Adult articular cartilage culture system and effects of IL-1β

by

Wei Wang

Submitted to the Department of Electrical Engineering and Computer Science
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Author .....
Department of Electrical Engineering and Computer Science
May 17, 1996

Certified by .....
Martha L. Gray
Associate Professor
Thesis Supervisor

Accepted by .....
Chairman, Departmental Committee on Graduate Students

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Abstract

Previous research in this lab has established a calf epiphyseal cartilage culture system and looked at effects of a particular cytokine, interleukin 1β on cartilage. In addition to the findings of increased tissue degradation and decreased tissue regeneration, evidenced by accelerated release of matrix components and reduced uptake of essentials, it is also observed that as IL-1β is allowed to diffuse into the extracellular matrix from the periphery, observed degradation of tissue nevertheless centers around the blood vessels embedded inside the matrix. Although vasculature is regularly present in young cartilage tissues, mature cartilage has no blood vessels. Therefore an older culture system without vasculature will help elucidate effects of IL-1β on cartilage specific chondrocytes, with no complication from other cell types.

The first purpose of this research, therefore, was to define such a culture system. This task involves modifying several experimental protocols, and conducting control experiments to define parameters for such a system. It was found that both chondrocyte and GAG concentrations are much lower in mature cartilage than in younger tissues, in addition to the much smaller overall ECM volume. Although less populous, chondrocytes in adult bovine articular cartilage have comparable synthetic capability as calf of new glycosaminoglycan (GAG) molecules. Furthermore, in culture with 20% FCS, adult cartilage loses proportionally much more GAG every day than calf, averaging about 5% of overall GAG content.

Given the average parameter values and their variances, the statistical paired-t test was adopted. As sample size is directly related to the width of confidence intervals, minimum sizes were calculated for an experiment to be able to detect any possible significant changes due to treatment. From one of the control experiments, it was found that at least 40 cylindrical cartilage disks have to be included in analysis to see a deviation in bulk GAG content from control on the order of 10% due to treatment. Such numbers were obtained for other parameters as well. Treatment protocols of sufficient sample sizes were then designed to ensure conclusive results can be drawn.

The second purpose, after the control experiments, was to study the effects of IL-1β on the metabolism of cartilage proteoglycans. It was found that at the concentration of 500 ng/ml, IL-1β has significant impact on mature cartilage matrix,

both severely repressing new generation and promoting increased loss of tissue GAG molecules. Accelerated degradation was detectable less than 20 hours after the first introduction of IL-1β. GAG synthesis is only 13% of control after 3 days of IL-1β treatment.

The third purpose was to understand the correspondence of spatial distribution between introduced IL-1β and GAG loss, through histological staining. It was found that although the spatial pattern of GAG loss was as expected, no clear difference was observable between control and treatment. Unlike younger cartilage with localized GAG loss following IL-1β treatment, mature ECM releases GAG over the entire volume, making it harder to discern from histological stains.

Thesis Supervisor: Martha L. Gray
Title: Associate Professor

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Chapter 1

Introduction

Articular cartilage is a major connective tissue type providing low-friction and wear-resistant bearing surfaces for load redistribution of the underlying bones. It is avascular and aneural in nature with less than 5% cell volume. The resident cells, chondrocytes, are loosely distributed throughout the extracellular matrix (ECM). Although sparse, these cells are mostly responsible for maintaining and regulating a rather complex network of macromolecules, cytokines and growth factors. This task is accomplished through the art of intricate balancing and feedback systems. For example, chondrocytes adjust rates of synthesis for new matrix macromolecules according to the current status and need of ECM. At the same time, they synthesize and release degradative enzymes to break down the macromolecular structure for growth and development. In healthy joints, these two processes take place simultaneously and synergistically to fulfill mechanical supporting functions of articular cartilage [52, 49].

Another balance exists through harmony of contributing forces in ECM. With close to 80% of wet weight in water and under physiological pH, the numerous negative charge groups on the proteoglycans (PG) dissociate to generate strong electrostatic repulsion as they exist in close proximity. The resulting swelling pressure relates directly to the compressive resistance of cartilage critical to its function. At the same time, the integrity of the matrix structure is maintained by tension in the collagen network, holding the swelling PG's together. Therefore, the well-being of cartilage as a functional mechanical buffer relies on sustained balances of many different factors

of biological, mechanical and chemical in nature [40].

In joint diseases such as degenerative osteoarthritis (OA) and inflammatory rheumatoid arthritis (RA), accelerated loss of charge bearing components out of ECM from heightened protease activities results in joint swelling and decreased stiffness. In this case, unfortunately articular cartilage behaves much like a unstable system. Once degradation prevails, chondrocytes seem to have little power to regain the balance, and the state of deterioration can only get worse.

Through *in vitro* explant culture studies, many events occurring during healthy and diseased cartilage have been characterized. Chondrocytes have been shown to modify their biosynthetic behaviors following changes in matrix environment, in terms of new macromolecule synthesis and matrix degradation. In controlled explants, elevated levels of chondrocyte synthesis, even net deposition of matrix materials following biological and/or mechanical stimulation have been achieved. It is the long term goal that through better characterization of cartilage remodeling mechanisms, possible approaches will be adopted to intervene joint degradation processes [28].

As joint modulating factors are vast and interconnected, explant cultures are developed to offer more defined systems and more meaningful interpretation of cartilage behavior with limited sources. This research defines an adult articular cartilage culture system as an alternative to the existing young tissue system in this lab. The non-vascular nature of more mature cartilage offers the advantage of no complication from multiple cell types, a difficulty encountered in interpreting behavior of younger cartilage. This explant culture is subsequently treated with interleukin 1, an important cytokine believed to be a major player in matrix degeneration. Responses are characterized in terms of changes in bulk material properties, cellular activities, and spatial distribution profiles.

1.1 Cartilage Extracellular Matrix

Articular cartilage contains two major structural components, proteoglycan and collagen. The collagen network (mainly type II) renders shape and tensile strength to the

ECM, while proteoglycan provides the ability to undergo reversible deformation and to withstand compressive load bearing. The well being of the matrix depends upon both the integrity of collagen network and the quality and quantity of embedded proteoglycan. The responsibility to maintain the matrix rests almost entirely on the few resident chondrocytes. As the cells are distant from each other and away from direct stimulation, there must exist signalling mechanisms by which chondrocytes respond to changes within the cartilage matrix during growth and development. Cytokines and growth factors are likely to be important modulators in cartilage because of their presence in ECM, their receptors on chondrocytes, and the correlation of their activities to changes in matrix properties. Equally important are changes in mechanical loading in modifying material properties of ECM, as cartilage is constantly under loading and changes in loading conditions (such as loss of gravity) result in changes in ECM.

1.2 Previous Research

Among the many different types of cytokines and growth factors, interleukin 1β (IL-1β) is believed to be a key player in cartilage degeneration as it is involved in both reduced synthesis and accelerated degradation, through two independent pathways [34]. As a local modulator, IL-1β can be synthesized by chondrocytes [7] and have receptors on them [5].

IL-1β suppresses synthesis of cartilage specific collagen types [27], and has inhibitory effects on proteoglycan synthesis [19]. This lab has used radioactive precursor incorporation as means of monitoring chondrocyte synthesis and their responses to IL-1β and other changes in the environment [50].

IL-1β is also capable of up-regulating the production and activation of various enzymes while simultaneously down-regulating various enzyme inhibitors. With elevated concentration and activity, these enzymes, notably stromelysin and the yet unknown 'aggrecanase', proceed to chop off structural and functional ECM components. Observable consequences include increased release of macromolecule glycosaminogly-

can (GAG) fragments out of the ECM, and increased matrix swelling.

Besides being affected by cytokines like IL-1β, the primary function of cartilage as load-bearing material requires that chondrocytes monitor and adjust to changing mechanical environments. Alterations in biosynthesis due to compressive loading have been studied under many protocols [28]. Generally, static compression reduces both protein and GAG synthesis, while dynamic loading can enhance, reduce, or have no effect on cartilage explant depending on loading conditions. However, mechanical effects to matrix degradation are not well known, neither is the extent of chondrocyte involvement [3].

This thesis was motivated by results of an ongoing study in this lab. The objective of the study was to examine the combined effects of loading and IL-1β on cartilage metabolism, as arthritic joints are simultaneously under the influence of both. Using an *in vitro* immature cartilage culture system, this study showed that IL-1β induced degradation is dramatically reduced under concurrent static compression, suggesting loading status is a very important determinant in how biological factors are interpreted. Also, it was found that the diffusion of IL-1β through ECM is the rate-determining step of PG degradation, despite its relatively small size [47]. The much lower diffusivity compared to comparably sized macromolecules implies possible binding of IL-1β to matrix components, and have significant implication in understanding how cytokine-mediated cartilage degradation occurs and how mechanical loading could alter this process.

The spatial profile of PG degradation was also studied. It was found that GAG loss occurs progressively from the perivascular regions in this younger cartilage culture, contrary to the progression of introduced IL-1β. This result suggests possible involvement of multiple cell types from the vasculature. Blood vessels, while present in immature cartilage, are absent in mature cartilage tissue, hence the interest in studying adult articular cartilage explants, and effects of IL-1β induced degradation in such culture.

1.3 Thesis Objectives

The specific objectives of this thesis are:

1. What are the biochemical composition and metabolic behavior of adult bovine articular cartilage explants, and how is it different from immature cartilage?

2. What are the animal-to-animal and site specific variances for the above properties, and the implication of such variation on methods of analysis?

3. Whether this culture system can be used for cartilage studies, judging from the availability of tissue and the required sample size for conclusive results?

4. How does treatment of IL-1β affect joint properties, such as biochemical composition, biosynthesis and degradation, and whether such changes correspond to previous observed roles of IL-1β?

5. What is the spatial degradation profile following IL-1β treatment, and how does it compare to those seen in younger tissue?

Chapter 2

Cartilage Biochemistry and Mechanics

Articular cartilage is a specialized connective tissue covering the ends of long bones in synovial joints functioning as load bearing materials. The mechanical properties of articular cartilage come from the biochemical composition and metabolic behaviors of the chondrocytes. Although major structural constituents of have been characterized long ago, understanding of cartilage biomechanics requires knowledge of interactions of numerous contributing forces in a very complicated network. The major component of adult cartilage is water, about 70% of total wet weight. Almost equal amounts of collagens and proteoglycans comprise most of the remaining mass, each contributing about 10-20% of wet weight [49]. Cartilage specific cells, chondrocytes, occupy only 2-10% of tissue volume. Due to the lack of blood vessels, nerve fibers and direct cell-cell contact, cellular communications occur through diffusion of signalling factors through ECM. The same mechanism is also used for nutrient and waste transport.

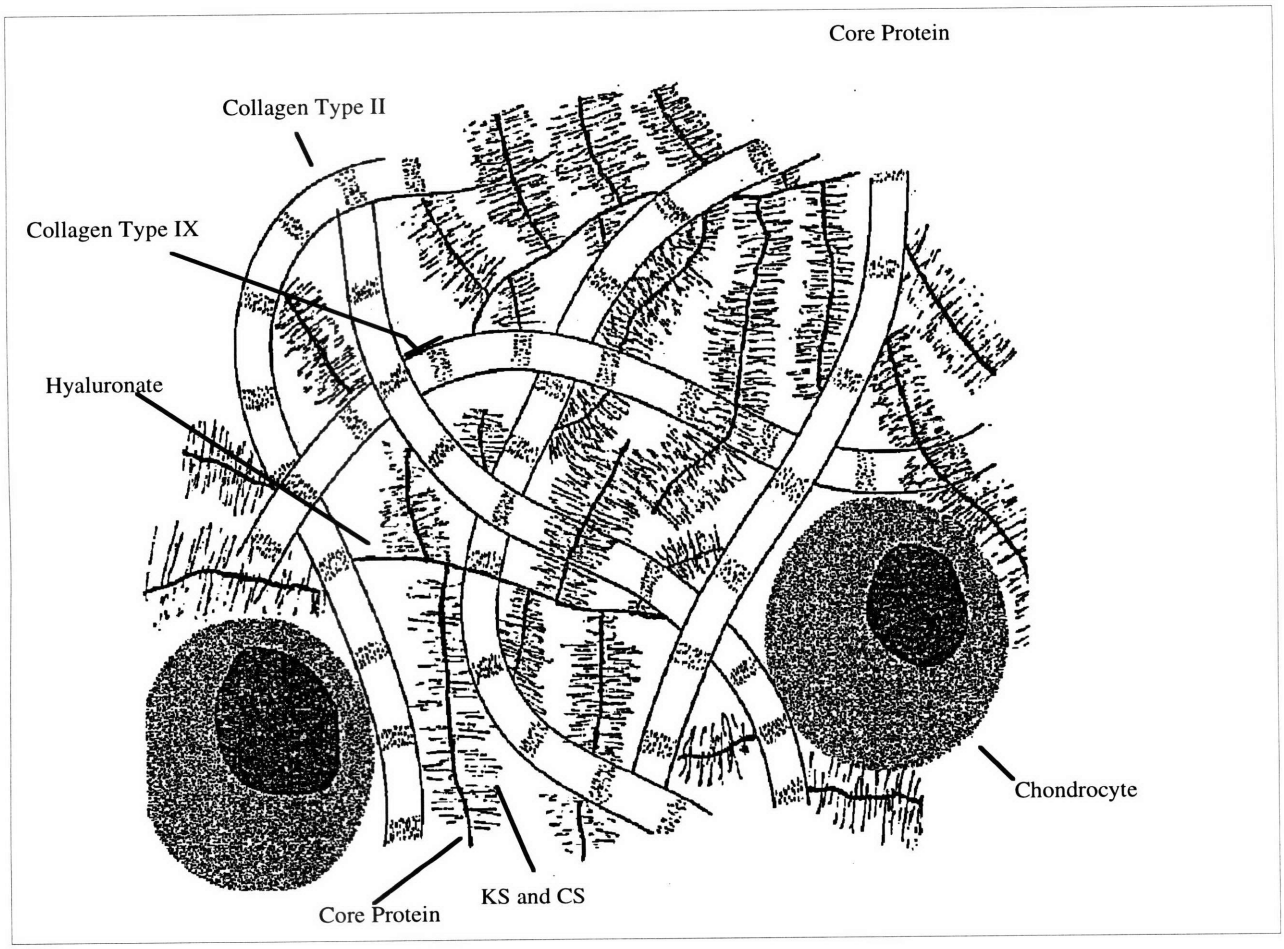


Figure 2-1: Major Components of Articular Cartilage Extracellular Matrix

2.1 Major matrix components

2.1.1 Collagen

Collagen is a major protein type in the body, comprising more than 30% of all protein mass. It is organized into insoluble fibers of great tensile strength to function as the major stress bearing component of the underlying tissue [60].

Like other proteins, the primary structure of collagen consists of amino acids. However, almost one third of the amino acids in collagen are glycines, the smallest amino acid with only a single H atom as the side chain. About 1000 amino acid groups are aligned to form one left-handed α chain, with the typical *Gly-X-Y* repeats. In this triplet, X is most likely proline, and Y 4-hydroxyproline. Three such α chains intertwine into a right-handed superhelix of molecular weight on the order of 300 kDa. This hierarchy of fiber bundles alternately twisted in opposite directions is thought to be one of the primary sources of high tensile strength critical to collagen function, as it is able to convert longitudinal tension to a much easily supported lateral compression on an almost incompressible triple helix. Glycine has to occur every third position because that is where the α helices go through the triple helical center, too congested for any larger amino acid type. Many collagen amino acid side chains are hydroxylated to facilitate interchain hydrogen bonding, critical at stabilizing the tertiary structure. For example, proline is modified by prolyl hydroxylase to hydroxyproline, in which ascorbic acid is required for enzymatic activity [38].

Several collagen types are present in articular cartilage (Table 2.1). Type II collagen is the major structural collagen in cartilage, comprising 95% of total collagen in adult hyaline cartilage [38]. It further assembles through quarter-staggering and crosslinking to form collagen fibrils. Fibrils then interact with proteoglycan to define the material properties of extracellular matrix. Unlike in other connective tissues, single collagen fibrils are scattered seemingly randomly in cartilage. Several other collagen types are also present in cartilage. Type IX (1%) is very important in joint remodeling. Its covalent association with type II collagen molecules strengthens and stabilizes the network, and is broken down during normal matrix growth, repair and

Table 2.1: Collagen Types Present in Articular Cartilage †

Collagen	Formula	% total	Properties or Possible Functions
Type II	$[\alpha_1(II)]_3$	80-90%	Forms fibrils and provides tensile strength to cartilage collagen network.
Type VI	$\alpha_1(VI)\alpha_2(VI)\alpha_3(VI)$	1-2%	Forms network of fine fibrils between larger fibrils.
Type IX	$\alpha_1(IX)\alpha_2(IX)\alpha_3(IX)$	10%	Present on surface of type II collagen fibrils. May play a role in cross-linking fibrils to each other or other ECM molecules.
Type XI	$\alpha_1(XI)\alpha_2(XI)\alpha_3(XI)$	5%	May play a role in determining type II collagen fibril diameter.

†Adopted from D.D.Dean [5]

also abnormal cartilage destruction [6, 31].

The biosynthesis of type II collagen involves first the formation of procollagen, which has propeptides of about 100 residues on both the *N*- and *C*- terminals of the collagen molecule. The presence of propeptides are necessary for the proper assembly of the triple helix. The Pro and Lys residues are hydroxylated to Hyp, 3-hydroxy-Pro, and 5-hydroxy-Lys in RER, as part of the post-translational chemical modification. This event precedes the folding of the three polypeptides, which requires the involvement of Hyp for stability.

2.1.2 Proteoglycan

Proteoglycan (PG) is the other major structural component in cartilage. While collagen defines the major framework, proteoglycan gives matrix the ability to undergo reversible deformation. Proteoglycan is a subcategory of glycoproteins, the nomenclature for compounds associating both protein and carbohydrates. In this case, PG is the covalent association of a polypeptide core protein with one or more glycosaminoglycans (GAG) to form a group of complex heterogeneous macromolecules. Most of the heterogeneity comes from affiliating different GAG chains and varying numbers of them. These GAG chains usually account for over 90% of PG's total mass [49]. There are several types of PG found in cartilage, but the most abundant and structurally important one is aggrecan, the aggregating proteoglycan (Figure 2-2).

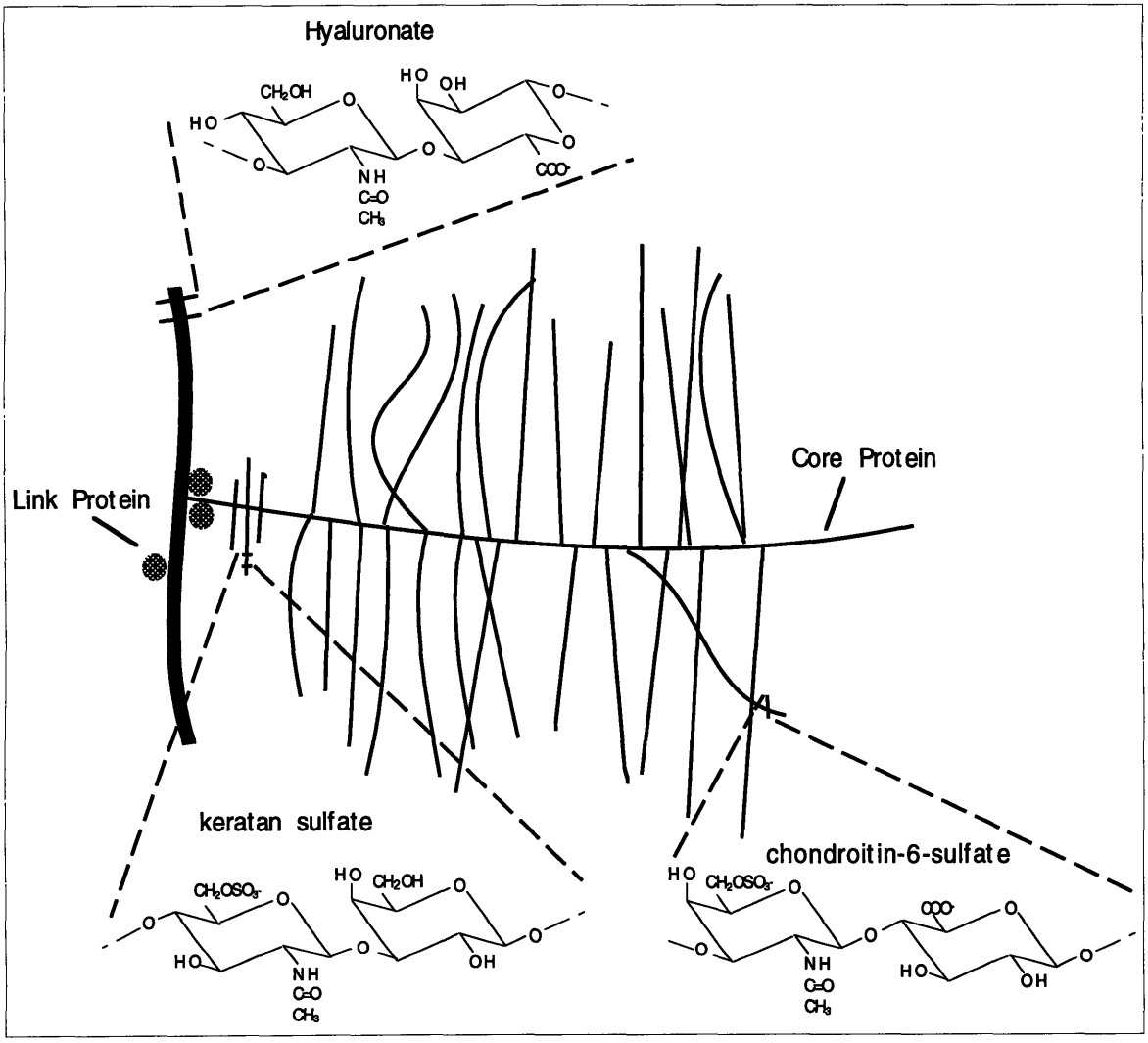


Figure 2-2: Schematic of Aggrecan Structure and interaction with hyaluronan. Many chondroitin sulfate and keratan sulfate groups are covalently associated with a core protein. Each core protein is then noncovalently associated to a long chain hyaluronic acid molecule stabilized by link proteins.

The core protein of aggrecan is a 210 kDa polymer with three globular domains (G1, G2 and G3) and two extended segments (E1 and E2) [25]. G1 is the first domain at the *N*-terminal, and the site of attachment to hyaluronan, a independent long chain GAG. G2 has a similar structure to G1, and is separated from it by a 21 nm extended region called E1. The long extended domain following G2 is called E2. This region of roughly 260 nm long is where the majority of GAG chains are covalently attached. At the *C*-terminal lies the G3 domain, a distinctive structure with unknown functions. In aged cartilage matrix, this domain is usually lost due to cleavage by proteases within E2. Varying lengths of E2 is another sources of heterogeneity for PG sizes in mature cartilage.

A glycosaminoglycan molecule is defined as a unbranched polysaccharide chain usually of alternating uronic acid and hexosamine residues. There are two major GAG types in cartilage, both of which are covalently linked to the core protein. Chondroitin sulfate (CS) is the major GAG type in ECM. It consists of repeating *D*-glucuronic acid and *N*-Acetyl-*D*-galactosamine disaccharide units. Each hexosamine residue is also sulfated, either at the 4' or 6' positions. Under physiological pH, chondroitin sulfate chains are highly anionic, resulting from deprotonation of both carboxyl and sulfate residues. Keratan sulfate (KS) is also a negative charge contributing source. The repeating disaccharides are *D*-galactose and *N*-Acetyl-*D*-galactosamine instead. However, since *D*-galactose is neutral at physiological pH, KS contributes one charge per disaccharide.

Aggregates of core proteins and GAG's are not randomly distributed in the ECM. They are noncovalently connected to each other through another important GAG, the hyaluronic acid (HA). It is the backbone for up to 200 aggrecan units to bind at their G1 domain, alternatively named the hyaluronic acid binding region (HABR). Neither of the disaccharide units in hyaluronate, *D*-glucuronic acid and *N*-Acetyl-*D*-glucosamine, are sulfated [51]. The association of aggrecans to HA is facilitated and enhanced by link proteins, the resulting conglomerate has mass on the order of 5 x 10^7 to 5 x 10^8 [49].

Biosynthesis of proteoglycan starts with the assembly of the protein core. Varia-

tions in its amino acid sequence are results of alternative splicing of exons on a single gene product. Then the addition and modification of GAG chains occur. Having no standard template or sequence to follow, glycosylation is rather inhomogeneous. GAG chains are added one at a time through UDP-sugar intermediates. For example, in the case of CS chain addition, xylose is first attached to serine (or threonine) by xylose transferase (through UDP-xylose), followed by the addition of two galactose residues, and then alternating addition of *N*-acetyl galactosamine and glucuronic acid. Sulfate incorporation occurs after the synthesis of the unsulfated GAG chains, and in the Golgi Apparatus through a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) intermediate. In the case of CS, both the 4 and 6 positions of *N*-acetyl galactosamine can be sulfated, and it has been shown that older cartilage synthesizes more 6-sulfated CS chains.

