

**EFFECT OF FLUID FLOW ON TISSUE-ENGINEERED
CARTILAGE IN A NOVEL BIOREACTOR**

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The Academic Faculty

by

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EFFECT OF FLUID FLOW ON TISSUE-ENGINEERED CARTILAGE IN A NOVEL BIOREACTOR

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To my wife Amy, son Nolan, parents Janet and Vin, and brother Nate ...

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Do-do-do, do-d'do ... Do-do-do, do-d'do ... Yeah down by the river, down by the banks of the river Charles ...

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LIST OF SYMBOLS

b	bioreactor flow chamber width
h	bioreactor flow chamber height
Q	bioreactor volumetric flow rate (mL min ⁻¹)
τ	wall shear stress (dynes cm ⁻²)
$^{\circ}$	degrees
g	gravity
σ	stress
ε	strain
ρ	density
μ	viscosity
E^*	dynamic compressive modulus (MPa)
E'	dynamic compressive storage modulus (MPa)
E''	dynamic compressive loss modulus (MPa)
E_Y	tensile Young's (constant strain rate) modulus (MPa)
$N=$	sample size
L	bioreactor length
L_e	entry length
Re	Reynold's Number

LIST OF ABBREVIATIONS

ACT	autologus chondrocyte transplantation
AMV	avian myeloblastosis virus
ANOVA	analysis of variance
BAC	bovine articular chondrocytes
bFGF	basic fibroblast growth factor
BME	β-mercaptoethanol
BMP	bone morphogenic protein
C	Centigrade
cDNA	complimentary DNA (library)
cm	centimeters (10^{-2} m)
CO ₂	carbon dioxide
COMP	cartilage oligomeric matrix protein
COX	cyclo-oxygenase
cT	cycle threshold
DMA	Dynamic Mechanical Analysis
DMEM	Dulbecco's Modified Eagle's Medium
DMMB	dimethyl methylene blue
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum ("serum")
FDA	Food and Drug Administration

FFT	fast Fourier transform
g	grams
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hours
HCl	hydrochloric acid
H&E	hematoxylin and eosin
Hz	Hertz (cycles per second)
IgG	immunoglobulin G
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IL-1	interleukin 1
ITS	insulin, transferrin, selenium
kPa	kilopascals (10^{-3} Pa)
L	liter (1000 mL)
mg	milligrams (10^{-3} g)
MgCl ₂	magnesium chloride
min	minutes
mL	milliliter (cm^3)
mm	millimeters (10^{-3} m)
MMP	matrix metalloproteinase
mN	milliNewtons (10^{-3} N)
MPa	Megapascals (10^6 Pa)
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cells
μm	micrometers (10^{-6} m, “microns”)

μg	micrograms (10 ⁻⁶ g)
N	Networks
nm	nanometers (10 ⁻⁹ m)
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OATS	osteocondral autograft transplant surgery
OD	optical density
Pa	Pascals
PBS	phosphate buffered saline
PG	proteoglycans
PGA	polyglycolic acid
PG-E ₂	prostaglandin E ₂
PI	protease inhibitor
PLA	polylactic acid
PLLA	poly-L-lactide
PRG4	proteoglycan 4
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RNA	ribonucleic acid
RT	reverse transcriptase
SafO	Safranin-O
SD	standard deviation
sec	seconds
SEM	standard error of the mean

sGAG	sulfated glycosaminoglycans
TGF- β	transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinase

SUMMARY

When damaged through either trauma or disease, articular cartilage exhibits little to no capacity for self-repair, thus prompting surgical intervention to alleviate joint pain. Damaged articular cartilage can degenerate to an arthritic state, leaving the subject with a painful and debilitating condition. Few clinical interventions have proven effective in restoring joint function over the long-term, prompting the exploration of new strategies including tissue engineering. Cells are combined with growth factors in an environment suitable for matrix production and assembly into *in vitro* derived articular cartilage tissue. Tissue engineering offers the promise of providing an “off-the-shelf” tissue with the matrix composition and mechanical properties similar to that of native tissue which can be implanted into a defect site with the hopes of restoring function. The cells and bioactive factors within the tissue maintain normal tissue homeostasis and grow with the subject, ideally offering a permanent, life-time solution and improved quality of life.

A tissue-engineered articular cartilage construct with the matrix composition and mechanical properties near that of native tissue has yet to be produced. While many strategies to achieve this goal exist, a dominant one is the use of bioreactor systems. Besides being amenable to large-scale bio-processing, bioreactors can provide a mechanical stimulus and/or improve mass transport in order to stimulate tissue development. Mechanical stimuli applied *in vitro*, such as compression, tension and shear, are inspired by the *in vivo* setting, as articular cartilage resides in a complex and demanding mechanical environment. Reproducing this environment is hypothesized to condition neo-tissue to become more like native tissue while developing *in vitro*.

The *overall objective* of this thesis is to employ a novel flow bioreactor to improve matrix composition and mechanical properties of cartilage constructs for load-bearing applications. Improving the matrix components and mechanical stability of the tissue to be more similar to that of native tissue may improve the ability of the tissue to function *in vivo*. Our *central hypothesis* is that exposure of three-dimensional (3D) scaffold-free, tissue-engineered cartilage constructs to physiologically-inspired forces in a novel bioreactor system will enhance cartilage matrix synthesis, leading to more robust mechanical properties.

The novel, dual-chambered, parallel-plate bioreactor system was developed to provide a quantifiable, steady shear stress stimulus to a slab of scaffold-free engineered cartilage. While fluid-induced shear stress has been used in other bioreactor systems, this is the first study involving a parallel-plate bioreactor with a block of neo-tissue. The parallel-plate nature of this system is significant because when properly designed, a pre-determined level of stimulus is applied to >95% of the tissue's length. This is in stark contrast to spinner flask-like or perfusion systems which apply an unknown or widely heterogeneous shear to the tissue [1, 2]. Perfusion systems are particularly difficult to analyze as fluid flows through a complex maze of pores in scaffold-based tissue. To facilitate analysis of shear stress on the engineered tissue, a scaffold-free tissue was used in our system. Tissues of this type have the added clinical benefit of possessing matrix components derived solely from the cells. In addition, this bioreactor possess two chambers that are separated by the tissue, allowing for multiple stimuli (biochemical or mechanical) to be applied in a differential fashion for potential induction of heterogeneity, similar to the native cartilage-to-bone transition.

In Chapter 3, the bioreactor design is presented along with a feasibility study. Shear stress was applied to the developing scaffold-free tissue in order to improve the matrix composition and tensile mechanical properties. After only three days of continuous shear stress stimulus, a significant increase was measured in type II collagen and total collagen content as well as in the tensile mechanical properties in shear-exposed tissues (0.1-Pa) when compared to static controls. Tissues were stained throughout for type II collagen and sulfated glycosaminoglycans (sGAG), without evidence of type I collagen deposition. Type I collagen should be specifically avoided in engineered articular cartilage constructs, as this protein is well known to not be present in normal tissue and is relegated to fibrocartilaginous or degenerative tissues [3]. Only the tensile mechanical properties were tested in this system, as compressive analysis was not reliable given the thin nature (~300 μm) of the tissues. While this study showed feasibility of the bioreactor to support and stimulate engineered cartilage, the matrix composition and mechanical properties still fell short of that seen in native tissue.

In order to build upon these results, the duration of flow was extended out to seven days, aiming to further improving the matrix composition and mechanical properties. As detailed in Chapter 4, while the mechanical properties, type II and total collagen content were again increased in shear-exposed tissues compared to controls, the beneficial effects came at the expense of several detrimental effects. A distinct band of type I collagen was noted on the shear-exposed surface of the tissue as well as a depletion of sGAG from the matrix. An increasing release of sGAG to the media was also evident over time and varied with increasing shear stress. Type I collagen was noted in the static controls as well, but significantly increased with the application of shear stress. Co-

localized with this band of type I collagen were flattened, fibroblast-like cells reminiscent of a type I collagen-producing phenotype as opposed to the rounded, type II producing cells throughout the rest of the matrix. In this study, an additional flow group was added at a two-decade level (0.001-Pa) below that of the previously used flow group (0.1-Pa). This group was added to not only account for differences in media volume between static and flow-exposed groups, but also to begin to examine any dose-dependent effects of shear stress on tissue development. Also in this study, quantitative PCR was instituted to analyze the gene expression profile of the tissues over time. Generally, type I and type II collagen were transiently up-regulated as early as 24 h in the highest shear group compared to the others. This gene expression profile was corroborated by the quantitative protein and qualitative histology data. Taken together, these studies showed that shear stress magnitude and duration modulates matrix composition and tensile mechanical properties.

With a solid understanding of how fluid flow and shear stress modulates the matrix composition and mechanical properties of engineered cartilage, the lack of thickness associated with the tissues was addressed next. Two benefits of thicker constructs (1-2 mm) are that they (1) are more clinically relevant and thus add impact to the study, and (2) are amenable to compressive testing. Therefore, Chapter 5 describes the effects of seeding density and time in culture on matrix composition and compressive mechanical properties of engineered cartilage of clinically relevant thickness. In this study, tissues were created in small “cell-culture inserts” which are essentially a static, 1/10th scale version of the bioreactor environment. While increasing the seeding density did increase the thickness of the tissues, it was not in a linear dose-dependent manner,

indicating a loss of efficiency with increasing seeding density. In fact, the lowest seeding density performed as well as any other group or better in terms of sGAG and collagen production on a per-cell basis. Compressive mechanical properties were improved over time in nearly all seeding densities, with the greatest dynamic modulus appearing at the three week time point of approximately 325 kPa at seeding densities of 4X and 6X. One of the most interesting results of the study came about when the increased seeding densities were tried in the bioreactor: at higher seeding densities (6X and 8X greater than normal), tissues formed blebs and wrinkles and easily delaminated off the membrane. Hypothesizing that the pores of the membrane were saturated with matrix, membranes with larger pore sizes were used and found to improve attachment and gross appearance of the tissues. Thus, this change (*i.e.*, increasing the pore size of the membranes used in the bioreactor) was instituted immediately for future bioreactor flow studies.

In the final series of experiments as described in Chapter 6, thicker tissues (~1 mm) were developed in the bioreactor and subjected to multiple levels of shear stress using steady and intermittent waveforms. Tissues were analyzed for matrix composition as well as tensile and compressive mechanical properties after three and seven days of flow, to parallel that which was examined in earlier, thin-tissue studies. Generally, the effects of shear stress and time in culture were similar for many metrics of matrix composition and mechanical properties, but to a lesser extent. This could perhaps be attributed to the time required for the thicker tissues to develop and mature. In an effort to exceed the beneficial results obtained in thin tissues while avoiding the detrimental effects, intermittent flows were examined. Tissues were subjected to flows alternating between different levels of shear stress for multiple duty cycles. Unfortunately, no

synergistic effects were seen with the intermittent flow, with most intermittent conditions scoring in between the two different shear levels. On a more positive note, these results underscore the fidelity with which one can use shear stress to affect the development of engineered cartilage.

In conclusion, this work produced a novel bioreactor system capable of modulating the matrix composition and mechanical properties of tissue-engineered cartilage. Results also support the notion that bioreactor-mediated mechanical conditioning can be beneficial to the acceleration of tissue development in an *in vitro* setting. Undoubtedly, the full potential of this bioreactor system has yet to be fulfilled. For example, compressive or hydrostatic pressurization can be used in lieu of or in conjunction with shear stress to condition the tissues. The use of growth factors and / or scaffolds to produce a cartilage or osteochondral construct have yet to be explored in this system, but the versatility inherent in the design warrants further exploration. Additional future directions are discussed in Chapter 7, with the ultimate goal being the development of a clinically relevant tissue-engineered articular cartilage construct for the restoration of function.

CHAPTER 1

INTRODUCTION

Due to its relative avascularity, low cellularity and lack of an undifferentiated cell reservoir, articular cartilage has a very limited capacity for self-repair when damaged either through trauma or disease [4]. Articular cartilage impairment and the resultant reduced joint function affects millions of people at a substantial cost. In the U.S. alone, approximately 6% of adults over 30 years of age are afflicted with osteoarthritis of the knee and 3% in the hip [5]. When accounting for factors such as lost wages in addition to traditional medical care, the costs associated with arthritic conditions exceed \$65 billion per year [6]. Surgical techniques have been developed to address small, focal lesions, but critical size defects remain without a viable solution. Tissue engineering efforts produce cartilage-like tissue *in vitro* containing living cells in the hope of replacing damaged cartilage and restoring joint function. However, tissue-engineered cartilage constructs lack both stable integration into surrounding tissues and mechanical properties necessary to withstand the demanding, complex *in vivo* loads. The *in vitro* production of cartilage constructs with inferior matrix composition and mechanical properties is often attributed to static culture [7]. Bioreactor systems have been developed to overcome the mass transport limitations of static culture and for the application mechanical stimuli. We have developed a novel dual-chambered flow bioreactor system for static and dynamic flow cell cultivation. This bioreactor's dual-chamber design provides separate nutrient media compartments above and below the developing tissue. In addition, the tissue is subject to fluid-induced shear stress, which has been shown to modulate chondrocyte matrix synthesis [8, 9].

Our *central hypothesis* is that exposure of three-dimensional (3D) scaffold-free, tissue-engineered cartilage constructs to fluid-induced shear stress in a novel bioreactor system will enhance cartilage matrix deposition, leading to more robust mechanical

properties. The *overall objective* of this project is to employ a novel flow bioreactor to improve matrix composition and mechanical properties of cartilage constructs for load-bearing applications. Improving the matrix components and mechanical stability of the tissue to be more similar to that of native tissue may improve the ability of the tissue to function *in vivo*.

Specific Aim 1: Develop a novel dual-chambered flow bioreactor and test the effects of short-term flow on scaffold-free 3D cartilage constructs.

