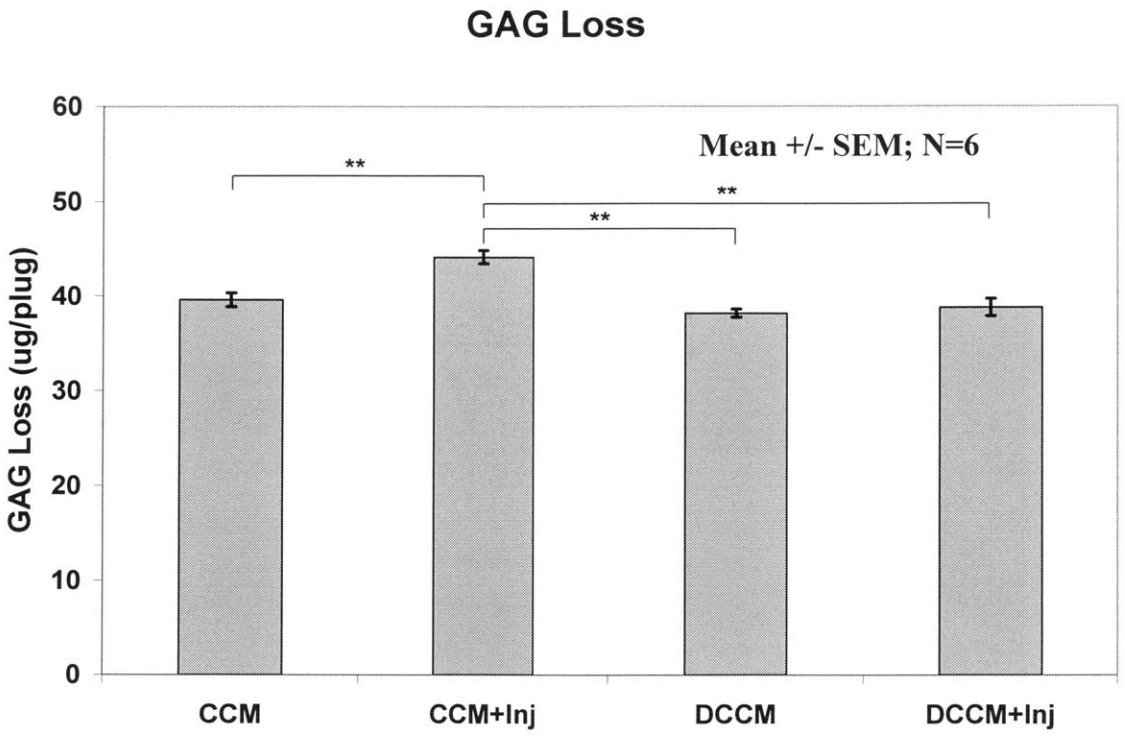

Figure 13: GAG loss for cartilage cultured for four days in 10% FBS medium that had previously been conditioned for three days by one 3mm punched piece of cartilage that was either alive or dead. Cartilage was killed by freezing in the minus 80° C freezer. Cartilage was injured to 50% strain at a strain rate of 1mm/sec. '**' represents p value of <.002 by a two sided student t test.

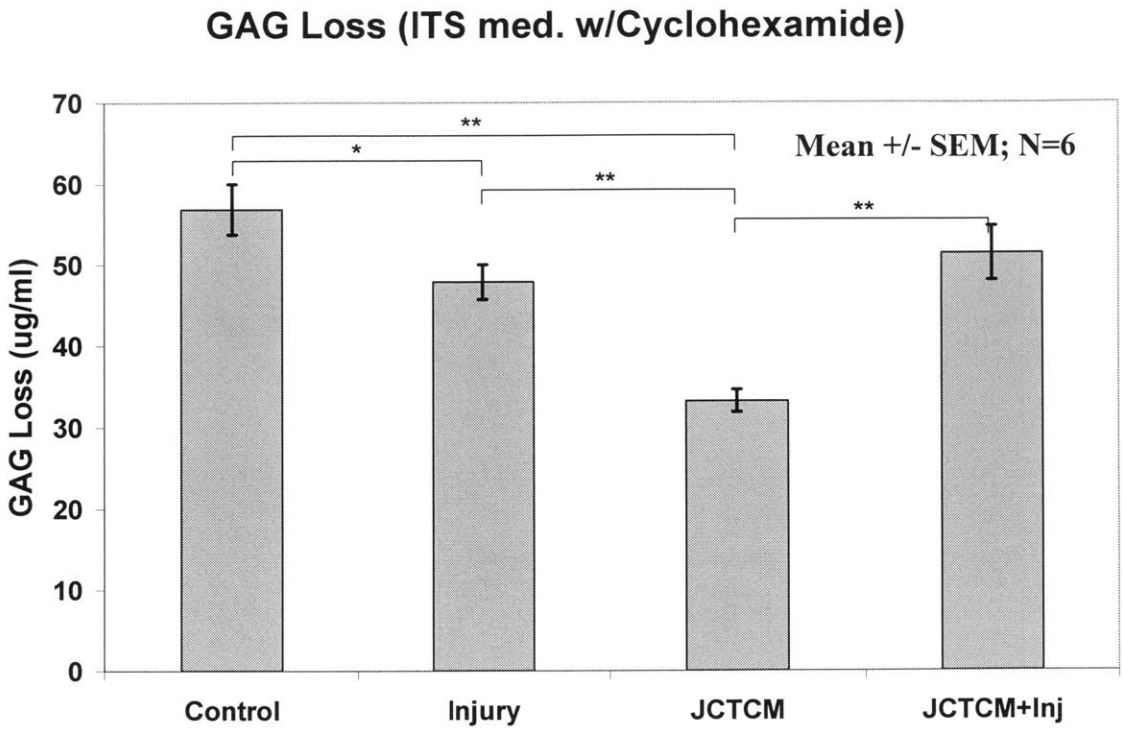

Figure 14: GAG loss for cartilage cultured in conditioned and unconditioned ITS medium supplemented with 200μg/ml of cyclohexamide over four days. Cartilage was cultured in medium containing cyclohexamide for 6 hours prior to injury. Injury was to 50% strain at a strain rate of 1mm/sec. Conditioned medium was made by culturing two 3mm punched pieces of joint capsule tissue/milliliter of ITS medium for four days. '*' represents p value of <.05, '**' represents p value of <.002

Appendix B: Addition Joint Capsule Tissue Data

Appendix B contains additional data from characterization of the joint capsule tissue that was not included in Chapter 5.

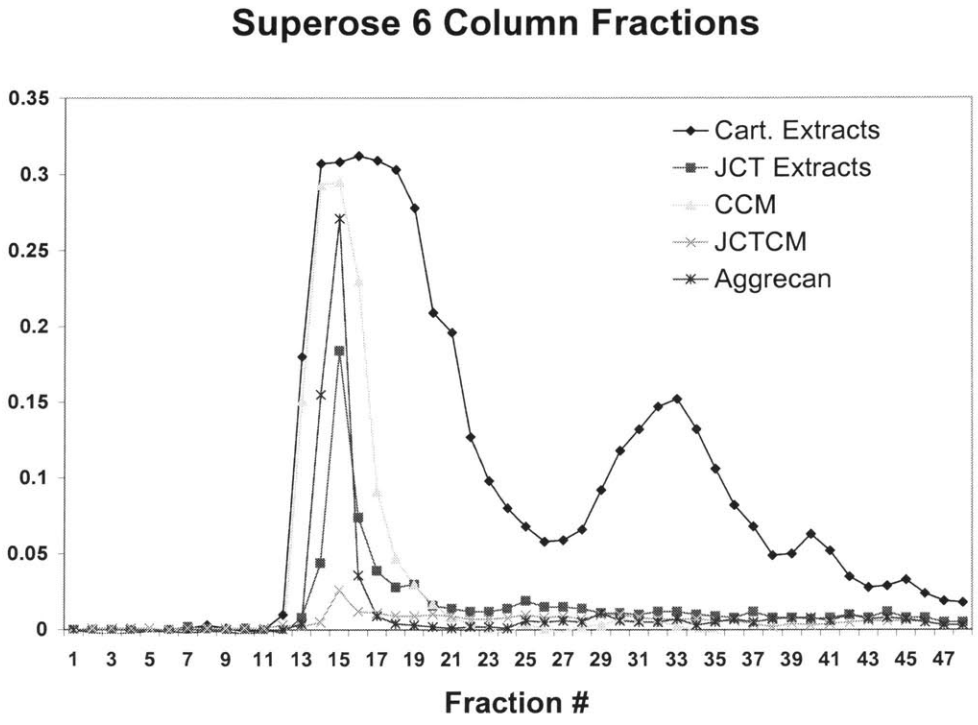

Figure 1: DMMB Assay of Superose 6 Column fractions for cartilage tissue extract, joint capsule tissue extract, cartilage conditioned medium, joint capsule tissue conditioned medium and pure aggrecan.

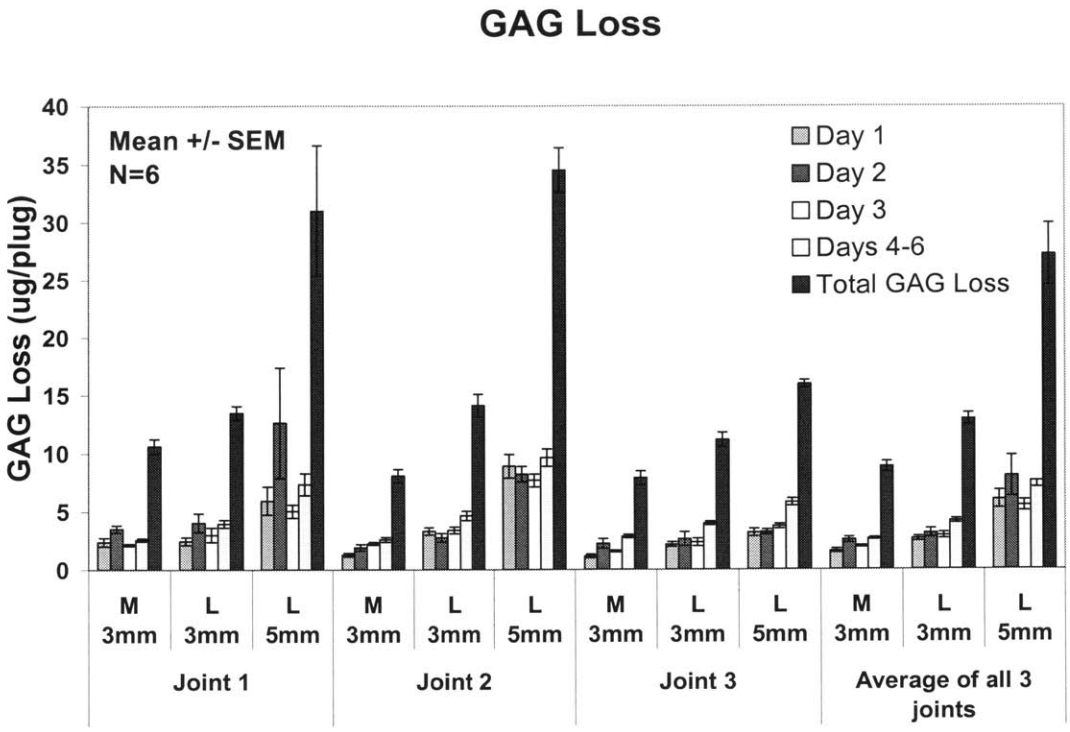

Figure 2: GAG loss for days 1,2,3,4-6 as a function of the joint that it was harvested from, the location within the joint and the size of the punch. Joint capsule tissue pieces were cultured in .5ml of 10% FBS medium. The medium was changed at every time point.

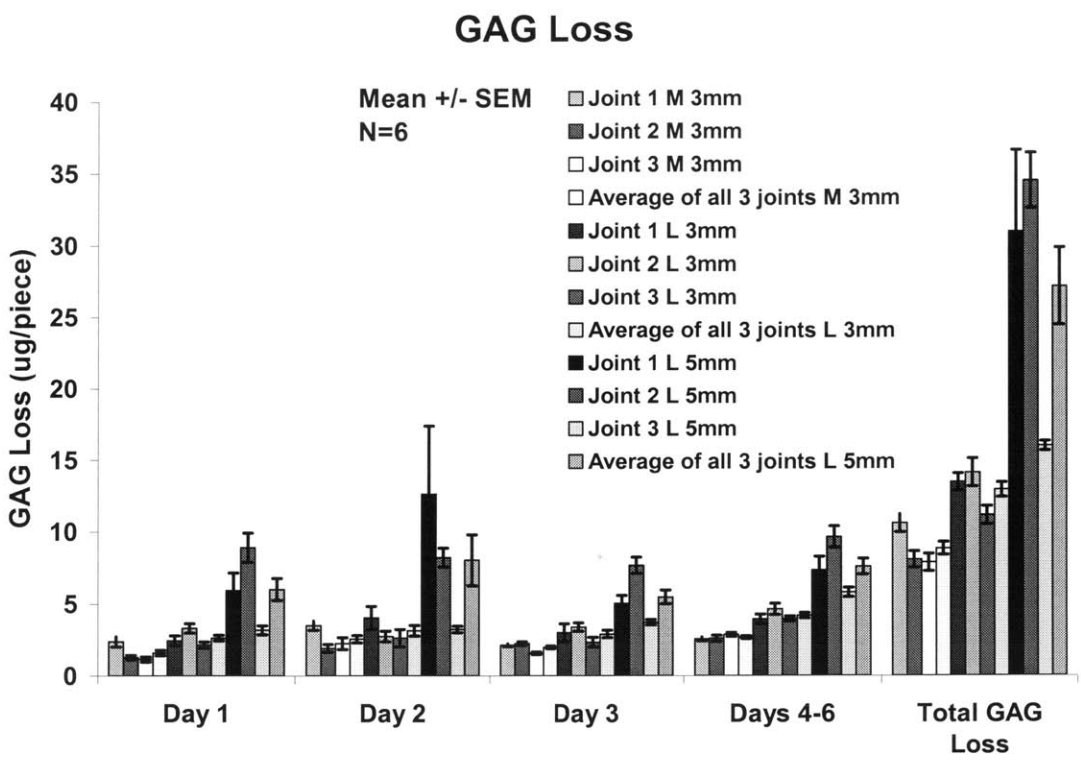

Figure 3: GAG loss for each joint, location and size as a function of the day after harvest. Joint capsule tissue was incubated in .5ml of 10% FBS medium. Medium was changed after every time point.