Since HA is not synthesized on an existing core protein, a different mechanism is used. An HA molecule is initiated in the inner plasma membrane, and the growing chain is extruded into the extracellular space. The precursors are still the UDP-sugars, but since HA doesn't go through Golgi complex, no sulfate groups are added.

2.2 Cartilage Mechanics

Articular cartilage biomechanics, i.e. how this important joint support material works, depend very much on the existence and maintenance of numerous contributing forces. On the one hand, these opposing forces provide functionality and allow remodeling. On the other hand, excessive imbalance results in loss of material properties and joint destruction.

2.2.1 Force Balance in ECM

The first order force balance in ECM is ionic in nature. Both collagen and proteoglycan are electrolytes capable of association/dissociation of their charge groups at different pH. For collagen, as well as protein cores of PG, the ionizable groups come from the amino and carboxyl residues (roughly 250 each in every collagen molecule).

Although most of them have pK's far away from the physiological pH, the entire collagen molecule is pretty much net isoelectric [52]. Therefore, collagen is not a significant contributor in ionic force balance. The story is totally different for proteoglycans. Their polysaccharide components account for almost all the net charges in many connective tissues. As mentioned above the charge groups in PG are the carboxyl groups (HA and CS) and sulfate groups (KS and CS), both capable of deprotonating to become negative charge carriers. Since the pK's for these groups are very low (between 2 and 4 observed), all of them are ionized under physiological conditions, resulting in the large net negative fixed charge density in ECM. The name 'fixed' comes from the fact that these charges are immobilized on the long GAG chains and in a network of collagen fibrils. The 'free' mobile charges, for example the much smaller hydronium (H_3O^+) and sodium ions (Na^+), then partition themselves across ECM and synovium boundaries to maintain bulk electroneutrality. As we can see, the densely packed negative charges create a strong swelling pressure to expand, only to be limited by the framework of collagen network and any imposing mechanical loading. It has been shown that if allowed, the PG network can swell up to 5 times its size in ECM [25]. This strong swelling pressure is the source of compressive resistance offered by ECM. One thing to note though. Articular cartilage has an active matrix, not only in the sense that its components are constantly regenerated, but also that the water content can change under loading or stress relaxation. As water content is changed, as under compression, the concentration of ions changes, so does their interaction, or the characteristic Debye length. The mobile charges change their partition accordingly. Therefore, dynamic equilibrium and ionic charge balance are keys to matrix function.

Aside from ionic interactions, other noncovalent forces, such as hydrogen bonding are also very important. For example, bridged water molecules between amino acid hydroxyl groups, in particular between 4-hydroxyprolyl residues, associate to help stabilize the triple helix. But much stronger covalent cross-linking is believed to be ultimately responsible for the tensile strength of collagen and the structure it forms. Since there is no cysteine residues in the fibril-forming collagen polypeptides, the

usual means of cross-linking – disulfide bonding is not present in collagen. Rather a different mechanism is employed involving lysyl oxidase. With the help of this Cu-containing enzyme, lysyl residues are converted to their corresponding aldehydes: allysines, which subsequently undergo aldol or aldimine condensation to covalently link structural components. The extent of cross-linking increases with age, which is why single collagen molecule is only obtainable in very young animals.

Various ionic, covalent and noncovalent forces interact and balance to give articular cartilage its unique material properties and function. Indirectly but ultimately, chondrocytes are responsible for producing macromolecules and enzymes to maintain such a balance. In growth or disease, one or more processes become dominant to allow changes to occur. However, if balance is not restored, matrix integrity and function will be undermined, leading to destruction.

2.2.2 Cartilage Degradation

In articular cartilage, collagen types II and IX are major sources of structural integrity of the ECM, by containing adequate amount of water and by resisting tensile force and osmotic pressure of proteoglycans. Type IX collagen acts as the interfibrillar "glue" that stabilizes and lends cohesion to collagen network of normal articular cartilage [6]. This cohesive framework physically traps aggregating proteoglycans, and provide binding surfaces for small non-aggregating proteoglycans like decorin and fibromodulin. However, this network is not covalently constructed, i.e. there is no covalent linkage between two of the most important cartilage components, collagen and proteoglycans. Therefore, extensive cleavage is not required to break down a system under constant swelling pressure. Little alteration in the meshwork (loosening of interfibrillar association) by limited collagenolysis, or alternatively through cleavage at the E1 region of proteoglycan, can result in significant loss of GAG diffusing out of the matrix and compromised material properties [30].

Matrix degradation can be enzymatic or non-enzymatic. Among enzymatic agents, matrix metalloproteinases (MMPs) have been the focus in literature as they are very much active under physiological pH levels present in ECM [48, 11]. Other proteases

and glycosidases, as well as nonenzymatic agents such as oxidative radicals can also be involved in matrix degradation [57].

It has been shown in OA cartilage, the content of natural type II collagen reduces significantly [10], presumably following increased hydration and denaturation by MMPs. Also, electron microscopic studies have demonstrated damage to collagen fibrils in RA [53]. Among the metalloproteinases, interstitial collagenase (MMP-1), gelatinase (MMP-2) and neutrophil collagenase (MMP-8) [59] have all been shown to cleave collagen [30], specifically at a single locus on the 3 α-chains of type II collagen. Another important endogenous enzyme is stromelysin (MMP-3), which can cleave at the *N*-telopeptide cross-linking sites of type II collagen, but not within the triple helix [6]. However, it can cut all three α chains of type IX collagen. Beyond direct actions, stromelysin is also involved in the activation processes of other proteases [18, 1].

Cleavages on PG occurs mostly on the protein core between G1 and G2 [13], and in the E2 region where CS and KS chains are attached [44]. Once the peptide bonds are broken, the entire structure not attached to HA is free to diffuse, and many ends up outside of ECM. Several types of MMPs, including stremolysin and collagenase have been shown responsible for many cleavage products [5]. However, one of the major cleavage site within E1 is still not accounted for [8].

Activities of proteases are very low in healthy joints [5], and there exist many protective measures to prevent cartilage from excessive degradation. Requirements of cytokine stimulation and activation from proforms are two examples. IL-1 discussed below has been shown to involve in both processes.

2.2.3 Interleukin 1

Interleukin 1 (IL-1) is a key member of the cytokine family, a collection of polypeptide hormones that mediate boy's response to various changing conditions. Similar to other cytokines, IL-1 level is negligible unless activated by some specific process, such as the immune response; and it conveys different messages depending on the types of target cells [7]. IL-1, and several other growth factors are particularly important in adult articular cartilage, as it has no means of communication through blood vessels

or nerve fibers, and cells are remote to each other.

The term IL-1 refers to two biochemically distinct but structurally related members, IL-1α and IL-1β. Although both share the same set of receptors with almost identical affinity, IL-1β is believed more dominant in extracellular space with pI near 7 [12]. Although IL-1β is active in extracellular compartment, it has no signal peptide sequence, common to proteins secreted out of cell membranes [7]. The proforms are converted to be biologically active through the actions of serine proteases, particularly elastase and plasmin. The mature polypeptide of IL-1β has molecular weight of 17.5 kDa.

Chondrocytes have been shown to produce IL-1β, as well as a host of other cell types [54]. Although small amounts of IL-1β may be present in cartilage under normal conditions, its production and presence is dramatically increased in degenerative pathology of the joint such as OA [21, 19]. Several endogenous sources capable of inducing IL-1β production have been categorized [55]. *Cartilage and bone debris*, either from a traumatic events or from age-related accumulation, may cause the secretion of IL-1β and initiate catabolic processes. *in intro* evidences have also shown that *macromolecules of cartilage* such as PG and different collagen types, when exceeding a concentration limit, can initiate IL-1β production. *Immune complexes*, such as immunoglobulins, have been detected in increased concentration in OA than in healthy joints, implying potential role in the induction of IL-1β. Other exogenous sources, such as synovial tissue and neutrophil leukocytes, may also introduce IL-1β into ECM [9].

IL-1β has many target cell types, including chondrocytes [54]. The structure and post-binding signal transduction have not been fully characterized. One possibility is that the IL-1β/receptor complex gets translocated to the nucleus upon binding, where it interact on the level of transcription [56]. Another possibility is the use of a signal transducing complex in the plasma membrane that in turn activates a second messenger system like cAMP. In general, cells stimulated with IL-1β demonstrate increasing cytosolic calcium levels, higher sodium/potassium ion fluxes, and increased protein kinase activities.

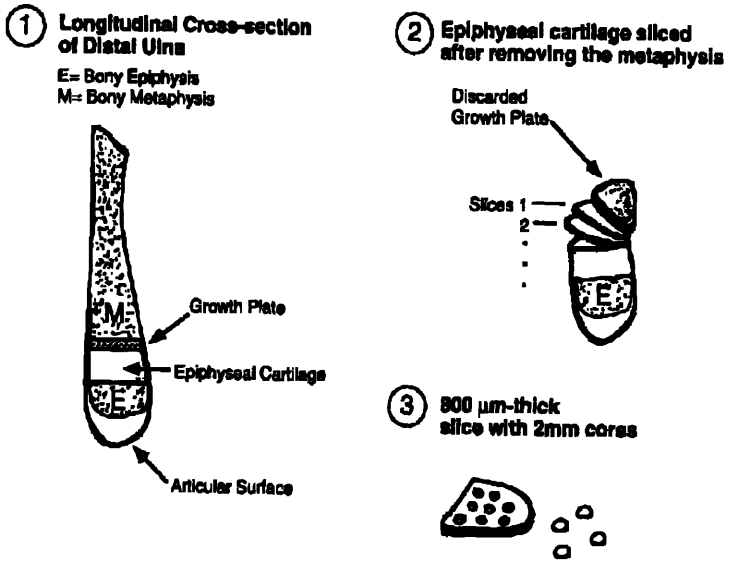
2.3 Tissue Culture Systems

In order to study cartilage and chondrocyte behaviors and properties, tissue culture systems are used to better isolate and characterize individual responses. Here tissue culture system refers to controlled *in vitro* culturing of native tissue to preserve tissue and cell viability and presumed similar behavior as is *in vivo*. In cartilage research, many aspects of tissue property and function have been studied through explant cultures. Following sterile harvesting, live tissue can be obtained and cultured in defined media containing essential nutrients, including amino acids, vitamins and minerals. It was found that for long term cultures, some growth factors and proteins in serum, e.g. fetal calf serum (FCS) are essential at keeping metabolic rates comparable to those *in vivo* [46]. FCS is more effective in stimulating metabolism than adult animals [46], and insulin-like growth factor is believed to be the active component [14]. After harvesting, it takes four to five days in culture before cartilage samples reach a constant metabolic state. In literature, for PG, this state is defined as constant PG levels in ECM as the result of the rates of synthesis and catabolism of PGs being equal [43].

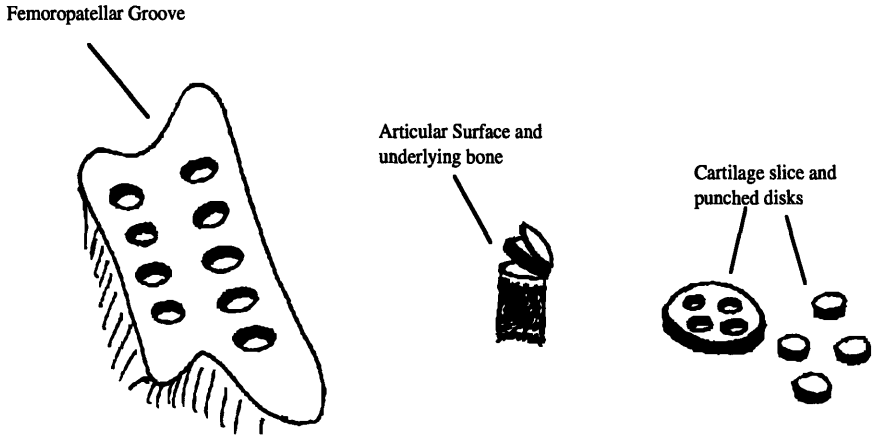
2.3.1 Calf Epiphyseal System

The calf cartilage culture system in this lab takes samples from the distal ulnae of newborn calves (A. Arena Co., Hopkinton MA). Epiphyseal cartilage in between the growth plate (metaphysis end) and the bony epiphysis is sectioned to desired thickness (0.8 to 2 mm) (Figure 2-3). Then 2 to 3 mm-diameter disks are punched from the slices and incubated in culture media under $37°C$ and 5% CO_2. The culture medium consists of low glucose Dulbecco's Modified Eagles Medium supplemented with additional 0.4 mM proline and 0.1 mM non-essential amino acids, with daily supplements of 1% fetal calf serum, 4 mM L-glutamine and antibiotics.

It was found that calf epiphyseal cartilage contains by volume, about 7% blood vessels and 10% cells, with the remaining space occupied by water and the macromolecules [2]. Chondrocyte density is estimated at 133,000 cells per mm^3 [58]. Mea-



Harvesting of Calf Epiphyseal Cartilage, from Nada Boustany.



Harvesting of Adult Articular Cartilage.

Figure 2-3: Schematic of cartilage tissue harvest. The three steps are named *coring*, *slicing*, and *punching*. Usually multiple slices of 0.8-2 mm thick can be obtained from each epiphyseal cartilage core, but only one slice of 0.6-0.8 mm from adult articular surface. In order to obtained cylindrical disks with parallel surfaces, the first few μm's are discarded. For articular cartilage tissue harvest, only sources of the femoropatellar groove is shown. Cartilage explants are also taken from the two condyles and the patellae.

sured DNA concentration has mean of 0.015 $\mu g/mg$ wet weight. Measured GAG density varies according to anatomical locations, increasing closer to the epiphysis. The values range from 40 to 80 mg/mL tissue water. However, because of the relatively large volume taken by vessels and cells, this calculation of GAG concentration underestimates the real ECM GAG density by about 15-20% [2].

This epiphyseal cartilage culture system was selected mainly for reasons of tissue abundance. Furthermore, the cartilage has relatively higher chondrocyte density than mature cartilage, providing metabolically more active specimens. The explants are relatively easy to harvest and culture, and are typical of hyaline cartilage in terms of metabolism and material properties. However, as mentioned above, young cartilage is generally vascularized regardless of the site of explant, with possibilities of complication from multiple cell types.

2.3.2 Adult Articular System

Mature articular cartilage is presumably more representative of aging tissue and more prone to arthritis. Although bovine articular cartilage has been successfully harvested and cultured in this lab, difficulties in handling did not make it a routine practice (Figure 2-4). Such difficulties include it being less cellular and consequently less metabolically active; harder to harvest due to less tissue abundance and harder bones; and harder to culture due to higher rate of GAG loss. As a consequence of difficulty in harvesting, the longer time tissue needs to be exposed, and the more likely to to have an infection. Moreover, variations both in terms of different joints in one animal and from one animal to another are rather high, making large sample size a necessity before making meaningful conclusions.

From the literature, mature cartilage have chondrocyte density on the order of 47,000 cells per mm^3 [58], 4-5 times lower than young cartilage, a number confirmed later in this study. The effects of serum in culture have been studied. Without serum, chondrocytes settle at a much lower level of synthesis. With an optimal 20% of FCS, synthetic rates have been observed similar to those *in vivo* [46]. However, cellular outgrowth in long term cultures have been observed with 20% addition of

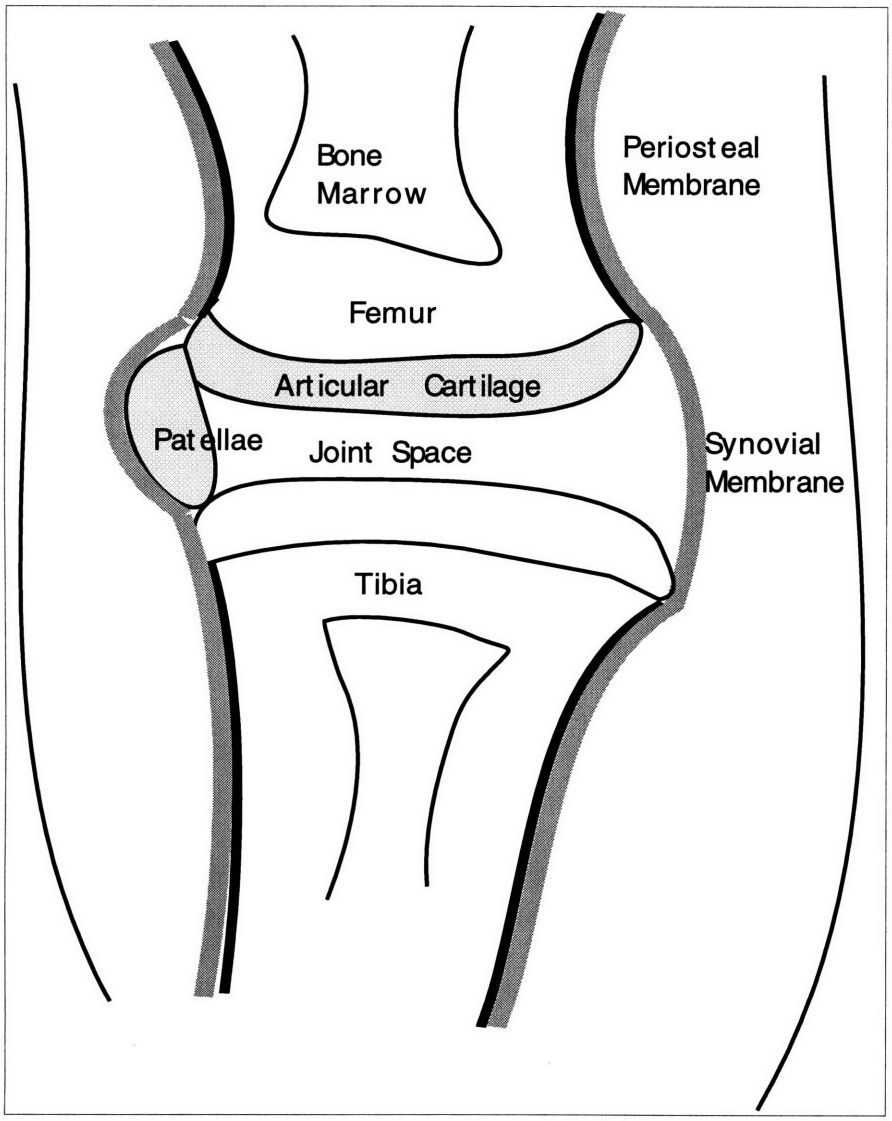


Figure 2-4: Schematic of adult bovine femur-tibia joint used for harvesting cartilage tissues. Articular cartilage is taken from femoropatellar groove, femoral condyles and the patellae (areas shaded).

FCS [16, 32]. On the other hand, GAG concentration is not significantly different in the two systems, although PG turnover is much faster in mature cartilage, with mean half life of 10 days compared to over 20 days in younger tissue [26].

2.3.3 Other Cartilage Culture Systems

Bovine adult articular and calf epiphyseal cartilage cultures are not the only ones studied. Many other sources of cartilage are reported in literature. Selected few are summarized in the following table. Most people use DMEM or Ham's F12 media, with supplements of serum, non essential amino acids (NEAA), l-glutamine, ascorbate, proline and antibiotics. Although these supplements enhance explant culture, their compositions are important in quantitative characterization of cartilage metabolic behavior, as discussed in the next chapter.

2.4 Effects of IL-1β on Cartilage

IL-1β is a multifunctional hormone, mostly in the up-regulation of cellular metabolism and increased expression of several genes coding for biologically active molecules. In cartilage, it is believed to be involved in both the inhibition of synthesis and the promotion of degradation. The inhibitory effect of IL-1 on the synthesis of proteoglycan is well established. The limited amount of proteoglycan still synthesized appears to have the same structure as those under normal conditions [21]. At sufficient concentration, IL-1 is able to bring about a complete inhibition of PG synthesis. Similarly, IL-1β reduces the rate of synthesis of major cartilage collagen types (II and IX), while promoting the synthesis of some non-cartilage collagen types (II and III) [21]. Elevation of degradation occurs through activation of proteases by IL-1β, particularly neutral metalloproteases stromelysin and collagenase [22]. During OA, where IL-1β level is higher, increased cartilage hydration and ultrastructural changes of collagen fibers are observed, indicating alterations in the collagen fiber network. The release of free proteoglycan fragments also increases significantly, causing loss of matrix components and compromised ability to withstand compressive loads.

Table 2.2: Cartilage Sources and Tissue Handling in Selected Literature.

Joint Source	Tissue Size	Principle Investigators	Major Interests	Comments
porcine laryngeal cartilage	N/A	T.E.Hardingham [45]	PG studies	Cartilage obtained fresh from the slaughterhouse, dissected free of adhering tissue and perichondrium, then shaved with a Stanley Surform. Immediately used for extraction of PG. Referenced by many.
bovine nasal septa	N/A	C.J.Handley [29]	PG studies	extracted in 4M guanidine HCl for 48 hours.
carpo-metacarpal joints of 4-5 month old calves	full thickness discs; 1-1.5mm thick, 5mm diameter	V.C.Mow [42]	mechanical compression studies	havested from central region of joint within 3-4 h after death. DMEM supplemented with BSA, NEAAs, glu, and antibiotics.
bovine metacarpo-phalangeal joint, 1-2 year old steer	50-150mg wet weight for each sample	C.J.Handley; V.C.Hascall [46]	PG Studies	Cartilage obtained within 2 h of death. Articular surfaces exposed under sterile conditions with rinses of sterile media to prevent drying. Incubated at $37°C$ with DMEM, 1g/l glucose and organic buffers and amino acids. Paper referenced by many.
bovine sesamoid bones from metacarpo-phalangeal joints of 6 month old calves	complete cartilage with bone attached	G.H.Korver [17]	Culture system for anatomically intact articular cartilage	medial sesamoid bones from MCP joints obtained within 2 h after slaughter, and washed with GBSS. Ham's F12 medium, supplemented with 10%FBS and antibiotics.
porcine metacarpo-phalangeal joints of adult pigs	N/A	J.T.Dingle [24]	Synovial catabolic factor study	Explants of articular cartilage from the metacarpals of young pigs (5-9 months old). Cultured in DMEM with 15% (v/v) heat inactivated normal rabbit serum.
bovine metacarpal of 6 month old calves	full length punched explants, 4 mm in diameter	D.Heinegard [35]	Mechanical Studies	Cartilage explants obtained within 2 h of sacrifice. Ham's F12 medium, no serum, with HEPES and supplemented with ascorbate and antibiotics.
bovine nasal cartilage	8mm cores, 1mm thick discs, highly uniform (60-70 mg)	J.Steinberg [23]	Tissue culture model of RA, PG studies	Bovine snouts obtained fresh at the time of slaughter. Nasal septum cleaned with betadine/soap solution, then placed in GBSS. Found microscopic vascular channels.
2 year old bovine nasal septa	2mm diameter, 25-35 mg	D.Heinegard [36]	PG studies	cartilage obtained at the time of slaughter, and rinsed with PBS. Most avascular region taken. Ham's F12 medium with glu, HEPES and antibiotics.
2 year old bovine femoro-patellar grooves	2 to 3mm diameter, 500 μm thick	R.Sah [32] and A.J.Grodzinsky	Growth factors and mechanical compression	1-2 slices from each core. Disks punched from the central area of slices. DMEM with NEAA, pro, ascorbate, and choices of sera or growth factors.
1-2 year old bovine knee joint, both sides of the patellar surface of the femur	five 8mm cores, mean thickness 1.5mm	J.J.Parkkinen [20]	Cyclic loading on PG synthesis	Knee opened under sterile conditions, cartilage cores obtained and attached to petri dishes with a tissue adhesive. Explants cultured for 2 days before experiment. Eagle's MEM with Earle's salts, supplemented with antibiotics, ascorbate and glu.

The recovery of cartilage after an IL-1 attack has also been studied. The increase in proteoglycan synthesis is very slow, requiring weeks to recover. In contrast, degradation induced by IL-1 returned to the normal level relatively quickly (3 days) [44].

It has become clear that the ultimate state of cartilage matrix does not depend upon a single factor, but the balance/imbalance of a variety of forces, including cytokines and their antagonists, procytokines and their activators, proteinases and their activators/inhibitors, and activator inhibitors, to just name a few. For example, IL-1 has been shown to be able to inhibit the production of tissue inhibitors of metalloproteases (TIMP) [5], altering the balance in metalloproteases and their inhibitors to favor degradation. It is also able to up-regulate (down-regulate) plasminogen activator (plasminogen activator inhibitor) to favor the activation of metalloproteases and itself [15]. The total balance of the entire complex network of hormones, enzymes and structural molecules are required for the well being of the matrix, and the lack of it, combined with limited ability of chondrocytic regulation, is the ultimate source joint degenerative diseases, like OA.