The *working hypothesis* is that short-term exposure of a scaffold-free, tissue-engineered articular cartilage construct to Poiseuille flow will specifically stimulate an increase in type II collagen synthesis and increase tensile mechanical properties. Tissue-engineered articular cartilage constructs will be grown *in vitro* in the bioreactor using a high-density, scaffold-free culture technique. Constructs will be subject to three days of continuous flow and compared to static culture controls. Elastic modulus and failure stress will be quantified in uni-axial tension. Portions of each construct will also be assayed for compositional variables such as water content, cellularity, type II collagen, type I collagen and proteoglycans.

Specific Aim 2: Test the effects of flow duration and shear stress magnitude on construct matrix accumulation, organization and mechanical properties.

The *working hypothesis* is that the long-term application of shear stress to these constructs will increase cartilage matrix components and tensile mechanical properties compared to short-term flow in a dose-dependent manner. Constructs will be subject to short term (three days) and long term (seven days) flow and assayed for cartilage matrix components and mechanical properties. Additionally, the dose dependency of shear stress on tissue development will be examined. Outcome measures for this aim will focus on more extensive characterization of the matrix component analysis (*e.g.*, PCR,

picrosirius red staining for collagen fiber orientation) and relating these findings to the mechanical properties.

Specific Aim 3: Engineer cartilage constructs of clinically-relevant thickness and test the effects of flow on construct matrix accumulation, organization and mechanical properties.

The *working hypothesis* is that higher seeding densities will create cartilage constructs of clinically relevant thickness (*i.e.*, 1-3 mm) and that the enhancements in matrix composition and mechanical properties seen in the first aim will be translated to these constructs when subject to shear. Compressive mechanical testing will become a primary outcome measure with these thicker constructs allowing for greater characterization of the tissue as well as additional understanding of the flow effects.

The proposed work is *significant* because tissue-engineered constructs that closely approximate the composition, organization, and mechanical properties of native articular cartilage may provide improved clinical treatment for repair of cartilage and osteochondral defects. The bioreactor system also provides a flexible platform technology for the study of three-dimensional engineered tissues. Our approach is *innovative* given the use of a novel dual chambered flow bioreactor that provides controlled biomechanical and biochemical stimuli to either side of tissue-engineered cartilage constructs.

CHAPTER 2

LITERATURE REVIEW

Articular Cartilage Structure and Function

Introduction

The Diarthrodial Joint and Cartilage Types

Diarthrodial (synovial) joints are those which allow for a large range of motion between encapsulated bones (*e.g.*, knee, hip, shoulder and fingers) [10]. These are distinct from the other human joints, fibrous (synarthroses) and cartilaginous (amphiarthroses), which have very little movement and are found in places such as the skull and pubic bones, respectively. Not necessarily within the joint, but nonetheless critical to stability of the joint, are various tendons and ligaments (*e.g.*, anterior cruciate ligament of the knee) [10]. In the diarthrodial joint, the system is surrounded by a fibrous capsule, the inner layer of which is the synovium. The synovium captures the waste products from the cellular components within the joint and also secretes the critical synovial fluid. The synovial fluid acts as the friction and wear-reducing lubricant in the bearing system, and is carrier of nutrients to the cells in the articular cartilage. This is crucial as mature human articular cartilage is devoid of a vascular supply [4]. Similar to dialyzed blood plasma, synovial fluid is comprised of mostly water (~85%), supplemented with hyaluronic acid (upon which the viscosity is based), other complex proteins and polysaccharides [11], as well as a high molecular weight glycoprotein recently found to be a major contributor to the lubricating ability of synovial fluid, aptly named lubricating glycoprotein-1 (LGP-1) or PRG-4 [12]. Also within the joint is articular cartilage, which lines the ends of the long bones and provides the nearly-friction free bearing surface for movement.

Articular and growth plate cartilage are types of hyaline cartilage, separated from the other two types of cartilage, fibrous and elastic. Hyaline cartilage is distinguished by the presence of type II collagen [13], the most abundant protein in the matrix accounting for 50-73% of the tissue's dry weight in articular cartilage [14]. Fibrocartilaginous structures such as the temporomandibular joint (TMJ) disc and the meniscus, are comprised mostly of type I collagen, secreted by fibroblasts and chondrocyte-like cells [3]. The structures of the nasal septum and ear are made of elastic cartilage, which as its name implies, contains the protein elastin [15].

Articular Cartilage

Function

Articular cartilage exists in the diarthrodial joint to minimize the joint stresses and surface friction encountered in every day life [14]. In response to these daily loads, articular cartilage functions by increasing the contact area, absorbing the force and therefore decreasing the applied stress [14]. Even still, one of the more fascinating qualities of articular cartilage is how it can act as nearly frictionless bearing surface for decades without significant wear, especially under slow, cyclic loading conditions and compressive loads of three to six times the body's weight [16]. For instance in the hip, stresses measured during normal gait range 4-6 MPa, the leg-lifting of a 26-lb weight increases the stress to 13.7 MPa [17] and can even reach as high as 20 MPa [18]. The durability of this tissue comes in part from the fact that the majority of the applied load is supported by interstitial fluid, consequently protecting and shielding the solid matrix from the brunt of the loads [14]. This fluid pressurization can lower the frictional coefficient to 0.0075 [10], which is reduced further by the presence of a lubricating film such as synovial fluid [18].

However, when damaged either through trauma or disease, disruption of any constituent in the joint structure (especially to the articular cartilage) results in loss of proper function. An imbalance of function leads to a positive-feedback loop causing additional pathological changes to the cartilage and surrounding bone [19]. As adult articular cartilage has a low intrinsic ability for self-repair, these changes can quickly progress to an arthritic condition, resulting in pain, loss of mobility and reduction in quality of life [19].

Structure

Articular cartilage is a relatively acellular, aneural and avascular tissue, comprised of one cell type (chondrocytes) and is essentially an immiscible, biphasic material consisting of a solid matrix and interstitial fluid [20, 21]. Representing the fluid component is mostly water (~75% of the tissue's total wet weight) and dissolved ionic species of electrolytes [21]; the remaining solid matrix is comprised of type II collagen fibrils (~65% by dry weight), proteoglycans (~25% by dry weight) and other non-collagen proteins [22]. In-depth discussions of the chondrocytes, interstitial water and solid matrix within articular cartilage appear in the sections that follow.

The average thickness of human articular cartilage is 1.69 mm, ranging from 0.22 mm and 3.65 mm (in the femur [23]) and can be classified into four zones according to depth through the thickness [14]. In Zone I, or the Superficial Zone, the matrix possesses its highest water and collagen content (~84% wet weight and ~86% dry weight respectively), but the lowest proteoglycan content (~15% dry weight) [24]. This zone is where joint articulation occurs and represents ~10% of the total thickness [14]. The type II collagen in this zone, deposited by more flattened chondrocytes, is aligned parallel with the articular surface, giving a higher tensile modulus to this zone [25]. The chondrocytes also secrete aggrecan and superficial zone protein (SPZ), which aids in the articulation joint motion. In Zone II, the Transitional Zone, the chondrocytes assume their

more characteristic rounded phenotype, indicative of the increased presence of Golgi, ER and mitochondria and subsequent synthetic activity [26]. Here, the collagen is more randomly oriented and has a decreased concentration than Zone I. However, Zone II has the highest concentration of proteoglycans, approximately 25% of the dry weight [24]. Zone III, the Middle/Radial Zone, has collagen fibers oriented perpendicular to the articular surface and is anchored into the calcified cartilage layer (“tidemark”). The largest diameter collagen fibers reside here, and chondrocytes are stacked in a columnar orientation [14, 26]. Lastly, the calcified cartilage zone represents Zone IV, and as the name suggests, is where the articular cartilage transitions into the underlying subchondral bone in the form of calcified cartilage and calcium salts [14]. There are minimal amounts of collagen and water in this zone [24], and the chondrocytes are smaller and minimally metabolically active [26]. Hypertrophic chondrocytes secrete type X collagen and promote calcification of the collagen [13].

Chondrocytes

Chondrocytes are the only cell type found in adult human articular cartilage and only occupy approximately 1-10% of the total tissue volume of human articular cartilage [14, 26]. With increasing age, the density and synthetic activity of the cells decrease, with the synthesis reaching a minimum at 20-30 years of age [13]. Despite their relatively low concentration, chondrocytes are responsible for producing and replacing all the matrix molecules (classified into collagens, proteoglycans and non-collagenous [27]) that give rise to articular cartilage’s unique mechanical properties and function [26]. In order to better sense the environment, integrins and cilia attach to the surrounding extracellular matrix to transduce mechanical signals [14, 26]. The chondrocyte metabolic and catabolic responses are highly sensitive to mechanical loading [27]. Consequently, most chondrocytes generally have a pronounced ER and Golgi apparatus to satisfy this metabolic duty [26]. These differentiated cells originate from mesenchymal stem cells

(MSC) during embryogenesis, at which point they continually divide and secrete matrix proteins. While these chondrocytes build the articular matrix, sub-populations of chondrocytes arise: peripherally located cells continue to secrete matrix (collagen, proteoglycans, *etc*). Mature chondrocytes assume the characteristic rounded morphology, cease proliferating and become embedded in matrix. Terminally differentiated chondrocytes become “hypertrophic,” swelling in size and laying down a matrix that will eventually be calcified and turn to bone.

Similar to the tissue-level zonality of articular cartilage, there is a cellular-level zone structure around the chondrocyte: the pericellular, territorial and inter-territorial domains. The pericellular region (~2 μm encapsulating the chondrocyte) is characterized by very little type II collagen, and high concentrations of type VI collagen [28], decorin [29] and aggrecan [30]. Aggrecan also is characteristic of the territorial and inter-territorial regions, but this molecule is more intact in the territorial, but more degraded in the inter-territorial [13]. These cellular-level zones are more pronounced depending on which tissue-level zone is being analyzed. For example, the inter-territorial region is distinctly evident mostly in the deep zone, superficial and transition zones are comprised mostly of territorial matrix [13].

Solid Matrix: Collagens and Proteoglycans

Articular cartilage is composed mainly of type II collagen, with other collagens such as type V, VI, IX, and XI representing less than 10% of the total collagen combined [13, 31]. Type X collagen is present in hypertrophic / calcified areas of cartilage [13]. Type II collagen – as its root word *kōlla* (Greek, meaning glue) suggests – forms a fibrillar network throughout the matrix, with fiber diameter dependent on depth into the tissue: 20 nm in the superficial zone, 70-120 nm in the deep zone [13]. Composed of three identical left-handed helical $\alpha_1(\text{II})$ chains composed of the glycine-proline-X repeat (where X is usually hydroxyproline), the type II collagen fiber is formed by the right-

hand triple helix via intermolecular cross-links (“tropocollagen”). Fibers polymerize extra-cellularly into fibrils via intra-molecular cross-links in a staggered configuration [31]. In the fibril bundle, type IX collagen covalently linked to type II collagen (anti-parallel) adds stability to the network assembly, and its degradation signals the onset of osteo- and rheumatoid arthritis [32-34]. Type IX collagen is different from other collagen types in that it is also considered a proteoglycan due to the inclusion of a GAG chain attached to one of its collagen chains [35]. Type II and type IX collagen are markers of differentiated chondrocytes and are essential to the matrix function [36] due to these interconnections. These type IX collagen cross-links, occurring at the fibril surface, are pyridinium cross-links (between pyridinoline residue) [34], the same that comprise the interfibrillar connections at the telopeptide regions within type II collagen [37]. Also at the fibril surface and aiding in nucleating the self-assembly of the fibrillar network is type XI collagen [38, 39]. The collagen network is then arranged into a mesh – the alignment of fibers dependent on zone (discussed previously) – and provides the framework for other tissue residents, namely water, the chondrocytes, and proteoglycans.

Proteoglycans, the other major constituent beside collagen in the solid matrix, consist of a protein core and a “bottlebrush” series of glycosaminoglycan (GAG) chains. Smaller proteoglycans (decorin and biglycan) account for approximately 10% of the proteoglycans in cartilage, whereas larger aggregates such as aggrecan ($1-4 \times 10^6$ Da) and versican contribute 50-85% [31]. A model of proteoglycan structure, aggrecan has a protein core linked (via link protein) onto a hyaluronan (HA) backbone at the HA binding domain (G1, N-terminal). The protein core extends outward, with the second globular domain (G2) connecting G1. Here exists several distinct regions, rich in different GAG structures such as chondroitin sulfate (~100 chains) and keratan sulfate (~50 chains). Terminating the core protein is the C-terminal domain, named G3. The entire GAG chain is 200-400 nm long extending outward from the HA backbone. Significant variations are seen in the amount, length, and type of GAG chains that reside on the protein core [31].

Versican, similar in size and structure, is expressed at much lower levels than aggrecan. Decorin and biglycan are also similar in general structure (*i.e.*, protein core with GAG chains), but of much smaller size, ~100,000 Da molecular mass. While aggrecan has many GAG chains, decorin and biglycan possess one and two chains respectively, consisting of dermatan and chondroitin sulfate. Their exact functions are still being elucidated, but it is evident that they interact with the collagen framework, possibly involved in the formation of microfibrils [13].

Embedded within the chondroitin sulfate and keratan sulfate chains are the fundamental sulfate (SO_3^-) and carboxyl (COO^-) groups. As will be discussed in the Interstitial Water section, the fixed negative charges of these groups are vital to determining the mechanical properties and function of articular cartilage. The “swelling pressure” created from these charges comes from the influx of counterions (*e.g.*, sodium, potassium, *etc.*) to maintain electroneutrality.