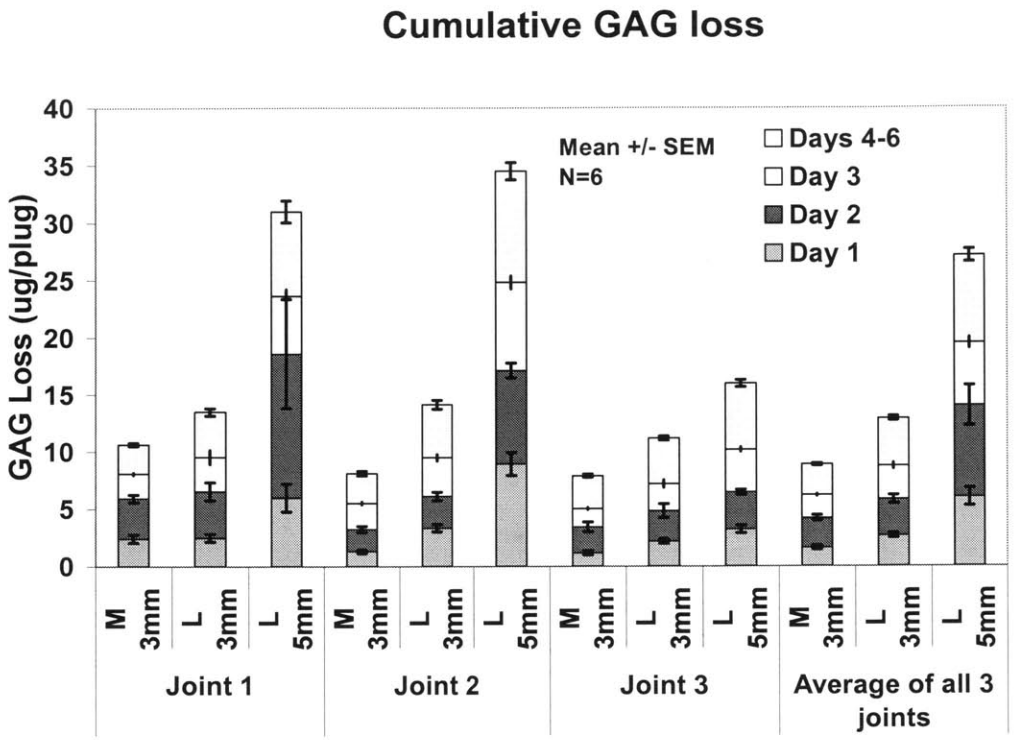

Figure 4: Cumulative GAG loss for each joint, location and size. Each bar is broken down into the amount of GAG loss that was contributed at each time point. Joint capsule tissue was cultured in .5ml of 10% FBS medium. Medium was changed at each time point.

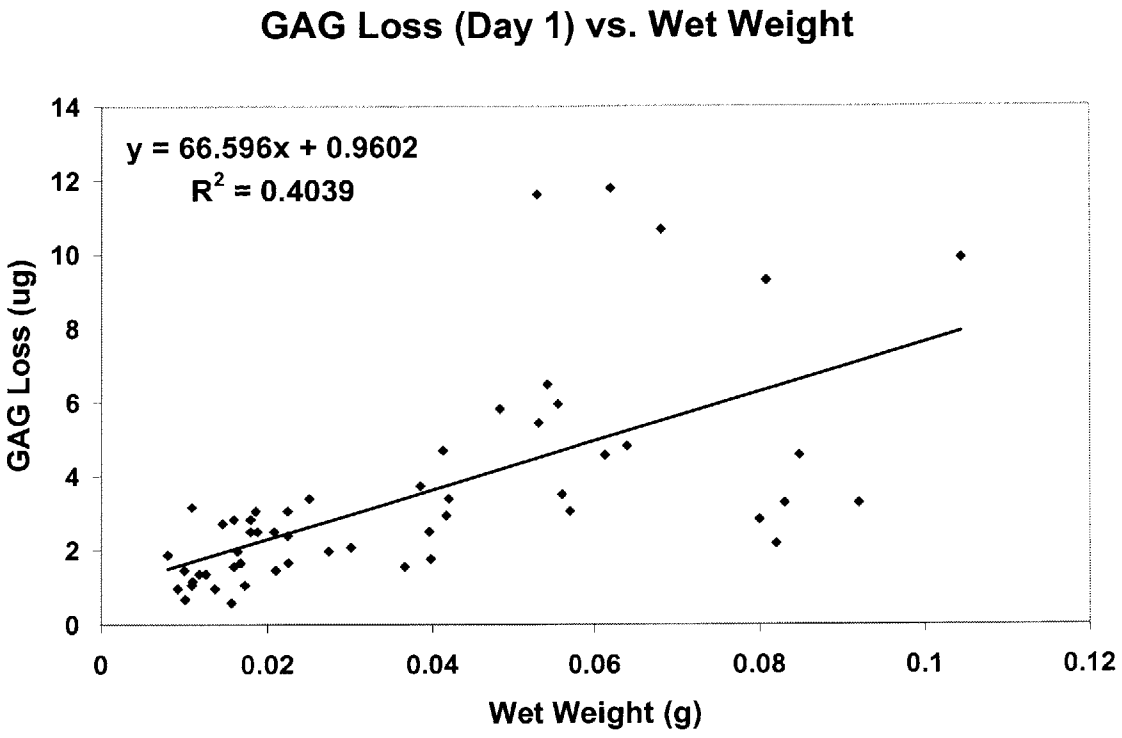

Figure 5: GAG loss for day 1 as a function of joint capsule tissue wet weight. Wet weight was measured on the day of harvest. Joint capsule tissue was then cultured for 24 hours in .5ml of 10% FBS medium after which GAG loss to the medium was measured. Data is fit with a linear regression.

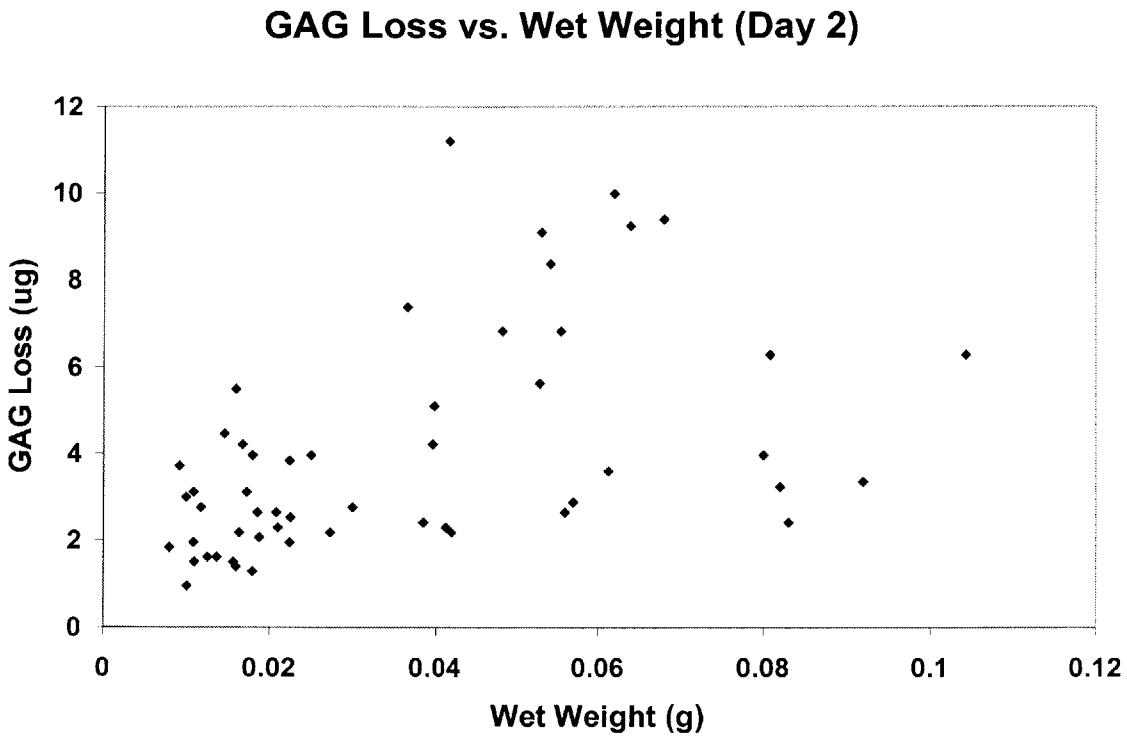

Figure 6: GAG loss on day 2 after harvest as a function of joint capsule wet weight. Joint capsule tissue was harvested from the medial and lateral side of three joints and punched with either a 3mm or 5mm dermal punch. Wet weight was measured immediately after being punched. Joint capsule tissue was then cultured in .5ml of 10% FBS medium for 24 hours at which time the medium was changed and joint capsule tissue was cultured for an additional 24 hours after which GAG loss was measured.

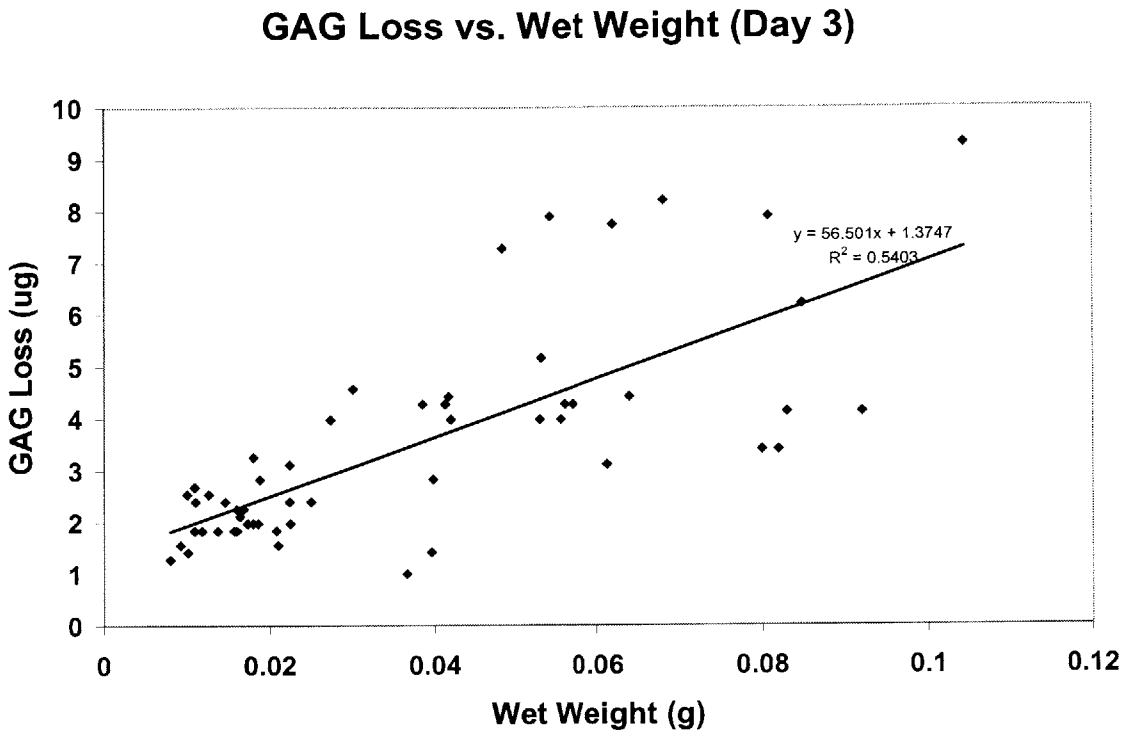

Figure 7: Joint capsule tissue GAG loss on day 3 after harvest as a function of joint capsule tissue wet weight. Joint capsule tissue was harvested from the medial and lateral side of three joints and punched with either a 3mm or 5mm dermal punch. Wet weight was measured immediately after being punched. Joint capsule tissue was then cultured in .5ml of 10% FBS medium. Medium was changed every 24 hours for the first three days and GAG loss was measured at each medium change. Data is fit with a best fit line obtained by a linear regression.

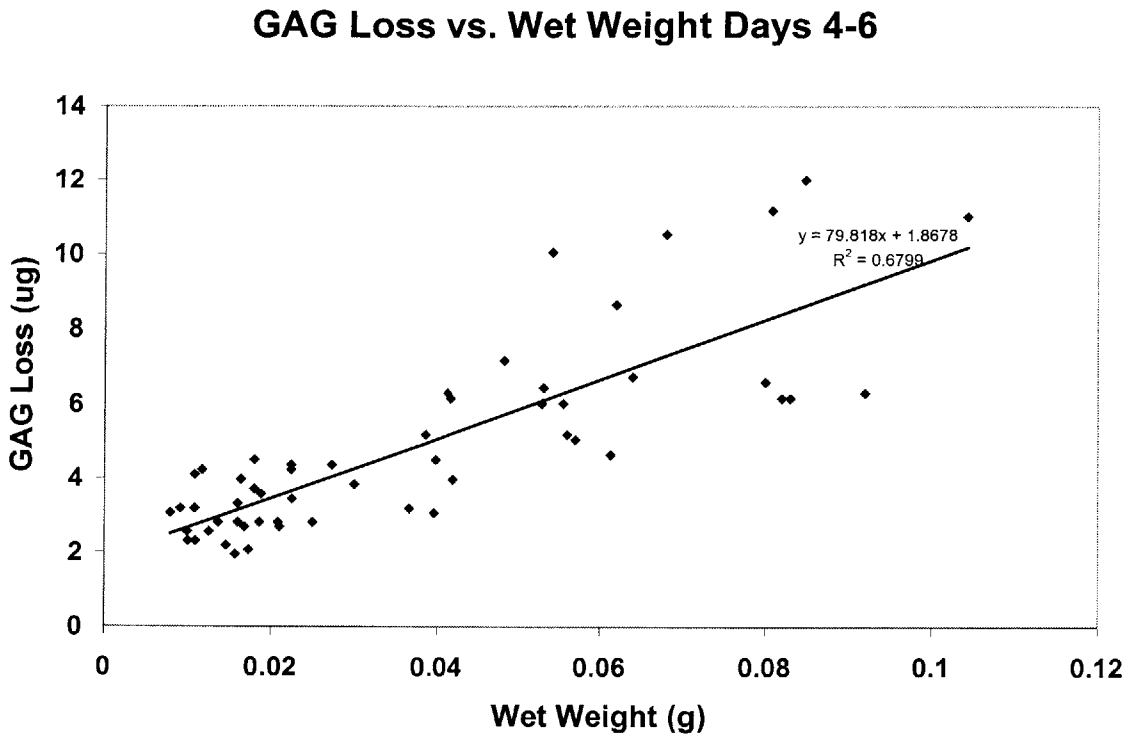

Figure 8: Joint capsule tissue GAG loss for days 4-6 after harvest. Joint capsule tissue was harvested from the medial and lateral side of three joints and punched with either a 3mm or 5mm dermal punch. Wet weight was measured immediately after being punched. Joint capsule tissue was then cultured in .5ml of 10% FBS medium. Medium was changed every 24 hours for the first three days. For days 4-6 the medium was left to culture in the same medium for the three days. At the end of day 6 GAG loss for days 4-6 was measured. Data is fit with a best fit line obtained from a linear regression.