Chapter 3

Materials and Methods

3.1 Materials

Hanks Balanced Salt Solution (HBSS) and Dulbecco's Modified Eagle's Medium (DMEM) with 25 mM HEPES were purchased from GIBCO (Grand Island, NJ). DMEM was also purchased from JRH (Lenexa, KS), as was fetal calf serum (FCS). Non-essential Amino Acids (NEAAs), *l*-glutamine, antibiotics (10,000 units penicillin, 10 mg streptomycin, 25 μg/ml amphotericin B in 0.9% NaCl) and papain were bought from Sigma (St.Louis, MO). Hoechst 33258 dye was from Hoefer Scientific Instruments (San Francisco, CA). Calf thymus DNA standards and shark chondoitin sulfate standards were also from Sigma. Radioactive sulfate was from New England Nuclear (Boston, MA), and radioactive proline from Amersham (Arlington Heights, IL). Ruthenium Hexammine Trichloride (RHT) and 8% glutaraldehyde were from Polysciences (Warrington, PA), and sodium cacodylate was from Fluka Chemie AG (Buchs, Switzerland). There are two sources of recombinant interleukin 1β, one courtesy of Professor Lee Gehrke, and the other from Cistron Biotechnology (Pine Brook, NJ).

3.2 Cartilage Explant and Culture Condition

Intact femur-tibia joint of an adult cow were obtained from a local abattoir (A. Arenas Co., Hopkington MA) on the day of slaughter. Under sterile conditions, the joint was opened, and cartilage tissues were exposed from three types of joint surfaces, femoropatellar groove (FPG), two femoral condyles (FC), and the patellae (P). The four joint surfaces and underlying bones were separated with a hand saw to be later mounted on the drilling vise. During this process (20-30 min) rinsing solution was applied continuously, prepared from HBSS and 1% (v/v) antibiotics. Once separated, cartilage surfaces were immediately covered with plastic wrap and placed on ice. On the vise, 3/8 inch diameter cores were drilled perpendicular to the articular surface. They were 2-3 mm long with less than 1 mm of cartilage at one end. Visibly damaged or arthritic cartilage were avoided. Cores were placed in culture dishes filled with HBSS and antibiotics and labeled of their relative positions. After drilling, Thickest possible (600 μm-800 μm) cylindrical cartilage slices were cut out with parallel surfaces using a sledge microtome (American Optical, Buffalo NY), and again placed in HBSS with antibiotics. The first few μm from the articular surfaces were discarded, so were those close to the tide mark. Four cylindrical disks of 3 mm in diameter were then obtained from each slice using a dermal punch (Miltex, Lake Success NY). Each disk was immediately transfered to a 24-well culture dishes containing exactly 0.5 ml supplemented media, and pre-incubated at $37°C$ with 5% CO_2 for at least 30 min. Culture media was prepared by from base media stock and daily supplements. Base media stock was prepared immediately before each experiment and includes DMEM, 0.1 mM NEAAs and 0.4 mM additional *l*-proline. Daily supplements include 20% FCS [43, 32] and 1% each of *l*-glutamine, ascorbic acid, and antibiotics.

The entire process of harvesting, from exposure of articular cartilage to disks freeswelling in media, took 7-9 hours to complete for about 100 disks, during which time cartilage surfaces were either continuously rinsed with or immersed in HBSS and antibiotics. Generally about 10 slices can be obtained from the two faces of FPG; 6-8

slices each from the two FCs; and 5-6 slices from the patellae. During microtoming and punching, some slices (mostly from FCs) showed dramatic curling. Disks from such slices usually showed relatively large changes in wet weights from day 1 to the last day of experiment, and many of them yielded minimal chondrocyte activity at the end of experiments.

3.3 Measurements of Bulk Properties

3.3.1 Wet and Dry weights

Approximately 15-18 hours after cartilage harvest, all disks were weighed as sterile as possible on a microbalance (Mettler, Highstown NJ) to obtain initial wet weights. Each disk was then immediately transfered to another culture dish filled with $0.5ml$ newly prepared supplemented media. The process of weighing for each disk took less than 2 minutes. On the last day of the experiment, each disk was again weighed to obtain its final wet weight. Following lyophilization of 18-24 hours, dried disks were measured for their solid mass.

3.3.2 Tissue DNA and GAG

Papain was used to dissolve cartilage disks in preparation of DNA and GAG measurements. 125 μg/ml papain solution was prepared with 5 mM cysteine hydrochloride, 0.1M phosphate buffered saline and papain. Each disk was added exactly 0.5 ml of the above solution, and left in $60°C$ water bath for at least 24 hours or until all tissues were dissolved.

The DNA content of each disk was determined using the bisbenzimidazole (Hoechst 33258) dye binding assay [37]. A SPF-500 spectrofluorometer (SLM Instruments, Urbana IL) was used for photometric measurements. This assay is based on enhanced fluorescence in high salt solution following binding of bisbenzimidazol with double stranded DNA. Duplicate $50\mu l$ cartilage digest solutions were aliquated for each disk, to which 2 ml Hoechst dye solutions were added. DNA dye solution was prepared

immediately before measurement, containing 0.1 μg/ml Hoechst 33258, 10 mM Tris, 1 mM Na_2EDTA, and 0.1 M NaCl. Fluorescence measurements as ratios to blank reference were recorded, and later converted to concentrations using calf thymus DNA standards. Six DNA standards were prepared ranging from 5 $\mu g/ml$ to blank (DI water). This procedure was repeated if the duplicates differed by more than 5%.

The GAG content of each disk was determined using dimethylmethylene blue (DMB) dye binding assay [33], with absorbance measurements performed on a 3B UV/VIS spetrophotometer (Perkin Elmer, Norwalk CT) [39]. Duplicate 20 μl tissue digests were aliquoted and added to 2 ml DMB dye immediately before measurements of absorption at 525 nm. Absorbances were converted to concentrations using shark chondrotin sulfate standards. Six GAG standards were prepared ranging from 1,000 μg/ml to blank (DI water).

3.4 Measurements of radiolabel incorporation

$Na_2S^{35}O_4$ and ^3H-proline incorporation rates were used as indicators of GAG and protein synthesis, respectively [28]. On the day of labeling, cartilage disks were incubated in base media stock, 1 mCi/ml $Na_2S^{35}O_4$, 10 mCi/ml ^3H-proline, and supplements. Final concentrations of radioactive precursors varied with total hours of incubation. It has been shown that radioincorporation increases linearly with time between 0.5 and 24 h [28]. For 18-24 hours of radiolabeling, 10 μCi/ml $Na_2S^{35}O_4$ and 20 μCi/ml ^3H-proline were used [32]. At the end of the radiolabeling period, each cartilage disk was washed six times sequentially in 1-2 ml HBSS for one hour. Previous studies have shown almost complete removal of unincorporated radiolabels and less than 5% loss of newly synthesized GAG out of ECM during the labeling period [28]. Cartilage disks were then digested with papain as discussed above.

For liquid scintilation counting, three aliquots of tissue digest solution, 40 μl each, were added scintilation cocktail containing 460 μl of Guanidine-HCl and 2 ml of Ecolume. They were mixed thoroughly till the cocktail became clear, before being counted. In order to estimate new GAG synthesis, radioactivities for incubating

Table 3.1: Sulfate Sources in Radiolabeling Media.

source	$[SO_4]^{2-}$ in source	% of final	final $[SO_4]^{2-}$	Comments
culture media	$0.8mM$	75.6%	$0.60mM$	
serum	$0.3mM$ [4]	20%	$0.06mM$	numbers for human sera. varies with batch.
antibiotics	$20mM$	1%	$0.20mM$	assuming streptomycin is entirely sulfated.
$S^{35} - Na_2SO_4$			$0.02mM$	varies with batch, but small compared to total.
total			$0.88mM$	

media were also measured. However, instead of 40 μl aliquot, 10 μl of 'hot' media and 30 μl of DI water were drawn, with addition of same amount of Guanidine-HCl and Ecolume. Original scintilation counts per minute (cpm) values were corrected for spillover across respective windows of the two isotopes using the program 'scintproc'. Two closest cpm's from the three aliquotes were further analyzed if they differ less than 5%.

In order to estimate synthesis from scintilation counts, it is necessary to know the exact concentration of precursor pool. For new GAG synthesis, the sources and concentrations of sulfate are listed in Table 3.1, yielding close to 0.88 mM of sulfate in final labeling media. FCS and radiolabel tracers may have different sulfate concentration for different experiments depending on particular batches and original activities, but the variations are small compared to the total concentration. The unexpectedly high sulfate content in antibiotics comes as the counter ion of streptomycin ($C_{42}H_{84}N_{14}O_{36}S_3$, M_r 1,457). Therefore, the 10 mg/ml streptomycin concentration in the antibiotic solution gives 2 mg/ml of sulfate, or 0.02 M.

Given cpm's for both media and tissue digest, and concentration of sulfate in the labeling media, tissue digest sulfate concentration can be calculated, and converted to amount of GAG synthesized assuming one newly synthesize GAG incorporates one sulfate group.

$$[SO_4]^{2-}(\text{tissue}) = \frac{0.88\text{mM} \cdot \frac{0.01\text{ml}}{2.5\text{ml}}}{\frac{0.04\text{ml}}{2.5\text{ml}}} \cdot \frac{\text{cpm}_{tissue}}{\text{cpm}_{media}} = 0.22\text{mM} \cdot \frac{\text{cpm}_{tissue}}{\text{cpm}_{media}} \tag{3.1}$$

$$\begin{aligned}\text{GAG synthesized(nmol)} &= [SO_4]^{2-}(\mu\text{mol/ml}) \cdot 0.5\text{ml} \cdot \frac{10^3\text{nmol}}{1\mu\text{mol}} \\ &= 0.22\text{mM} \cdot 0.5\text{ml} \cdot 10^3 \cdot \frac{\text{cpm}_{tissue}}{\text{cpm}_{media}} \\ &= 110\text{nmol} \cdot \frac{\text{cpm}_{tissue}}{\text{cpm}_{media}}\end{aligned} \tag{3.2}$$

In later analysis, GAG synthesis was normalized with time to nmol per 24 hour period, assuming linear incorporation.

A conversion factor is similarly calculated for proline incorporation. Media *l*-proline concentration is supplemented to be $0.5mM$, with negligible contributions from serum (20% of $0.161mM$ [41]) and ^3H-proline labels. So,

$$\text{protein synthesized(nmol)} = 62.5\text{nmol} \cdot \frac{\text{cpm}_{tissue}}{\text{cpm}_{media}}. \tag{3.3}$$

3.5 Measurements of media GAG

GAG concentration in media were measured as indicator of the extent of GAG release related to PG catabolism. Since there is no GAG disaccharides present in culture media before incubation with cartilage tissues, any GAG measured after incubation can only arise from release out of ECM. GAG assays were performed with a microplate reader (VMAX, Molecular Devices) using 96 well dishes. Four $20\mu l$ aliquots each for each media collection were added to 200 μl of DMB dye solution. Closest two readings less than 5% different were averaged and converted to GAG concentration similar to tissue GAG measurements. The same shark chondroitin sulfate standards were used but with lower concentration (125 μg/ml to blank).

3.6 Tissue Fixation and Staining

On the day of fixation, designated cartilage disks were moved from incubating media directly to 1 ml fixation solution with RHT for 4.5 hours at room temperature. Fixation solution contains 2% (v/v) glutaraldehyde, 0.05 M sodium cacodylate, 0.7% (w/v) RHT, with pH of 7,4 (adjusted with 0.1 M HCl). RHT was added 10 min prior to fixation. They were then moved to 4 $^{\circ}C$ to continue fixing for 12 hours. After 12 hours, disks were allowed in the same fixation solution but without RHT for 2-3 days at 4 $^{\circ}C$. Washing buffer containing 0.1 M sodium cacodylate (pH of 7.4, osmolarity of 330 mosmol) was prepared and fixed tissue was washed four times sequentially for 4 hours before storing in 70% ethanol solution.

After sectioning to 5 μm thick slices and mounted on glass slides by Pathology Services (Cambridge, MA), cartilage tissues were stained with 0.1% toluidine blue solution. The entire process included deparaffinization and rehydration, staining, and dehydration, and took over 3 hours. Stained tissue slices were then mounted with coverslips using Permount, and laid flat for 1 day before photomicroscopy.

Chapter 4

Results and Discussion

4.1 Control Studies and Data Analysis Methods

Control studies were performed to obtain parameters for this adult culture system, to estimate sample variations, and to define proper data analysis methods. This information will be needed to properly design actual treatment protocols. For example, before actual treatment, means of sample selection and statistical analysis need to be determined. Also estimates of proper sample size are important to determine to how many tissues to harvest and whether multiple treatment within each experiment is feasible. Before showing results, the means of statistical analysis is explained first.

4.1.1 Statistical Analysis

SAMPLE MEAN AND SAMPLE VARIANCE

There are two different types of mean or average we can obtain when we make measurements on a population of size N. If the measurement is made over the entire population (size $n = N$), it is called the population mean μ, otherwise the sample mean $\bar{X}$ ($n < N$), because limited samples are taken from the entire collection. Although the formula for calculating the two are identical, statistical interpretations

are quite different for the two numbers.

$$\mu = \frac{\sum X_i}{N} \tag{4.1}$$

$$\bar{X} = \frac{\sum X_i}{n} \tag{4.2}$$

$$\sigma^2 = \frac{\sum (X_i - \mu)^2}{N} \tag{4.3}$$

$$s^2 = \frac{\sum (X_i - \bar{X})^2}{n-1} \tag{4.4}$$

By definition, μ is the true mean of the population of interest, while $\bar{X}$ is the mean of the particular sample of size n, and used as a point estimator of μ. According to the central limit theorem and sampling distribution, when samples of size n are repeatedly drawn from the population, and $\bar{X}$'s calculated, the probability density function ($\mathcal{P}$) will be normal (Gaussian) if the population is normal, or tend toward normality as n increases. Furthermore, as n is increased, the variance of $\bar{X}$ $\sigma^2_{\bar{X}}$ distribution decreases. Given normally distributed population with true mean μ and variance σ^2, the distribution of all possible sample means from sample size n, has normal distribution for large n, and first and second moments of the following:

$$\mu_{\bar{X}} = \mu, \tag{4.5}$$

$$\sigma^2_{\bar{X}} = \frac{\sigma^2}{n}. \tag{4.6}$$

As population statistics are seldomly known, sample variance s^2 is used as the best estimate of σ^2. Therefore, $\sigma^2_{\bar{X}}$ is best estimated from:

$$s^2_{\bar{X}} = \frac{s^2}{n}. \tag{4.7}$$

where $s_{\bar{X}}$ is the *standard error of the mean* (SEM). For the measurements in this writing, mean±SEM ($\bar{X} \pm s_{\bar{X}}$) are reported unless otherwise noted.

Student's t distribution and Hypothesis Testing

Knowing the $\bar{X}$ sampling distribution $\mathcal{P}_{\bar{X}}$, hypothesis testing can be conducted to provide information as to how close the experimental sample mean $\bar{X}$ is to the first moment of all sample means $\mu_{\bar{X}}$, which is identical to the population mean μ for large enough n. However, for small sample size ($n < 30$) the density function is not Gaussian but a similarly bell-shaped Student's t distribution. For control and treatment experiments involving adult articular cartilage, sample sizes are all smaller than 30, so this distribution is used and probability values taken from standard statistics reference books [61].

Testing of significance, or alternatively hypothesis testing involves calculating sample statistics followed by inference of the probability of such statistics in a specified interval of a known distribution. For example, when analyzing effects of IL-1 treatment on overall tissue GAG content, the purpose is to know the true concentration difference between control and treated disks (μ_{C-T}). Therefore hypotheses are written to test whether experimental value significantly deviates from null hypothesis of no difference, and the probability of such deviation:

$$H_0 : \mu_{C-T} = 0$$

$$H_1 : \mu_{C-T} \neq 0 \tag{4.8}$$

First, the distribution function of μ_{C-T} for H_0 is described as the t curve of mean 0, variance s^2_{C-T} and size n. Sample statistic $\bar{X}_{C-T}$ is then calculated, and located on the distribution curve. If this sample statistic is outside a pre-specified probability interval (indirectly chosen through level of significance α), the null hypothesis will be rejected. Otherwise, the experiment is inconclusive at detecting any significant difference.

Level of Significance and Confidence Interval

As mentioned above, in order to test hypothesis, a probability interval from H_0 has to be determined before actual experiments by choosing a proper level of signifi-

cance α. With a small enough interval (large enough α), any result can be significant, but the significance of the statement is consequently lowered. For a two sided hypothesis testing (sample statistic possible to occur on both sides of the H_0 value), the probability of any sample statistic falling inside this interval is exactly $1-\alpha$ for true H_0. Consequently, if experimental sample statistic falls outside of this interval, then the probability of rightfully rejecting H_0 will be $1-\alpha$. The predetermined level of significance for all the following statistical analysis is $\alpha = 0.05$.

Alternatively, a confidence interval (CI) using results from experimental statistics can be calculated to make same conclusion. Defining the two limits as h and l, they are calculated from the following:

$$l = \bar{X} - t_{\alpha(2),n-1} \cdot s_{\bar{X}} \tag{4.9}$$

$$h = \bar{X} + t_{\alpha(2),n-1} \cdot s_{\bar{X}} \tag{4.10}$$

where $t_{\alpha(2),n-1}$ refers to the t score for a two-sided test with sample size n. CI is equally distant from $\bar{X}$, and the width of such distance is determined from $s^2_{\bar{X}}$ and the level of significance. This interval has the same $1-\alpha$ probability of containing the H_0 value if it is true, and this probability of stating otherwise if the H_0 value falls outside. Although not explicitly written out, sample size n directly affect the width of CI, through values of both $t_{\alpha(2),n-1}$ and $s_{\bar{X}}$. The larger the sample size, the smaller of both values, thus the smaller the CI. CIs are presented below as means of judging H_0.

Two-sample vs. Paired-sample Hypothesis Testing

When the means of two populations are compared, as in the case of concluding effects of IL-1 treatment on cartilage tissue, either two-sample or paired-sample hypothesis testing can be used depending on sampling conditions. The two-sample method refers to gathering experimental statistics from two independent populations, and then use these statistics for hypothesis testing. But if the two populations are correlated, the paired-sample method will be better at making comparisons. In this

case, the hypotheses are very similar to the one-sample case discussed previously.

For example, for the experiments studying IL-1, all the cartilage disks were cultured with identical media except half of them had IL-1 while the other half did not. After calculating the means ($\bar{X}_C$ and $\bar{X}_T$) and variances ($s^2_{\bar{X}_C}$ and $s^2_{\bar{X}_T}$) of the two groups. The two-sample hypotheses are written as:

$$H_0 : \mu_C - \mu_T = 0$$

$$H_1 : \mu_C - \mu_T \neq 0 \tag{4.11}$$

Assuming two normal populations with equal variances, the two confidence limits are:

$$l = (\bar{X}_C - \bar{X}_T) - t_{\alpha(2),(2n-2)} \cdot s_{\bar{X}_C - \bar{X}_T} \tag{4.12}$$

$$h = (\bar{X}_C - \bar{X}_T) + t_{\alpha(2),(2n-2)} \cdot s_{\bar{X}_C - \bar{X}_T} \tag{4.13}$$

where $\bar{X}_C$ and $\bar{X}_T$ are the calculated means for control and treatment groups respectively, $t_{\alpha(2),(2n-2)}$ is the t score for two sided probability of α and sample size of both control and treated being n. The standard error of the difference between the means, $s_{\bar{X}_C - \bar{X}_T}$ is calculated according to the following:

$$s^2_{\bar{X}_C - \bar{X}_T} = \frac{s^2_{\bar{X}_T}}{n} + \frac{s^2_{\bar{X}_C}}{n}. \tag{4.14}$$

On the other hand, for paired-sample t tests, a new mean population difference is defined, $\mu_d = \mu_C - \mu_T$. The hypotheses are desired to test whether this difference is significantly away from zero.

$$H_0 : \mu_d = 0$$

$$H_1 : \mu_d \neq 0 \tag{4.15}$$

Similar to the one-sample method, CIs are given from the following:

$$l = \bar{d} - t_{\alpha(2),n-1} \cdot s_{\bar{d}} \tag{4.16}$$

$$h = \bar{d} + t_{\alpha(2),n-1} \cdot s_{\bar{d}} \tag{4.17}$$

where $\bar{d}$ is the calculated mean of the differences and $s_{\bar{d}}$ is the standard error of the differences. However, sample size n in this calculation is different from that of the two-sample method. n for a paired-sample method is the total number of differences, or if two experimental numbers are used to calculate one difference, n is half of total number of disks. In the following experiments, four numbers were used to calculated each difference, therefore n's were only one quarter of overall number of cartilage disks.

Another fact is that although the mean difference between control and treatment is calculated differently for the two methods. The results are identical, as only the sequence of linear operators is switched:

$$\bar{d} = (\bar{X}_C - \bar{X}_T) \tag{4.18}$$

But no similar statement can be made for the variances, or the width of CIs.

Local Averaging

When calculating each difference for the paired-sample t test, four samples instead of two were used. Coming from the many difficulties in adult tissue handling and culturing, the desire was to help detect and avoid non-representative measurements. This practice is named local averaging.

In this case, the four cartilage disks taken from each cylindrical slice were randomly grouped into two, and only one pair was assigned IL-1 treatment, again through random selection. The two disks within each pair were cultured identically. During analysis, the measurements within each pair were first averaged before differences were taken across the two pairs. If significant deviation exists within the pairs, there may be possible problems in tissue handling. For example, if some disks were infected or otherwise lost cellular viability, local averaging would indicate a large difference in incorporation rates within the pairs (low incorporation is defined as comparable to levels of blank, or less than 100 cpm in either ^{3}H or ^{35}S windows). Subsequently,

measurements from such disks were not included in further analysis, as they were not representative of normal functional cartilage tissues.

$$\bar{d} = \frac{\bar{X}_{C_1} + \bar{X}_{C_2}}{2} - \frac{\bar{X}_{T_1} + \bar{X}_{T_2}}{2} \tag{4.19}$$

4.1.2 DNA and GAG Concentration in Adult Cartilage

For measurements in the control studies, cartilage disks were harvested on day 0 and cultured for 3 days in freeswelling conditions with supplemented media including 20% fetal calf serum. On day 3 (the fourth day after harvest), all disks were changed into radiolabeled media for 18-24 hours as described in chapter 3. After six sequential washings to remove free radiolabels, tissue samples were lyophilized and digested with papain solution, before aliquotes were measured for desired properties. Additionally, on days 1 and 4, the wet weights of each disk were measured, respectively named initial and final wet weights.

For DNA and GAG contents in each disk, tissue digests were aliquoted and measured photometrically against standards of known concentration. The numbers were also normalized to initial wet weights for differences in cut size.

In order to compare, another experiment cultured cartilage disks from one to two week old calf femoropatellar groove, under identical conditions as applied to adult tissue for 6 days, including addition of 20% FCS. Furthermore, calf epiphyseal cartilage measurements were also adopted from experiments by Gregory Allen, although culture conditions are different in such experiments (7 days of culturing with 1% heat inactivated FCS).

The DNA concentrations after 3 days of culture are shown in Figure 4-1 for two experiments (different animals), and compared to that of calf. All values are averages (μg/mg) $\pm$ *SEM* for the three joint surfaces and the overall. Sample sizes for each average are indicated in the parenthesis. It is obvious that DNA concentrations in adult tissue differ significantly from animal to animal. There is also significant differences among the three joints. Furthermore, cell contents can vary widely from disk to disk, as in Figure 4-2. No one joint surface has consistently higher/lower cell

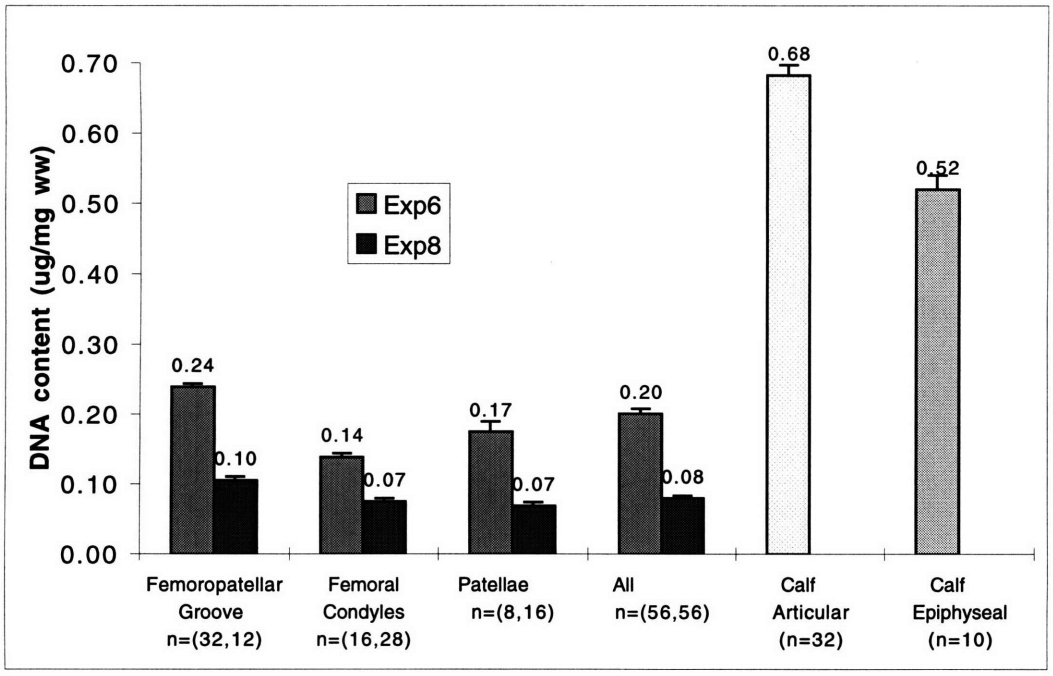


Figure 4-1: Average DNA contents normalized to wet weight, mean±SEM. Exp6 and Exp8 were experiments with adult articular cartilage; calf articular cartilage was cultured from the femoropatellar groove; and calf epiphyseal data was adopted from Greg Allen.