Interstitial Fluid: Water and Ions

As previously discussed, articular cartilage can be viewed as a biphasic material, with a solid matrix phase and a fluid phase, comprised mostly of water (58-78% of the wet weight, which varies with thickness through the tissue) and dissolved inorganic salts (*e.g.*, sodium, calcium, chloride, potassium [40]). Roughly 30% of this water resides within the collagen fibers, the other 70% trapped by the proteoglycans [31, 40]. This interstitial fluid functions to not only provide nutrients to the tissue, but also to absorb much of the compressive loads of the joint [41]. Joint loads are cushioned by the hydrostatic pressure within the matrix. This pressure is created by the interstitial water and salt ion entrapment by the highly (fixed) negative charge of the proteoglycans and subsequent resistance to flow [42]. In fact, more than 90% of the compressive stress is absorbed by interstitial fluid pressure under certain theoretical and experimental loading conditions [42]. One important aspect of articular cartilage is its effective pore size, in

the range of 20 angstroms to 65 angstroms. At this pore size, along with the compacted collagen fibrils along the upper surface, creates a relatively low hydraulic permeability on the order of 10^{-14} m/Ns [43]. This allows only water to pass through and consequently concentrates the other proteins and polysaccharides in the remaining synovial fluid under compression. This aspect serves as a crucial point in modeling joint fluid dynamics, and is thought to dominate the material properties of the tissue [44]. Thus, the molecular characteristics and interactions of interstitial fluid give rise to unique fluid flows and hydrostatic pressures, absolutely critical (if not central) to the mechanical integrity of articular cartilage [20, 40].

Articular Cartilage Mechanics

Material Properties: Mathematical Modeling and Mechanical Testing

Articular cartilage is a heterogeneous, anisotropic material that has unique mechanical properties suited to withstand the complex and demanding loads seen *in situ*. The mechanical properties of articular cartilage have been the topic of innumerable works and have spawned a great many mathematical models and testing protocols to better understand how the tissue responds to load. This section will detail the most important aspects of articular cartilage mechanics against the backdrop of the models and tests used in elucidating the underlying mechanics. Not to be discussed is the historical progression of models (*e.g.*, linear elastic, single phase viscoelastic) and tests which founded the basis for the current, more detailed and complex ones, as the former simply do not describe many of the responses of articular cartilage under physiologic situations.

The response of articular cartilage to an imposed load can be divided into three separate categories: time-independent, time-dependent, and swelling properties. Division into these three categories is based on the three material phases of articular cartilage: solid matrix, interstitial water and ionic species [24]. Simply put, the time independent

properties describe the solid matrix alone and are based on models and tests assumed to be at equilibrium; *i.e.*, when interstitial water has stopped flowing through the matrix. Here, the matrix is described as an incompressible porous-permeable solid [45]. Time-dependent studies and models (biphasic [20]; solid matrix and water) do take into account interstitial flows and are consequently more complex, mathematically involved, but better describe the tissue's response. Lastly, the large amount of negative charge balanced by counter-ions gives rise to a swelling pressure that also dictates mechanical properties and even more complex models (triphasic [21]; matrix, water and ions).

Flow Independent

In flow independent studies, the critical measurements are taken at equilibrium; *i.e.*, when interstitial water has stopped flowing. Commonly used equilibrium parameters are the Young's modulus, compressive aggregate modulus, shear modulus and Poisson's ratio [31]. Similar to the directionality of the forces applied to articular cartilage *in vivo*, the usual testing protocol is performed under compression, tension or shear.

In tension, the contribution to the mechanical stiffness is predominately due to the collagen fibers [46], as this rope-like molecule is biochemically suited to resist tensile strains. In fact, studies have shown that the tensile modulus and collagen network stiffness is independent of proteoglycans [47, 48] and a highly significant correlation exists between collagen-to-proteoglycan ratio in normal human cartilage [49, 50]. A wide range of tensile Young's moduli have been found, varying between 1 and 30 MPa, with factors such as species, age, location on joint, joint type, depth into tissue, orientation with respect to split lines (in fatigue testing as well [51]), degree of cross-link and donor health [31, 52] influencing the modulus.

Properties in tension can be measured by way of an equilibrium stress-strain or constant strain rate (dynamic) experiment; in either case, a force-displacement curve is generated, converted to a stress-strain plot and from this the properties are calculated.

The stress-strain relationship is linear for small strains (<15%) [31]. Young's modulus (E_Y) is defined as the linear (elastic) region of the curve and ultimate strength as the maximum stress obtained prior to failure. The specimen to be tested is usually cut into the shape of a dogbone so that the stress concentration and failure point occur at a known cross-sectional area. In some constant strain rate measurements, a two-parameter model is used to describe the recruitment of collagen fibers [31]. Both of these test systems show similar ranges and age/health/location/*etc.* variations while closely correlating with collagen fiber properties, although the modulus found in dynamic tests tends to be higher than the equilibrium case. Reporting the modulus as dynamic or equilibrium is therefore important to avoid confusion.

The equilibrium response in compression differs significantly from that of tension, both from a testing, modeling and physiologic point. Most commonly, the confined compression apparatus is utilized: a cartilage cylinder is placed in a radial confining chamber, resting on top of a rigid, impermeable platen, all bathed in a neutral buffer solution. A load (or displacement) is applied to the cartilage face and transmitted through a porous filter so that fluid may exude. Only normal displacement of the tissue is allowed as the confining chamber prevents lateral expansion. Consequently, the confining chamber aids in bearing load which gives meaning to the mechanical parameter of interest, the aggregate modulus, H_A . The displacement at equilibrium under several step loads (creep) or measured load at equilibrium under several step displacements (stress-relaxation) is used for generation of the stress-strain curve, from which H_A is calculated. Similar to tensile measurements, properties gleaned from confined compression studies show variations in a host of variables (*e.g.*, age, matrix composition, health, *etc.*) [53]. However unlike tensile measurements, compressive properties show a positive correlation to proteoglycan content [31] and an inverse correlation with water content [53]. More recently, collagen has shown to have a positive correlation with the compressive properties as well [54].

Under pure shear conditions with small strains, negligible hydrodynamic pressures or volumetric changes occurs, allowing for flow independent measurements to be made [31]. In response to an instantaneous angular displacement, a shear stress is imparted onto the tissue, which decays over time until equilibrium is reached. Y.C. Fung's quasi-linear viscoelastic theory [55] can be applied to yield a equilibrium shear modulus, which has been found to be approximately 0.2 MPa [31]. A dynamic test can also be run, as a sinusoidal angular displacement is exerted on the tissue over a range of amplitudes and/or frequencies. The dynamic nature of the test allows for interesting mechanical properties to be discerned: besides the dynamic shear modulus G^* , the storage modulus G' , loss modulus G'' and phase angle ϕ can be calculated. The storage modulus is indicative of the elastic storage energy, the loss modulus is proportional to the dissipated energy and the phase angle represents the ratio of the energy dissipation to energy storage within the tissue. Not surprisingly, the relative contributions from collagen and proteoglycans to the material properties are more convoluted in this system, but a positive correlation between shear modulus and collagen content has been found [31]. Similar to the case for tension, dynamic material properties tend to be greater than those observed in the equilibrium setting.

It should be noted that dynamic studies of the above discussed form are not limited to shear, but are applicable to compression as well. Similar parameters are produced in dynamic compression, such as the dynamic compressive modulus, E^* , compressive storage modulus, E' and compressive loss modulus, E'' . One confounding factor in compression though is that of "lift-off" where the oscillating platen loses contact with the articular surface. This can be visually observed in the traces of the load where a plateau is visible at the peak. Also, the total harmonic distortion of the sinusoid can be measured. Dynamic mechanical properties are also dependent on sample dimensions. Use of a dynamic frequency sweep can be viewed as a more physiologically relevant

testing protocol since the experimental tissue can be subject to a similar range of frequency loads as that witnessed in normal, every day life [56].

Flow Dependent & Biphasic Theory

As has been previously discussed, the mechanical behaviors displayed by articular cartilage are dictated in great part by the flow of interstitial water and its effect on the solid matrix. The biphasic model, developed by Van C. Mow and his co-workers, mathematically describes and very accurately predicts the response of cartilage to a defined displacement. Mow originally constructed this model in an attempt to explain some ultrastructural properties of cartilage (surface undulations) but was later used to explain the load bearing nature of interstitial fluid in the joint. Mow deviated from many of the previous models in that a more fundamental approach, based on first principles, was taken to modeling the joint mechanics. The motivation for designing this model was in part to address the feature of viscoelastic creep, stress-relaxation, and load bearing mechanism of interstitial fluid seen in native articular cartilage [44]. It was hypothesized that the viscous nature of the interstitial fluid flow through the articular cartilage defined the viscoelastic mechanical response. Many of the previous theories assumed that cartilage is a linearly elastic solid, but these types of materials do not display viscoelastic creep or stress-relaxation – phenomena consistently seen in cartilage. Consequently, choosing a constant value for many of the mechanical properties in a model (*i.e.*, Young's modulus, Poisson's ratio) is a poor assumption and limits the value of the models.

One of the most important parameters to be developed from the biphasic model is the time constant, T , which governs the kinetics of creep and stress relaxation in compression [31]. For example, the time constant for healthy cartilage is 3X that of osteoarthritic tissue indicating that normal tissue takes 3X longer to reach equilibrium. This suggests the role of fluid pressurization in shouldering the joint loading contributes

more to load bearing in normal tissue than arthritic. This model underscores the importance of fluid pressurization – in fact, using empirical data, this model shows that the stress developed by fluid drag (interstitial water flow) is 30X higher than the stress required to compress the tissue [31]. Indeed, massive complexity is required of this model in order to explain this intricate testing system: the stress profile is highly non-linear as the fluid efflux and solid matrix compaction are heterogeneous through the tissue. Equilibrium is eventually reached after fluid and matrix redistribution in stress relaxation. Despite the complexity of this model, many responses of the tissue are explained, and thus emphasize its utility.

Swelling & Triphasic Theory

Not considered in any of the previous models is the fact that the interstitial fluid within the tissue is actually at a higher pressure than the ambient [31]. This phenomenon arises from the high amount of negative charges stemming from the proteoglycans being offset by counter-ions. The total ion concentration within the tissue is higher than that outside the tissue, giving rise to a higher intra-tissue pressure, known as the osmotic pressure [57]. This osmotic pressure causes a swelling pressure within the articular cartilage.

The triphasic theory, also developed by Mow and his co-workers, adds an ionic phase onto the biphasic model [21]. Consequently, both the swelling effects dictated by the ionic species and the biphasic viscoelastic effects borne of the fluid and solid phases are accounted for, including the streaming potentials induced by electrokinetic effects. Simply stated, the model predicts that the total axial stress at the loading platen is the sum of three stresses: that of the elastic solid matrix (biphasic), the chemical expansion and the osmotic swelling pressure. The triphasic and biphasic models have been compared against empirical data and agree well except in the case where bathing salt solution concentration is shown to have an effect of the material properties [58].

Regulation of Cartilage Metabolism by Mechanical Factors

It is well accepted that a wide array of biomechanical factors affects the integrity of articular cartilage, including trauma, obesity (static overloading), disuse and joint instability [59]. Such factors cause detrimental changes to the cell population, matrix composition (loss of proteoglycans, increase in enzymatic breakdown) and mechanical properties (compressive and tensile) [60-63]. Interestingly, moderate degrees of exercise have been shown to positively influence (maintain or improve) articular cartilage integrity [64, 65]. To examine the mechanisms behind translation of the mechanical load into a biochemical signal, *in vitro* and *ex vivo* systems have been developed to simplify the complex *in vivo* loading situation. Studies examined here are discussed according to loading modality (compression, tension and shear) and their phenomenological effects as well as implications for mechanotransduction. Both tissue level and cell-level experiments are discussed as such as the conclusions drawn must be made according to the system. For example, tissue level studies (explants or cells in gels) allow for analysis of mechanical factors' influence in a controlled setting while preserving the cell-matrix interactions. However, this can also complicate the number of variables available for analysis, prompting a need for cell-only experiments. Both static and dynamic loading modalities are examined where applicable. Discussed here are loading systems that apply compression, shear and tension, as these are three physiologically-inspired loading regimes. While important carriers of mechanical signals, hydrostatic pressure, electric fields, fluid transport and electromechanical transduction are not discussed here for the sake of brevity.

Compression

Generally speaking, static compression has been found to have inhibitory effects while dynamic compression produces positive effects in terms of articular cartilage

maintenance. For example, in bovine explant culture, tissues were found to have increased proteoglycan synthesis (by radiolabeled ^{35}S -sulfate incorporation) when subjected to unconfined dynamic compression, dependent on amplitude, frequency [66] and radial location in the explant [67]. Furthermore, dynamic compression can have a “chondro-protective” effect by reversing detrimental effects (nitrite and prostaglandin- E_2 secretion) produced by specific antagonists (IL-1 β) [68, 69]. In the presence of certain growth factors (*e.g.*, IGF-1), the combination of growth factor and dynamic loading can even produce synergistic biosynthetic effects [70]. Not surprisingly, tissue subject to supra-physiologic loads over a short duration (impact), display immediate and delayed cell death [71].

Explants under the influence of static compression on the other hand, were found to release proteoglycans in a load-dose dependent manner [72] and down-regulate expression of aggrecan and type II collagen [73]. However, studies that examine the time-course of gene expression have found a increase in mRNA levels for aggrecan, type II collagen and link protein after 8h of 50% compression, followed by a reduction in expression levels below that of free-swelling controls by 24 h, a result more commonly reported [74]. In this same study, a sustained expression of critical MMPs (-3, -9, -13) was noted over time [74]. Chondrocytes cultured in gel systems (alginate) responded similarly to the explants in static compression by reducing aggrecan production [75] and type I, type II and aggrecan mRNA [76]. Molecules involved in joint lubrication as well, (*e.g.*, PRG4) are likewise negatively and positively affected by static and dynamic compression [77]. Another common theme to static and dynamic compression is that their effects are dependent on location (depth) and stage of maturation (age) of the tested sample [78]. Taken together, it is clear that compression yields very different effects based on the time-varying nature of the mechanical loading (*i.e.*, static or dynamic).