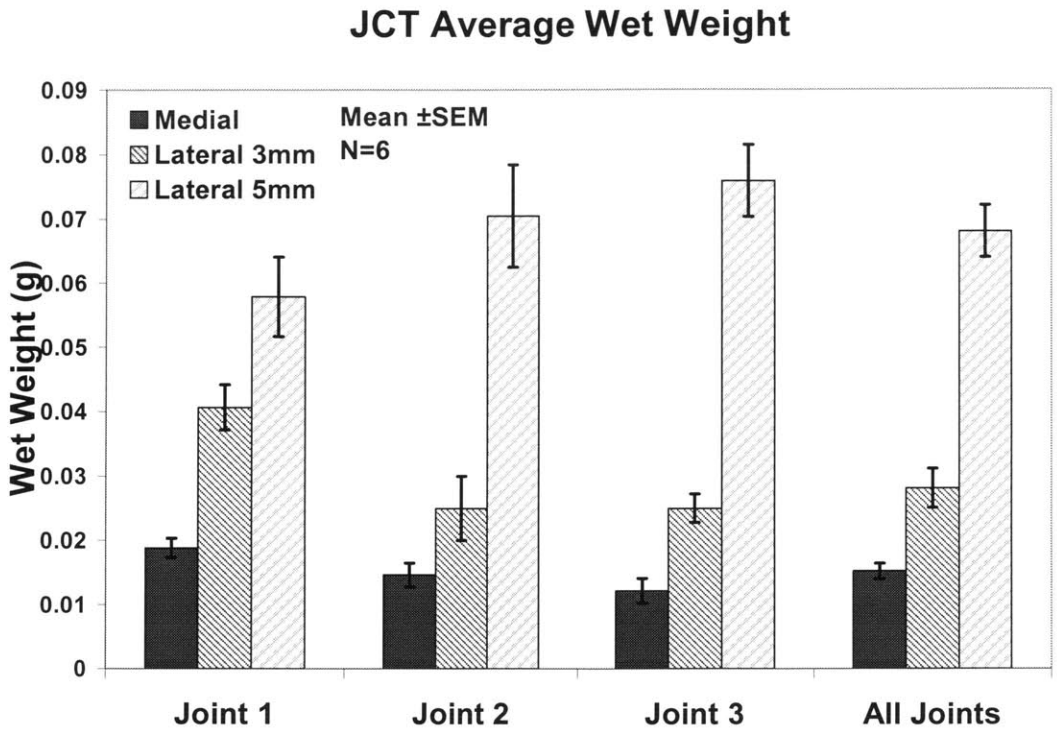

Figure 9: Joint capsule tissue wet weight by joint of harvest, side of harvest and size of punch. The plot shows that the average wet weight for a given side and size of punch can vary from joint to joint. Joint capsule tissue was harvested from the medial and lateral sides of three different joints and punched with either a 3mm or 5mm dermal punch. Wet weight was measured immediately after being punched.

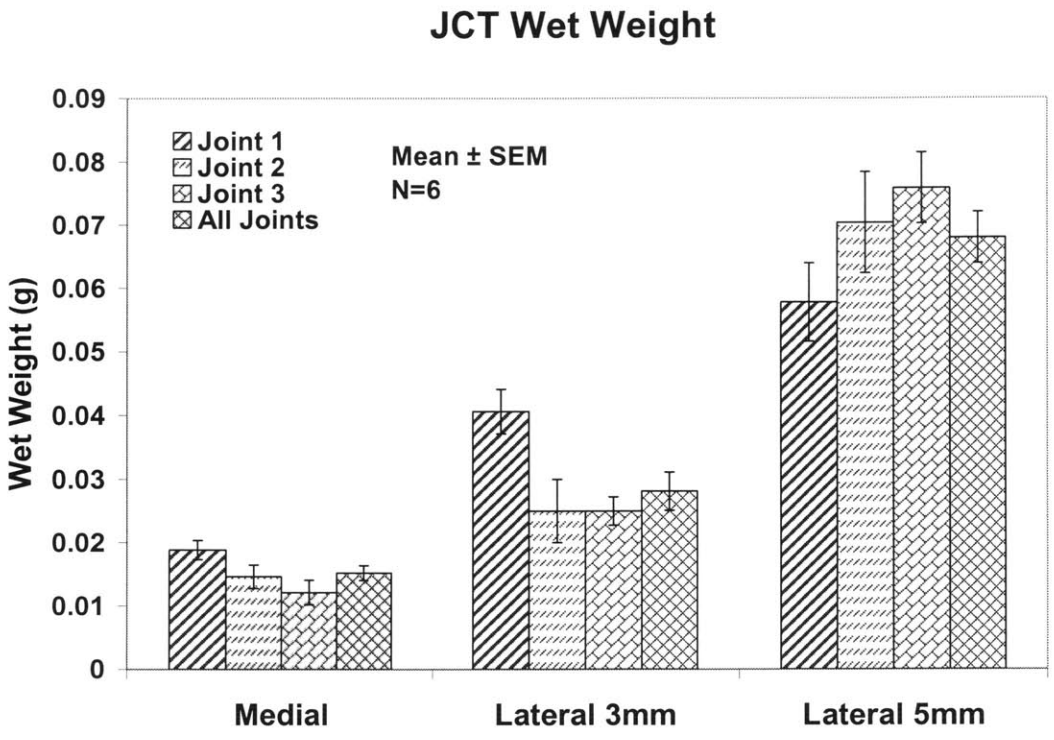

Figure 10: Joint capsule tissue wet weight as a function of location, size and joint. Data is organized to show the variation that exists for the same punch size and harvest location amongst different joints. Joint capsule tissue was harvested from the medial and lateral sides of the joint and was punched with either a 3mm or 5mm dermal punch.

Appendix C: Harvesting Methods

1) Harvesting Cartilage/Bone Cores

The first step in harvesting the cartilage/bone cores is to collect all of the necessary tools and autoclave those that need to be autoclaved. Table 1 shows the tools that need to be collected and which ones need to be autoclaved before hand for this step in the harvesting process.

Table 1: Tools needed for cartilage/bone cores harvest

To be autoclaved	Other tools
Forceps	Holder for joint
Teeth forceps	Scalpel blades
Scalpel holder	24 well plate
Squirt bottle for PBS	Paper to cover counter
	Knife with black handle
	Hammer

The next step is to put on the proper attire. This includes a lab coat, gloves, safety glasses and a mask. This is to keep blood and flying pieces of tissue off of clothes and skin as well as to protect the cartilage from becoming infected. It is also important to change gloves often to prevent contamination as well as keep things clean.

Once the tools are autoclaved and PBS is prepared, it is time to begin harvesting. Cartilage is harvested from the femoropatellar groove of 1-2 week old calves. The joints consist of the entire leg with the exception of the hoof which has been removed. The first step in preparing the joint for tissue harvest is to clean the tissue away from the bone near

the femoral head as shown in Figures 1c and 1d. The next step is to remove the femoral head by using a hacksaw as shown in Figures 1e and 1f.

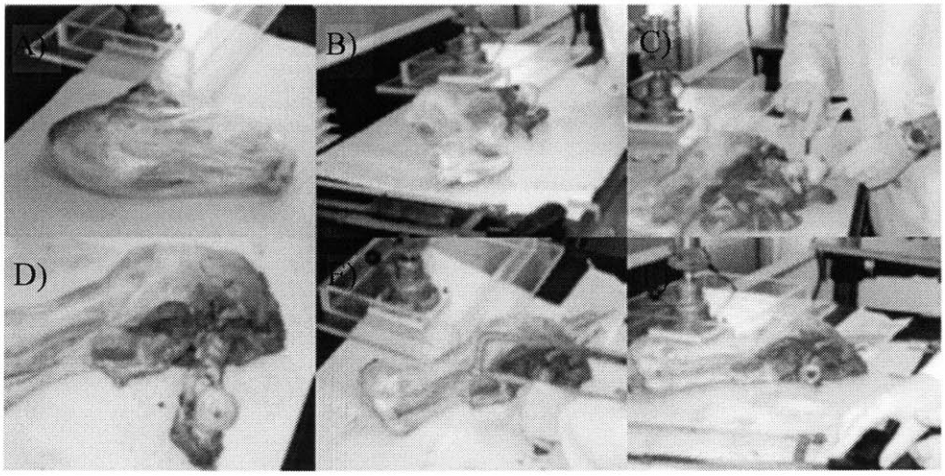

Figure 1: A) Joint as it arrives in lab. B) Joint out of plastic bag. C) Cleaning tissue away from femoral head. D) Joint with tissue removed from around femoral head. E) Removing femoral head with hacksaw. F) Joint with femoral head removed.

Once the femoral head has been removed, the joint is placed into the holder as shown in Figures 2a, 2b and 2c. The joint is held in the holder by three screws that are tightened using a hex wrench. The screws are tightened until the bone cracks. Once the joint is secure in the holder, the lower portion of the leg is removed from the upper portion of the leg by making an incision in the knee joint just above the meniscus as shown in Figure 2d. It is important to change your gloves regularly. Next the ligaments and tendons which hold the joint together in the middle are cut, followed by those on the outside of the joint, Figure 2e. Finally, any additional tissue which is holding the joint together is removed, Figure 2f, leaving just the upper portion of the joint, Figure 2g. At this time it is usually best to change scalpel blades. Once the bottom half of the joint has been

removed the remaining tissue covering the femoropatellar groove is removed as shown in Figure 2h leaving the groove completely exposed, Figure 2i. While removing the tissue covering the groove it is important to try and not let it touch the groove as you are removing it.

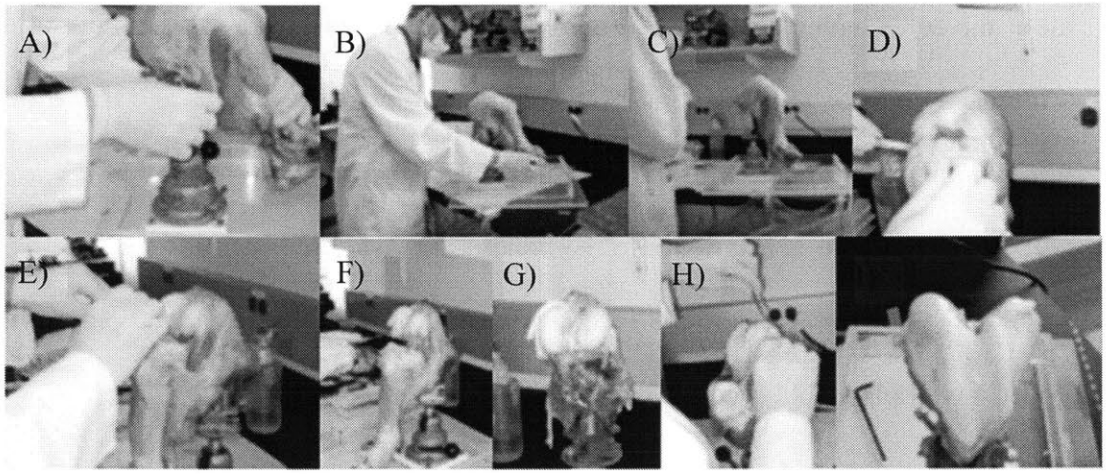

Figure 2: A, B, C) Joint Being secured in holder. D) Initial incision to separate the top portion of the joint from the bottom portion of the joint. E) Removal of ligaments and tendons which hold the joint together. F) Removal of additional tissue which connects bottom portion of joint to upper portion of joint. G) Upper portion of joint. H) Removal of tissue to expose femoropatellar groove. I) Exposed femoropatellar groove.

A drill press fitted with a 9mm diameter hollow drill bit, Figure 3a, is used to drill out cartilage/bone cores from the femoropatellar groove. Four to five cores are taken from the medial side of the joint (the larger side) and three to four cores are taken from the lateral side of the joint (the smaller side) as shown in Figure 3c. In order to get usable cores, the holder is used to position the joint such that the surface of the groove that is to be drilled is as perpendicular to the drill bit or parallel to the floor as possible, Figure 3b. This gives the cartilage/bone cores a flat surface which is easier to slice and has less

waste. It is possible to start taking cores from either the top or the groove or the bottom of the groove with equal success. It is important to leave enough room between the cores so that the path of the drill bit doesn't overlap beneath the surface of the joint leaving only partial cores. The joint is repositioned for every core taken to ensure a flat surface. If it is difficult to loosen the handle on the holder by hand, use a hammer to loosen it. Once all of the cores have been drilled, Figure 3c, a knife is used to break them out of the bone, Figure 3d and 3e. This is done by leaving the joint in the holder and inserting a knife through the cartilage and into the bone just below the ridge on the side of the joint, Figure 3d, once the knife is inserted into the bone it is twisted to break off the core from the bone, Figure 3e. The cores are then removed using forceps, Figure 3f, and placed into a well of a 24 well plate filled with PBS, Figure 3g.