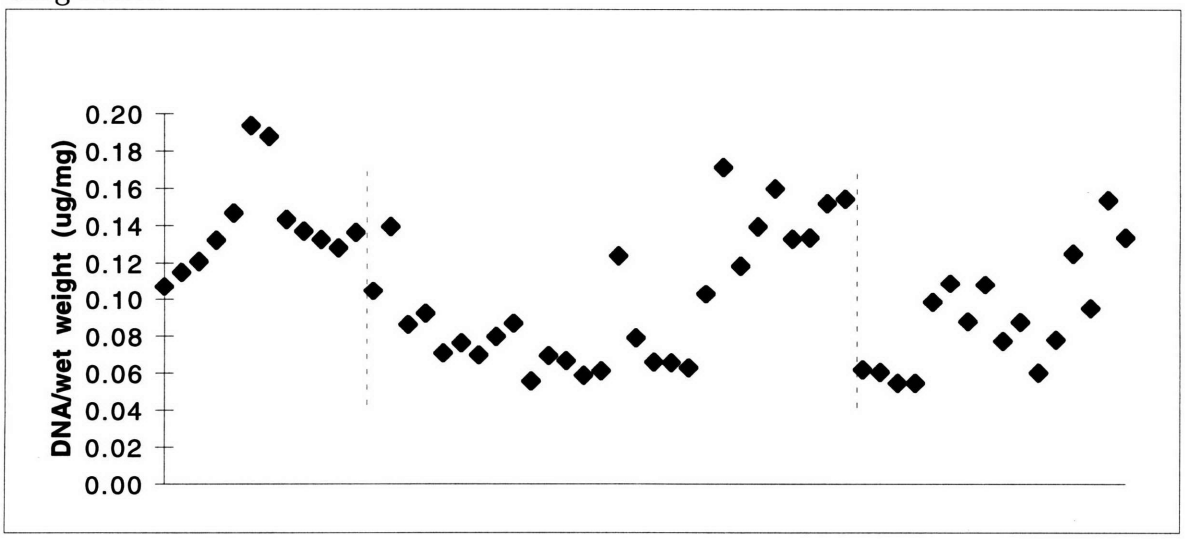


Figure 4-2: DNA contents for each disk (Exp8), normalized to wet weight. From left to right, the dotted lines separate the femoropatellar groove, the femoral condyles and the patellae in that order, and four disks in each slice are arranged adjacent to each other.

content than the others either, although the femoropatellar groove in both experiments shown happens to have the highest values. DNA content in adult cartilage is very much lower than that in younger tissues. Usually a factor of 4-5 is observed, a difference comparable to other reports in literature [58].

Similar trend exists for bulk GAG content as well (Figure 4-3). With identical culturing conditions, calf articular cartilage disks had several fold higher GAG concentration following 6 days of culture than adult tissue following 4 days of culture. This is also true when comparing GAG concentrations on day 0, averaging 55.4±2.6 for calf articular and 27.7±2.1 for one of the adult experiments. However, unlike calf tissue, the variance of GAG measurements in adult is rather high (Figure 4-4).

4.1.3 Sensitivities of DNA and GAG Measurements

With such high variation, it will not be appropriate to assume all disks have almost identical properties. And if there is indeed some difference in biochemical composition between control and IL-1β treatment, it will be difficult to differentiate an effect of treatment alone from the inherent high variation of the tissue itself. From the discussion earlier, when comparing two point estimators of population means, the higher the variances, the wider the CIs, and the harder it will be to conclude significant results even if the alternative hypothesis H_1 is true. Although intuitively increasing sample size should narrow CIs, there are many quantitative questions to answer before meaningful treatment protocols can be designed. These questions to be answered from control studies are listed below:

- If IL-1β indeed has a significant impact on a property we measure ($\mu_C \neq \mu_T$), using the two-sample t test, how significant (in terms of percentage change) does the impact have to be to be detected?

- How does increasing sample size help narrowing CI, quantitatively, how big n has to be to detect a 10% treatment deviation from control?

- Given the large difference among joint surfaces, which one (or two, three) is best for treatment studies?

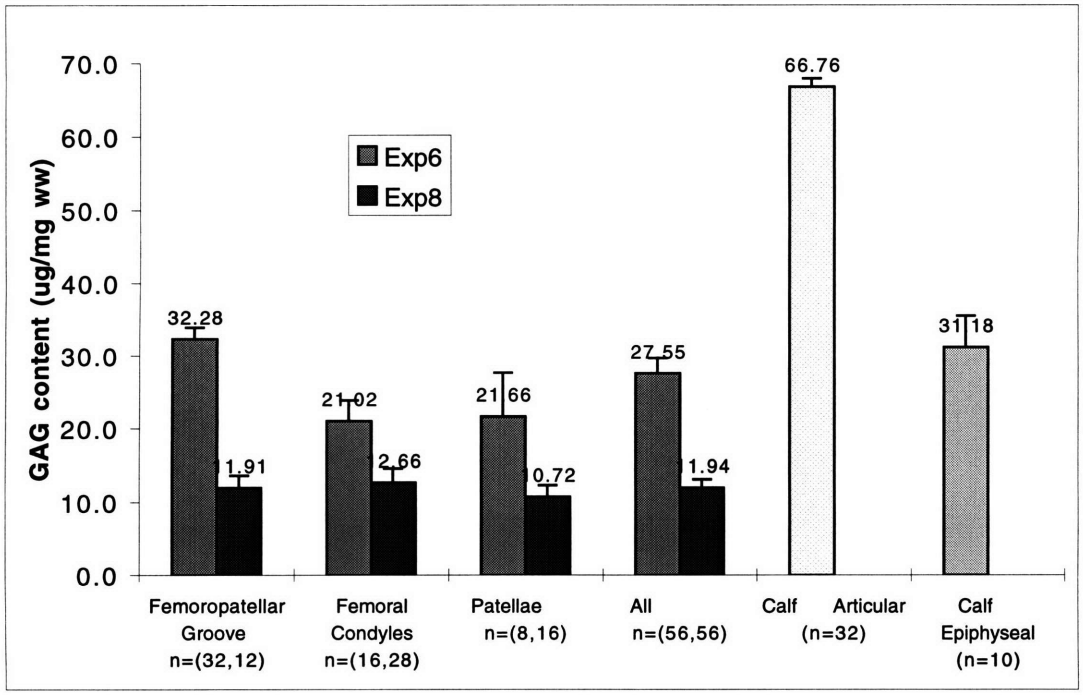


Figure 4-3: Average GAG contents normalized to wet weight, mean±SEM

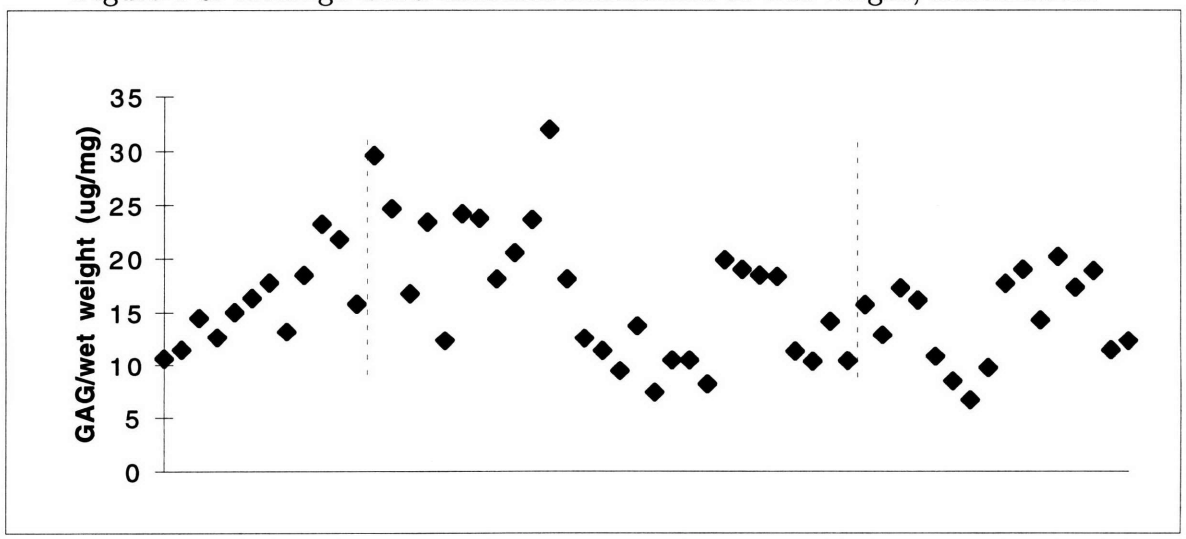


Figure 4-4: GAG contents for each disk (Exp8), normalized to wet weight. From left to right, the dotted lines separate the femoropatellar groove, the femoral condyles and the patellae in that order, and four disks in each slice are arranged adjacent to each other.

- Harvesting cartilage from multiple origins certain is one way of enlarging n, but is it going to help?
- What about the dependence in cartilage tissues from nearby loci, will paired-sample t test offer better analysis?
- If it does, what are answers to all the questions above?

To answer these questions, an empirical approach was adopted using data from control experiments. First, cartilage disks from each experiment were randomly assigned to two groups of equal sizes, C_1 and C_2, mimicking treatment experiments. For each slice, two disks were in C_1 and the other two in C_2. Then two-sample confidence intervals were calculated for each group for the parameters of interest, such as average DNA content for each group. In this case, the true hypothesis is known:

$$\mu_{C_1} - \mu_{C_2} = 0 \tag{4.20}$$

since both groups were treated identically without IL-1. In addition, half the width of each CI indicates exactly the smallest change detectable in each direction for that parameter, assuming actual treatment groups have similar variances. Since limits of CI have units of that measurement, it is normalized to the mean of the measurement to obtain a unitless quantity expressed in percentage, the *sensitivity*. This number indicates the smallest percentage change for any treatment protocol to claim statistically different results from the control, i.e. to reject the null hypothesis: $\mu_C - \mu_T = 0$.

$$\text{sensitivity} = \frac{h - l}{2 \cdot \bar{X}} \cdot 100\% \tag{4.21}$$

Two-sample confidence intervals and sensitivities for DNA concentration are shown in Figure 4-5 for Exp8. The intervals are given as high-low lines calculated from:

$$l = (\bar{X}_{C_1} - \bar{X}_{C_2}) - t_{0.05(2),(2n-2)} \cdot s_{\bar{X}_{C-1} - \bar{X}_{C_2}} \tag{4.22}$$

$$h = (\bar{X}_{C_1} - \bar{X}_{C_2}) + t_{0.05(2),(2n-2)} \cdot s_{\bar{X}_{C-1} - \bar{X}_{C_2}} \tag{4.23}$$

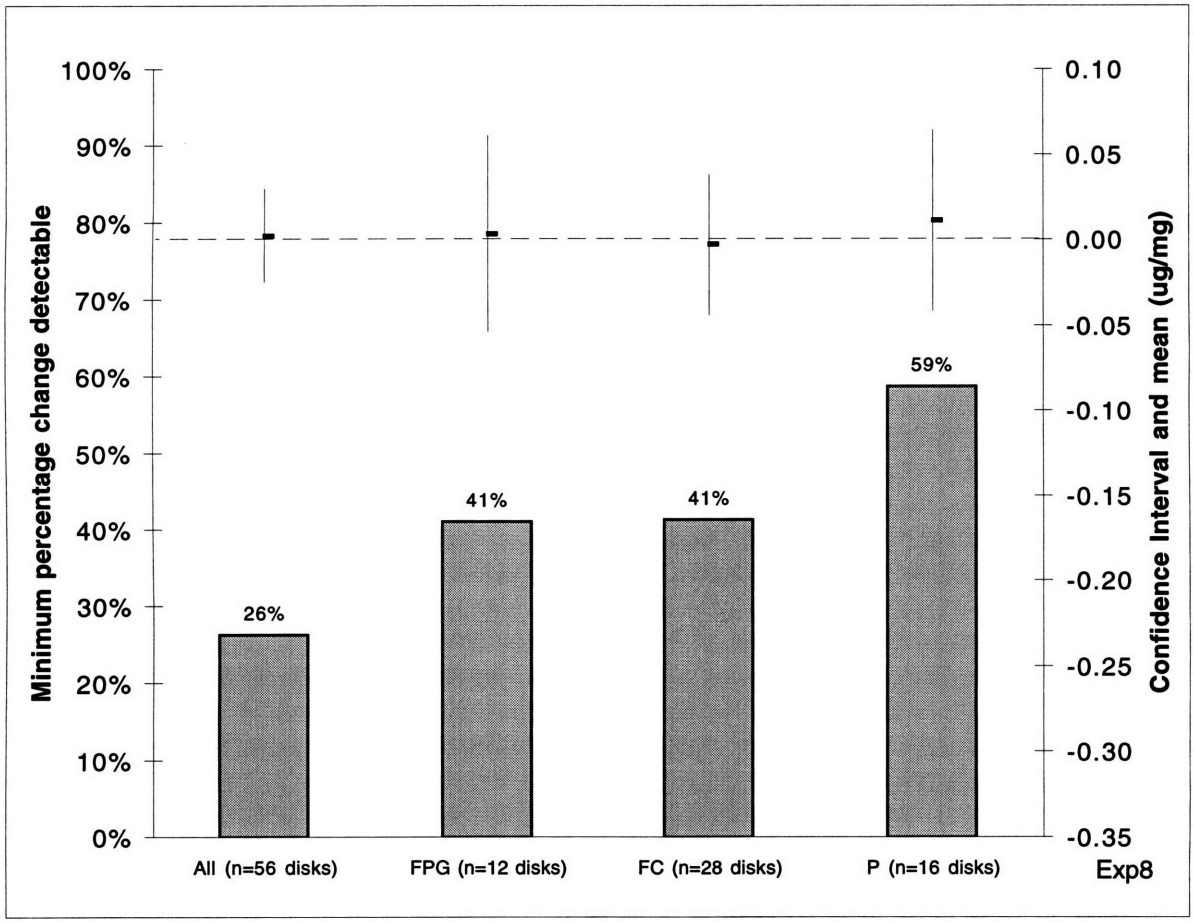


Figure 4-5: Two-sample CIs and sensitivities for DNA contents, columns and left axis show sensitivities. Lines and right axis show ranges of CI for $\bar{X}_{C_1} - \bar{X}_{C_2}$, tickmarked at the means. FPG=Femoropatellar Groove; FC=Femoral Condyles; and P=Patellae.

where n is number is cartilage disk in each joint surface or overall. Consistent with the true null hypothesis, all the CIs include 0, which should be the case 95% of the time. Also, despite the differences, inclusion of all samples gave the smallest sensitivity. However, even the smallest is huge. The implication is that even if IL-1 is indeed effective, the changes have to be greater than 26% to be discoverable. This is really discouraging, as it would take more than 89 samples to be able to see a 10% deviation from control.

As a second approach, based on the observation that the four disks in each slice tend to smaller variations than inter-slice variations, the paired-sample confidence intervals are calculated. As mentioned earlier, local averaging is also applied. As a consequence, n was reduced to only one quarter of the original size in the two-sampled t test. However, the reduction in sample size was more than compensated by reduced variation in the differences, indicating smaller variations within each slice. The results are shown in Figure 4-6. Only numbers for FPG and combining all three joint surfaces are shown. The sensitivities for two-sample t test are shown again for these two grouping. With the paired t test and local averaging, the sensitivity for including all samples dropped significantly to 9%, and for FPG to only 10%. Obviously, the difference pairs ($d_i = X_{i,C_1} - X_{i,C_2}$) had much smaller variance ($s_{\bar{d}}^2$) than the pooled variance from the two sample variances ($s_{\bar{X}_{C_1}-\bar{X}_{C_2}}^2$).

Another conclusion from the control experiments is that although the three joint surfaces have different compositions, the inclusion of all three gives the lowest sensitivity with the paired-sample t test. Once again, although the absolute values of each disk may be quite different, the differences in each slice are comparable enough to warrant benefits from increased sample size. Figure 4-7 shows the comparison of CIs obtained from different grouping, FPG, FC and P individually, and all three included. Values for two experiments are included.

Very similar results are obtained from GAG concentration data. Sensitivity is the lowest when all samples are included and when pairing is applied (Figures 4-8 and 4-9), confirming the method is appropriate for analyzing adult cartilage culture measurements. When using data from all three joint surfaces and the paired-sample

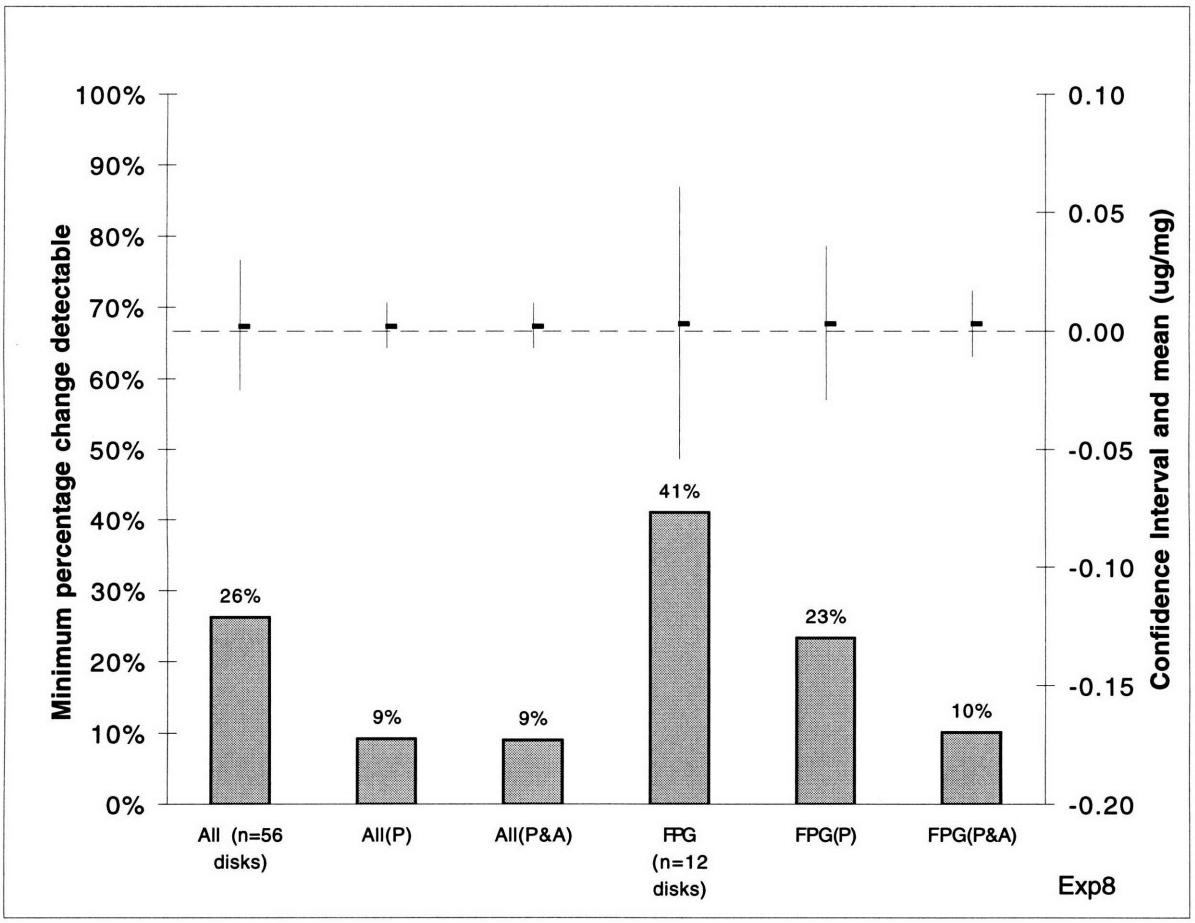


Figure 4-6: CIs and sensitivities for DNA contents, columns and left axis show sensitivities. Lines and right axis show ranges of CI ($\bar{X}_{C_1} - \bar{X}_{C_2}$ for two-sample and $\bar{d}_{C_1-C_2}$ for paired-sample), tickmarked at the means. P=with pairing; P&A=with pairing and local averaging; otherwise two-sample. FPG=Femoropatellar Groove.

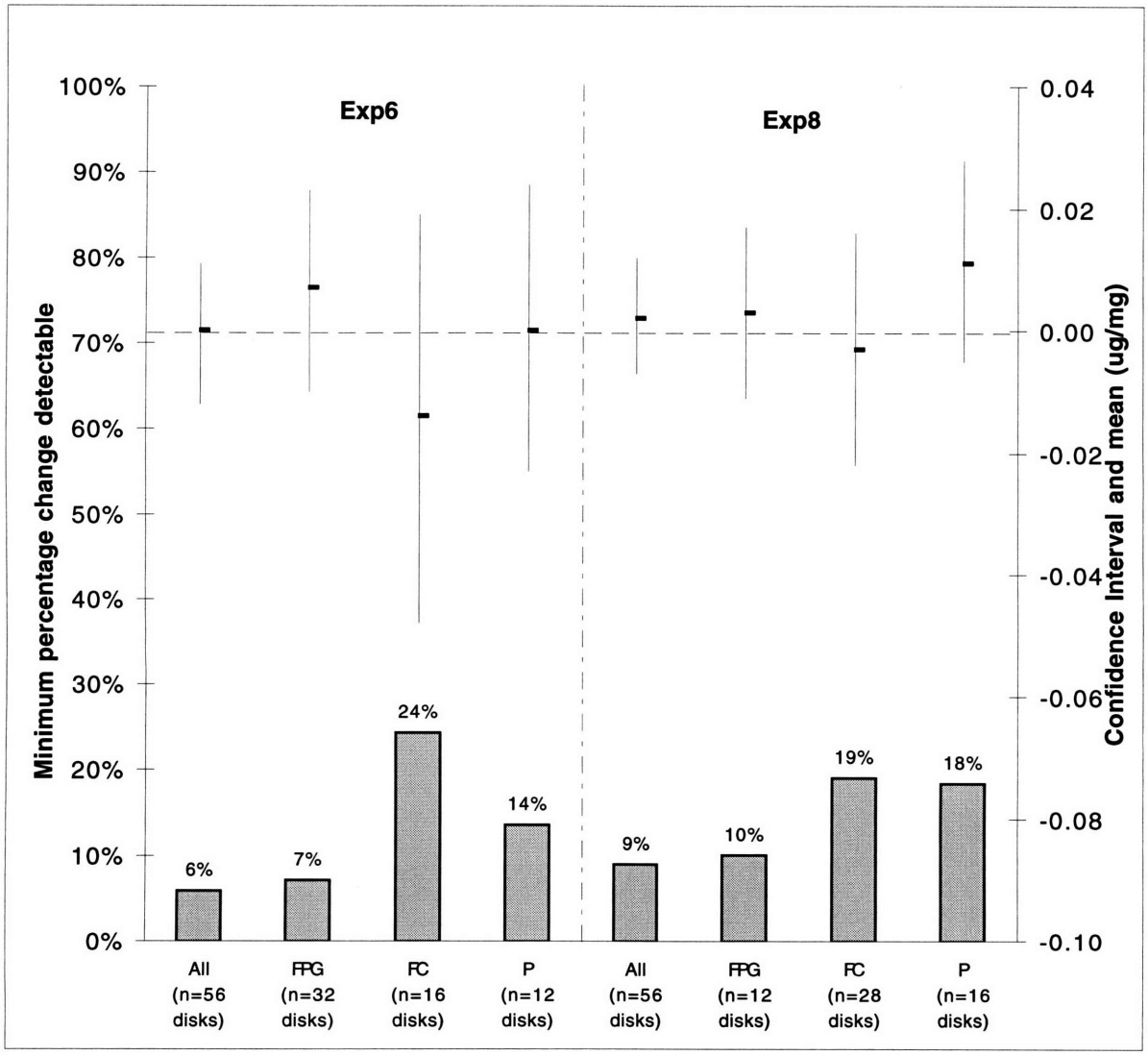


Figure 4-7: Paired-sample CIs and sensitivities for DNA contents, columns and left axis show sensitivities. Lines and right axis show ranges of CI for $\bar{d}_{C_1-C_2}$, tick-marked at the means. FPG=Femoropatellar Groove; FC=Femoral Condyles; and P=Patellae.