While these phenomenological studies are important, understanding the mechanism behind the observed effects is the ultimate goal. In static compression, the

solid matrix is compacted as fluid exudes, and consequently there is an increase in fixed charge density [79]. Following tissue compaction, the effective pore size of the matrix is reduced, which could hinder physical transport of nutrients and waste products. On the other hand, dynamic compression gives rises to other phenomena such as hydrostatic pressure, fluid flow and streaming potentials [79-81]. Interleukin-1 (IL-1) has been implicated in the static compression signaling pathway leading to proteoglycan and collagen-based degradation [82], as have the transcription factors *c-fos* and *c-jun* [74]. In both static and dynamic cases, the cell, nucleus, ER and mitochondria volumes change as the matrix enveloping these entities deforms and alters the osmotic balance, potentially leading to mechanotransduction [83, 84]. Further confounding the elucidation of a link between mechanical load and mechanotransduction is the fact that applied loads result in spatially heterogeneous stress and strain fields within the tissue. Finite element models have been formulated to aid in discerning regional effects [85].

Shear

Tissue shear experiments are particularly useful because there is no volumetric change, interstitial fluid flow or induced pressure gradients, limiting the number of variables in the system and isolating the effects of matrix and cell deformation [85]. Explants exposed to a dynamic sinusoidal shear also have been found to increase synthesis in terms of proteoglycans and protein (by radiolabeled ^3H -proline) [86, 87]. Proteoglycans and protein were increased 25% and 50%, respectively in dynamic shear at frequencies between 0.01 and 1.0 Hz [88]. In this study, the distribution of this biosynthesis appeared uniform in both the radial and vertical directions. By subjecting the tissue to pure shear and thusly removing fluid flow as a variable, the synthetic response can be attributed to cell and matrix deformation.

On a cellular level, many studies have been performed using chondrocytes in monolayer culture and exposing them to fluid-induced shear stress. With only cells in the

system, many cell-signaling events can be isolated, although the results have to be carefully interpreted as chondrocytes exist in tissue as rounded cells, whereas in monolayer they are flattened and may respond differently than *in situ* [89]. These flow studies are also sensitive to flow frequency [90], as most of the fluid is delivered by pulsatile peristaltic pumps. Early studies examining fluid-induced shear stress ($16\text{-dyne/cm}^2 = 1.6\text{ Pa}$) on high density monolayers of human chondrocytes showed cells aligning with flow and increasing the production of proteoglycans (and size) in the matrix and that released into the media after 2 and 3 days [8, 91]. In the same experiment, PGE_2 release was also increased, along with increased expression of TIMP-1 over time. A separate but related experiment showed an increase of the osteoarthritic marker interleukin-6 (IL-6) and expression levels after 48h and 24h of flow at 16 dyne/cm^2 [92]. Furthermore, nitric oxide release increased with time and magnitude of applied fluid shear alongside proteoglycan synthesis increases [89]. It was determined in this study that this biosynthetic pathway was related to G-protein and phospholipase C activation [93]. Others have implicated Ca^{2+} store release by IP_3 as a potential signaling mechanism [94, 95]. Further down the signaling stream, the anti-apoptotic factor *bcl-2* was found to decrease with increasing shear stress magnitude, whereas the pro-apoptotic markers membrane phosphatidylserine and nucleosomal degradation were increased [96]. Other signaling molecules such as ERK1/2 (specific MAPKs) are up-regulated in response to shear, independent of Ca^{2+} mobilization [97].

Tension

While articular cartilage is primarily loaded in compression, tensile forces can develop in the matrix when loaded [98]. Chondrocyte seeded gels showed a reduction in proteoglycan (but not protein) synthesis at certain displacements, indicating a potential threshold effect [99]. In an oscillatory system, bovine chondrocytes responded with a decrease in proteoglycan as well as protein synthesis. This was coupled with an increase

in cell proliferation and altered cell morphology [100]. Somewhat contrary however, chick sternal chondrocytes grown in scaffold-free, self assembled matrix showed an increase in proteoglycan and DNA synthesis in a cAMP-dependent manner when subject to oscillatory tension at 0.2 Hz [101].

Global conclusions drawn from the *in vitro* experiments mirror those of the *in vivo* situation. Static loading (*e.g.*, obesity or disuse) is a destructive pathway, while dynamic loading in a normal, physiologic range (*e.g.*, moderate exercise, not traumatic) promotes maintenance of healthy tissue. Elucidation of the mechanotransduction pathways is a topic of numerous academic pursuits, but the pathways are still not completely clear.

Damaged Articular Cartilage, Arthritic Conditions and Treatments

Arthritis: Condition, Prevalence and Impact

Affecting more than 30 million Americans alone – and hundreds of millions of people worldwide [102] – osteoarthritis (OA) is the most common form of arthritis and the leading cause of disability worldwide [103]. Treatments for arthritis (both osteoarthritis and rheumatoid) cost an estimated \$95 billion per year, which does not even account for additional costs such as lost productivity [104]. This degenerative joint disease significantly reduces one's quality of life based on reduced mobility and joint pain; the impact extends to their families and careers [105]. The risk associated with OA disability in the knee is as great as that for cardiovascular disease and greater than any other medical condition in elderly people. Risk factors of OA include age (strongest predictor), obesity (body mass index), physical activity level, genetics [6, 102], and sport-related trauma, which is generally believed to progress to OA [106]. Injuries leading to OA can be acute or chronic in nature. The most common site of osteoarthritis are (in

order) knee, hip and hand, but other areas such as the shoulder, spine, feet and ankles are also affected [103]. Two types of OA exist: primary OA, where the tissues no longer properly response to normal joint loading, and secondary OA, stemming from excessive loading [107].

Changes to the Tissue, Response to Injury

As has been noted, cartilage has a very limited capability to repair itself [106] and if injuries are left untreated, the condition can progress to OA. The onset of OA is marked by matrix degradation outpacing matrix synthesis [108]. Cytokines and proteases produced by chondrocytes, synovium and sub-chondral bone degrade the matrix [107]. Proteoglycans are lost from the matrix, the collagen fiber network is damaged and cell death follows [106]. Similar effects are seen in response to high stress impacts (trauma) [41]. Collectively, this creates an abnormal state of stress in the cartilage system. The tissue enters a positive feedback loop where collagen ultrastructure breaks down, surface permeability increases, lubrication mechanisms and nutrient pathways become altered, and further cell death all lead to accelerated degradation of the tissue [109]. Characteristic clinical features of an OA-affected joint are abnormal and degraded cartilage, inflamed synovium and altered bone structure [110]. Within the articular cartilage, fragmented type II collagen, aggrecan and cartilage oligomeric matrix protein can be found as biomarkers indicative of OA [110].

Based on the severity of damage, a multitude of clinical treatment options exist, with the end goal of restoration of function. Treatments options are based off graded scales with respect to location, size (area) and depth of the defect (assessed arthroscopically [111]) or joint space narrowing and bone attrition (radiography [112]). The following sections describe the treatment options based on clinical evaluation and will serve as a background for the motivation of tissue-engineered articular cartilage.

Intervention for Cartilage Damage

If insignificant damage has occurred, joint function can be well restored using non-invasive strategies. Up until recently, such indications were widely treated by physicians with non-steroidal anti-inflammatory drugs (NSAIDs), most commonly the cyclooxygenase-2 (COX-2) inhibitors. The more popular COX-2 inhibitors were Bextra® (valdecoxib) and Celebrex® (celecoxib), as well as Vioxx® (rofecoxib) marketed by Pfizer and Merck Pharmaceuticals, respectively. These drugs, while very successful in terms of alleviating joint pain, were found to have a higher incidence of “cardiac events” and were consequently pulled from the market among demands from the FDA [113]. “Nutriceuticals” such as glucosamine and chondroitin sulfate are not regulated by the FDA as they are considered supplements and not drugs and are available over the counter. As a result, few well-designed / controlled studies have examined their effects, but some double-blind, placebo-controlled randomized trials did suggest that glucosamine was safe and modestly effective in reducing arthritis-derived pain [114-116]. Viscosupplementation (*i.e.*, an injection of high-molecular-weight hyaluronan directly into the knee meant to simulate the lubricating abilities of synovial fluid) has been found to have similar beneficial effects as NSAIDs [117], but without the cardiac events implication.

Frequently, minor tissue damage (*e.g.*, Stage I superficial fibrillation[111]) must be treated with a minimally-invasive operation such as arthroscopy. During arthroscopy, the extent of damage can be viewed better while loose bodies can be cleaned out of the knee using a washout [118]. However, the ability of arthroscopy, washout, lavage, or debridement to stop the progression into an OA state is not clear.

Larger defects (*e.g.*, Stage II/III $\sim 1.3\text{cm}^2$ [111]) that have progressed to full-thickness lesions are candidates for a more invasive clinical intervention such as microfracture [119, 120] or abrasion arthroplasty [121]. In these techniques, the subchondral barrier to the marrow cavity is breached in order to produce bleeding: this

bleeding allows for an influx of clotting and growth factors and stem cells which together for a fibrocartilaginous clot in the area of damaged cartilage[119-121]. The formed tissue, however, possesses type I collagen which is not normally present in hyaline cartilage and does not impart proper function [122, 123]. With the inability of these methods to properly restore function, the autologous chondrocyte transplant (ACT) procedure was developed to meet this unmet clinical need [124]. Here, autologous chondrocytes are harvested from a core of patient's tissue, expanded *in vitro* and then re-implanted into the defect site under a periosteal flap [124]. It is unknown what the relative contributions are from the periosteal and chondrocyte cell populations towards tissue regeneration. Currently, this ACT procedure is commercially available as Carticel™ and is marketed by Genzyme Biosurgery. Clinical outcomes with this technique have been reported as “good to excellent” in humans with large (1.5-12.0cm²), full-thickness articular cartilage defects after 2- to 9-years of follow-up [125]. Not surprisingly, this procedure has fared well in younger patients [126]. Although promising, this procedure remains expensive and patients are not recommended to resume high impact activities until 15-18 months [127]. Perhaps most importantly, ACT has reduced absenteeism and disability in patients, making the direct costs worth the savings in indirect costs and economic burden [128].

The promising results obtained by the cell-derived healing of ACT, both in terms of regenerated tissue and economic impact, could be improved if the tissue was more hyaline-like and allowed for the patient to return to normal activities faster. Both goals could theoretically be accomplished with a tissue-engineered construct, which promises an articular cartilage-like tissue (with respect to matrix composition and mechanical properties) with near off-the-shelf availability. Current tissue engineering strategies will be explored in great detail in the next section.

More invasive procedures such as allograft, (autologous) mosaicplasty [129, 130] or total knee arthroplasty / replacement have been used to provide a “new” load bearing

surface almost immediately. Cartilage allograft suffers the same drawbacks as other allograft tissues (*e.g.*, quality of tissue, risk of infection, elicitation of an immune response[131]). Mosaicplasty can greatly increase donor site morbidity [132] and has yet to be proven as a worthwhile treatment based on clinical studies or an extensive body of animal studies [106]. Arthroplasty, while an excellent treatment for an older population, is not recommended for younger, more active patients due to the limited lifetime of the parts.

Current Tissue Engineering Strategies

Engineered Construct Requirement, Parameter Space, “Basic” Constructs

As discussed in the previous section, no optimal treatment for full-thickness, Stage II/III articular cartilage defects is currently available for a variety of reasons. Theoretically, tissue-engineered constructs can fill that unmet medical need because of their multi-faceted advantages. The target tissue-engineered construct should possess the following qualities, all of which have been discussed at length in the previous sections:

- A *cellular* component to encourage remodeling
- Similar *matrix composition* to native tissue, such as type II collagen (and other collagens) and proteoglycans in the proper amount and spatial distribution
- Similar *biomechanical* properties (compressive, tensile, shear) to native tissue that allows the patient to return to normal activities sooner than other treatments
- Ability to properly *integrate* into the host defect site to discourage repeat visits, loosening, and aiding in resuming an active lifestyle
- Minor to no *immune* response
- “Off-the-shelf” *availability* to medical institutions for prompt implantation

⇒ Combination of all these factors to *alleviate joint pain and restore function in a timely, economic fashion*

A myriad of approaches exist to tissue engineering of cartilage; this literature review aims to categorize the most notable contributions into discrete topics.

Cell Type

Candidate cell types have been mostly limited to either committed chondrocytes or mesenchymal progenitor cells from a variety of species. Frequently, immature committed chondrocytes used to generate engineered tissues *in vitro* are not of human origin. These “model systems” take advantage of the availability and ease of use of non-human cells (*e.g.*, bovine), but results gleaned from these systems have the caveat that they may not be able to be translated into a human, clinically-relevant model in a straightforward manner. Model systems are popular because immature primary cells are available at sufficient quantities and tend to perform relatively well due to the maintenance of their differentiated state and young age compared to adult cells [133]. Committed chondrocytes from articular, auricular, and costal regions have been shown to possess similar ability to generate neo-tissue and integrate with surrounding tissue *in vivo* [134]. Expansion of chondrocytes requires plating these normally rounded cells onto tissue culture plastic where they flatten and tend to lose their differentiated function, becoming more fibroblast-like [135-137]. Using expanded cells has historically produced inferior constructs compared to primary cells. Avoiding expansion in tissue-engineered efforts is a double-edged sword: while the *in vitro* constructs are easier to grow (in terms of cell availability and potency in producing matrix), it ignores what is sure to be a problem in translation from research into a commercialized product. This is especially true of the case (perhaps the most clinically relevant case) where autologous adult cells are used with intent to generate an implantable neo-tissue (*e.g.*, Carticel™).