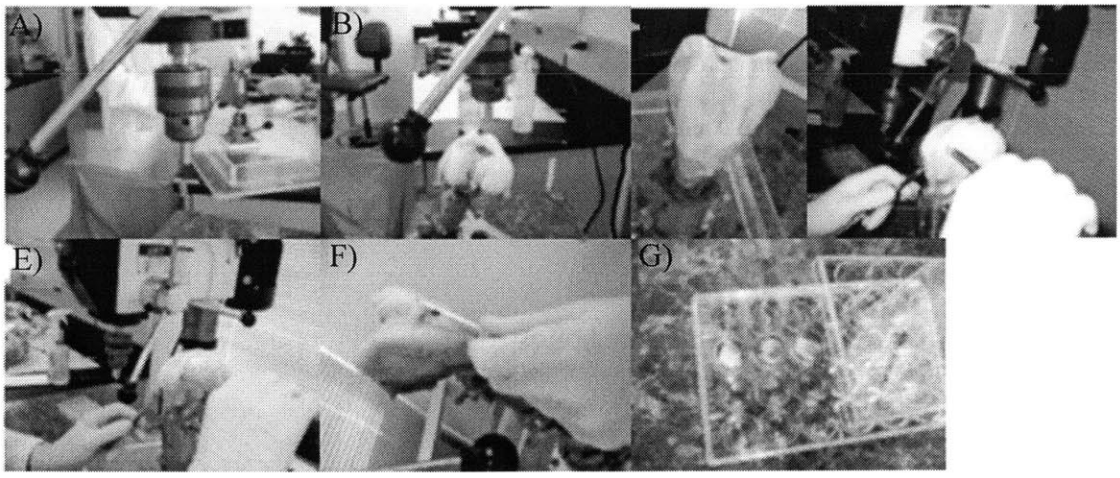

Figure 3: A) Drill used to obtain cartilage/bone cores. B) Joint surface ready to be drilled. C) Joint that has been completely drilled. D,E) Insertion of knife into joint to remove cartilage/bone cores. F) Removal of cartilage/bone core using forceps G) 24 well plate with three cartilage/bone cores in PBS.

2) Slicing the cartilage/bone cores to obtain 1mm thick cartilage disks.

Table 2 shows the items that need to be collected and autoclaved before slicing.

Table 2: Tools needed before slicing

To be Autoclaved	Other tools
Aluminum Foil	Petri dish
2 Bent forceps	Micrometer
Cartilage holder	kimwipes

A microtome is used to slice the cartilage/bone cores into 1mm thick disks. First a small kimwipe is ripped into long shreds, Figure 4b. One piece of kimwipe is then wrapped around the bone portion of the cartilage/bone core as shown in Figure 4c. This helps keep the core more secure in the holder. The cartilage/bone core is then placed into the holder so that only cartilage is exposed and then placed into the microtome, Figures 4d, 4e and 4f. It is important to make sure that the holder is secure in the microtome. Next the most superficial layer of cartilage is removed. This is done by placing the turn crank at 6 o'clock and pulling the blade across the top of the cartilage. The lever is then turned two rounds until it once again reaches the 6 o'clock position, Figure 4g. The blade is once again pulled across the top of the cartilage until a full circular disk of cartilage is cut off. Once this has happened, it is time to begin taking 1mm thick slices. To do this, turn the crank one full rotation and then turn the crank again until it reaches about 4:30. Slowly pull the blade across the cartilage in a controlled movement, Figure 4h, 4i and 4j. Use a Petri dish to catch the cartilage as you slice it and measure the thickness of the cartilage using the micrometer, Figure 4k. It is important to measure the slice in multiple directions to ensure that the entire slice is of uniform thickness. For the next slice turn the lever two full turns and then a little more to the 5:30position and take a slice. For the next slice do two full turns plus a little extra to get to 6:00 to 6:30 depending on whether the previous slice was thicker than 1mm or thinner than 1mm. It is important to adjust

the exact amount the lever is turned to how the cartilage is slicing. If the slices are consistently too thick, turn the lever less, if they are too thin, turn the lever more. Sometimes the slices will be too thick if the cartilage is loose in the holder so it is important to make sure that the cartilage is very tightly held and that one does not try and take a slice too quickly. It is important to use a controlled motion. Each slice is placed into individual wells filled with PBS of a 24 well culture plate. After coring or slicing it is possible to store the cartilage in the refrigerator for a few hours, however, it is important to let it warm back up to room temperature before attempting to punch or slice it. .

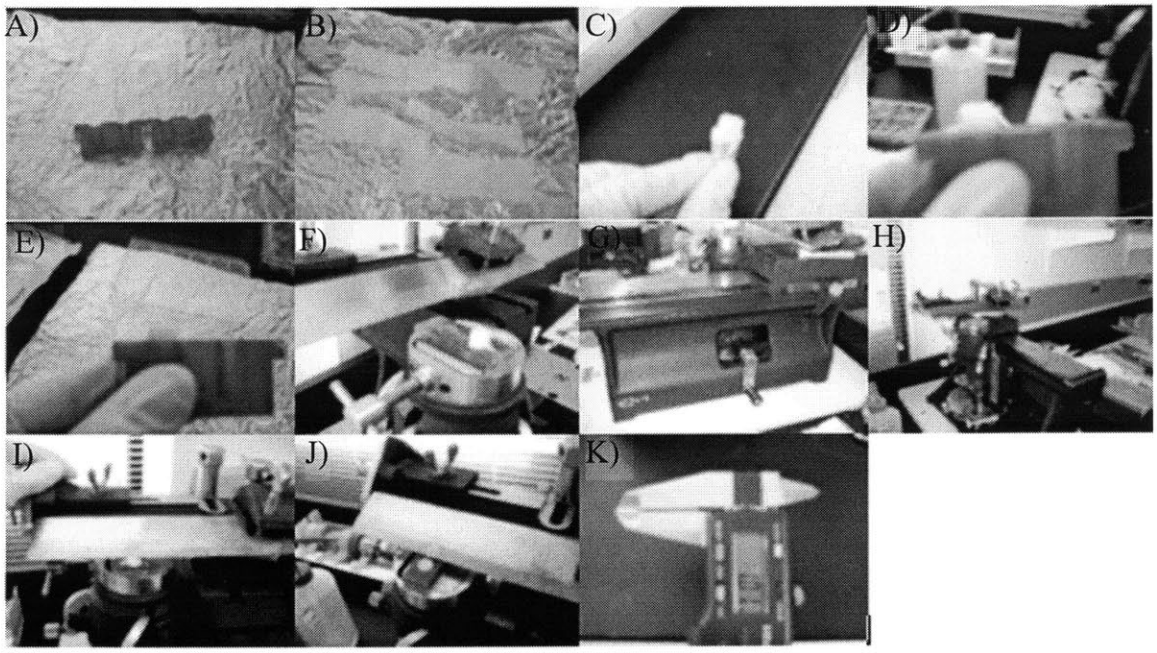

Figure 4: A) Holder for cartilage bone cores. B) Torn kim wipes. C) Kim wipe wrapped around cartilage/bone cores. D,E) Cartilage/bone core placed in holder. F) Holder with core placed in microtome. G) Microtome lever at 6 o'clock position. H,I, J) Taking a slice of cartilage using the microtome. K) Measuring the thickness of the cartilage slice using a digital micrometer.

3) Punching the Cartilage:

Table 3 shows the tools that need to be collected and autoclaved before punching as well as the solutions that need to be made up.

Table 3: Things to organize before punching cartilage

To be Autoclaved	Other tools etc.
Spatula	Petri dish
3mm Punch	24 or 48 well plate
Plug remover	Medium

A sterilized 3 mm dermal punch is used to obtain 3mm punched pieces of cartilage. One cartilage slice is transferred onto a normal size Petri dish. The dermal punch is then used to punch out four to six 3mm diameter pieces of cartilage, Figure 5a and 5b, which are then transferred into a well filled with 1ml of 10% FBS medium in a 48 well plate for four punches or into a well filled with 2ml of 10% FBS medium in a 24 well plate for 6 punches. The number of punches obtained from each slice is dependent on the number of groups needed for an experiment and whether or not the experiment involves injury. Usually for injury experiments only four punches are taken from each slice. These punched plugs are then allowed to equilibrate for two to five days before intervention by being kept in an incubator at 37°C and 5% CO_2.

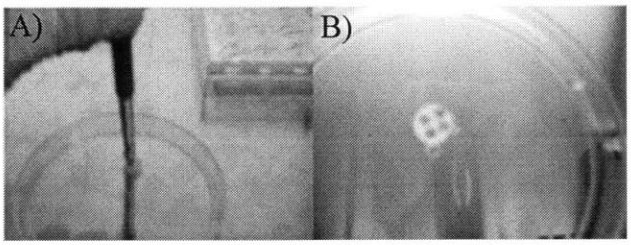

Figure 5: A) Cartilage slice being punched with 3mm dermal punch. B) Cartilage slice after it has been punched four times.

4) Harvesting Joint Capsule Tissue

Table 4 shows the tools that need to be collected and/or autoclaved prior to harvesting joint capsule tissue.

Table 4: Tools and solutions to organize and make before harvesting joint capsule tissue

To be autoclaved	Misc. other stuff
3 forceps (with teeth)	Petri dishes
2 scalpel holders	PBS
3mm or 5mm punch	Medium
Plug remover	48 well plate
Squirt Bottle for PBS	Small joint holder

Joint Capsule tissue is the tissue that encloses the inside of the joint. The joint capsule tissue becomes infected much more easily than the cartilage so it is important to harvest the joint capsule tissue in the hood in order to decrease the risk of infection. To do this the joint is placed into the holder and then transferred into the hood, Figure 6a. A sterile scalpel and forceps are used to clear away the outside membranes and to remove the lower portion of the leg as described in the first section. Once the lower portion of the joint has been removed, new scalpels and forceps are used to remove the tissue that is medial and lateral to the joint, shown in Figures 6b, 6c and 6d. It's important to change scalpel blades often during these initial steps and gloves if necessary. The harvested

pieces of joint capsule tissue are placed in a Petri dish containing just enough PBS to make sure that the tissue does not dry out, Figure 6e. These pieces of joint capsule tissue are punched in the same way as the cartilage using a sharp, sterilized 3mm or 5mm dermal punch, Figure 6f. In this case one or two pieces of punched joint capsule tissue are cultured in 1ml of medium. As was shown earlier in the thesis in order to see marked effects of the joint capsule tissue it is important to condition the medium for at least three days at a concentration of at least 1 piece/1ml.

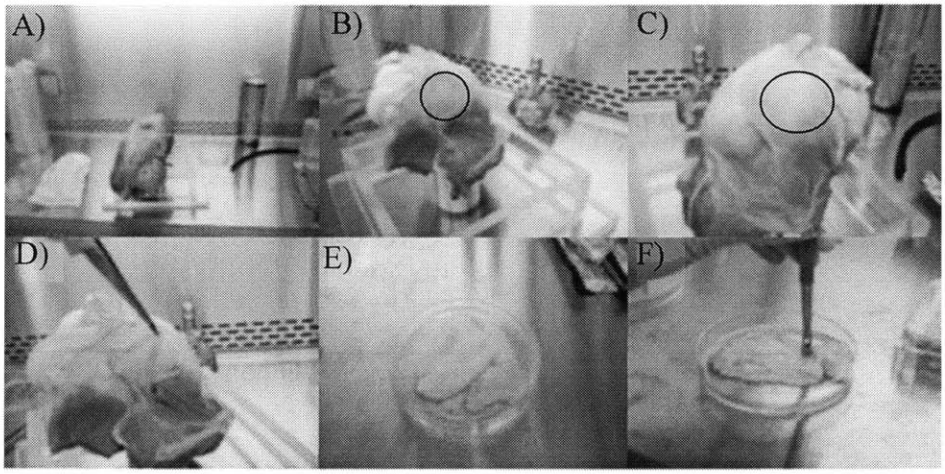

Figure 6: A) Joint in the hood. B) Medial side of the joint with joint capsule tissue circled. C) Lateral side of the joint with joint capsule tissue circled. D) Removal of joint capsule tissue from the medial side. E) Harvested joint capsule tissue in PBS in a standard Petri dish. F) Joint capsule tissue being punched with 3mm dermal punch.

For larger versions of the pictures found in this appendix see Appendix E.

Appendix D: Solutions and Protocols

Part 1: Solutions

10% FBS Medium

	e.g. for 10ml
90% DMEM- Low Glucose	9ml DMEM
10% FBS	1ml FBS
100X Hepes Buffer	100µl Non Essential amino acids
100X Non-essential Amino acids	100µl Hepes Buffer
100X PSA	40µl Ascorbate
250X Ascorbate	40µl Ascorbate
250X Proline	40µl Proline

ITS Medium

	e.g. for 10ml
100 % DMEM	10ml DMEM Low Glucose
100X ITS	100µl ITS
100X Hepes Buffer	100µl Hepes Buffer
100X Non-essential Amino acids	100µl Non Essential amino acids
100X PSA	100µl PSA
250X Ascorbate	40µl Ascorbate
250X Proline	40µl Proline

PBS

	e.g. 100ml
DI Water	90ml DI Water
10X PBS w/o Mg^{2+} and Ca^{2+}	10ml PBS w/o Mg^{2+} and Ca^{2+}
100X PSA	1ml PSA

Pro K

	e.g. 20ml
Tris HCL	19ml Tris HCL
20x ProK stock	1ml ProK stock

Rx Wash

PBS

.8mM sodium sulfate

1mM proline

Radiolabel

ITS or 10% FBS Medium

To label bovine tissue want a concentration of 10μCi/ml of radiolabel

^{35}S-Sulfate-measures proteoglycan synthesis

^{3}H-Proline-measures protein synthesis

Step 1) Determine how much medium is needed for the experiment and add 1.5 ml to that number for standards and a little extra cushion.