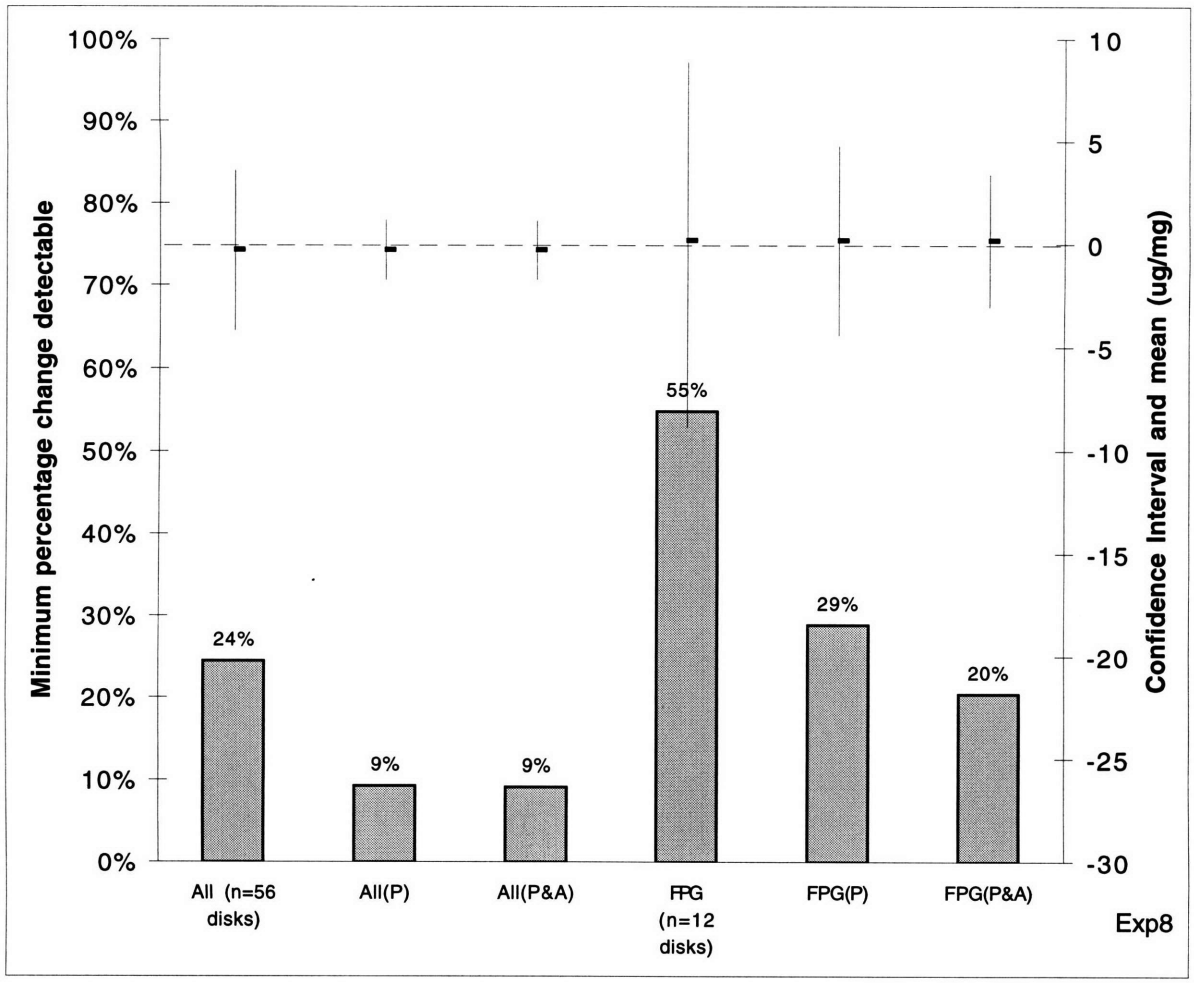


Figure 4-8: CIs and sensitivities for GAG contents, columns and left axis show sensitivities. Lines and right axis show ranges of CI ($\bar{X}_{C_1} - \bar{X}_{C_2}$ for two-sample and $\bar{d}_{C_1-C_2}$ for paired-sample), tickmarked at the means. P=with pairing; P&A=with pairing and local averaging; otherwise two-sample. FPG=Femoropatellar Groove.

t test, the same variation in treatment groups will allow 8% difference to be detected in bulk GAG concentration.

In the calculation of sensitivities and subsequent generalization, a few assumptions were made. First of all, variance of treatment group was assumed to be same as that of the control and treatment groups, for lack of better information. Also, in order to generalize to future experiments, similar variance was assumed from animal to animal. Finally, the CIs were calculated from a group of 56 cartilage disks. With everything else being equal, it is expected that sensitivities will be lower if more cartilage disks are harvested. To summarize, the number of cartilage disks required to detect at least

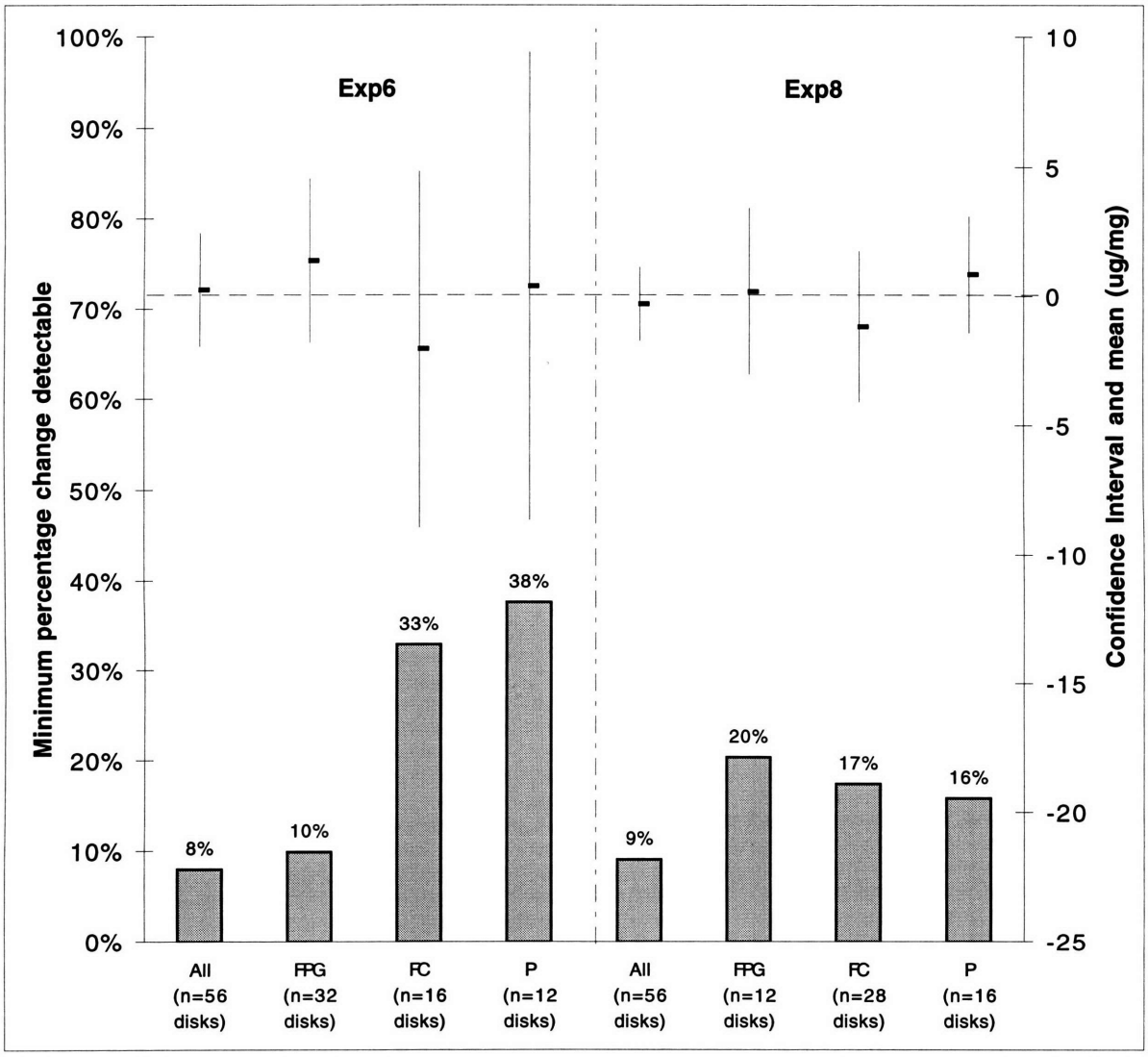


Figure 4-9: Paired-sample CIs and sensitivities for GAG contents, columns and left axis show sensitivities. Lines and right axis show ranges of CI for $\bar{d}_{C_1-C_2}$, tick-marked at the means. FPG=Femoropatellar Groove; FC=Femoral Condyles; and P=Patellae.

Table 4.1: Minimum numbers of cartilage disks to have low than 10% sensitivity, using both pairing and local averaging.

Numbers calculated from	Exp6	Exp8
Bulk DNA	28	48
Bulk GAG	40	48
^{3}H incorporation	60	44
^{35}S incorporation	48	68
Initial wet weight	40	56
Final wet weight	20	16
Dry weight	16	16
Water percentage	12	12
GAG release day 1	60	32
GAG release day 2	256	56
GAG release day 3	212	48
GAG release day 4	256	100
Actual total number of disks	56	56

a 10% deviation from control is listed in Table 4.1 for the two experiments and for all the properties measured. Initial wet weights were measured on day 1. Final wet weights and dry weights were measured immediately after the experiments. Water percentages were calculated from final wet weights and dry weights:

$$\text{Water percentage} = \frac{\text{Final wet weight} - \text{Dry weight}}{\text{Final wet weight}} \cdot 100\% \tag{4.24}$$

Measurements of radio-incorporation and media GAG releases will be explained later.

4.1.4 Changes of DNA and GAG content during culture

It has been reported in literature that chondrocytes grow significantly in number with supplements of 20% FCS. One article showed increases of 195% from day 1 to day 20 of culture [32], despite the fact that mature cartilage cells normally do not divide [49]. One of my experiments studied whether significant cell growth happens during 8 days of culturing adult cartilage. Similarly, changes in bulk GAG content during the same culture period was also measured.

When harvesting cartilage disks, besides the usual four cylindrical disks from each slice, an additional block of tissue was cut out, weighed and immediately digested with papain. DNA and GAG contents were measured as described before, and labeled DNA_0 and GAG_0. The other disks were incubated in media supplemented with 20% serum for 8 days before being similarly digested, measured and averaged within each slice to get DNA_f and GAG_f. After normalization to wet weights, DNA and GAG contents before and following culturing were compared using the paired-sample t test.

After 8 days of culture, there is no statistically detectable difference between DNA_0 and DNA_f in the adult cartilage explants cultured, with a sensitivity of 19%. The same result is true following identical treatment to calf articular cartilage for 6 days (12% sensitivity). However, the story is different for GAG content. In both adult and calf, PG metabolism failed to maintain tissue GAG homeostasis. In adult cartilage, the 8 days of culture saw an average 37% drop in bulk GAG concentration, while calf tissue gained 14% after 6 days. The significance of such changes will be discussed later in the context of PG turnover.

4.1.5 Synthetic Rates

Chondrocyte viability and synthetic activities were measured through uptake of radioactive precursors. $Na_2S^{35}O_4$ incorporation was used as indicator for GAG synthesis, and ^3H-proline incorporation for protein synthesis. As discussed in chapter 3, liquid scintilation counts per minute for ^{35}S and ^{3}H were then converted to amounts of GAG and protein synthesized.

To measure synthetic activities, after 3 days of culturing, tissues were incubated in supplemented media containing radioisotopes for 18-24 hours. It has been shown that during this time frame, incorporation rate is linear and incorporated radioactive precursors are mostly on the macromolecules [28]. Incorporation rates for both GAG and protein are shown in Figure 4-10 for two adult experiments and compared to measurements of calf. Radio-incorporations are expressed as *nmol* of new GAG disaccharides synthesized per μg of DNA during a 24 hour period (mean±SEM), in order to normalize for differences in cell content. Similar to bulk DNA and GAG

measurements, radioactive precursor incorporation can differ significantly from animal to animal, and from joint to joint. However, there is no dramatic differences in synthetic activities of new GAG between adult and calf articular cartilage under the same culturing conditions. This does not mean comparable amounts of GAG are generated in the same time interval though, as adult tissue has much less cells to do the work. Although incorporation data from calf epiphyseal cartilage is also included in Figure 4-10, it is not appropriate to make comparison as it is cultured with much less serum, and components in FCS (believed to be IGF-1) has been shown to significantly stimulate synthetic activities [14].

Assuming that one sulfate group is incorporated into each disaccharide of newly synthesized GAG disaccharides, the average 6-8 nmols of sulfate incorporation per day per μg DNA transforms to about 6-8 nmols of new GAG disaccharides, or 3-4 μg ($M_r \simeq 500$) synthesized on the day of measurement, as adult cartilage disks have close to 1 μg of DNA each.

4.1.6 Rate of GAG Release

To estimate rate of PG degradation, after media change every day, old media were saved and frozen at $-20°C$ until the end of the experiment. Then all media samples were analyzed together using the DMB dye binding assay similar to bulk GAG measurements.

The amount of GAG remaining in disks during culture is shown in Figure 4-11. Absolute amounts of GAG release daily were normalized to total GAG content (GAG_t), defined as the sum of final GAG content (GAG_f) and all releases ($\sum R_i$). For the two adult experiments (Exp6 and Exp8):

$$GAG_t = \sum_{i=1}^{4} R_i + GAG_f \tag{4.25}$$

$$[\%\text{remaining}]_n = \frac{GAG_t - \sum_{i=1}^{n-1} R_i}{GAG_t} \cdot 100\% \tag{4.26}$$

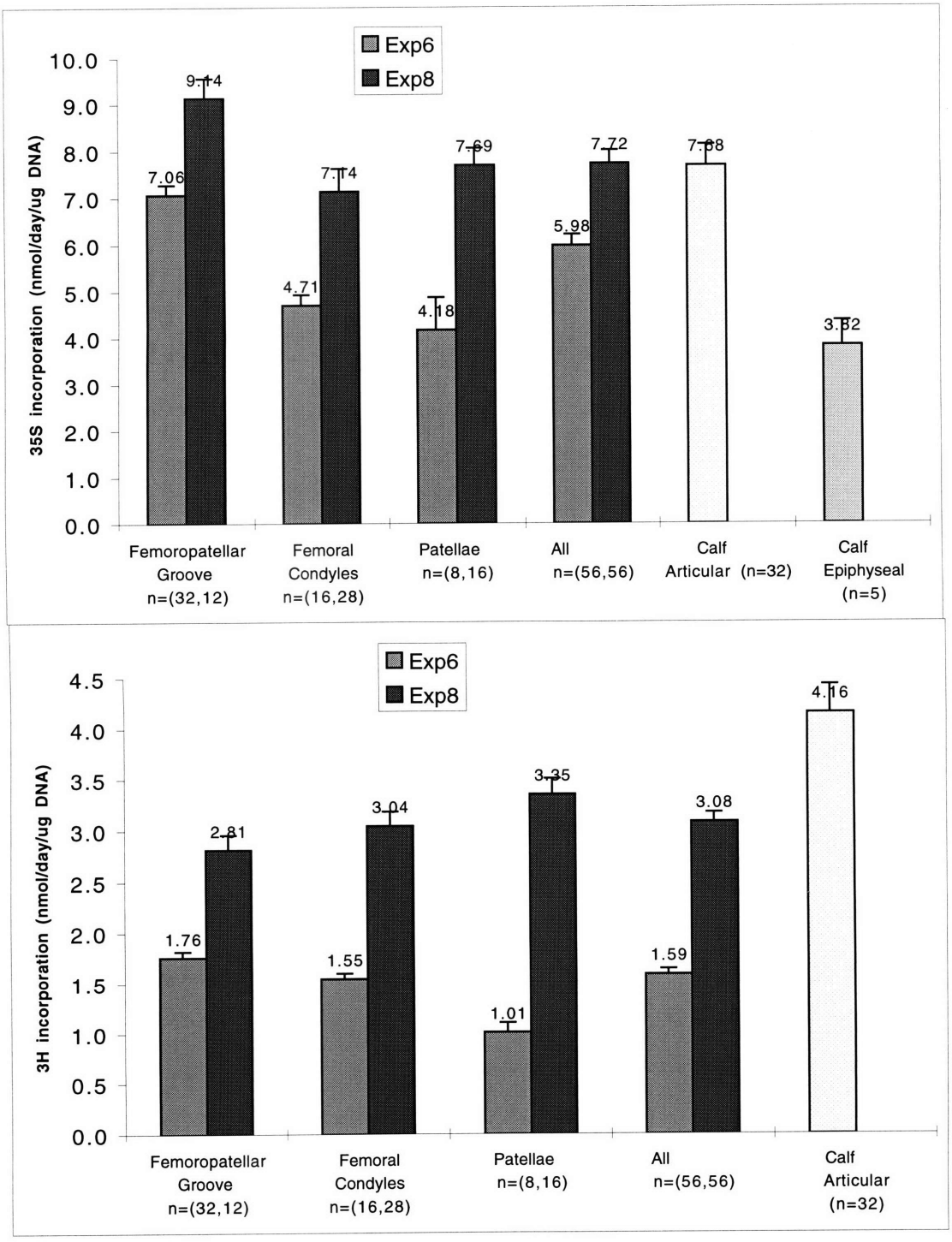


Figure 4-10: Average ^{3}H- and ^{3}5S incorporation rates in adult articular, calf articular, and calf epiphyseal cartilage explants, in $nmol/24h/\mu g$ DNA (mean±SEM). Numbers in parenthesis indicate sample size.

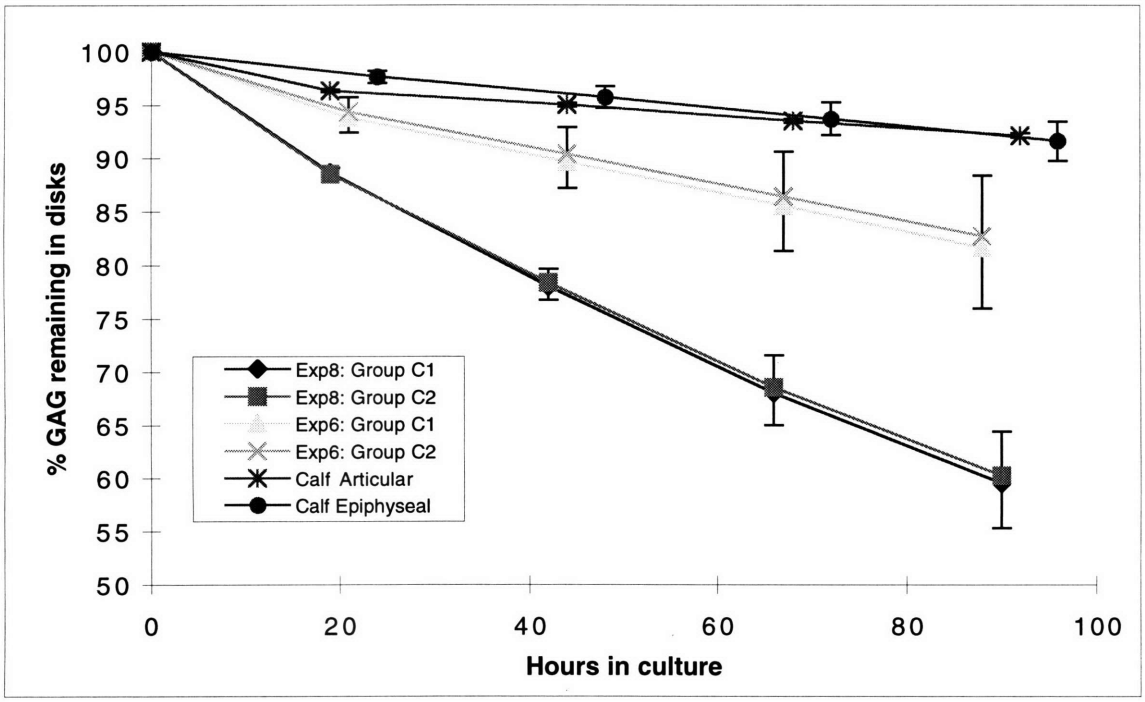


Figure 4-11: Average remaining GAG content in cartilage disks as percentage of GAG_t, mean±SEM. For adult tissues, measurements of two randomly selected groups (C_1 and C_2) are shown. For comparison, release data from calf articular (n=32) and calf epiphyseal cartilage (n=4) are also shown.

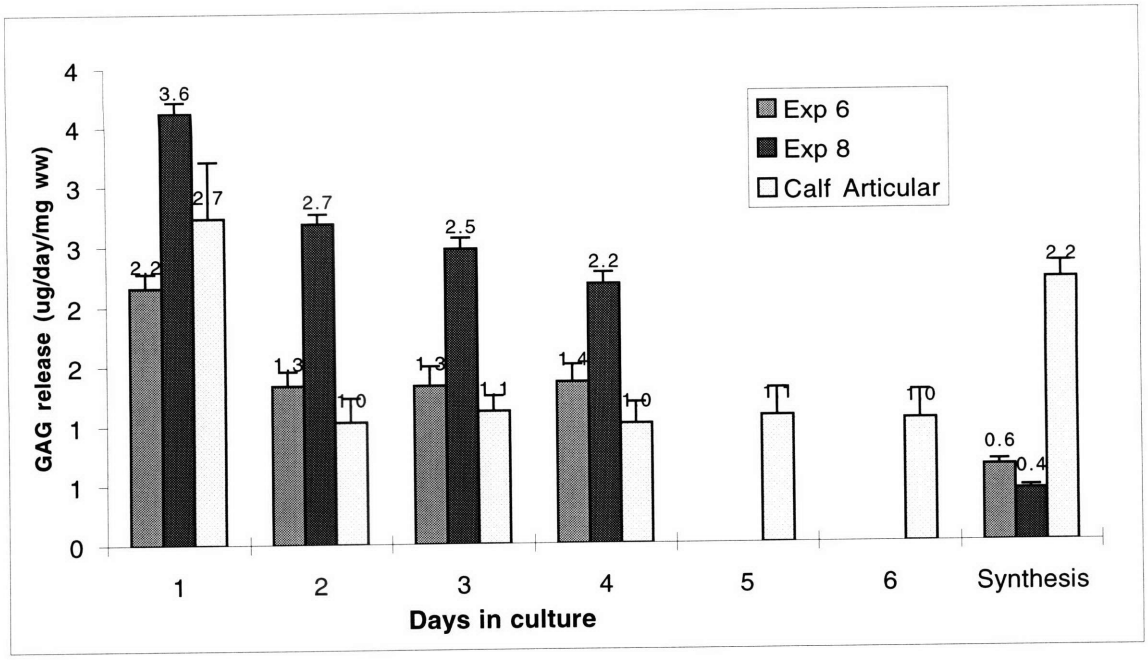


Figure 4-12: Amount of GAG released during four days of culture for adult cartilage, and 6 days of culture for calf articular cartilage; and compared to amount of GAG synthesized on the last day of experiment, in μg/24h/mg wet weight.

Once again, animal to animal difference is high, but the differences between the two randomly partitioned groups are nonsignificant. In both adult and calf, ECM usually releases significantly more GAGs during the first day of culture. and appears mostly linear after that. GAG release from mature matrix is proportionally much higher than either calf articular or epiphyseal cartilage. Absolute amounts of release normalized to per 24 hour period are shown in Figure 4-12, and compared to synthesis on the last day of experiment. Once again, calf articular cartilage releases less GAG despite the much richer pool of overall GAG in its ECM. It is also interesting that under identical culturing conditions of 20% FCS, chondrocytes in the younger tissue synthesize more GAG in one day than the amount lost, resulting in net gain of overall GAG. On the contrary, cells in adult cartilage are only able to synthesize GAG at a fraction of the amount lost.