Much research has been devoted to improving the quality of expanded cells (regaining differentiated function) by culturing the cells in an environment which promotes the characteristic rounded, chondrocytic phenotype. This appears to be crucial for proper differentiated function [135]. Success has been obtained in experiments that suspend the cells in a gel/bead such as alginate [136, 138], expand the cells in the presence of the growth factors FGF-2 [139] and TGF- β 1 [140, 141], and pellet the cells into a scaffold-free 3D culture [137], among others.

Alternatively, others have explored the use of pluripotent cells that can be expanded in culture and then differentiated into a committed chondrocyte. Mesenchymal stem cells (MSCs) are involved in normal human growth and development; upon condensation and signaling by various growth factors, these precursor cells form cartilage during limb formation [142]. MSCs isolated from adults have been shown to differentiate into both bone and cartilage *in vitro* [143] (including from adult humans [144]), making them attractive for chondral or osteochondral [145] tissue engineering. *In vitro* differentiation of MSCs down a chondrocyte pathway usually requires specific chondrogenic growth factors: TGF- β , BMP-2, GDF-5, insulin, β -glycerophosphate and dexamethasone have been used in MSC chondrogenesis [146, 147]. Chondrogenesis of MSCs can also be enhanced by mechanical stimulation (*e.g.*, compression) similar to that seen *in vivo* [148-150]. While not to the extent of differentiated adult chondrocytes, MSCs also possess a limited capacity to undergo passaging and maintain differentiation potential, although inclusion of growth factors such as FGF have greatly improved these shortcomings [151]. Periosteal cells have also demonstrated the ability to undergo chondrogenic differentiation and generate cartilage-like neo-tissue in an *in vivo* rabbit model [152].

Growth Factors

The media formulation used in maintenance of chondrocytes in engineered tissues remains nearly constant across many groups. Growth media is based on a buffered base media (usually DMEM), supplemented with several necessary factors such as L-glutamine, amino acids, sodium pyruvate and ascorbic acid (vitamin C for collagen formation). Recently, a more stable form of ascorbic acid, ascorbic acid-2-phosphate has been used due to its increased longevity in media and acceleration of pro-collagen to collagen [153]. Also included in media is either a serum (*e.g.*, fetal bovine serum, FBS) or a serum-free substitute as in insulin-transferrin-selenium (ITS) in various concentrations, although 10% FBS is generally most common. 20% FBS has beneficial effects in terms of matrix production [138, 154], but has the drawbacks that this is an expensive and variable (inherent animal-to-animal) supplement. Other growth factors used in cartilage tissue engineering are in conjunction with progenitor cells (previously discussed) or osteo-induction in osteochondral constructs (discussed later).

Scaffolds

In addition to the requirements listed in the introduction of this section, scaffolds must also be biodegradable and thus not elicit an immune response, or improperly change the local environment (*e.g.*, pH) upon release of their degraded products. The scaffold must also permit sufficient nutrient and waste diffusion or convection by proper design of the pore architecture so that cells throughout the matrix remain viable. Ideally, the scaffold would possess or encourage cell-mediated attainment of mechanical properties, satisfactory for *in vivo* implantation. The following section is a brief review of scaffolds commonly used in cartilage tissue engineering. There exist innumerable scaffold selections when one considers the variable parameters such as polymer material, (co-) polymer concentration, pore size, open area, thickness, architecture, persistence/degradation, surface modifications, *etc.* Since many types of scaffolds have

been used in conjunction with cartilage tissue engineering, the most pertinent and representative examples are discussed below.

3D Polymeric Scaffolds

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their derivatives are commonly used in cartilage tissue engineering, most notably by the Vunjak-Novakovic group. This group has found PGA to support formation of cartilage tissues very well in terms of matrix composition when cultured in bioreactor systems [155]. Tissues formed are predominantly type II collagen with type IX collagen and proteoglycans taking up the remainder of the dry mass [36]. While these PGA systems fared relatively well in terms of recapitulating the properties of native tissue, this group has moved to a more dynamic mechanical conditioning of their constructs to further improve the properties [156]. Interestingly, when this group directly compared different scaffolding materials, they found that the culture environment (*i.e.*, bioreactor versus static culture) was the most significant predictor of quality matrix composition [157]. Similarly, others have found a significant benefit of bioreactor culture in PGA scaffolds [158, 159] and PLLA scaffolds [160]. PGA/PLA copolymers have been combined in different ratios in order to customize the degradation characteristics of the copolymer scaffold and analyzed when implanted *in vivo* [161]; limited data suggests that altering the degradation can affect quality of the implant. Direct comparison of collagen PGA, PLA-PGA copolymer and nylon scaffold showed similar performance in terms of proteoglycan and collagen synthesis. However, PGA showed the highest proteoglycan synthesis while collagen mesh showed the highest collagen synthesis [162]. Both of these meshes showed similar matrix composition when cultured after 5 weeks in a bioreactor system when compared to static controls [162], again indicating the importance (and perhaps dominance) of the dynamic culture conditions compared to scaffold material.

Gel systems

A logical choice of scaffold for the tissue engineering of a cartilage implant is collagen, as this is similar to the native matrix. Mizuno *et. al*, in a type I collagen sponge, showed that BACs formed cartilaginous tissue, but performed better in the static configuration relative to perfused culture. Expression of type II collagen and aggrecan, as well as proteoglycan synthesis and retention in the matrix were all reduced after two weeks in the perfused cultures [163]. However, these same researchers showed an increase in matrix synthesis when the collagen sponges were subject to cyclic hydrostatic fluid pressure stimulation [164]. In a similar system, Fujisato *et. al* found that inclusion of bFGF enhanced matrix production [165], a growth factor not included in the Mizuno studies. Wakitani *et. al* showed promising *in vivo* healing with MSC and periosteal cell-seeded collagen sponges in a full-thickness rabbit femoral study [152].

As natural healing in the microfracture model is mediated by fibrin clotting, fibrin has shown promise as a scaffold for tissue repair in its ability to maintain differentiated chondrocyte function [166]. Fibrin is clinically attractive due to its low cost, ease of use, safety and availability [167], but has been shown to limit cellular infiltration [168]. Minimal *in vivo* success has been achieved in the presence of inhibitors of fibrinolysis [169], but others have found significant cell mediated toxicity and immunologic response [170].

Agarose and alginate are also attractive scaffold materials as these polysaccharides readily form a gel that can entrap and maintain the rounded phenotypes of the chondrocyte. Mauck and co-workers have greatly furthered the field of cartilage tissue engineering in BAC-seeded agarose gels, which are subject to dynamic mechanical loading [171]. This groups and others have taken advantage of the ability of agarose to be easily cast into a regular shape and outfit into a chamber for dynamic application of beneficial mechanical stimuli [80, 171]. In an early study, Mauck *et. al* showed that the equilibrium modulus of agarose and alginate cell-seeded slabs can be modulated by their

volume concentration [171]. These gels are also amenable to making anatomically-shaped constructs due to their phase transition from a freely flowing gel into a solidified gel [172]. While these *in vitro* results are promising, *in vivo* application of agarose remains suspect due to its poor resorption properties and evidence of eliciting an immune response [173, 174].

Although not as common, hyaluronan [1, 157, 175, 176] and chitosan [177-180] has been explored as vehicles for chondrogenesis and tissue engineering in both static and bioreactor environments. The reader is directed to an excellent review of scaffolds for the tissue engineering of articular cartilage by Frenkel and DiCesare for a more in-depth review [174].

Scaffold-free

In order to circumvent many of the issues associated with scaffolds, scaffold-free constructs have been created by simply seeding cells at a high density ($\sim 2 \times 10^6$ cells/cm²). Cells stack on top of each other, sufficient for maintenance of the rounded, chondrocytic phenotype, and a neo-tissue is produced on the order of weeks. The groups of Rita Kandel (Univ. Toronto) and Jack Lewis (Univ. Minnesota) pioneered this field using bovine [181] and rabbit [182] primary chondrocytes, respectively. Tissues generated using the scaffold-free technique attained tensile moduli on the order of MPa after only three weeks in culture [183]. Others producing scaffold-free tissues have found similar success in generating tissues comprised of type II collagen, proteoglycans and other chondrocyte-specific markers (*e.g.*, type IX collagen) [133, 184]. Mainil-Varlet *et. al* implanted ~ 1 mm thick porcine-derived scaffold free tissues into the knee of adult minipigs with improved morphological appearance, organization, integration and noticeable remodeling compared to controls (empty defect) [185]. Masuda *et. al* invented an efficient process for passaging cells while maintaining their phenotype prior to creating a scaffold-free construct. Here cells are grown in alginate, expanded in culture,

released, and then seeded as a scaffold-free construct [138]. Scaffold-free tissues produced from this alginate recovered chondrocyte (ARC) method consist of mainly type II collagen [138].

Functional Tissue Engineering

The term “functional tissue engineering” has become more popular with respect to cartilage tissue engineering efforts in recognition of the biomechanical role served by these tissues [186]. Implied in functional tissue engineering is the notion that tissues which normally serve a biomechanical role *in vivo* could benefit from mechanical stimuli or other physical factors when being engineered *in vitro*, especially in the case of articular cartilage [4].

Static Culture

It is generally accepted that 3D tissues grown in static (no fluid flow) conditions result in inferior tissues in terms of matrix composition and mechanical properties. This is also the case for articular cartilage constructs [14, 157, 183]. Static culture will remain as a useful modality for the culture of monolayer cells and expansion, but it is doubtful that it will be effective in producing a thick, robust articular cartilage tissue *in vitro*. Diffusion alone is not sufficient for native tissue to survive and therefore an engineered cartilage worthy of implantation will not tolerate static conditions either.

Hydrodynamic Bioreactors

Spinner Flasks, Rotating Wall, Concentric Cylinder and Perfusion Systems

Consequently, a number of bioreactor systems have been developed which increase the fluid convection through and around engineered tissues. Dynamic conditions (fluid flow or direct deformation) affect developing tissues by increasing the overall mass transport and providing a mechanical stimulus, both of which have been shown to be

potent modulators of chondrocyte metabolism [7, 157, 158, 171, 187-192]. Bioreactors are also more amenable to bioprocess by allowing for on-line measurement and adjustment of pH, oxygen and other critical parameters. In its simplest form, a bioreactor can be created from a spinner flask with engineered tissues suspended within the flow field. While engineered tissues reminiscent of articular cartilage were formed, the tissues were heterogeneous in terms of cell density and matrix deposition (sGAG); an annulus of fibroblast cells was evident, indicative of a shear field not well tolerated by the outermost chondrocytes [155]. With the evidence that sub-optimal bioreactor fluid regimes exist, Vunjak-Novakovic *et. al* compared static flasks (no flow), spinner flasks (turbulent flow), and rotating vessels (laminar flow; where the engineered tissue is in a state of perpetual free-fall) [7, 193]. Here, the static vessels produced “small and fragile” constructs, the spinner flasks generated constructs with a fibrous outer capsule and the rotating flasks developed tissues with the best matrix composition, mechanical properties and morphology [7, 193]. Collectively, this suggests that dynamic – but not turbulent – culture conditions are necessary for proper tissue growth.

This important conclusion was seen to influence new, more well-designed bioreactors, in terms of fluid flow properties and amenability to bioprocess, such as the concentric cylinder bioreactor [189, 194]. Multiple PLLA scaffolds are adhered to an inner bob, which is stationary relative to an outer bob. As the outer bob rotated concentrically around the inner bob, with the space between the bobs primed with nutrient media, laminar shear stress is applied to the tissues. In fact, the flow field around the tissues is so well distributed that tissue are seeded by simply adding cells into the bulk media, resulting in a seeding efficiency of 95% after 24h. Shear stress (as dictated by RPM of outer bob) was found to have an effect on cell number, sGAG and collagen production over time [194].

In order to ensure delivery of nutrients to cells within a cell-seeded scaffold, perfusion systems have been developed to force media through the pores of a scaffold.

These systems cause greater mass transfer through the tissue as well as shear stresses. Depending of the scaffold geometry, the shear stress magnitude applied to the cells within the construct can vary widely [1, 2, 189, 195] making conclusions about the applied shear difficult to make. Regardless, considerable success has been achieved in perfused bioreactor systems by improving cell number, sGAG and collagen content [158]; but this is highly dependent on the system and parameters (*e.g.*, flow rate, shear stress). Others have found significantly reduced expression of type II collagen and aggrecan in perfused culture compared to non-perfused controls [163]. More complex perfusion systems, such as those which oscillate media through scaffolds and improve seeding homogeneity, may be required for efficient development of tissue [196].

Compression Systems

Another approach to improving the mass transport and mechanical stimulation of developing tissues is through the use of compression systems. Certainly, this is an intuitive solution as one of the most common loading modalities of native tissue *in situ* is through compression. A substantial body of work from Mauck, Hung and Ateshian (Columbia University) exists examining the effects of dynamic compression of cell-seeded agarose discs. In a 2000 study, a one hour of 1 Hz frequency compression followed by one hour off, three times a day over 4 weeks resulted in a six-fold increase in equilibrium aggregate modulus, as well as significant increases in proteoglycans and collagen [171]. Similar beneficial effects of dynamic loading have been seen by others, even with different offset compression and applied frequencies [75, 80, 197]. This dynamic loading was found to have different effects on mechanical properties and matrix composition depending on the seeding density [198] and inclusion of growth factors [199]. In fact, addition of the known mediators of biosynthesis, TGB- β_1 or IGF-1, combined with dynamic loading resulted in a synergistic effect on mechanical properties of cell-seeded alginate constructs [199]. However, others using a different loading

protocol (alternate day loading) did not see any effect on protein synthesis, but did observe a benefit to proteoglycan production (as well as release into the media) [200]. Given the respective roles of proteoglycans and collagen in compression, the result of no increased protein (collagen) synthesis may not be surprising. It must be stressed that the nature of these loading protocols is dynamic and not static; besides the implications previously discussed in explant experiments, engineered tissues undergoing static compression similarly show reduced expression of type I and II collagen [76] as well as decreased synthesis rates of proteoglycans and proteins [75, 76, 197]. Furthermore, dynamic loading has been shown to have a negative effect on some engineered tissues (*e.g.*, fibrin based), indicating that the effects may be scaffold or vehicle specific [201].