Step 2) Use excel spread sheet to calculate how much sulfate is needed since it has a relatively short half life. Make sure that the calibration date is correct.

Step 3) Add sulfate to the medium first and mix well. Take out .5ml of labeled medium and set aside for use as a standard.

Step 4) Add proline to the medium, mix well and take out .5ml for use as a standard.

Step 5) Add desired amount of medium to culture plate and incubate samples in labeled medium for between 6-24 hours. Record both start and stop times for accurate analysis of the results.

Step 6) End the radiolabel by making up Rx Wash solution (see above) and adding between .25-.5 ml into the wells of a 48 or 96 well plate. Wash eat cartilage plug three times in the Rx Wash solution for at least 10-15 minutes each wash.

Step 7) Make up enough Proteinase K solution so that each plug can be digested in 1ml and label 2ml microcentrifuge tubes while the plugs are sitting in the Rx Wash solution.

Step 8) Add 1ml of Proteinase K solution to each microcentrifuge tube.

Step 9) Once the wash is completed, transfer each plug to the appropriate microcentrifuge tube. Make sure that the plug is completely submerged in the Proteinase K solution.

Step 10) Place microcentrifuge tubes in the 60° water bath for two days.

Step 11) After two days check to make sure that there are no pieces of tissue floating around in the microcentrifuge tubes. Vortex all of the microcentrifuge tubes. If there were small pieces of tissue floating around, put them back into the water bath and check on them in 24 hours. If the cartilage is completely digested move on to step 12.

Step 12) Pipette 100µl of digest into plastic vials used for the scintillation counter and add 2ml of ethyl alcohol and cap. Do the same for the medium standards that were saved.

Step 13) Vortex vials. The solution should appear cloudy and then turn clear.

Step 14) Run empty bottles through the scintillation counter to make sure they are clean. To do this, open the program for the scintillation counter and select the desired isotopes. Select one count for a time of 0:10 and choose the correct user name.

Step 15) Once the blanks have been run, remove any bottles that have a count higher than 20.

Step 16) Place plastic vials with the samples into the glass bottles. Select desired isotopes, specify file name and count for 3:00.

GAG Assay

To Make GAG standards you will need:

~1ml DMEM or PBE

2mg/ml GAG Stock

Transfer about 1ml of DMEM to a 1.5ml centrifuge vial and take 7 small Centrifuge tubes and line them up

Put 180µl of PBE or DMEM and 20µl of GAG in the first vial

Put 100µl of PBE or DMEM in the rest of the vials.

Mix up solution in first Vial and transfer 100µl to the second vial, mix this up well and transfer 100µl to the third vial and so forth.

DO NOT TRANSFER ANYTHING TO THE LAST VIAL THAT SHOULD BE JUST DMEM OR PBE

This will give you standards of 200µg/ml 100, 50, 25, 12.5, 6.25 and 0

You only need to use the standards for 100 and down

Assay

Pipette 20µl of sample or standards into each well of the clear 96 well plates for Maxy.

Next, use the multi-channel pipette to add 200µl of DMMB dye to each well.

Put the plate in the machine and run it by opening the icon that says Maxy. Select 520nm for the wavelength and select run.

How to avoid medium evaporation for accurate GAG loss analysis

Evaporation can be a major problem when performing GAG analysis as even a small amount of evaporation can change the outcome by making a significant difference insignificant or vice versa. This is an especially large problem when culturing cartilage in small amounts of medium such as .25ml or .5ml or when culturing cartilage for extended periods of time in the same medium. Because GAG is measured as a

concentration it is important to do everything possible to eliminate evaporation. Evaporation occurs in the outside wells of plates whenever they are cultured in the incubator. To combat this problem, put at least an equal amount of DMEM in the outside wells of the culture plate. DMEM filled wells should form a ring around the experimental wells. This way, DMEM will evaporate instead of the medium.

DNA Assay

To make DNA standards you will need Tris HCl and the DNA standard stock solution which is at a concentration of 10μg/ml. The table below shows the amount of each solution that needs to be added to each eppendorf tube to make standards of 10, 5, 4, 3, 2, 1 and 0 μg/ml.

Standard Concentrations	Tris HCL	DNA Stock (10μg/ml)
10 μg/ml	0 μl	200 μl
5 μg/ml	100 μl	100 μl
4 μg/ml	120μl	80 μl
3 μg/ml	140μl	60 μl
2 μg/ml	160 μl	40μl
1 μg/ml	180 μl	20 μl
0 μg/ml	200μl	0 μl

To make 50ml of the DNA dye, mix 5 ml of 10X TEN in 45 ml of DI water and add 5μl of Hoescht dye. It is not advisable to make less dye than this as it is difficult to accurately pipette out amounts of less than 5 μl.

To measure DNA for cartilage explants, digest them in proteinase K following the same protocol as for a scintillation count. Once the explants are digested, vortex them and

transfer 20µl of each sample to individual wells of the black 96 well microtiter plates found by victor, the plate reader. Once the standards and samples have been added to the plate, add 200µl of the DNA dye to each well. Next, place the 96 well plate into the plate reader and open the icon labeled Victor^2. Once it has opened, click on the icon that looks like a stoplight with a wand. A list of protocols should appear. Click on the folder Steph and select the file DNA. Click on next. A picture should now appear of the 96 well plate. If any of the wells are empty, select them by clicking on them. There should be an option to either measure them or label them empty. If they are empty, choose empty. This saves time as the wells are being measured. Once all of the empty wells have been labeled as empty, click on next. The next screen provides the option of entering text notes into the output file. Enter any important information here about what is being run. When finished, click on next and then finish. The plate will now be read. Once the machine is finished reading the plate, click on the middle icon which looks like a sheet of paper. This will display the output file. Once the output file is displayed click on file and select export. Now name the file and save it to the appropriate folder on the computer.

Guanadine Extraction and Ethanol Precipitation

To perform a guanidine extraction, prepare the tissue by freezing it in liquid nitrogen. It is important through the first part of this process that the tissue remains frozen the entire time. The tissue is then powdered using a pulverizer that is used in RNA extraction. Throughout the powdering process it is important to keep the pulverizer and the tissue cold. This is done by pouring liguid nitrogen over the pulverizer.

Once the tissue has been pulverized so that it forms a fine powder, the Guanadine solution can be added. 500 µl of GuHCl + PI solution needs to be added for each sample. A sample consists of four, 3mm X 1mm cartilage disks or two, 3mm punched pieces of joint capsule tissue.

To make 5ml, mix together the following solutions and powders:

	location	stock conc	amt needed	
Guanidine, 4M	RT	7M	2.857	mL
EDTA, 10 mM	4C	500 mM	0.100	mL
PMSF, 2 mM	RT	200 mM	0.050	mL
Benzamidine HCl, 15 mM	RT	1M	0.075	mL
6-aminohexanoic acid, 0.25 M *(aka ε-amino-N-caproic acid)*	chem shelf	(powder)	164.000	mg
pepstatin, 1 µM	aq, -20C	100 µM	0.050	mL
sodium acetate, 50 mM	RT	1M	0.250	mL
DI water			1.618	mL

After adding the Gu HCL + PI solution to the samples, tape the cryovials to the rocker in the refrigerator and start it. Leave them rocking for 48 hours in the refrigerator. After 48 hours spin down the samples and transfer the supernatant to fresh vials. Keep track of how much supernatant there is and add three times that volume of 200 proof ethanol + 5mM Sodium Acetate. Once the ethanol solution has been added, place the vials in the minus 4°C freezer overnight. Next, spin the tubes for 20 minutes on high speed in the microcentrifuge and discard the supernatant and dry the pellet. Once the pellet is dry, the guanidine extraction and ethanol precipitation are complete.

Appendix E: Blown up Harvest Pictures

The following pictures are a larger version of the pictures found in Appendix C. The Figure numbers are also the same as in Appendix C for easy reference.

Part 1: Harvesting Cartilage/Bone Cores

Figure 1A: Joint as it arrives in lab.

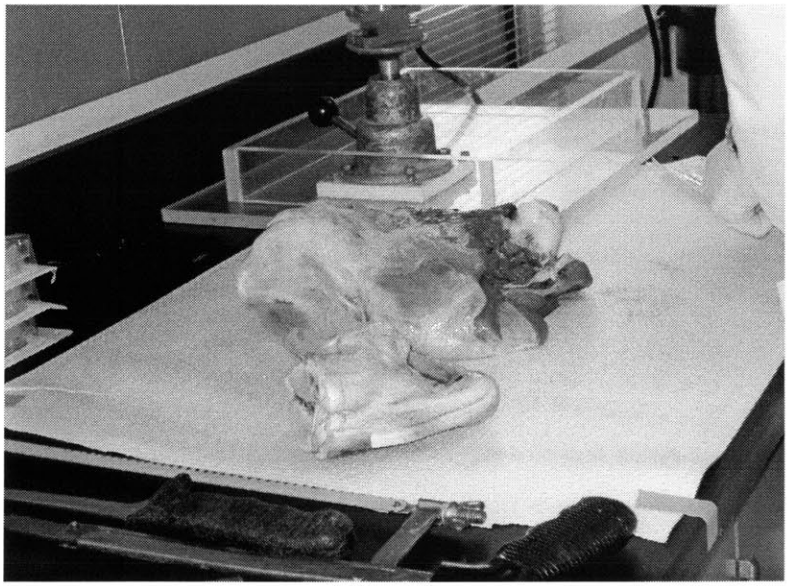

Figure 1B: Untouched joint outside of plastic bag.

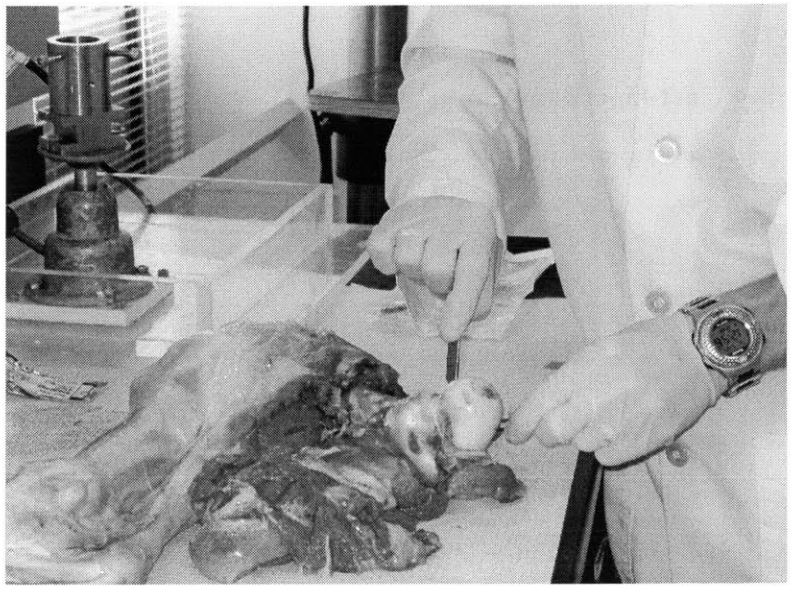

Figure 1C: Cleaning tissue away from the femoral head.

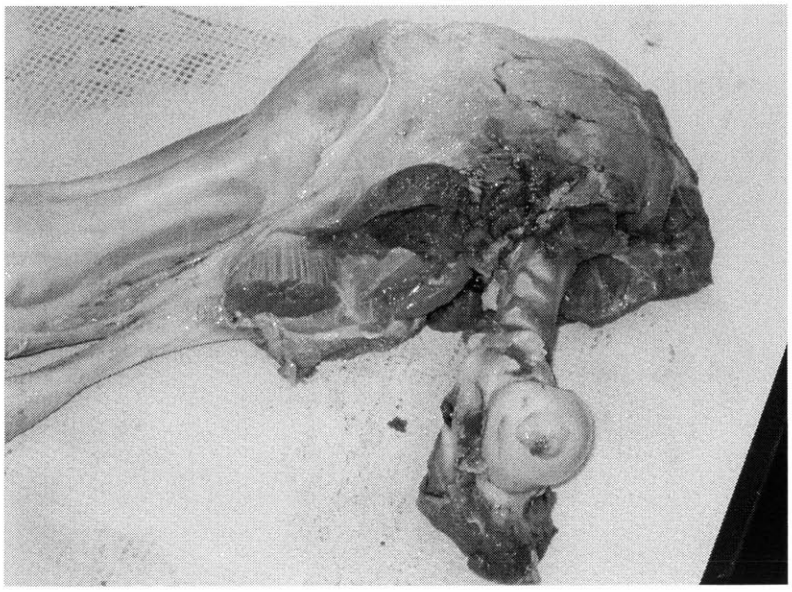

Figure 1D: Joint with tissue removed from around the femoral head.

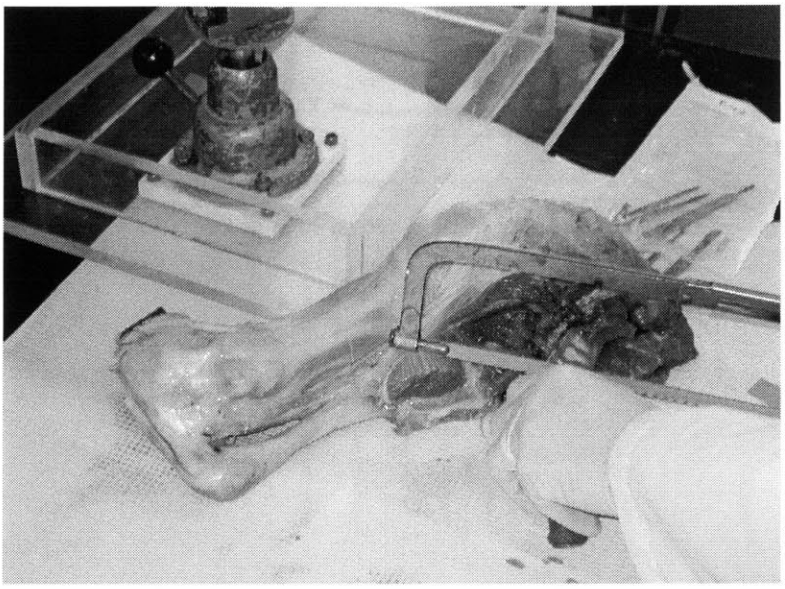

Figure 1E: Removal of femoral head with a hacksaw.