In order to compare relative extent of GAG synthesis *vs.* release throughout the entire culture period, an extra piece of tissue was harvested on day 0 as described previously to get GAG_0. Although two of the four cartilage disks were later treated

with IL-1β, only data from the two control disks in each slice were used in this study.

If PG metabolism is balanced during the culture period of 8 days, then net GAG content on day 0 (GAG_0) would not significantly differ from that of day 8 (GAG_f). However, it was found experimentally that GAG_f is significantly lower than GAG_0, indicating overall net loss of GAG content in adult cartilage explants (Figure 4-13).

$$GAG_f - GAG_0 = \sum_{i=1}^{8}(S_i - R_i) < 0 \tag{4.27}$$

where S_i and R_i are the amounts of synthesis and release during day i, respectively.

Furthermore, the amount of synthesis during the 8 day period is not negligible, either. Statistics reveal significant difference between GAG_t and GAG_0.

$$\begin{aligned} GAG_t - GAG_0 &= [GAG_f + \sum_{i=1}^{8} R_i] - [GAG_f - \sum_{i=1}^{8}(S_i - R_i)] \\ &= \sum_{i=1}^{8} S_i > 0 \end{aligned} \tag{4.28}$$

All the above statistics were done with the paired-sample t test using an α of 0.05.

Although on the last day of experiment, new GAG synthesized (0.28 $\pm$0.02 μg/mg, or $\sim$ 5% of GAG_0) averages less than 20% of those lost (1.49 $\pm$0.06 μg/mg, or $\sim$ 1% of GAG_0), it is obvious from Figure 4-13 that this rate can not be the average over 8 days. In order to make up for all the differences between GAG_t and GAG_0, higher synthesis has to have occurred earlier in the culture period.

For comparison, similar calculations for calf articular cartilage were done, obtaining values of GAG_0, GAG_f, GAG_t and day 6 synthesis S_6. They are plotted in Figure 4-14. Although S_6 of calf is almost 10 times higher than S_8 of adult, six days of such synthesis still would not make up for the difference in GAG_t and GAG_0, implying possible declines in synthesis of culturing calf tissue as well.

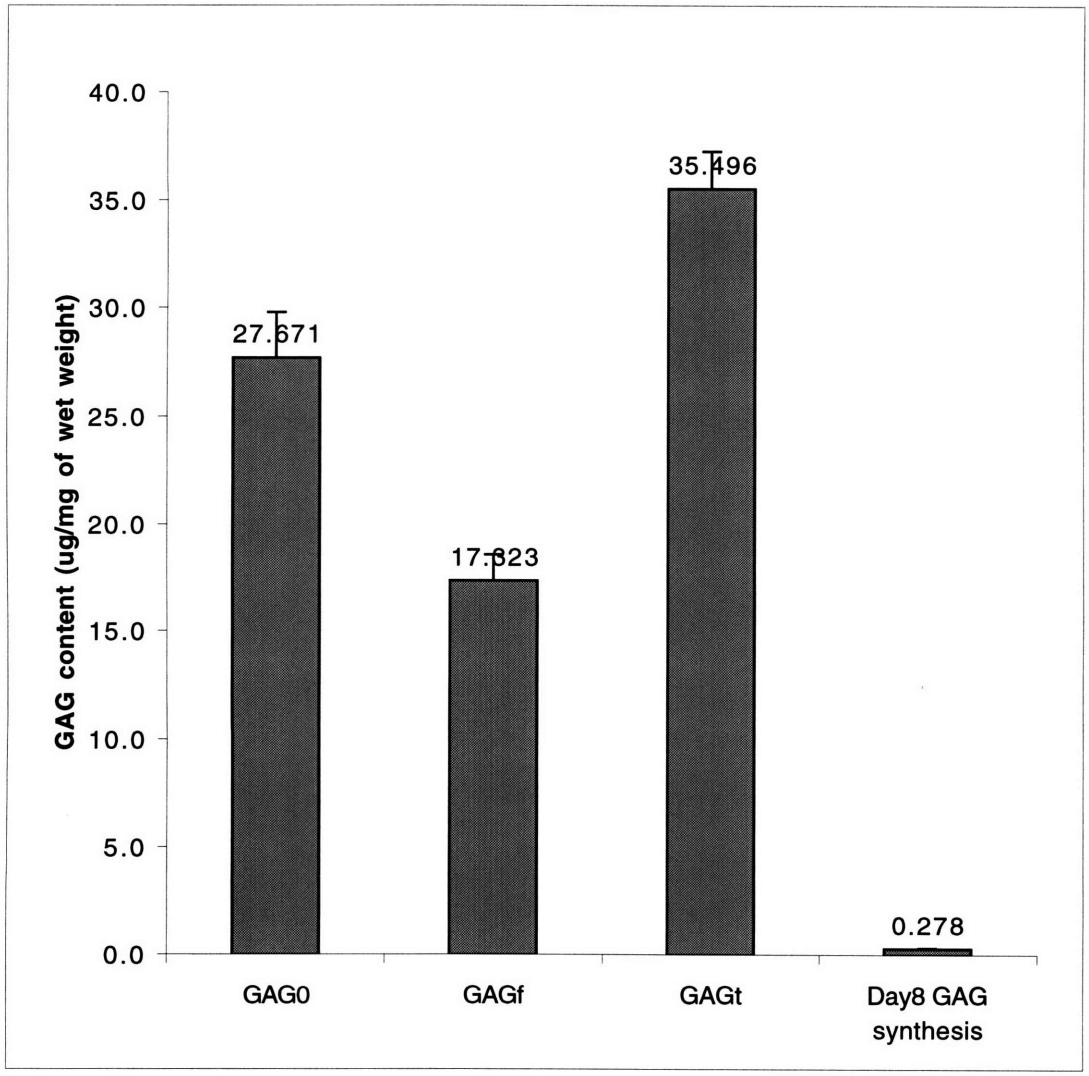


Figure 4-13: Comparison of GAG_0, GAG_f, GAG_t and S_8 (mean±SEM) in adult cartilage explants, where GAG_0 and GAG_f are the measured GAG contents on days 0 and 8; GAG_t is the calculated sum of GAG_f and 8 days of release; and S_8 is the GAG amount synthesized on day 8.

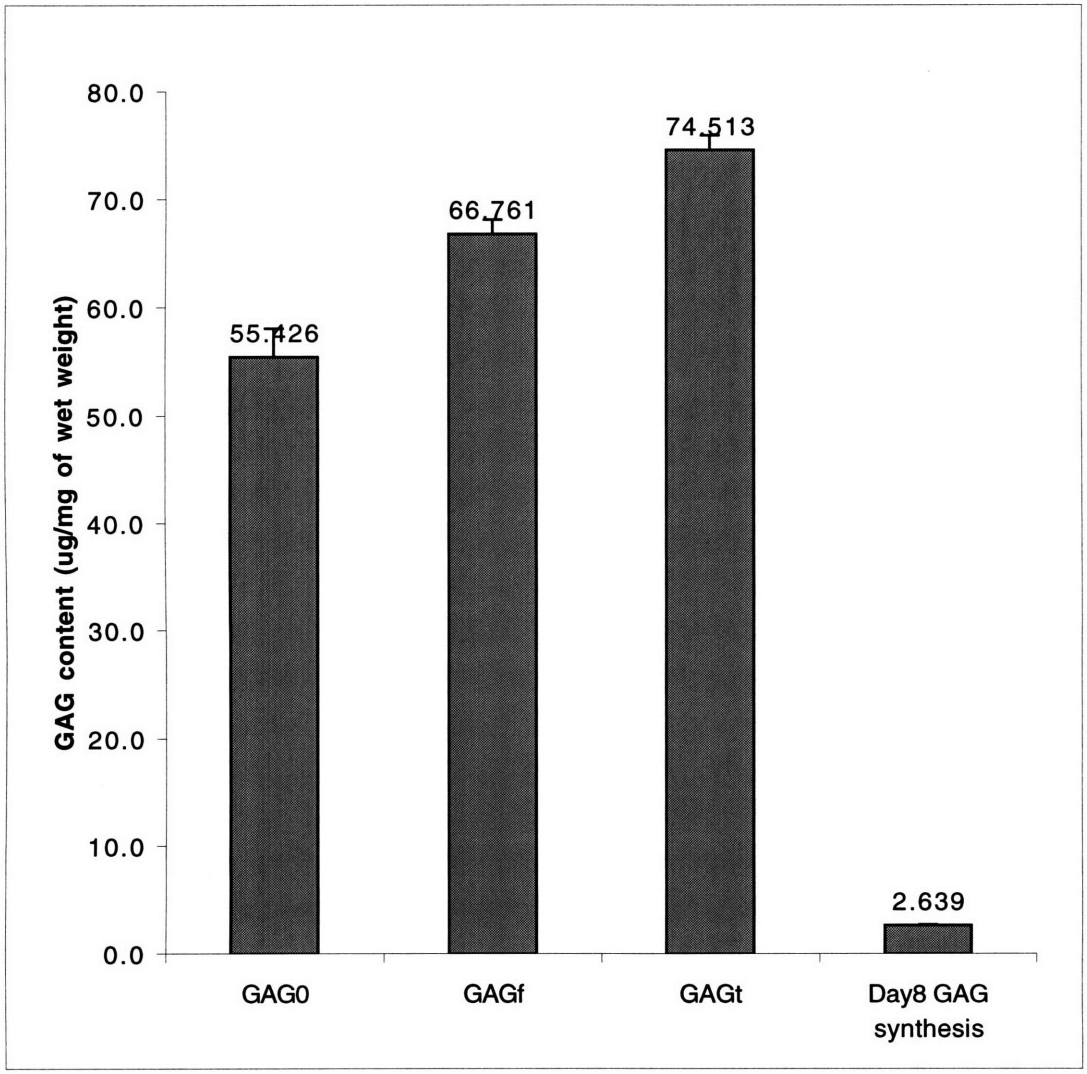


Figure 4-14: Comparison of GAG_0, GAG_f, GAG_t and S_6 (mean±SEM) in calf cartilage explants, where GAG_0 and GAG_f are the measured GAG contents on days 0 and 8; GAG_t is the calculated sum of GAG_f and 6 days of release; and S_6 is the GAG amount synthesized on day 6.

4.2 Effects of IL-1β on Adult Cartilage

To study effects of IL-1β on PG metabolism, two experiments (exp14 and exp16) were conducted where adult cartilage disks were harvested on day 0 and cultured for a total of 8 days with 500 ng/ml IL-1β applied on day 4. Cartilage tissue selection and data analysis protocols were as defined in the previous section. Briefly, two 3 mm diameter, 600 μm thick cylindrical disks were randomly selected out of each slice for IL-1β treatment, while the other two for control. Following experiment, paired-sample t tests and local averaging were used for data analysis.

Calf articular cartilage explants from the femoropatellar groove were cultured for 6 days under identical conditions as comparison (Exp17). There is, however, a difference in sources of IL-1β. Both Exp16 and Exp17 used IL-1β purchased in one batch from Cistron Biotechnology, while Exp14 used IL-1β generously given by Prof. Lee Gehrke of HMS and HST.

Effects of IL-1β on DNA content

Four days of IL-1β saw a slight but statistically significant decrease in bulk DNA content, to 92% of control in Exp16 (Figure 4-15). Result from another experiment (Exp14) was not conclusive due to high sensitivity (9.5%). A similar decrease to 88% of control level was observed for calf explants following only 2 days of treatment.

Effects of IL-1β on bulk GAG content

Tissue GAG content measured after 4 days of IL-1β treatment differs significantly between control and treated disks (Figure 4-16). GAG concentration in the two adult experiments decreased to 78% and 67% of control levels respectively. In calf, a reduction to 83% of control was observed after 2 days.

Effects of IL-1β on GAG synthesis

As mentioned earlier, changes in final GAG content may arise from two sources, changes in GAG release and new GAG synthesis. From radio-incorporation mea-

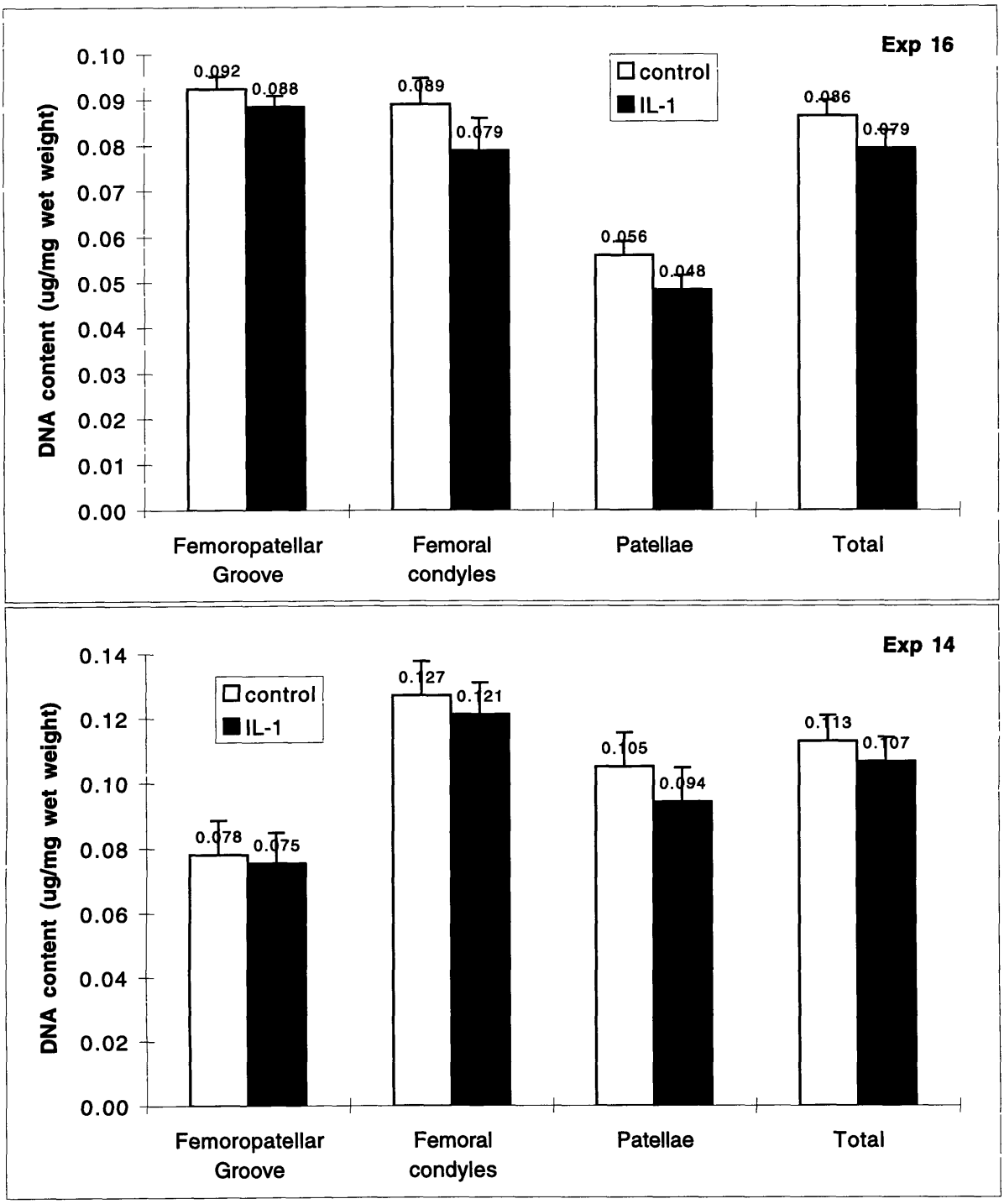


Figure 4-15: Average DNA contents in adult articular cartilage with and without IL-1β treatment, mean±SEM. Both experiments were cultured for a total of 8 days, with IL-1 treatment starting on day 4.

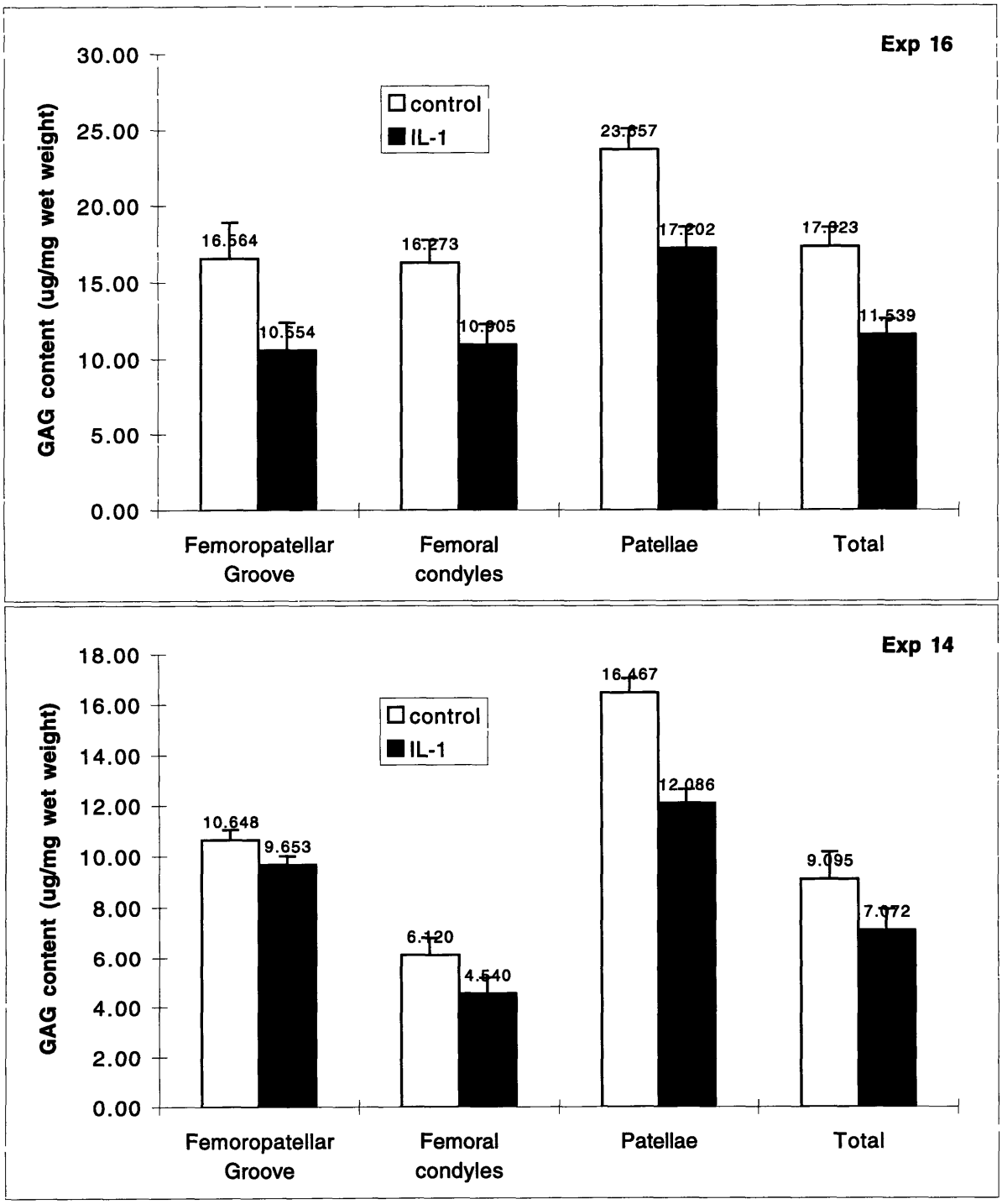


Figure 4-16: Average GAG contents in adult articular cartilage with and without IL-1β treatment, mean±SEM. Both experiments were cultured for a total of 8 days, with IL-1 treatment starting on day 4.

surements, it is obvious that GAG synthesis is dramatically reduced following IL-1β treatment. Three days after the start of adding IL-1β to their culture media, chondrocytes were synthesizing at rates as low as 27% and 13% of control in the two experiments (Figure 4-17). Compounded by the fact that adult tissue in culture already has synthesis rates lower than GAG loss in control samples, IL-1β brought almost a halt to new GAG synthesis, significantly expediting depletion of tissue GAG.

Effects of IL-1β on GAG release

At the same time, IL-1β also promotes increases in GAG release out of the ECM. This increase is already statistically significant 20 hours after IL-1β was first introduced. Figure 4-18 shows the percentage remaining GAG normalized to GAG_t for both control and treatment groups in Exp16. For the first three days, when the two groups were identically treated, no difference in GAG release was seen. During the first 20 hours after incubation with IL-1β supplemented media, cartilage disks on average released 2.6 times as much GAG as control. However, the next 20 hour period saw release rates going back to almost control levels. But heightened release is evident again during the next two days of culturing with IL-1β. Because overall GAG is decreasing in cultured cartilage disks, daily release amounts are normalized to total amount of GAG available in Figure 4-19. Once again, the day after treatment say huge increase in GAG loss.

$$[\%\text{GAG loss of total}]_n = \frac{R_n}{GAG_t - \sum_{i=1}^{n-1} R_i} \tag{4.29}$$

This pattern of dramatic increase in GAG release shortly after treatment followed by "recovery" to lower release levels, is seen in all the experiments with IL-1β. The significance and mechanism of such behavior is not yet clear. Usually the "recovery" is not lasting though. As the treatment protocol continues, elevated levels of GAG loss recurs.

Effects of IL-1β on Water Content

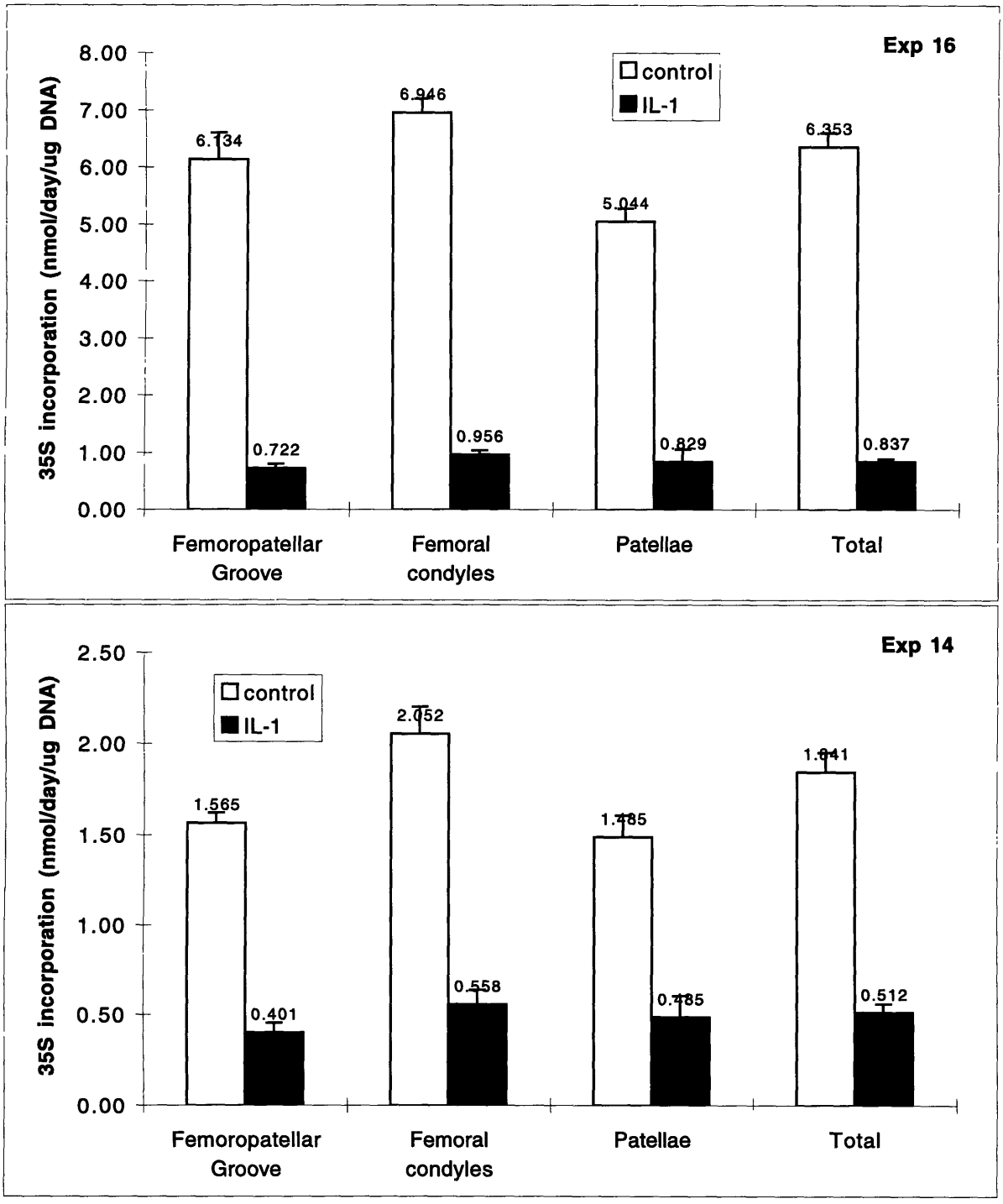


Figure 4-17: Average GAG contents in adult articular cartilage with and without IL-1β treatment, mean±SEM. Both experiments were cultured for a total of 8 days, with IL-1 treatment starting on day 4.