Shearing (Fluid and Direct) Systems

Loading in shear is perhaps the least common method of mechanical stimulation, although very promising results have been gathered in both fluid-induced and direct-contact shearing experiments. Scaffold free tissues have been subject to direct shear and compression and compared directly in the same study. Here, the sheared tissues resulted in a higher proteoglycan and collagen content (when normalized by wet weight) compared to the compressed or static tissues [190]. The equilibrium stress and modulus were also increased in these tissues [190].

Osteochondral and Anatomical Shaping

Many of the *in vitro* designed cartilage constructs are done so with the assumption that they will integrate with the surrounding tissue *in vivo*. However, as was previously discussed, integration of tissue radially is a major challenge facing the entire orthopaedic community, not just tissue engineers. In the tissue engineering community, many strive to reproduce the matrix composition and mechanical properties of native tissue, but evidence suggests that immature constructs or enzymatic treatment of surrounding tissue

may be more advantageous [202]. Implanting a mature construct – if proper integration is achieved – would (theoretically) allow the patient to resume normal activities sooner, thus making this a worthwhile pursuit.

Rapidly gaining popularity is the “osteocondral construct” strategy which aims to produce a mature construct with a high integrative potential, taking advantage of the repair potential of the sub-chondral marrow space. While adult cartilage exhibits a low propensity to remodel, the underlying subchondral bone and marrow cavity does allow for significant remodeling. Hence, a relatively new approach to cartilage tissue engineering is to use osteochondral tissues (*i.e.*, tissue with a bone and cartilage component) so that when implanted, the bony substrate can be placed in or adjacent to the marrow cavity and be actively remodeled. This would serve as an anchor for the rest of the chondral tissue.

These constructs now employ new strategies and possess new parameter spaces for fabrication of a “biphasic” construct. Scaffolds researched extensively for bone tissue engineering (*e.g.*, tri-calcium phosphate, polycaprolactone, *etc.*) are now being combined with cartilage tissue engineering strategies. One challenge for these tissues is the creation of a new biologic and mechanical interface between the cartilage and bone components. Some have chosen to simply suture the chondrocyte-seeded scaffold onto the bony scaffold [203, 204], encapsulating the two halves with fibrin [205] or gelatin [206], or press-fitting a chondrocyte pellet into the scaffold [145]. As these are relatively young pursuits, limited *in vivo* data exists, although lessons can be learned from analogous procedures such as OATS / mosaicplasty. Schaefer *et. al* found that their osteochondral constructs withstood physiologic loading over six months and integrated well with the underlying bone. However, lateral integration of the cartilage portion was disappointing.

Perhaps residing at the pinnacle of the cartilage tissue engineering is that of the anatomically shaped osteochondral construct, cultured under dynamic loading conditions. Hung *et. al* have produced osteochondral tissues in the shape of a human patella. Human

cadaver joints were used as templates for a computer-aided machining system to produce anatomically correct molds and bony substrates [172]. Cell-seeded agarose gels were cast on top of devitalized trabecular bone. Cartilage tissue was formed in terms of collagen and proteoglycans indicating an exciting show of feasibility for anatomically shaped engineered tissue. While this study was not performed under dynamic conditions, the application of stimulatory loads may enhance or accelerate development of the most realistic osteochondral tissue substitute to date.

CHAPTER 3

SHEAR STRESS INCREASES TYPE II COLLAGEN DEPOSITION AND TENSILE MECHANICAL PROPERTIES IN BIOREACTOR- GROWN TISSUE-ENGINEERED CARTILAGE¹

Introduction

When damaged by disease or trauma, articular cartilage has a very limited regenerative capacity due in part to its lack of a blood supply and undifferentiated cell reservoir, therefore typically requiring clinical intervention to restore pain-free function [124, 207]. In the United States alone, over 500,000 total joint replacements are performed annually [208], prompting the exploration of less invasive and perhaps longer lasting repair strategies such as tissue engineering. For clinical success, it has been suggested that tissue-engineered constructs may need to approximate the matrix composition, organization and biomechanical properties of native tissue in order to promote construct integration and load-bearing capability *in vivo* [192].

In native human articular cartilage, the two predominant matrix macromolecules are type II collagen and proteoglycans, which account for 50-73% and 15-30% of the dry weight respectively [14]. While physiological levels of proteoglycan content are feasible, tissue-engineered constructs for cartilage repair are typically deficient in type II collagen and lack the native tissue's zonal organization [155, 191, 192, 209]. Although the importance of replicating zonal organization has yet to be determined, the crucial role of

¹ Modified from: Gemmiti CV and Guldberg RE; *Fluid flow increases type II collagen deposition and tensile mechanical properties in bioreactor-grown tissue-engineered cartilage*. Tissue Eng, 2006. 12 (3): p. 469-79 with permission from Mary Ann Liebert, Inc.

the collagen network in determining the tensile, shear, and swelling properties of cartilaginous tissues is well established [31].

One approach to enhancing the matrix composition and mechanical properties of tissue-engineered constructs was through the use of bioreactors, which culture tissues *in vitro* under dynamic conditions. These dynamic conditions (fluid flow or direct deformation) affect developing tissues by increasing the overall mass transport of nutrients and waste and/or providing a mechanical stimulus, both of which have been shown to be potent modulators of chondrocyte metabolism [7, 157, 158, 171, 187-192]. As type II collagen is known to resist tensile and shearing forces [31], it may be hypothesized that applied shear stresses will promote chondrocyte-mediated deposition of type II collagen in order to counteract the shearing force, resulting in an enhancement of the tissue's mechanical properties. The effects of shear stress have been studied on chondrocytes in a variety of bioreactor systems using scaffold-based tissue-engineered constructs [1, 194, 210, 211]. However, the local shear stresses applied to constructs are either unknown or widely heterogeneous [1, 2, 189, 195], making correlations between shear stress and effects on construct matrix composition or mechanical properties difficult to assess.

Expanding on the field of bioreactor-mediated development of tissue-engineered cartilaginous constructs, we introduced a dual-chambered, parallel-plate bioreactor to apply a consistent level of fluid-induced shear stress to a scaffold-free construct. Scaffold-free techniques generate cartilage-like tissue by using a high seeding density (*i.e.*, 10^6 cells/cm²) which promotes maintenance of the rounded, chondrocytic phenotype [133, 140, 182, 185, 212]. In the hydrodynamic environment of the bioreactor, scaffold-free constructs are advantageous over scaffold-based constructs since the mechanical stimulus is more easily quantifiable due to the simple geometry and lack of biomaterial porosity. Furthermore, the parallel-plate design of the bioreactor allows for a consistent level of fluid-induced shear stress to be applied to more than 95% of the construct's

length. Fluid-induced shear stresses have been applied to chondrocytes in monolayer [92, 97, 213] and scaffold-based constructs before [1, 194], but never to tissue-engineered constructs in the highly-controlled, laminar fluid environment of a parallel-plate bioreactor system. The objectives of this study were two-fold: first, to establish feasibility with our novel bioreactor system in its ability to support three-dimensional culture of cartilaginous tissues, and second, to assess the effects of fluid flow on tissue-engineered cartilage using a scaffold-free construct design. We hypothesized that fluid-induced shear stress applied to the scaffold-free tissue-engineered construct would stimulate chondrocyte-mediated deposition of type II collagen. We further postulated that this additional deposition would result in an increase in tensile mechanical properties, indicating a functional improvement of the tissue.

Materials and Methods

Bioreactor

A custom dual-chambered, parallel-plate bioreactor was designed for controlled flow of three-dimensional tissue-engineered constructs. This parallel-plate bioreactor has similar features to those used previously to apply fluid-induced shear stress to cell monolayers [214-218], but here is being used to culture and mechanically stimulate three-dimensional slabs of engineered cartilaginous tissue. The basic design of the bioreactor is two parallel-plates separated by a defined distance that creates a flow channel, through which fluid is perfused. The flow channel is rectangular box with a set height, length and width. When fluid is flowed through the channel, the resulting fully-developed velocity profile is parabolic (Figure 3.1) with a fluid-induced shear stress (“wall shear stress”) applied at the surface of each plate, commonly referred to Poiseuille flow [219]. The wall shear stress (τ_w) is linearly related to viscosity and volumetric flow rate (1).

$$\tau_w = \frac{6\mu Q}{bh^2} \quad (1)$$

In this expression, τ_w is typically expressed in Pa, μ is the media viscosity in Pa-sec, Q is the flow rate in mL/sec, b is the flow chamber width in cm and h is the fluid gap height in cm. Since the tissue represents one surface of the parallel-plate, equation (1) is used to estimate the shear stress applied to the tissue under flow. For our bioreactor, dimensions were 1.27 cm wide (b) x 4.57 cm long (L) for a culture area of 5.8 cm² with a 350 μ m fluid gap height (h).

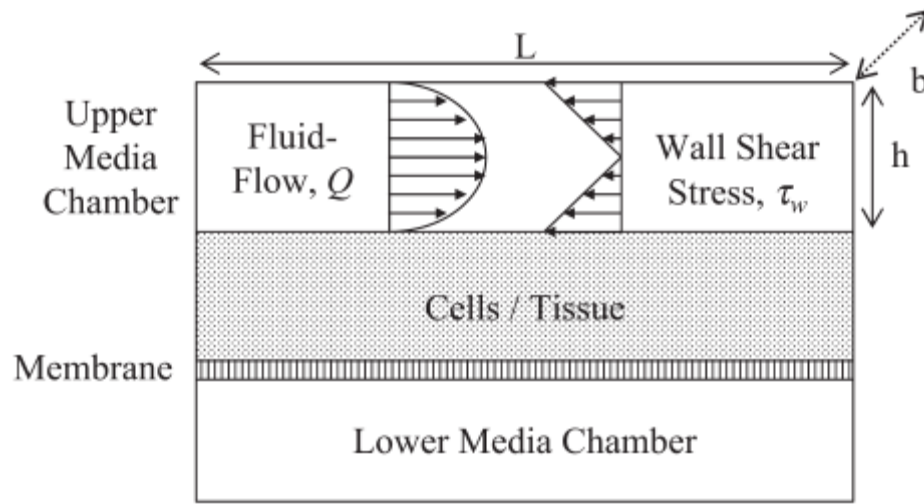


Figure 3.1 – Bioreactor Flow Schematic. The bioreactor (not to scale) is comprised of two media chambers, one above (“upper”) and one below (“lower”) the tissue, which resides on a semipermeable membrane. The upper media chamber is a rectangular box with dimensions length (L), height (h) and width (b , out of the page). Flow of nutrient media through the upper chamber results in a parabolic velocity profile when fully developed. Consequently, a shear stress is developed in the chamber which is maximal at the surface of the tissue, τ_w . A constant level of shear is applied to more than 95% of the tissue length (L).

These dimensions ensure that the resultant flow is parabolic and two-dimensional, laminar (non-turbulent), as expressed by the dimensionless Reynolds number (Re) [216, 220], and has a minimal entry length (L_e). To ensure that the flow remains parabolic and two-dimensional in shape, the ratio of the flow channel width (b) to the height (h) must

be much greater than one [216], which in our system is $(b=1.27 \text{ cm}) / (h=0.035 \text{ cm}) = 36.3$. For the flow to remain laminar, a Reynolds number of 62.1 was calculated (shown below), which is considerably lower than the 2000 threshold that classifies the fluid flow as laminar [216]. Reynolds number was calculated by using a viscosity (μ) of 0.70 cP, fluid gap height (h) of 350 μm and density (ρ) of 1 g/cm^3 (2).

$$\text{Re} = \frac{\tau_w \rho h^2}{6\mu^2} \quad (2)$$

Kinematic viscosity (μ) was calculated by measuring the dynamic viscosity of the nutrient media using a Cannon-Fenske dynamic viscometer (Fisher, Vernon Hills, NJ) and dividing by the media density. A wall shear stress (τ_w) of 0.2-Pa was chosen as the target shear stress. Stresses on this order have been reported to induce cellular proliferation and increase matrix synthesis in chondrocyte monolayer cultures [9]. For fully-developed flow (*i.e.*, the region in which the flow remains two-dimensional and parabolic) an entry length (L_e) is calculated [216] by (3) and compared to the total flow chamber length, L .

$$L_e \approx 0.05(h)(\text{Re})(3)$$

Using the Reynolds number calculated in (2), the entry length (L_e) was estimated to be 1.6 mm, or 3.5% of the total length. This indicates that the wall shear stress calculated by (1) is consistent over 96.5% of the bioreactor length, L . Tissue associated with the entry length was not included in the biochemical analysis.

Bioreactor parts were fabricated out of clear polycarbonate, purchased from the same vendor (McMaster-Carr, Atlanta, GA) unless otherwise noted. Assembly of the bioreactor is illustrated in Figure 3.2A. There are two media chambers in this bioreactor: an upper and a lower chamber, separated by a porous, track-etched, polycarbonate membrane (GE Osmonics, Minnetonka, MN) with 0.2 μm diameter pores and a nominal 10- μm thickness. The walls of the top piece and surface of the membrane form a well,

hereafter referred to as the upper chamber. The lower chamber is formed between the bottom surface of the membrane and the bottom piece. The membrane is held flat and taught by affixing it to a rigid stainless steel frame using a medical grade two-part epoxy while under radial tension. Due to the permeable nature of the membrane, cells have access to nutrient media in both the upper and lower chambers. The lower chamber is accessed through ports on the bottom piece, through which media can be delivered via a syringe or perfusion tubing lines. Cells and tissue can be cultured in either chamber, on either surface of the membrane, but were restricted to only the upper chamber for this study.