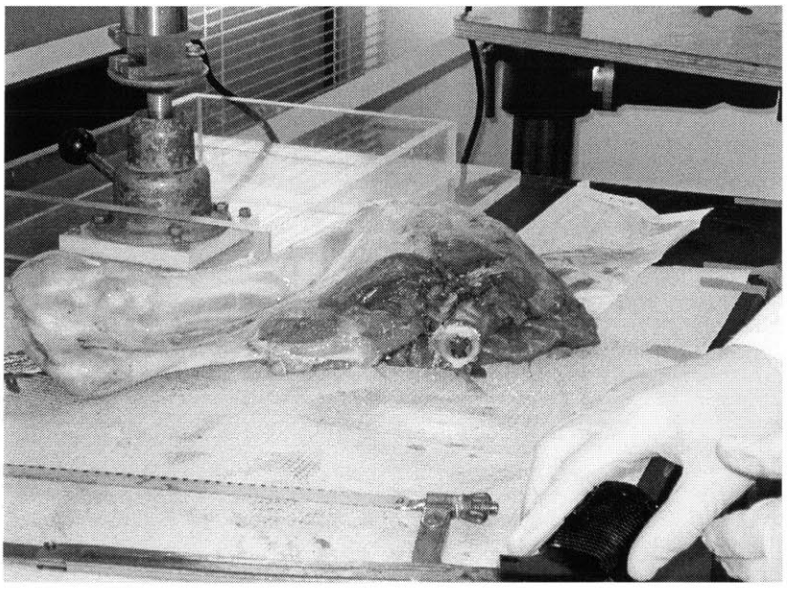

Figure 1F: Joint with femoral head removed

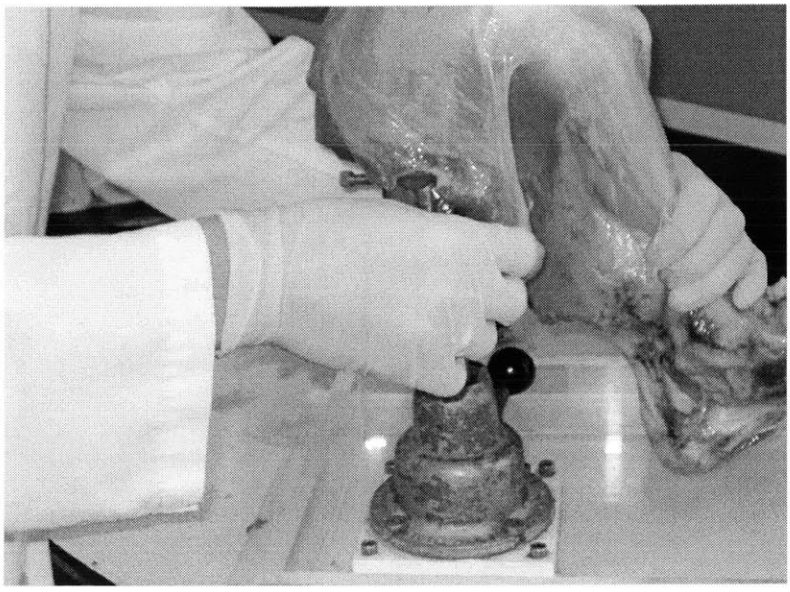

Figure 2A: Joint being secured in holder.

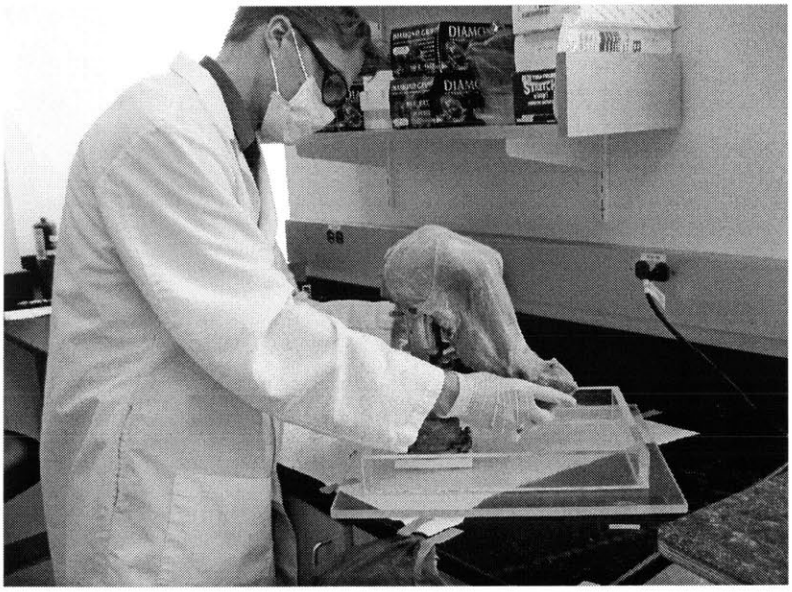

Figure 2 B: Joint being secured in holder.

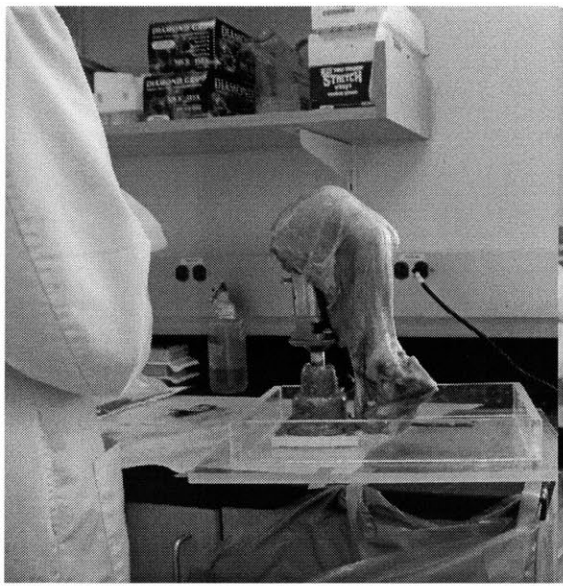

Figure 2C: Joint being secured in holder.

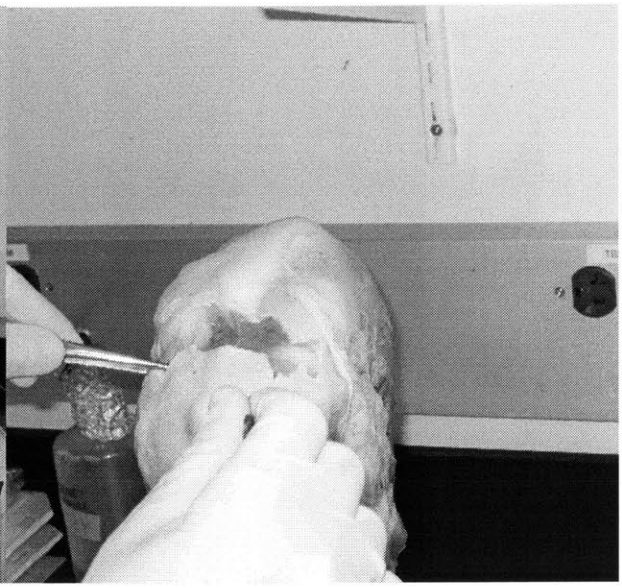

Figure 2D: Initial incision to separate the top portion of the joint from the bottom portion of the joint.

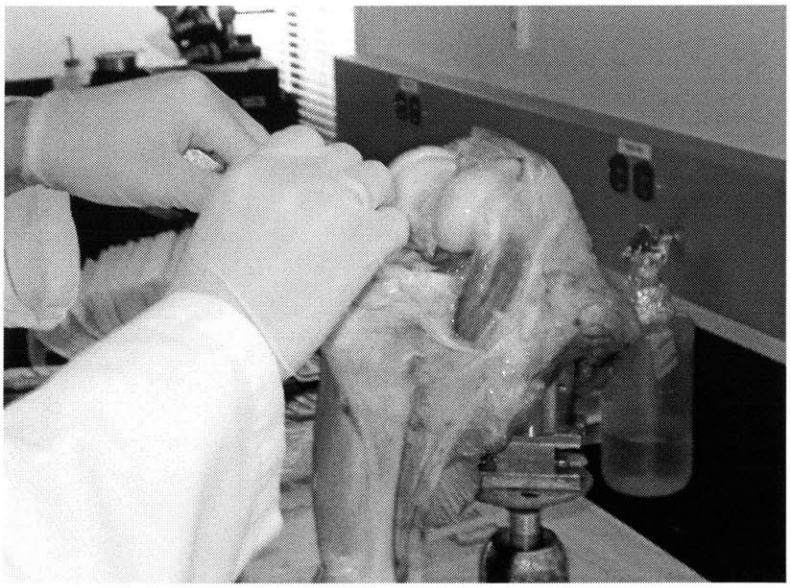

Figure 2E: Removal of ligaments and tendons which hold the joint together.

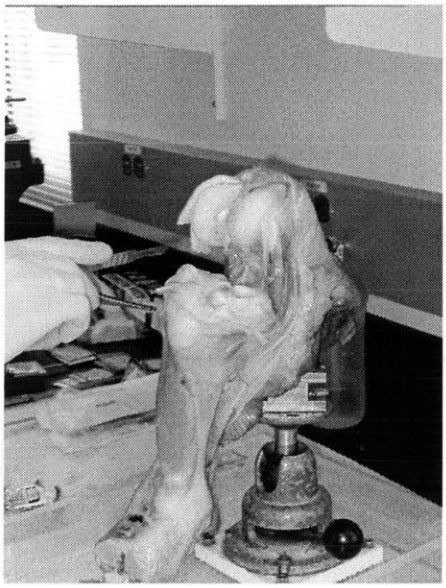

Figure 2F: Rmoval of additional tissue which connects the bottom portion of the joint to the upper portion of the joint.

Figure 2G: Upper portion of joint with condyles exposed.

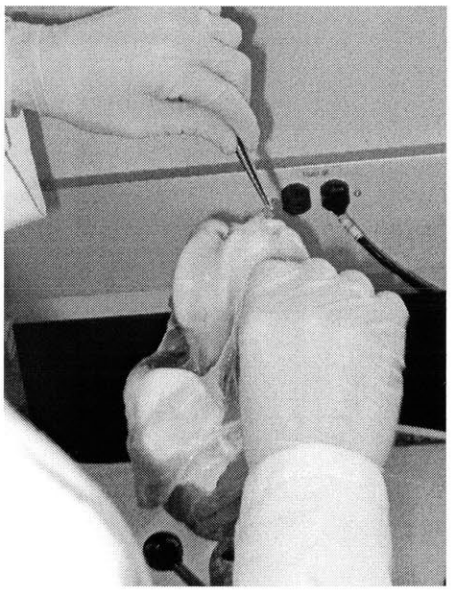

Figure 2H: Removal of tissue to expose the femoropatellar groove.

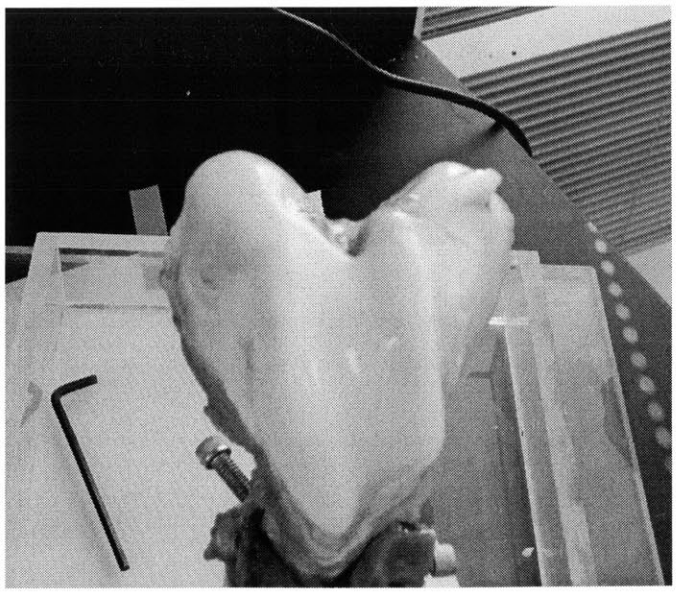

Figure 2I) Exposed femoropatellar groove.

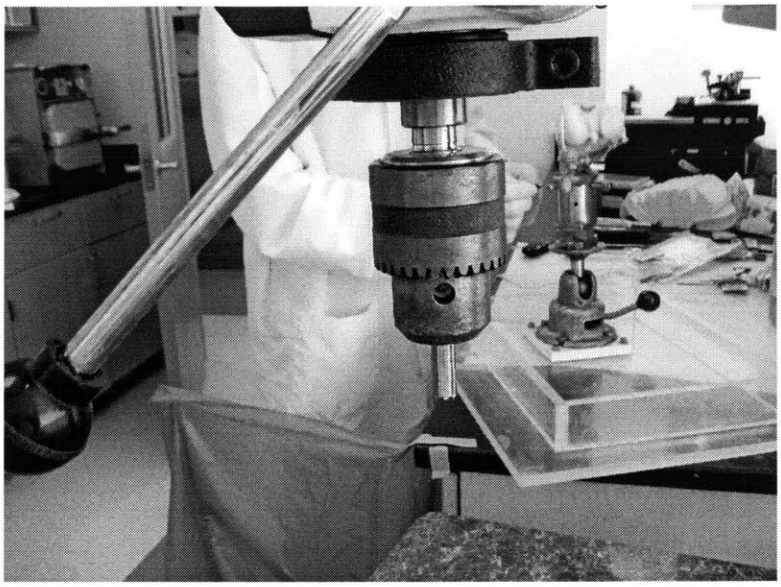

Figure 3A: Drill used to obtain cartilage/bone cores.