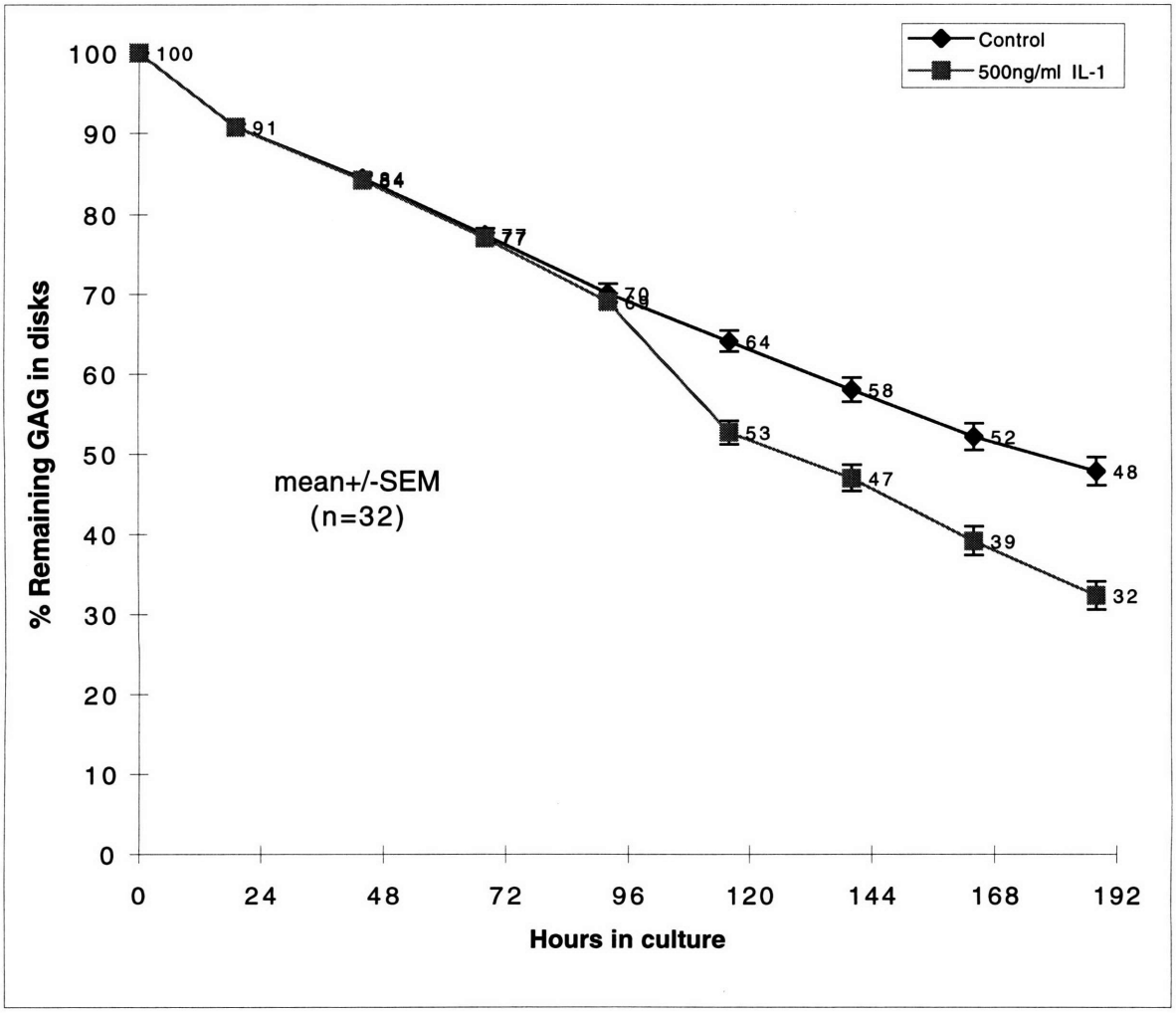


Figure 4-18: Average remaining GAG content in adult cartilage disks with and without IL-1β treatment, mean$\pm$SEM normalized to GAG_t. Data taken from Exp16.

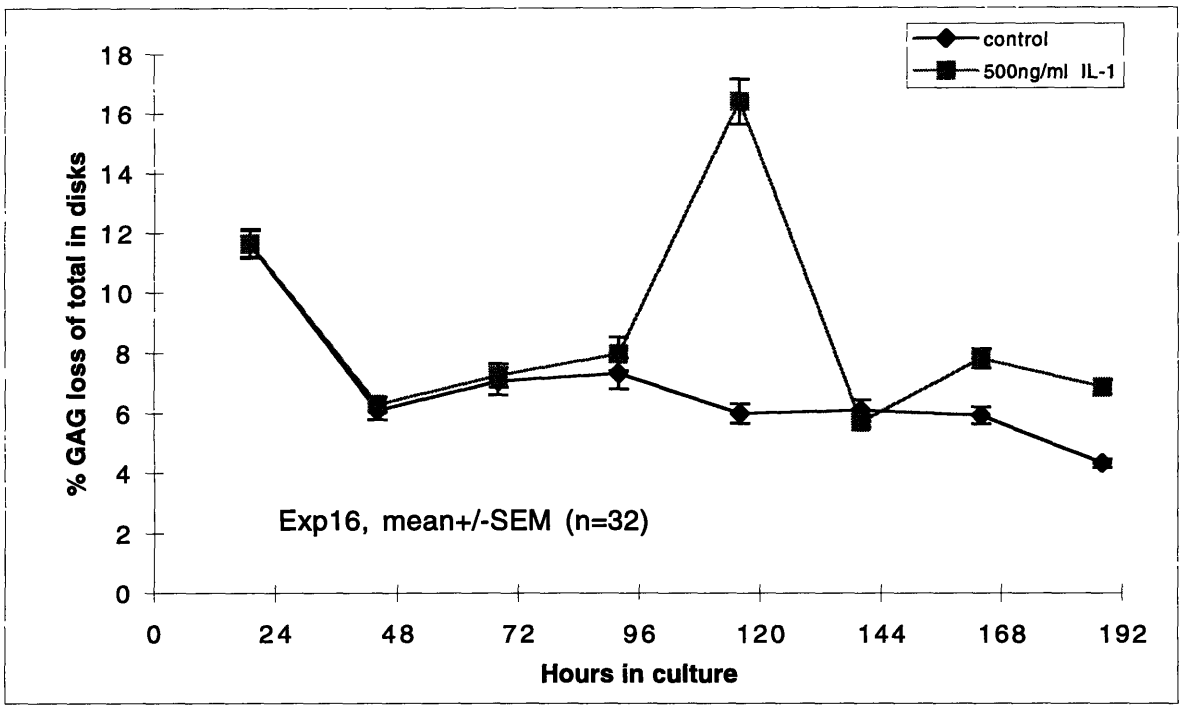


Figure 4-19: Average daily GAG loss in adult cartilage disks as percentage of bulk GAG content of the previous day, with and without IL-1β treatment, mean±SEM. Data taken from Exp16 and normalized to amount per 24 hour.

Measured wet weights on the last day of experiment also differ slightly between control and IL-1 treated disks. In Exp16, such wet weights dropped to 93% of control, while the other experiment had 7.4% sensitivity and was inconclusive. No statistically significant difference was seen from either dry weight measurements or calculated water percentage. It is likely that due to the net loss of GAG out of the ECM, not only does tissue dry weight decrease, the ability of the matrix to hold water also dwindles. Therefore overall water percentage remains essentially unchanged. The nonsignificant change in dry weight is likely the outcome of high sensitivity (7-9%). This speculation is somewhat confirmed from data in the calf articular experiment. There was again a reduction to 94% of control after 2 days of IL-1 treatment, but there was also a statistically significant decrease of dry weight, to 90% of control on average, with no net change in calculated water percentage.

Spatial Degradation Profile of IL-1β

Histological staining was performed for control and IL-1 treated disks in exp16. Starting on day 4, eight disks from two slices were placed in fixative solutions daily till the end of the experiment. These fixed cartilage disks were later sectioned, stained and observed for GAG distribution.

Due to the lower concentration in adult tissue, GAG staining was much weaker than typical calf tissue staining. Nevertheless, radial degradation profile is very much evident in disk both with and without treatment of IL-1β. However, there is no readily observable difference in GAG staining between IL-1β treated and control disks as both lose relatively large amount of GAG every day. Another difficulty may come from the structure of adult cartilage ECM. As there is no vasculature in adult tissue, besides significant radial loss of GAG, it is conceivable that GAG is also lost evenly throughout the entire volume, making it impossible to detect minor differences in overall staining intensity.

Chapter 5

Summary and Future Work

5.1 Thesis Objectives and Summary

WHAT ARE THE BIOCHEMICAL COMPOSITION AND METABOLIC BEHAVIOR OF ADULT BOVINE ARTICULAR CARTILAGE EXPLANTS, AND HOW IS IT DIFFERENT FROM IMMATURE CARTILAGE?

There is no single number of biochemical composition and metabolic behavior for adult bovine articular cartilage explants, as such properties vary widely from one animal to another, and within one animal from one location to another. Nevertheless, compared to younger tissue, mature cartilage ECM has less GAG content and cell population, in terms of both overall abundance and unit volume concentration. The relatively few chondrocytes present seem to have comparable rates of synthesis of

Table 5.1: Average values of DNA, GAG, ^3H- and ^{35}S-incorporation rate, and water percentage for cartilage disks in selected experiments without IL-1 treatment, in mean±SEM. All cartilage samples were cultured in 20% FCS. Bulk DNA, GAG and radio-incorporations rates were measured on the last day of each experiment. Sample sizes are included in parentheses.

	Exp6($n = 56$)	Exp8($n = 56$)	Exp14($n = 20$)	Exp16($n = 32$)	Calf($n = 32$)
DNA(μg/mg)	0.200±0.007	0.079±0.004	0.086±0.003	0.113±0.008	0.680±0.015
GAG(μg/mg)	27±2	12±1	17±1	9±1	67±1
^{3}H(nmol/24h/μg)	1.59±0.05	3.08±0.09	2.98±0.075	1.29±0.04	4.2±0.3
^{35}S(nmol/24h/μg)	6.0±0.2	7.7±0.3	6.35±0.2	1.8±0.1	7.7±0.4
Water percentage	79.2±0.5	83.7±0.5	83.3±0.7	75±2	83.1±0.4

new GAG and protein molecules as those in younger matrix, judging from similar radioactive precursor incorporation. As to the pace of GAG loss, numbers from matrix of older animal again have high variation, but they are all much higher than those from younger tissue. Typical rates are 5%-10% of GAG lost per day for adult and about 1%-2% for immature cartilage. As a consequence of low synthesis and high degradation, there was a net loss of GAG when culturing adult articular cartilage even with 20% serum. On the contrary, the same 20% serum resulted in a net gain of overall GAG content in calf articular cartilage. In either culture system no change in DNA concentration during the culture period was observed. Averages of some of the above parameters are summarized in Table 5.1.

WHAT ARE THE ANIMAL-TO-ANIMAL AND SITE SPECIFIC VARIANCES FOR THE ABOVE PROPERTIES, AND THE IMPLICATION OF SUCH VARIATION ON METHODS OF ANALYSIS?

It is obvious from Table 5.1 that most of the these measured properties vary significantly from animal to animal, sometimes exceeding 100% difference. Similar variations are seen at different locations in a single animal with no one joint consistently dominant in any property. However, it was experimentally proven that intra-slice variation in adult articular cartilage is much smaller than inter-slice variations, as samples are much closer to each other geographically. The subsequently chosen paired-sample hypothesis testing was expected to yield much better performance at detecting true differences due to experimental treatments, and was adopted as the method of choice in analyzing data from IL-1β treatments.

It was also found that although various properties differ sharply from one joint to another, when using the paired-sample hypothesis testing, inclusion of data from all three joints yielded the best sensitivity, benefiting from larger sample size. Therefore, when studying effects of IL-1β treatment on adult cartilage, samples were harvested from all three joints, and sample sizes were determined from the minimum numbers obtained in control experiments.

WHETHER THIS CULTURE SYSTEM CAN BE USED FOR CARTILAGE STUDIES, JUDGING FROM THE AVAILABILITY OF TISSUE AND THE REQUIRED SAMPLE SIZE FOR CONCLUSIVE RESULTS?

The minimum sample sizes for detection of at least 10% difference in treatment were provided in Table 4.1 for several properties. In the interest of studying PG metabolism, cartilage disks on the order of 60 seem to suffice for the above resolution. Experimentally, it is feasible to obtain 20-40 disks from the femoropatellar groove, 20-30 disks from each condyle, and 10-20 from the patellae. Therefore when cartilage samples are harvested from all three joints, the required sample size is possible to reach. If treatment turns out to have more dramatic changes in the properties of interest, such as the rates of synthesis as found out later, such a large sample size becomes a luxury.

HOW DOES TREATMENT OF IL-1β AFFECT JOINT PROPERTIES, SUCH AS BIOCHEMICAL COMPOSITION, BIOSYNTHESIS AND DEGRADATION, AND WHETHER SUCH CHANGES CORRESPOND TO PREVIOUS OBSERVED ROLES OF IL-1β?

First of all, at a level of 500 ng/ml, IL-1β has an impact on the chondrocyte population, reducing overall abundance by almost 10% after 4 days of incubation. The activities of the remaining cells, in terms of both protein and GAG synthesis, are dramatically reduced. Radiolabeled proline incorporation was decreased to only about half of control, while incorporated sulfate into macromolecular GAGs was down to less than 30%. At the same time, matrix GAG loss is elevated, presumably through increased production and activation of GAG-chewing proteases, and the loss of matrix mechanical integrity followed by collagen degradation. Less than 24 hours after the introduction of IL-1β, GAG release from adult bovine articular ECM is already statistically higher than control. The next few days of continuous culturing with IL-1β saw levels returning to near control levels initially, followed by slight but statistically significant increases again. As culturing stops at four days after IL-1 treatment, it is unknown whether such trend continues. As bulk GAG content keeps falling, the ability of the matrix to hold water declines, as evidenced in decreases in wet weight.

Table 5.2: Average changes due to IL-1β treatment, as percentage of control. Both experiments had sample size of 32 disks each for both control and treatment, and were cultured identically except days of IL-1β treatment. Numbers in parenthesis indicate sensitivities following non-conclusive testing.

	Adult(Exp16)	Calf(Exp17)
Bulk DNA	91%	88%
Bulk GAG	66%	83%
^{3}H incorporation	57%	13%
^{35}S incorporation	13%	6%
Initial wet weight	(2.2%)	(2.8%)
Final wet weight	93%	94%
Water percentage	(1.5%)	(1.1%)

Such changes are certainly to alter material properties of the ECM, although such measurements were not made in this study.

As comparison, calf femoropatellar groove articular cartilage explants were identically cultured and treated with 2 days of 500ng/ml IL-1β. Although results are all in the same direction, IL-1β effects on chondrocyte synthesis in immature cartilage is much more severe. Some of the measurements are summarized in Table 5.2. IL-1β effects on GAG loss are also more pronounced in calf matrix. Bulk GAG content was 83% of control after only 2 days of treatment with the same concentration of IL-1β, and GAG loss during the first 24 hours was more than 4 times the control level. During the second 24 hours, GAG loss decreased some, but still at a level more than twice that of control (Figure 5-1).

WHAT IS THE SPATIAL DEGRADATION PROFILE FOLLOWING IL-1β TREATMENT, AND HOW DOES IT COMPARE TO THOSE SEEN IN YOUNGER TISSUE?

Histological studies from adult cartilage cultures showed clear radial loss of GAG at the absence of any vasculature. Tissue GAG staining seems to qualitatively correspond with the calculated GAG content remaining in the cartilage disks, but it is hard to draw conclusions from IL-1β treated samples. Although significant difference was seen in the amount of GAG loss due to IL-1β, such difference was not clear in

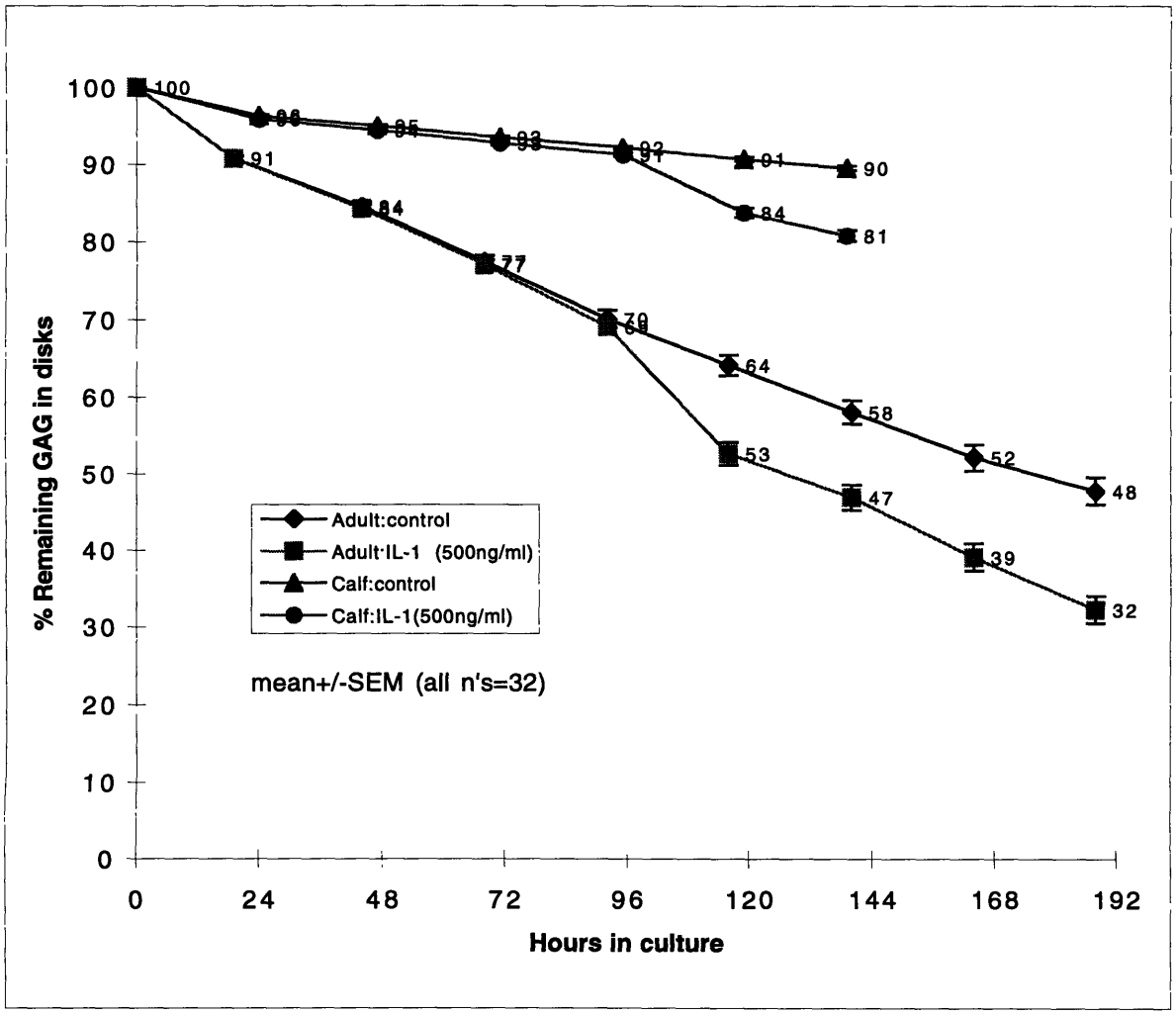


Figure 5-1: Average remaining GAG content in adult and calf articular cartilage disks with and without IL-1β treatment, mean$\pm$SEM normalized to GAG_t. Data taken from Exp16 and Exp17.

histological GAG staining. Several facts may help explain this result. First, unlike calf, adult cartilage explants lose a large amount of GAG even under control conditions, combined with less overall GAG on day 0, their staining is rather weak during the culturing period with IL-1β. Secondly, without the presence of vasculature and localized GAG loss in adult tissue, even the same amount of GAG loss per day would be harder to visualize over a much larger volume. It is clear from the diffusivity of IL-1β that in 20 hours it has enough time to reach almost all the cells. Furthermore, GAG loss is much more pronounced in calf matrix. While control disks from calf released on average about 1% of GAG_t daily, IL-1β treatment caused a first day release as high as 7% of GAG_t. As a contrast, adult matrix had about 6-7% loss every day without IL-1β, and a 16% drop with it (Figure 5-1).

5.2 Future Work

Obviously, cells in mature and immature cartilage behave differently under the influence of IL-1β. Those in younger tissue tend to respond more dramatically to stimulation by this cytokine, evidenced in both synthesis and degradation. It is clear from previous studies that at least the profile of GAG degradation implicates possible involvement of multiple cell types. Unfortunately this study is not able to distinguish whether chondrocytes in the two culture systems reacted differently to IL-1β or the presence of other cell types in younger tissue made the difference. However this study did show that the difference in responses to IL-1β is not due entirely to the amount of supplemented serum, as the same amount was used in culturing both explants.

There are several directions this research may take. All the cartilage explants in the above studies were cultured in freeswelling conditions, with no applied strain. This is unusual to cartilage ECM, as its primary function is to sustain loading. Therefore studies of chondrocyte behavior and matrix metabolism under the context of mechanical loading will be relevant. Some preliminary control studies with axial confinement ($\sim$ 1% strain) showed dramatic changes in both synthesis and GAG loss. Whether static mechanical compression will have similar effects on adult cartilage as it did

on calf, and whether dynamic or load release protocols can modulate chondrocyte synthetic behaviors, are possible topics of future research.

Along the grand scheme of research interests in this lab, cartilage cellular and metabolic behaviors can be studied under joint applications of both mechanical compression and IL-1β, as this is closer to conditions naturally occurring in arthritic diseases. Previous studies showed somewhat "alleviated" effects by IL-1β when cartilage explants were simultaneously loaded to a fixed strain. Whether such behavior can be repeated in adult cartilage is important as such information may likely help patients with arthritis determine the types and intensity of their exercises.

In line with the development of proper culturing systems, neither the adult and calf articular cartilage explants achieved balance in PG metabolism while in culture with 20% FCS. In order to make conclusions comparing parameters from different culturing periods, a true "steady state" metabolic condition will be very useful and needs further experimenting.

In order to better study the spatial and temporal patterns of IL-1β mediated GAG loss, some protocols of histological staining have to be revised to reflect the lower GAG content in adult tissue, such as sectioning to thick slices, or modifying the staining agent. Improvements in this resolution can help us better identify the profile of GAG loss immediately following IL-1β infiltration, and possibly the mechanism of "recovery" shortly after.

Appendix A

Effects of axial confinement on adult cartilage explants

Several control studies were started in order to understand the role of mechanical loading on adult cartilage PG turnover. Some of the results are shown below. The first experiment (Exp12) compares bulk biochemical measurements and GAG metabolism between freeswelling and axially confined cartilage disks.

Adult articular cartilage explants of same size and shape described in Chapter 3 were harvested and cultured in supplemented media with 20% FCS on day0. They were allowed in the freeswelling condition for 4 days. Starting on day 4, randomly selected half of all disks were moved into mechanical compression assemblies, and remained in these assemblies till the end of the experiment. The other half stayed freeswelling. One of these assemblies cultures 12 cartilage disks, and includes a 24 well culture dish, a cylindrical glass post about 4mm in diameter and 2-3cm in length that allows axial compression, and a set of metallic supporting structures to maintain desired strain levels on the combination of glass post and cartilage disk. For this experiment, no fixed strain was applied. The glass posts were only compressing the disks through their weights ($\sim$ 2g). A total of 86 hours were cultured, with media changed every 20-24 hours as usual, except cartilage explants were not allowed to move around during the procedures. Because of the resting glass posts, fluid flow was no longer possible through either of the two axial surfaces of cartilage disks, severely

affecting nutrient and oxygen transport. In an attempt to make such exchanges more efficient, all the mechanical compression assemblies were continuously shaken to maintain convection throughout the experiment. During the 86 hours of axially confined culturing for the treatment disks, the last 24 hours included radioactive precursor labeling, The control disks were identically labeled. Following the experiment, similar measurements and data analysis were done as described in the thesis.

Another experiment (Exp15) cultured cartilage disks identically except after 4 days of freeswelling, only 8 hours of axial confinement was applied before tissues were lyophilized. The media for this entire 8 hours included radiolabels.