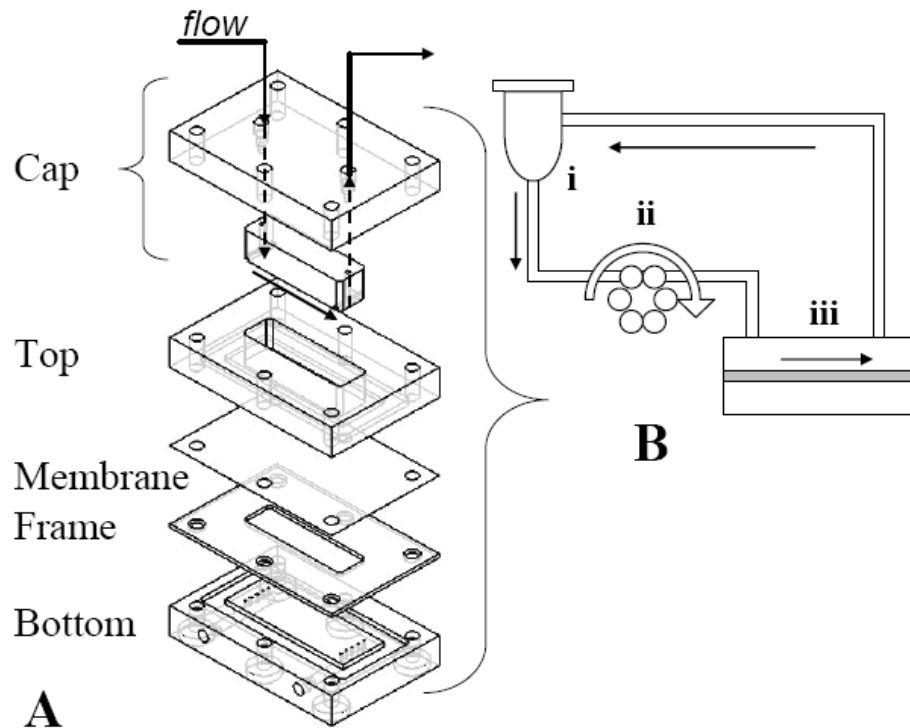


Figure 3.2 – Bioreactor Assembly (A) and Flow Circuit (B). The cultured tissue resides on the membrane, which is held in a flat, taught geometry after being epoxied to the frame. The top and cap form the upper media chamber above the tissue, while the frame and bottom form the lower media chamber. Luer-lock ports (not shown) are fitted onto the cap and bottom for media access and perfusion through the upper and lower media chambers, respectively. Six stainless steel machine screws (not shown) fasten the device together, compressing silicone gaskets (not shown) seated in the bottom, top and cap to create a liquid seal. Flow loop schematic (B): a bubble-trap / reservoir is fashioned by epoxying plastic-luer lock ports into a micro-centrifuge tube (i). A peristaltic pump (ii) drives media via silicone tubing lines from the reservoir to the bioreactor (iii), where the tissue is perfused. Media exits the bioreactor and completes the loop by returning to the reservoir.

In order to maintain sterility and allow for gas exchange, a non-sealing cover with an overhanging lip around the bioreactor perimeter is placed on the top piece during “static” culture (no-flow, control condition). In the “flow” configuration, the cover is replaced with the cap. The cap mounts directly onto the top and creates a fluid seal. On

the cap, there is a protrusion that extends into the upper chamber, creating the fluid flow chamber. Plastic shims of a defined thickness are placed in between the top and cap (outside the fluid path), creating a target fluid gap height (h) of 350 μm . Luer-lock fittings (Cole Parmer, Vernon Hills, IL) are screwed into the access ports in the cap, allowing for attachment of tubing lines. Fluid flow was driven by a digital Masterflex peristaltic pump and size #16 tubing (Cole Parmer, Vernon Hills, IL) connected in a loop between the inlet and outlet ports of the cap as well as a media reservoir made from a micro-centrifuge tube (Figure 3.2B). Dead volume was approximately 7 mL in the complete flow loop. Due to the pulsatile nature of the pumping action, it should be noted that the flow is classified as unsteady, but remains laminar.

Cell Isolation and Seeding

Harvest

Bovine articular chondrocytes were isolated from the femoral condyles and patellar groove from 2-4 week old calves (Research 87 Inc., Marlborough, MA). Full-thickness tissue cores were harvested under aseptic conditions and minced into 1-3 mm² pieces. Pieces were digested in a 0.2% type II-collagenase (Worthington Biochemical, Lakewood, NJ) in DMEM (Invitrogen, Carlsbad, CA) solution and incubated at 37°C on an orbital shaker for approximately 48 hours. Cells were pelleted by centrifuge, resuspended at 10×10^6 cells/mL in fetal bovine serum (FBS, Hyclone, Logan, UT) with 10% dimethyl sulfoxide (DMSO, Fisher, Fair Lawn, NJ) and frozen in liquid nitrogen in 1-mL aliquots. Full-thickness cores were also taken for biochemical analysis for comparison to tissue-engineered cartilage samples.

Experimental Design

Flow Experiments

At the time of seeding, cells were thawed and resuspended in DMEM supplemented with 10% FBS, 50 µg/mL ascorbic acid-2-phosphate (Sigma, St. Louis, MO), 10 mM non-essential amino acids (Invitrogen) and 10 mg/mL gentamycin (Invitrogen). Each bioreactor was seeded with 10×10^6 cells into the upper chamber with a viability >90% assessed by trypan blue (Invitrogen) and hemocytometer assay. Nutrient media was changed 24 hours after seeding to remove debris, then every 48 hours in both the top and bottom chambers. After two weeks of static culture, the cover was replaced with the cap for the flow experimental group. A subset of bioreactors remained static to serve as controls. Flow at 3 mL/min was applied for 72 hours continuously. Actual shear applied to each construct was back-calculated from (1) as the difference between the shim height and the tissue thickness, which was determined microscopically at the end of the experiment as described below. Although tissue thickness was consistent within samples, the applied shear stresses varied between 0.07 and 0.15 Pa due to thickness variations between samples. Media was changed for static and flow samples 36 hours after the start of flow at the midpoint of the experiment. Flow direction was also reversed at the 36 hour feed. All bioreactors, including the entire flow loop, were maintained in an incubator at 37°C, 95% relative humidity and 5% CO₂. At the end of the 72 hours, all bioreactor tissues (static and flow) were assayed for biochemical markers and mechanical properties. The three day flow experiment was repeated three times with four samples per group, producing a total of N=12 pairs of experimental flow and static control samples.

Matrix Composition Assays

Histology

For histological analysis, two 0.25-inch wide cross-sections were cut per sample, transiently rinsed in phosphate buffered solution (PBS, Invitrogen) and fixed in either 1% glacial acetic acid in 95% reagent alcohol (acid alcohol) or 10% neutral buffered formalin and stored at 4°C. Tissue was fixed for 24 hours, rinsed for 2 hours in PBS and then stored in 70% ethanol at 4°C until time of tissue processing. Samples were paraffin-embedded and 5 µm cross-sections were cut. Acid alcohol sections were used for immunohistochemistry, while formalin-fixed sections were used for hematoxylin and eosin (H&E) and Safranin-O staining. All experimental and control samples were stained with H&E, Safranin-O and immunostained for type I, type II and type IX collagen.

Immunohistochemical (IHC) methods utilized a monoclonal antibody for type I collagen (MAB3391, Chemicon Inc, Temecula, CA), polyclonal antibodies for type II and type IX collagen (II-II6B3 and B3-1 respectively, Developmental Hybridoma Studies Bank, Iowa City, IA), a biotinylated anti-mouse secondary antibody (BA 2001, Vector Laboratories, Burlingame, CA) and the Vecastain ABC alkaline phosphatase kit (SK 5100 and AK 5000, Vector). Antigen retrieval was achieved with a 10 minute digestion using Pepsin Solution (Invitrogen) at 37°C. For all histological stains, native bovine articular cartilage and tendon control specimens were included for both positive and secondary-antibody controls. From the IHC-stained samples, tissue thickness was determined using light microscopy and Northern Eclipse software (Empix Imaging, Cheektowaga, NY). Thickness was measured at 10X magnification at 250 µm intervals along the length of the sample and averaged. Only the middle six millimeters (corresponding to the width of the dogbone) were used for thickness measurements when calculating Young's modulus.

Quantitative Biochemistry

Tissue from flow and static samples, as well as native tissue samples obtained during the harvest, was quantified for wet and dry weight (after overnight lyophilization). From this, the water content was calculated. Tissues were digested at 4°C with pepsin (Sigma) for 72 hours followed by elastase (Worthington) for 24 hours, then assayed for type II collagen through a capture enzyme-linked immunosorbent assay (ELISA, Chondrex Inc., Redmond, WA) according to the manufacturer's protocol. ELISA samples were compared against type II collagen standards (supplied with kit) and read on a PowerWave spectrophotometer (Bio-Tek, Winooski, VT) at an optical density of 490 nm. Digested samples were further subject to overnight hydrolysis with 6N HCl at 110°C, resuspended in 0.25M sodium phosphate buffer, reacted with chloramine T (Mallinckrodt Baker, Phillipsburg, NJ) and p-dimethylaminobenzaldehyde (Mallinckrodt Baker) and read on the spectrophotometer at an optical density of 540 nm in order to determine the hydroxyproline content and converted to total collagen using a 7X scale factor [221]. Type II collagen and total collagen contents are expressed as average percentage of native tissue. The remaining tissue was digested at 60°C for 16 hours in 125 µg/mL proteinase K (Worthington), then assayed for DNA by Hoescht 33258 dye [222] (Sigma) and sulfated glycosaminoglycan content (sGAG) by dimethyl-methylene blue [223, 224] (DMMB, Aldrich). Hoechst dye samples were compared to calf thymus DNA standards diluted in 1X TEN and analyzed on a PerkinElmer HTS 7000 fluorometric plate reader (Wellesley, MA) at excitation/emission wavelengths of 360/465 nm. DMMB samples were compared to chondroitin sulfate standards diluted in 100 mM ammonium acetate and analyzed on the PowerWave plate reader at an optical density of 525 nm. Native tissue from the harvest was simultaneously analyzed by all of the above techniques. Total DNA and sGAG contents were normalized by the dry weight and expressed as a percentage of the dry weight $\pm$ the standard error of the mean (SEM).

Mechanical Testing

Tension

To determine if flow altered the mechanical properties of the tissue, tensile mechanical testing was performed and used to calculate Young's modulus and ultimate strength. To harvest samples, tissue was gently separated from the membrane and placed in 37°C DMEM. A section of tissue was stamped into the shape of a dogbone for tensile testing, weighed and placed back in media. Tissue not included in the dogbone was designated for histology or biochemical analysis. The gage length and width of the dogbone samples were 20 mm x 6 mm. Ends were placed in soft-tissue grips in a uniaxial mechanical testing system (DDL, Test Resources, Shakopee, MN) and stretched under a tensile load at a rate of 0.5 mm/sec until failure. Force was determined by a calibrated 5.6 lb load cell (Interface, Scottsdale, AZ) and recorded at 30 Hz along with strain data through the computer acquisition interface (MTestWr software, Interface). Stress (MPa) was calculated as the output force (in Newtons), divided by the cross-sectional area in millimeters (gage width multiplied by thickness, determined histologically). Strain was calculated as the output deformation (mm) divided by the gage length (mm). Young's modulus was calculated as the slope of the linear region of the stress/strain curve; whereas ultimate strength was determined as the maximum stress of the stress/strain curve prior to complete failure (Figure 3.3).

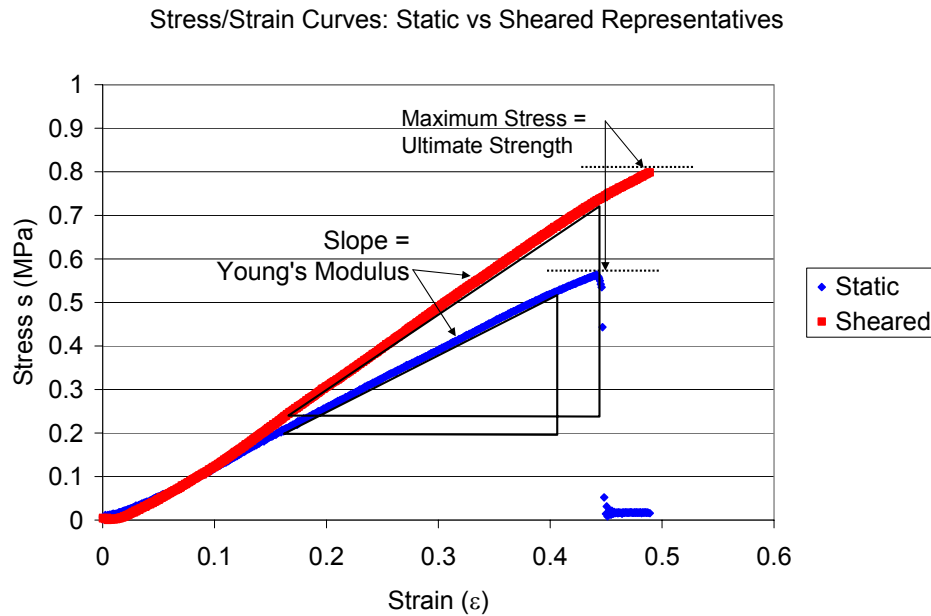


Figure 3.3 – Representative Stress/Strain Curves. Load and displacement were recorded for each static and flow-exposed sample under a tensile load until failure. From this, stress and strain were calculated and plotted as shown. Young's modulus (slope of the linear region) and ultimate strength (maximum stress obtained prior to failure) were calculated from the curves.