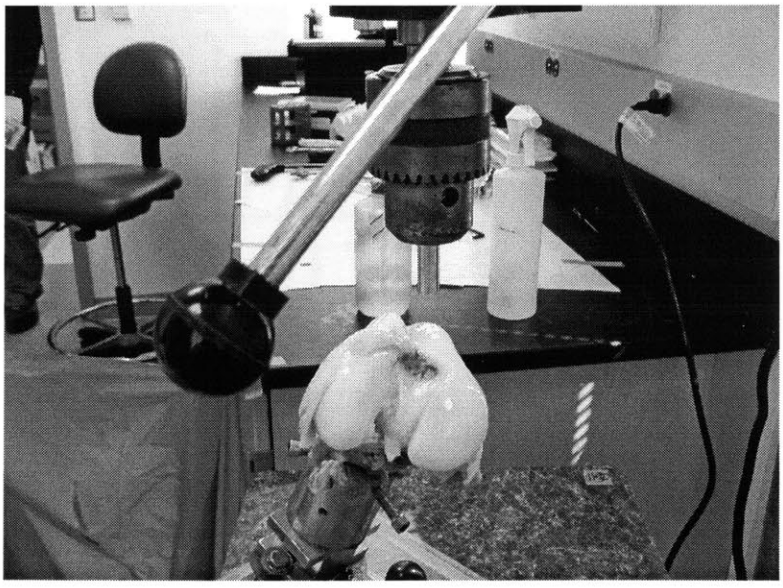

Figure 3B: Joint surface ready to be drilled.

Figure 3C: Joint that has been completely drilled.

Figure 3D: Insertion of knife into joint to remove cartilage/bone cores.

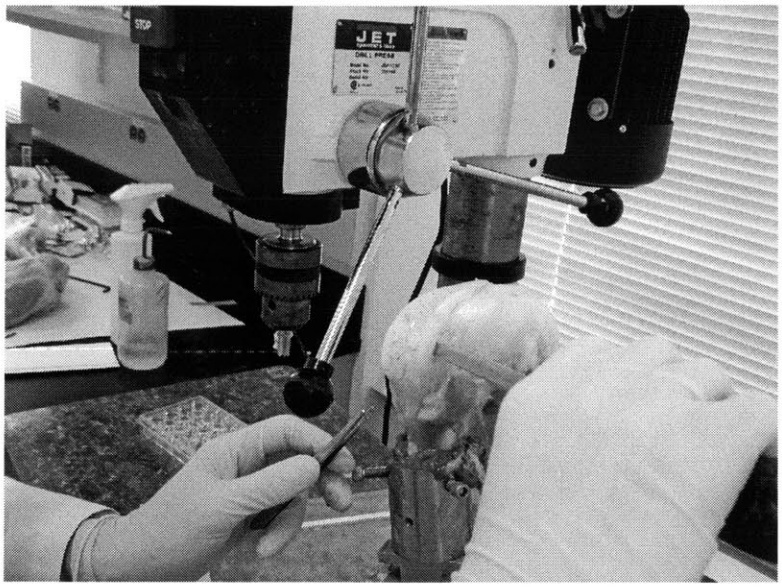

Figure 3E: Insertion of knife into joint to remove cartilage/bone cores

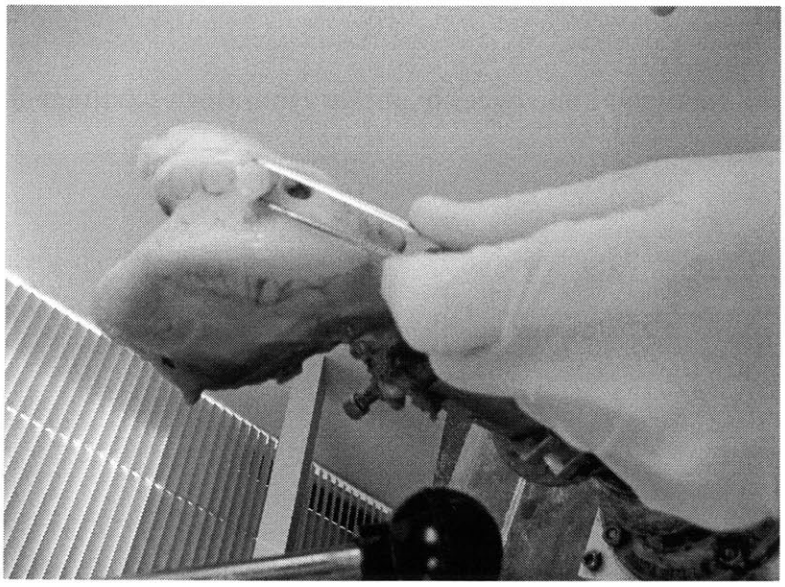

Figure 3F: Removal of cartilage/bone core using forceps.

3G) 24 well plate with three cartilage/bone cores in PBS

Part 2: Slicing the cartilage/bone cores to obtain 1mm thick cartilage disks.

Figure 4A: Holder for cartilage/bone cores

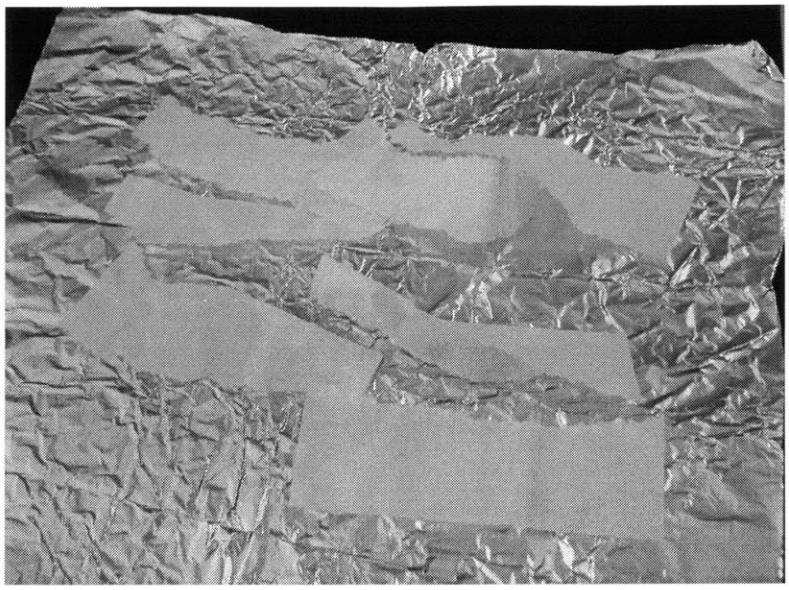

Figure 4B) Torn Kim Wipes used to wrap the cartilage/bone cores

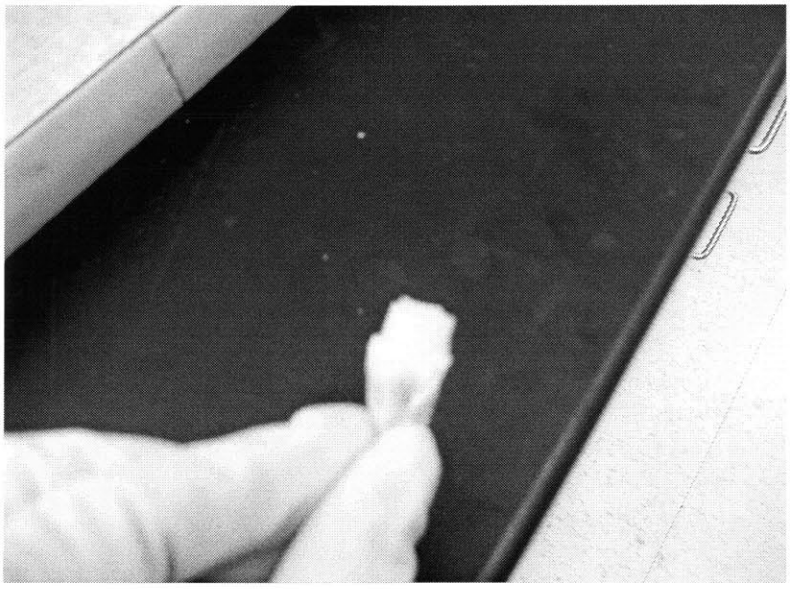

Figure 4C: Kim wipe wrapped around a cartilage/bone core.

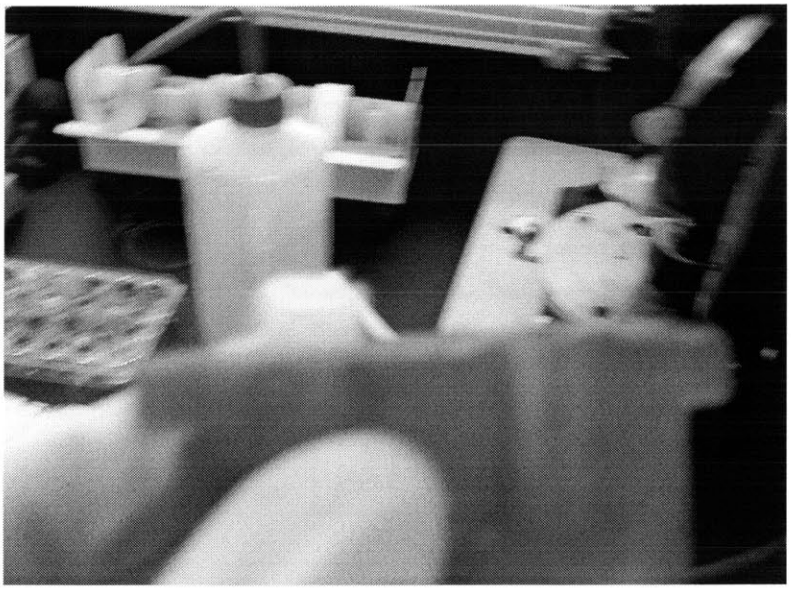

Figure 4D: Cartilage/bone core placed in holder

Figure 4E: Cartilage/bone core placed in holder

Figure 4F: Holder with core placed in the microtome and ready to be sliced.

Figure 4 G: Microtome lever at 6 o'clock position.

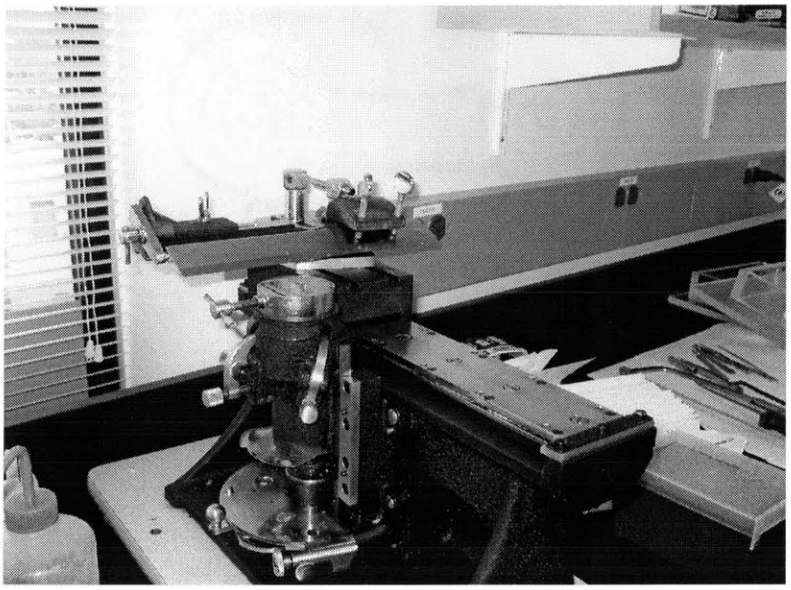

Figure 4H: Taking a slice of cartilage using the microtome

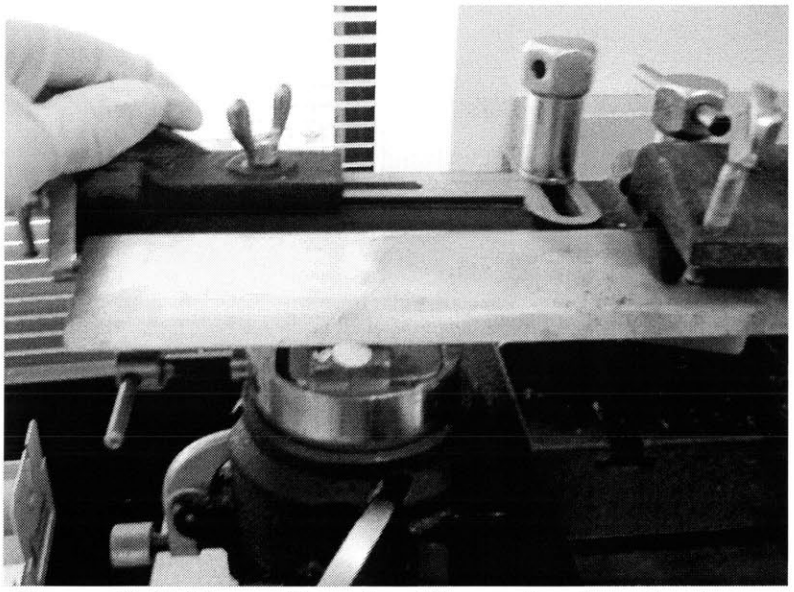

Figure 4I: Taking a slice of cartilage using the microtome.

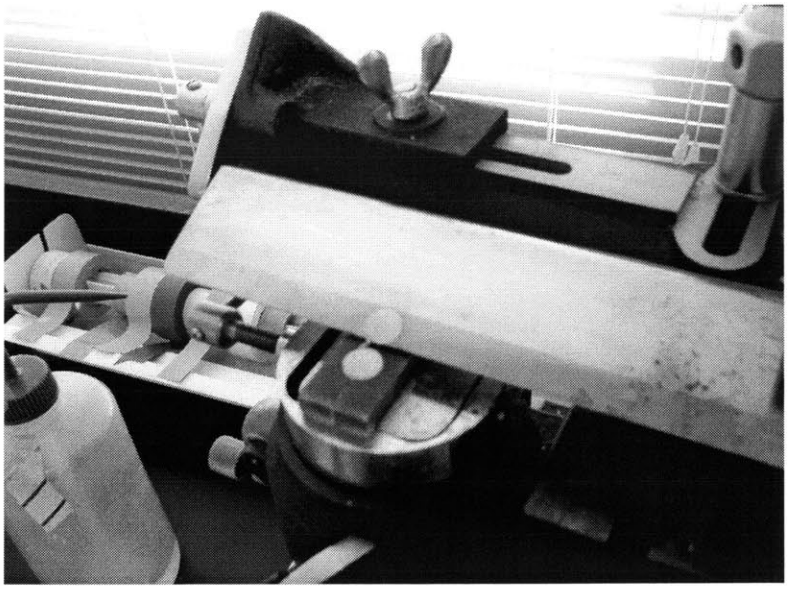

Figure 4J: Piece of cartilage on microtome blade after being sliced.