Some of the results from both experiments are shown in the following figures. There was no statistically significant reduction in bulk DNA content in either the short-term or the long-term treatment protocols (Figure A-1). Bulk GAG content was at 80% of control after 86 hours, but non-significant after 8 hours (Figure A-2). Part of the reduction in overall GAG content can be attributed to the dramatic decrease in new GAG synthesis, which is down to 78% of control after 8 hours of compression, and to only 45% following 86 hours (Figure A-3). At the mean time, rate of protein synthesis is reduced too. Both experiments showed statistically significant results, to 85% 8 hours into compression and 68% at the end of 86 hours (Figure A-4).

Rates of GAG loss were also studied. Adult cartilage explants after 8 hours of axial confinement did not statistically differ from freeswelling controls in the amount of GAG detected in media. But the results were significant 14 hours later, when compressed disks were found releasing 65% more GAG than controls. Percentage daily remaining GAG contents normalized to GAG_t are plotted in Figure A-5. Unlike IL-1β treatments, no obvious "recovery" pattern was observed. Continued application of fixed strain resulted in progressively more release of GAG from the ECM. In Figure A-6, daily percentage loss was plotted in terms of overall GAG contents in cartilage disks. Once again, while the control disks maintained relatively constant levels of GAG loss, axially confined disks were escalating the pace of GAG degradation. Furthermore, wet weights in the treatment group was also lower than those in the control, consistent with significant loss of GAG.

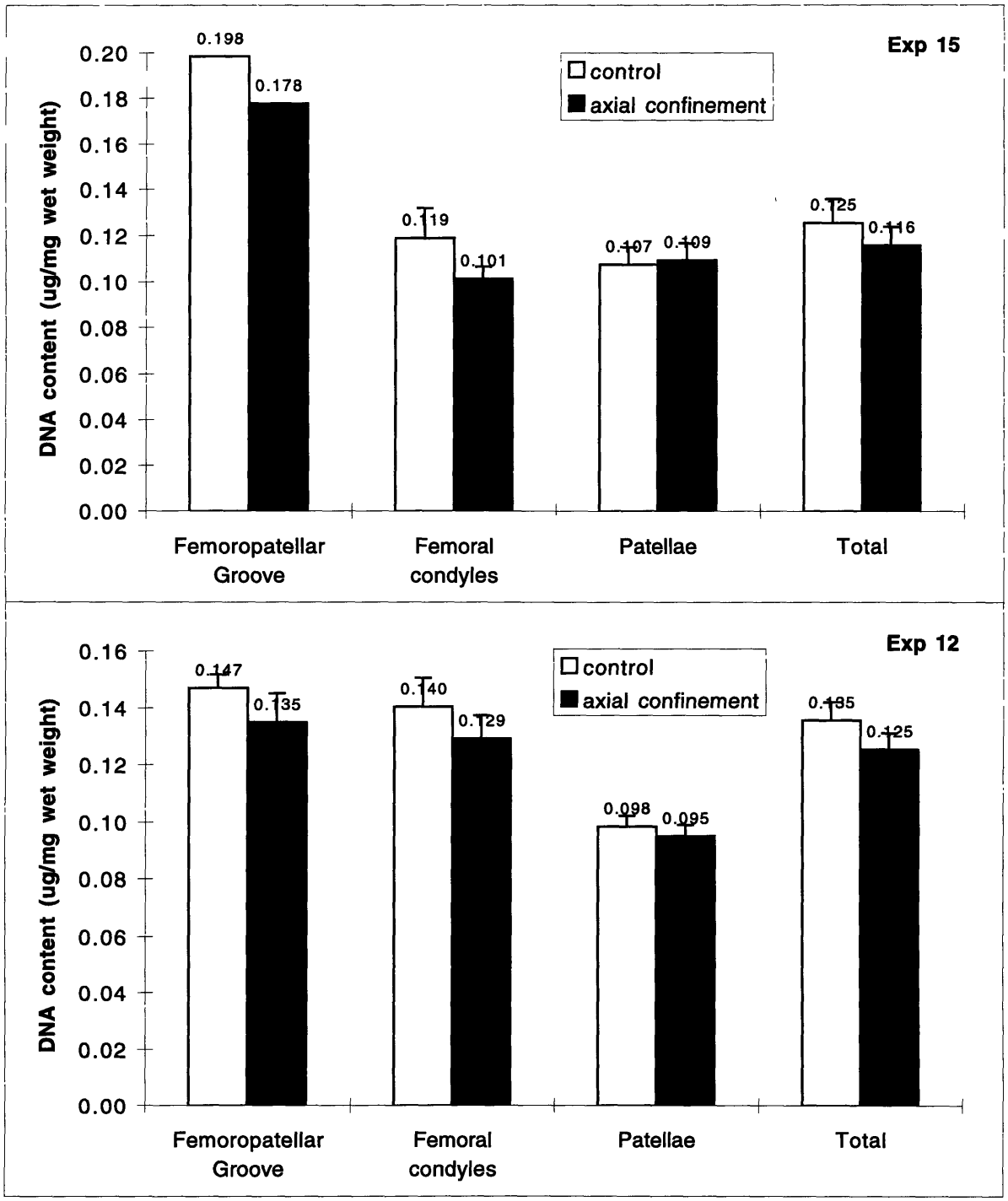


Figure A-1: Average DNA contents of adult articular cartilage in freeswelling and axially confined cultures, for 8 hours (Exp15,n=14) and 86 hours (Exp12,n=36), mean±SEM. Both experiments included 4 days of freeswell culture before applying compression. The estimated strain of compression is 1%.

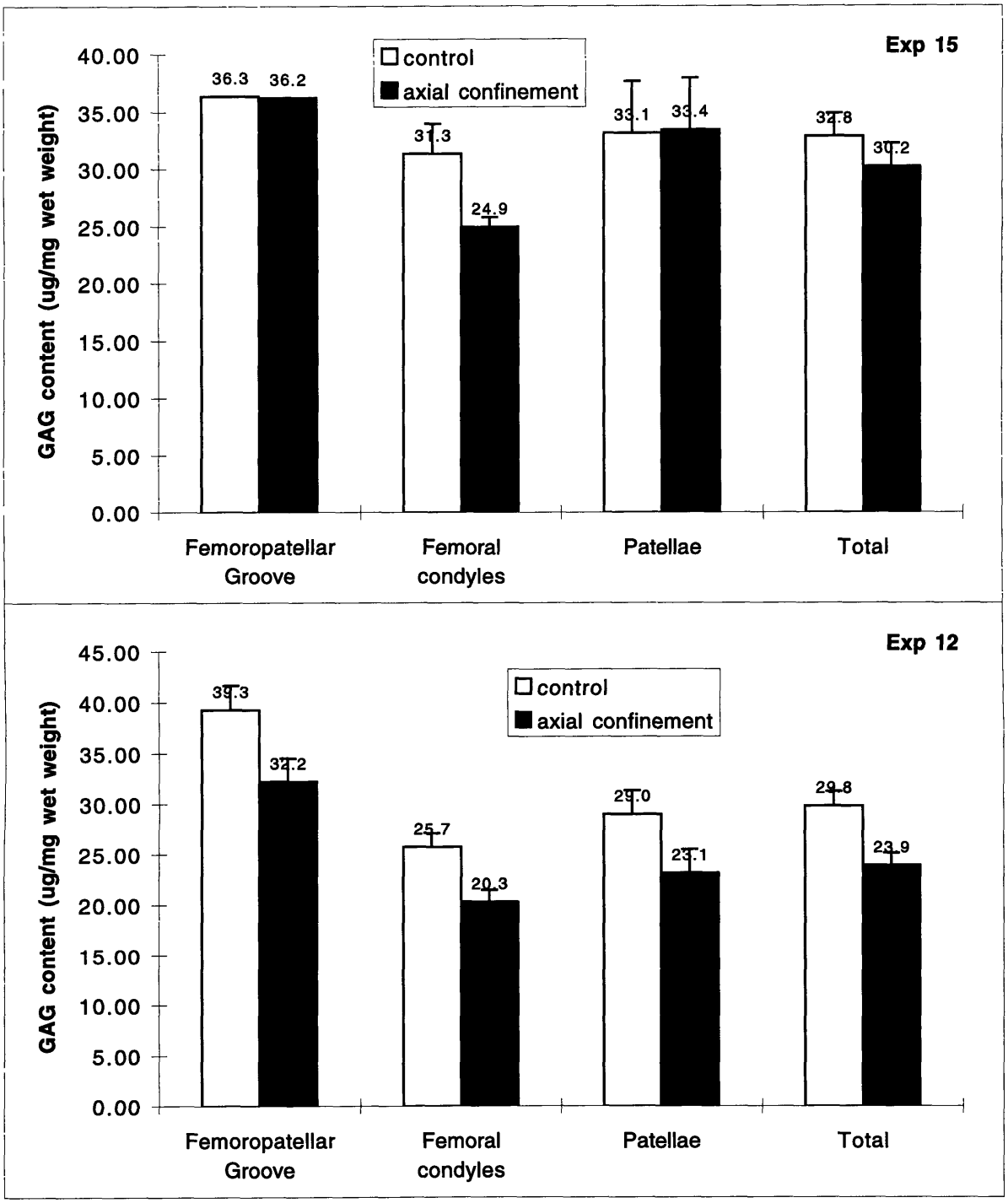


Figure A-2: Average GAG contents of adult articular cartilage in freeswelling and axially confined cultures, for 8 hours (Exp15,n=14) and 86 hours (Exp12,n=36), mean±SEM.

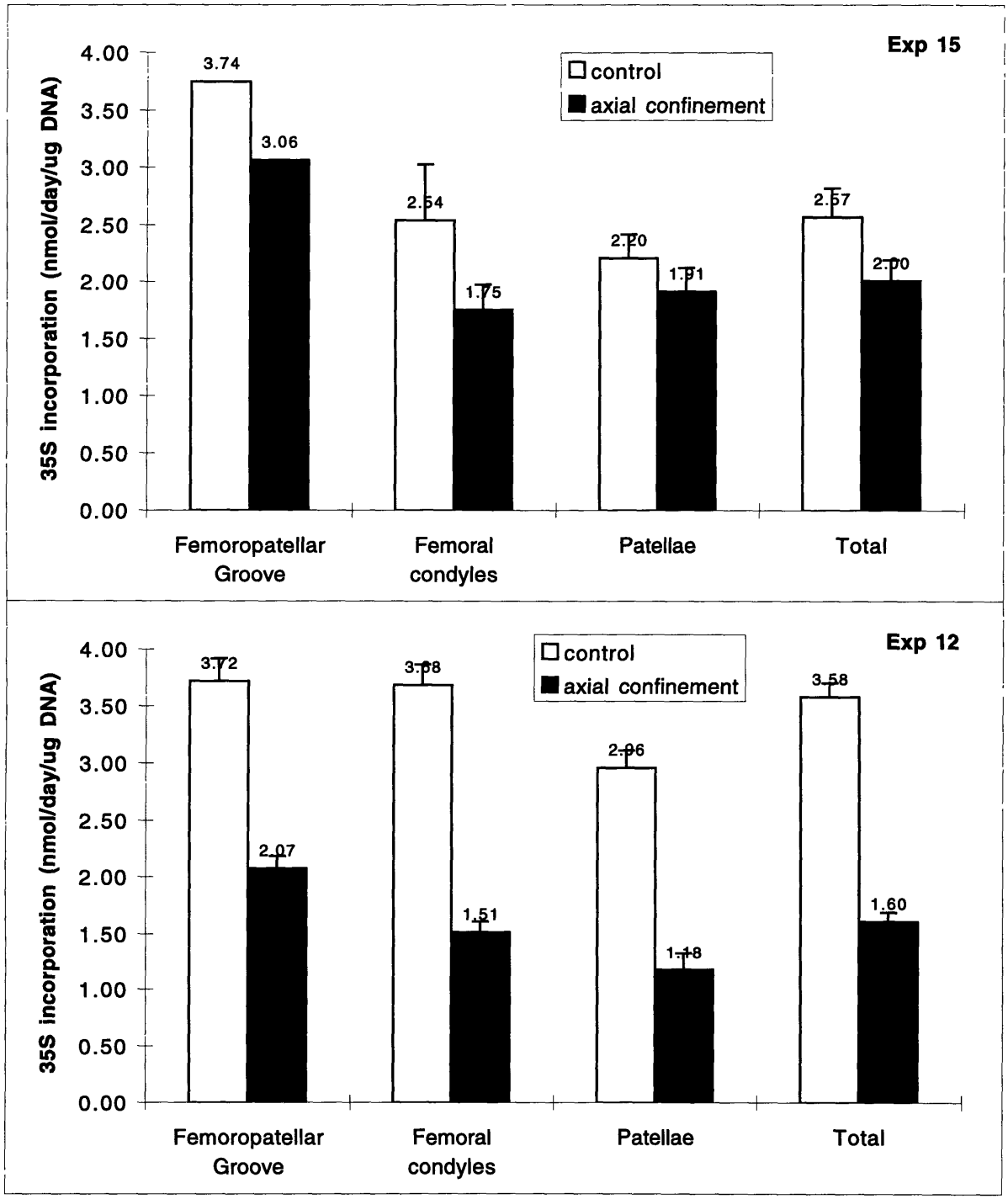


Figure A-3: Average ^{35}S-incorporation rates of adult articular cartilage in freeswelling and axially confined cultures, for 8 hours (Exp15,n=14) and 86 hours (Exp12,n=36), mean±SEM.

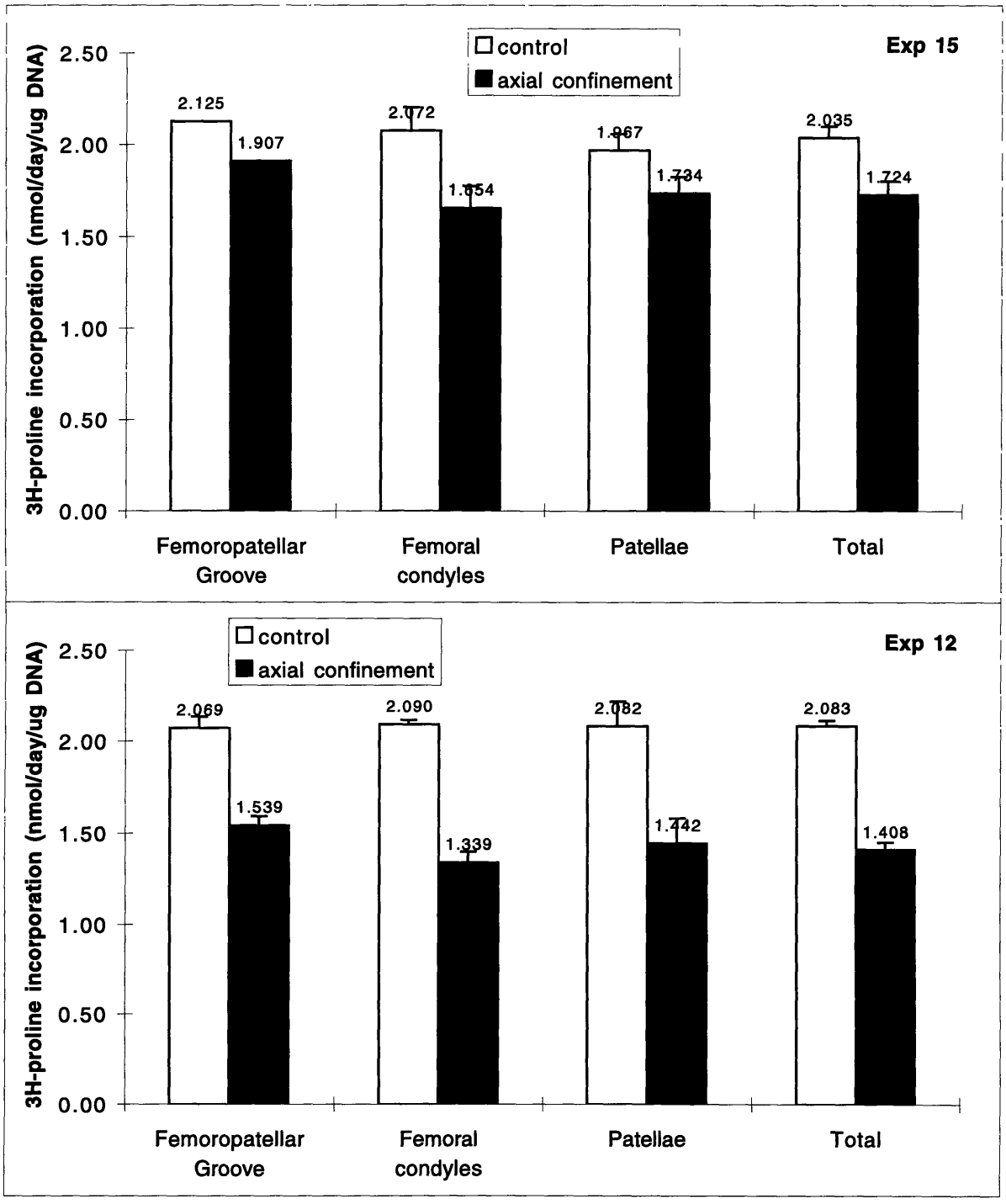


Figure A-4: Average ^{3}H-incorporation rates of adult articular cartilage in freeswelling and axially confined cultures, for 8 hours (Exp15,n=14) and 86 hours (Exp12,n=36), mean±SEM.

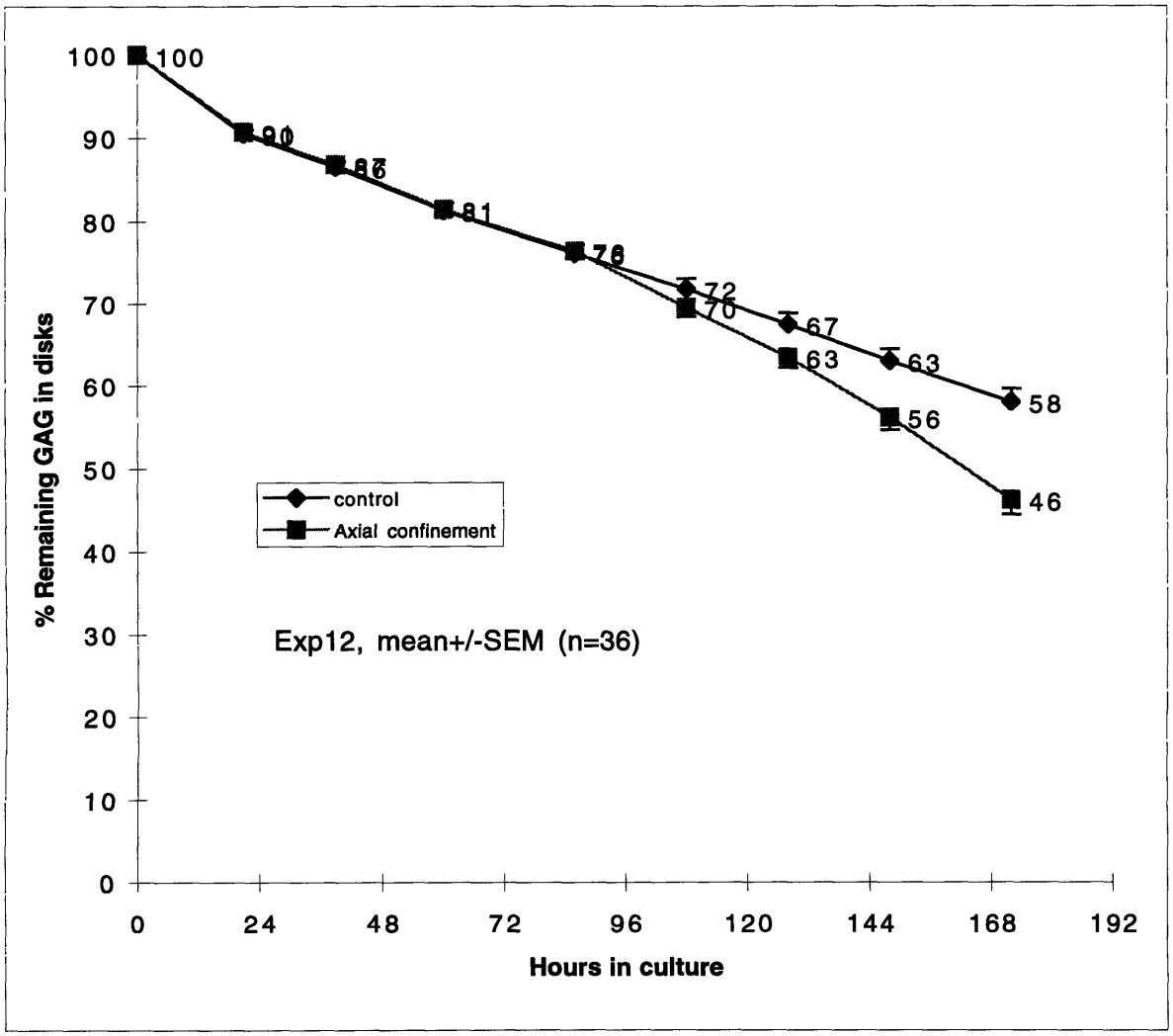


Figure A-5: Average remaining GAG content in adult cartilage disks between freeswelling and axially confined disks, mean±SEM normalized to GAG_t. Data taken from Exp12.

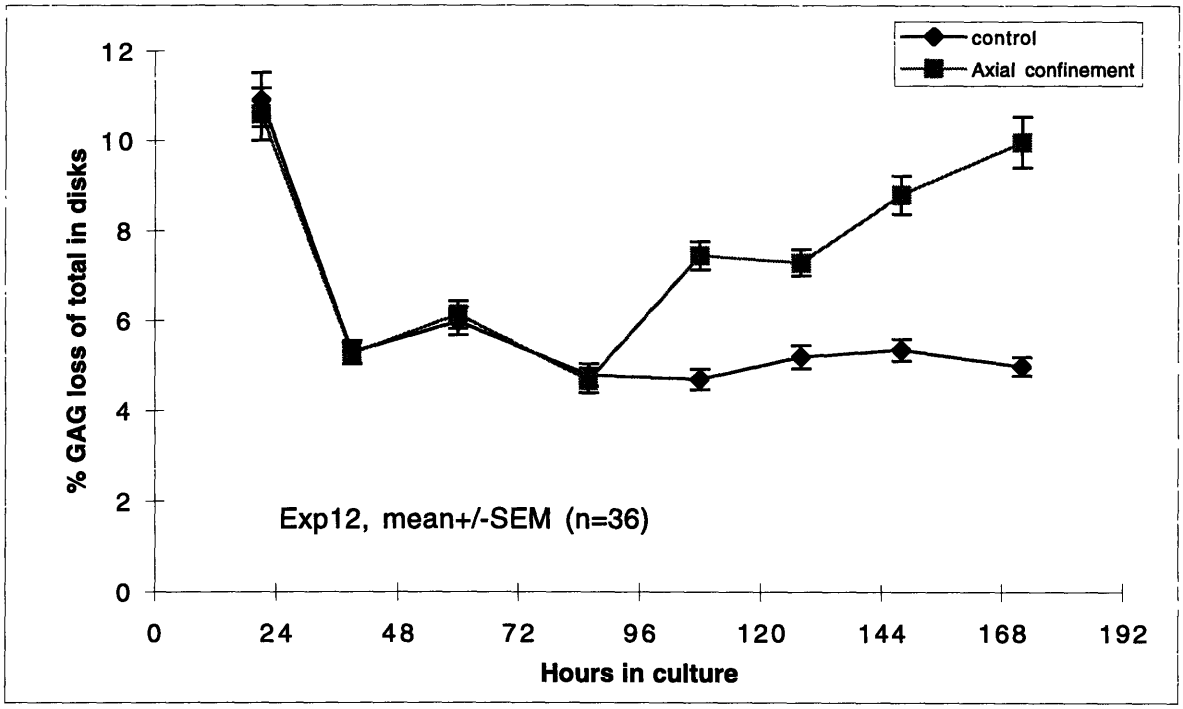


Figure A-6: Average daily GAG loss in adult cartilage disks as percentage of bulk GAG content of the previous day, from freeswelling and axially confined disks, mean±SEM. Data taken from Exp12 and normalized to amount every 24 hour.

Several difficulties make interpretation of the above results more complicated. As mentioned earlier, dramatic differences in the surface areas of nutrient exchange between freeswelling and axially confined specimens may itself have significant impact on the behavior of chondrocytes. Although no significant reduction in DNA content was observed, the question of whether all the cells were still alive awaits a better means of detection. On the other hand, the increase in GAG degradation is most likely the result of compression. The less than 1% strain or 3 kPa pressure is much less than the typical MPa range cartilage tissue usually experience. And despite the sharply reduced surface area, GAG release is very much higher.

A protocol of 50% mechanical compression was also experimented, comparing the above close to 0% compression to the fixed 50% strain based on original cut size. But the limited sample size did not reveal any significant results.

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