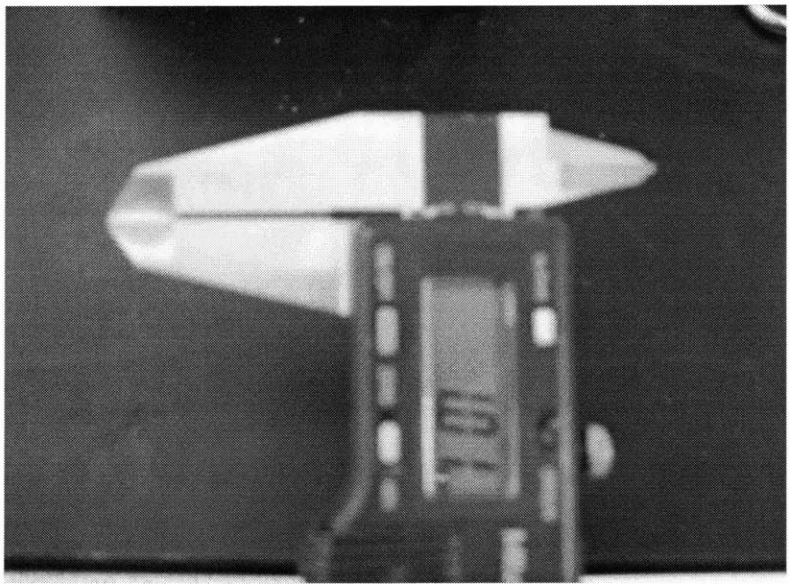

Figure 4K: Measuring the thickness of the cartilage slice using a digital micrometer.

Part 3: Punching the cartilage

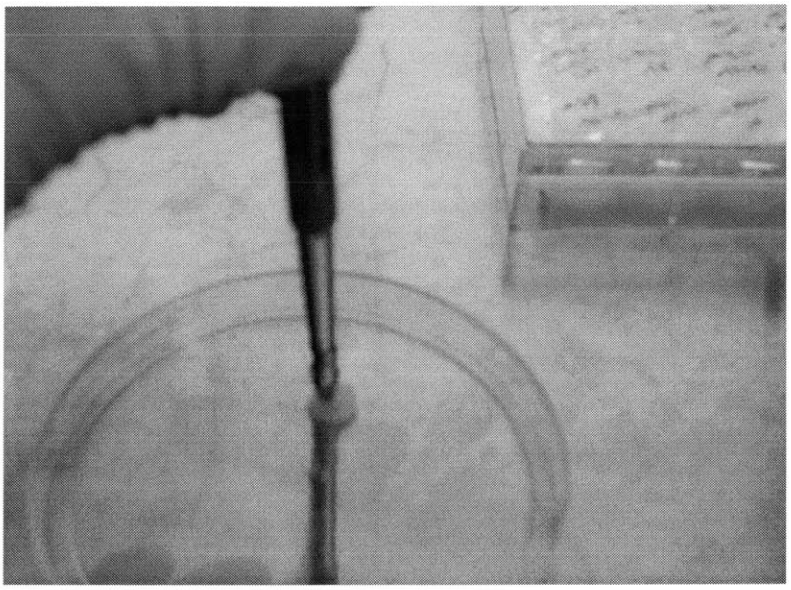

Figure 5A: Cartilage slice being punched with a 3mm dermal punch.

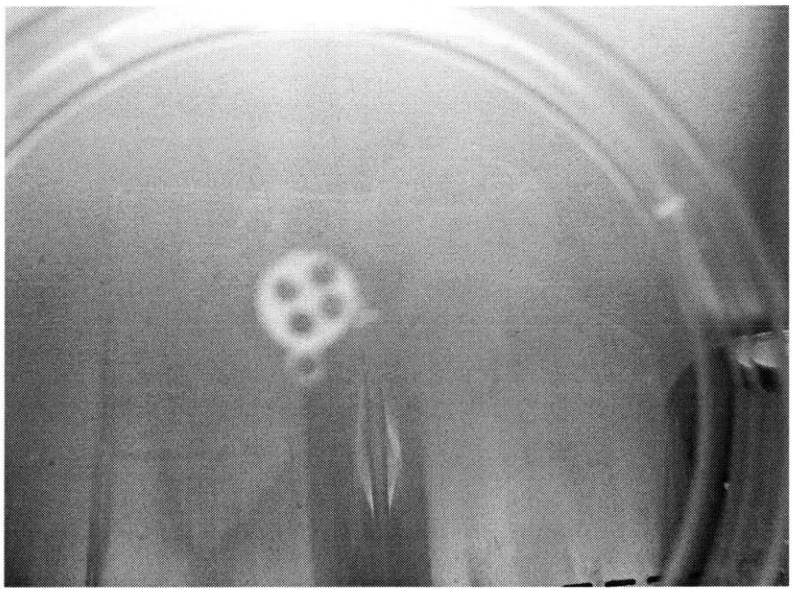

Figure 5B: Cartilage slice after it has been punched four times

Part 4: Harvesting joint capsule tissue.

Figure 6A: Joint in the hood

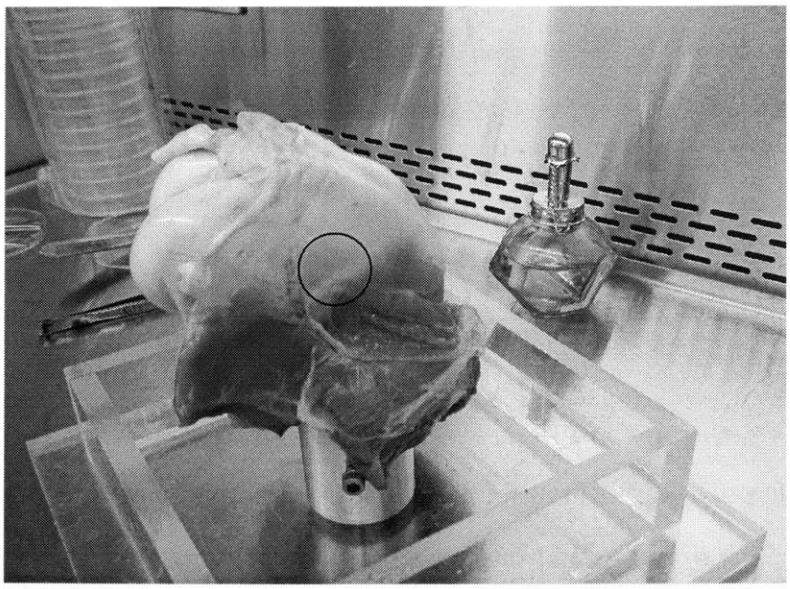

Figure 6B: Medial side of the joint with joint capsule tissue circled.

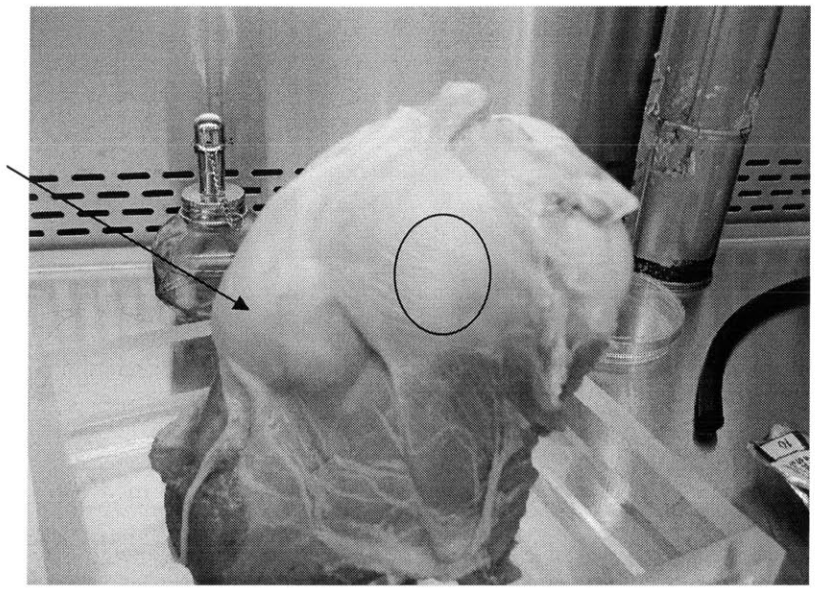

Figure 6C: Lateral side of the joint with joint capsule tissue circled.

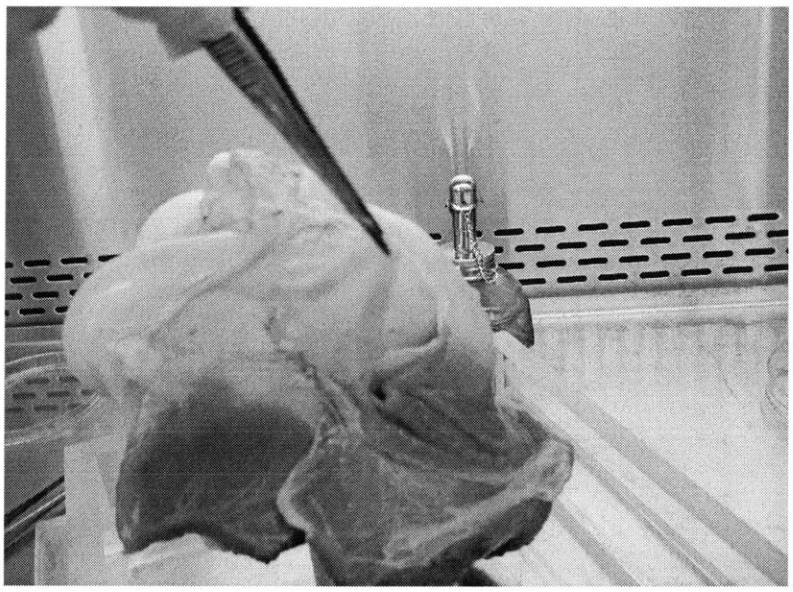

Figure 6D: Removal of joint capsule tissue from the medial side.

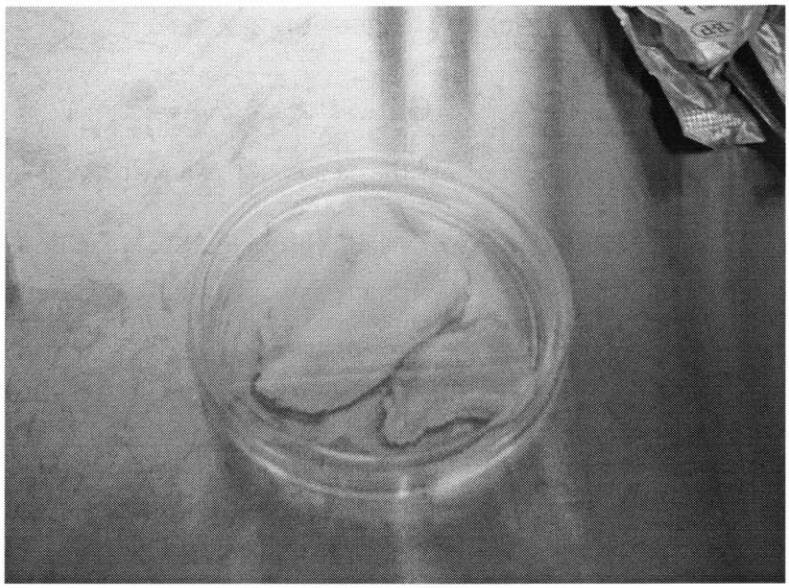

Figure 6E: Harvested joint capsule tissue in PBS in a standard Petri dish. Lateral side is the larger piece and medial side is the smaller piece.

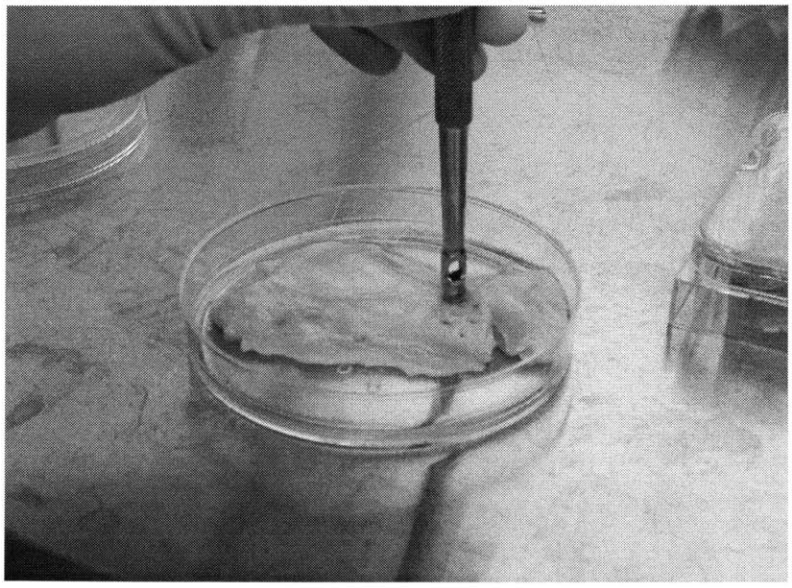

Figure 6F: Joint capsule tissue being punched with a 3mm dermal punch.

Acknowledgements

I am grateful for the help and support of my thesis advisor Alan Grodzinsky. I would like to thank all the members of the Grodzinsky lab who helped by teaching me laboratory techniques and provided constructive feedback when I would discuss my work with them. I would also especially like to thank my husband Robert for all of his love and support.