Statistics

All samples across all experiments were pooled into static (control) and flow (experimental) groups and are expressed as averages and standard error of the mean, unless otherwise noted. Statistics were analyzed by 2-sample t-test using Minitab software (State College, PA), with statistical significance assigned to $p \leq 0.05$.

Results

Histology

After two weeks of static culture plus the three days of additional culture, both static controls and experimental sheared cultures grew into cartilage-like tissue (Figure

3.4). Cells secreted extracellular matrix and attained an average thickness of 250 μm . There was no significant difference in thickness between static ($260 \pm 15 \mu\text{m}$) and flow cultures ($233 \pm 13 \mu\text{m}$). In both conditions, cells on the top of the construct assumed a flattened morphology while a dense band of rounded cells in the middle of the construct, were flanked by concentrated matrix. Small undulations were observed in the upper surface of both static and shear samples.

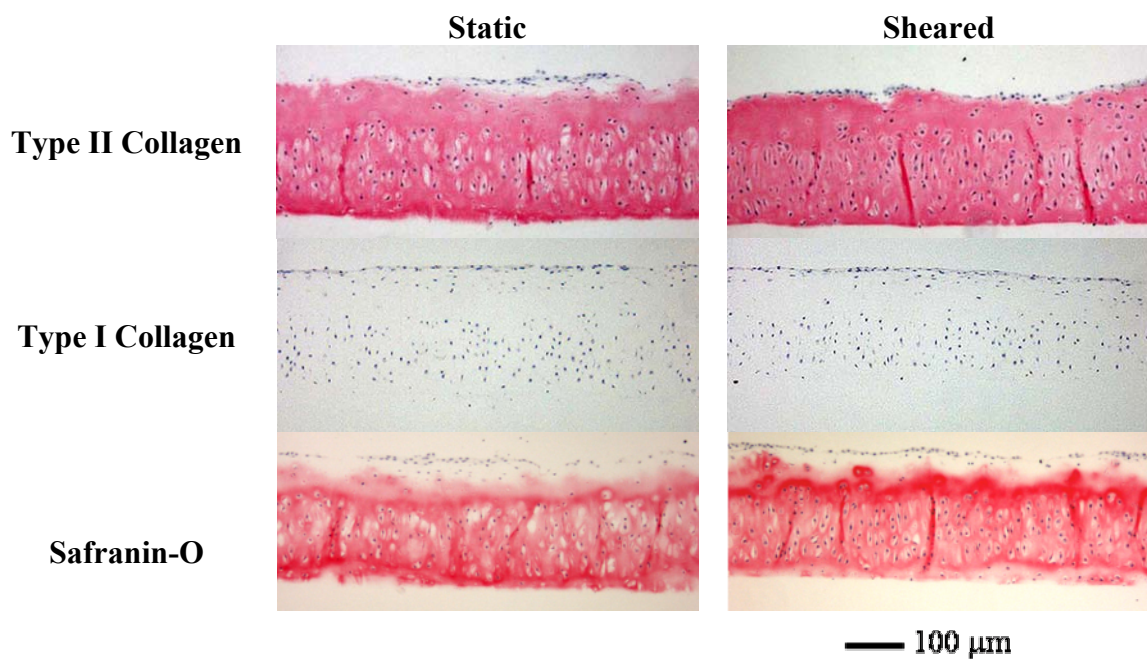


Figure 3.4 – Immunohistochemical Staining. Representative cross-sections of static and sheared tissue stained for type I, type II and type IX collagen. Original mag. = 10X; scale bar represents 100 μm .

Type II collagen, type IX collagen and sulfated glycosaminoglycans were evident throughout static and shear samples, indicating maintenance of a chondrocytic phenotype. Importantly, very little-to-no staining was seen for type I collagen in either group, with no qualitative differences observed between the groups. The type I collagen detected was relegated to small patches across the surface of the constructs, consistently co-localized

with flattened, fibroblastic cells. Although not pictured, the positive controls were appropriately stained for the markers of interest, while the secondary controls were negatively stained for all IHC samples.

Matrix Composition

Three days of shear had no significant effect on water, DNA or sGAG content. Water content was $90.1 \pm 0.3\%$ in static samples and $89.2 \pm 0.2\%$ with shear. DNA content averaged $0.83 \pm 0.12\%$ for static samples and $0.79 \pm 0.7\%$ for shear while sGAG content averaged $33.9 \pm 3.1\%$ for static samples and $36.6 \pm 3.8\%$ for shear.

While shear did not alter cellularity or sGAG production, type II collagen production was specifically and significantly up-regulated in the sheared group ($3.0 \pm 0.3\%$ per dry weight) compared to static controls ($1.3 \pm 0.3\%$), as indicated by ELISA assay (Figure 3.5). This effect was corroborated by a significant increase in total collagen content with shear ($9.2 \pm 0.4\%$) compared to static controls ($7.5 \pm 0.6\%$) as determined by the hydroxyproline assay (Figure 3.5).

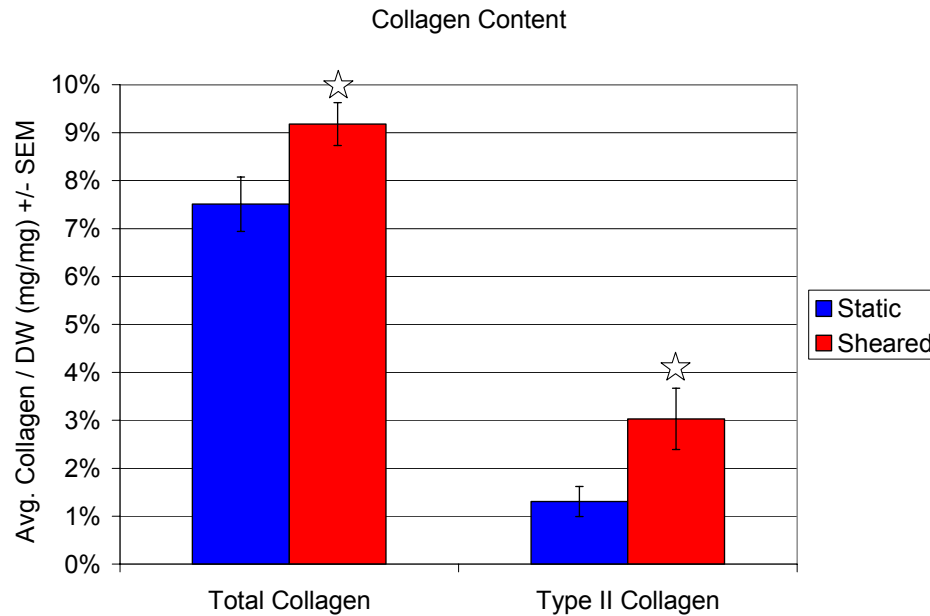


Figure 3.5 – Collagen Content. Total and type II collagen content (normalized by dry weight and native tissue) for static and shear conditions. Star represents $p \leq 0.05$ compared to static controls.

Mechanical Testing

To determine if shear had a concomitant effect on mechanical properties, tensile mechanical testing was performed. Both groups had a small toe-in region followed by a linear stress-strain curve to failure. A significant increase in Young's modulus (Figure 3.6) was observed in shear samples (2.28 ± 0.19 MPa) compared to static controls (1.55 ± 0.10 MPa), perhaps indicating functional incorporation of increased type II collagen into the assembled matrix. Ultimate strength was also significantly increased with three days of shear (0.81 ± 0.07 MPa) compared to static cultures (0.62 ± 0.05 MPa).

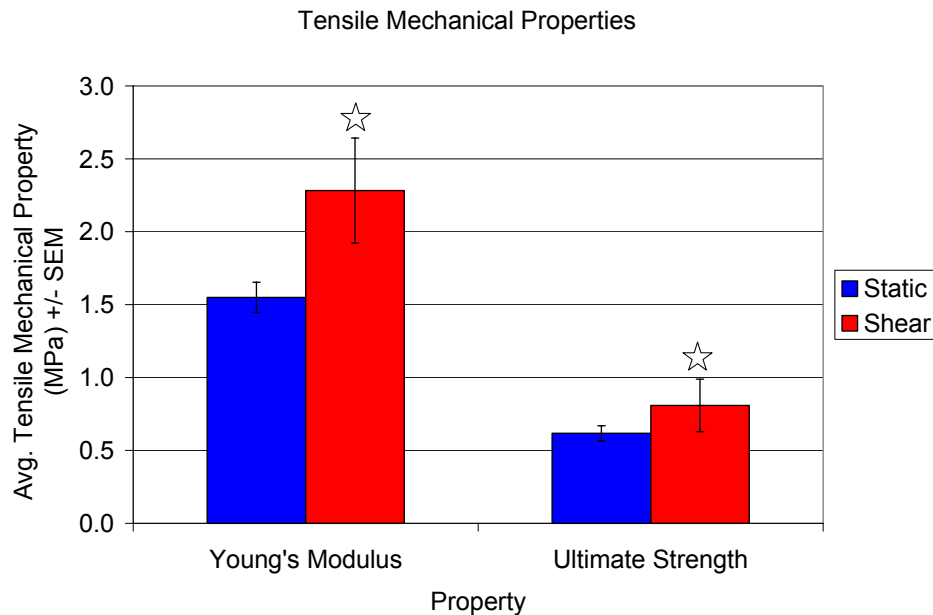


Figure 3.6 – Tensile Mechanical Properties. Young's modulus (left) and ultimate strength (right) for static and shear conditions. Star represents $p \leq 0.05$ compared to static controls.

Discussion

Current tissue-engineered articular cartilage constructs are generally deficient when compared to native tissue in terms of their mechanical strength and matrix composition, particularly type II collagen. Mechanical stimulation of tissue-engineered constructs in bioreactors has recently been explored as a means to improve matrix composition and mechanical properties to better resemble that of native tissue. Here, we investigate the effects of fluid-induced shear stress on developing tissue's matrix composition and tensile mechanical properties, delivered by a novel bioreactor system. We demonstrate that three days of shear stress significantly increased the total collagen amount, specifically that of type II collagen, as well as the tensile mechanical properties.

Compressive and shear forces have been applied previously to developing constructs in an attempt to mimic the mechanical cues native cartilage experiences *in vivo*

[1, 80, 190, 194, 197, 209, 225]. Given the need for increased type II collagen incorporation, it is envisioned that shear stress may aid in increased deposition of this crucial matrix protein, as it functions to resist tensile forces [31]. Application of shear stress as a mechanical signal to chondrocytes has been shown to be a potent modulator of cellular metabolism, and has been achieved through either direct deformation of the tissue [86, 88, 190, 191] (where an actuating surface directly contacts and deforms the tissue) or through bioreactor-mediated hydrodynamic culture [1, 2, 194, 195] [91, 97, 213, 226]. Bioreactor-mediated hydrodynamic environments (*i.e.*, those produced by spinner flasks, rotating wall, perfusion systems, *etc.*) apply shear stress to the tissue in culture, increases the mass transport and availability of nutrients throughout the construct due to enhanced convective fluid flow. However, it has been shown that bioreactor-grown constructs cultured under turbulent hydrodynamic conditions result in inferior tissues when compared to those cultured in laminar flow [187]. Such findings suggest that while hydrodynamic culture conditions may be beneficial to construct development, a well-defined and controlled fluid environment is necessary to encourage proper tissue growth [189, 194].

For these reasons, we chose to develop a novel parallel-plate bioreactor which not only removes the static boundary layer via convective fluid flow, but also applies a defined mechanical stimulus in the form of shear stress. Parallel-plate flow systems have been widely used to study the effects of flow on a variety of cell types [92, 214, 216, 218, 227, 228] because of their precise delivery of shear stresses as dictated by Poiseuille fluid mechanics [219]. Unlike many other parallel-plate bioreactors, our system is used to culture a three-dimensional tissue instead of a monolayer of cells. In our system, we estimate a consistent level of shear stress is delivered to the tissue surface over 96.5% of its length (see Equation 3). Additionally, our bioreactor adds another media chamber underneath the tissue to enhance availability of nutrients, perhaps facilitating growth of thicker, more homogenous tissues. Furthermore, this dual-chamber design has the

potential for induction of heterogeneity by using differential application of biochemical or biomechanical stimuli in the opposing media chambers.

Application of a consistent level of shear stress is enhanced by use of a scaffold-free construct in our bioreactor. Scaffold-free constructs have been shown previously to yield mechanically robust cartilaginous tissue [182] responsive to mechanical loading [190, 191]. While the bioreactor is amenable to scaffold-based technologies, use of a scaffold-free construct allows for a more precise fluid mechanics environment by circumventing the complicated fluid dynamics associated with scaffold-based technologies. Fluid flow through scaffold pores create local shear stresses which are either unknown or widely heterogeneous [1, 2, 195]. Complex numerical algorithms are therefore required to estimate these shear stresses; even still, these algorithms are inherently scaffold geometry-dependent, making correlating the mechanical stimulus and its effects on tissue development convoluted [1, 2, 195].

The results of the current study showing significantly improved matrix composition and mechanical properties with flow stimulation agreed well with previous studies which reported an increase in total collagen (hydroxyproline) with direct mechanical shearing forces in scaffold-free cultures [229]. However, we have further shown significant and preferential type II collagen accumulation and no distinguishable increase in type I collagen content. This is a crucial distinction, as cartilaginous tissues containing predominately type I collagen (*e.g.*, fibrocartilage) should be avoided for articular cartilage replacement, as it has been shown that these tissue do not attain the same mechanical properties *in vivo* [230]. While sparse type I collagen was detected along the surface of both static and flow samples, a thick, distinct fibrous capsule was not observed, which has been seen to develop in turbulent hydrodynamic environments such as that in a spinner flask [187]. The significant increase in type II collagen content is corroborated by the parallel increase in total collagen content. The shear-mediated increase in type II collagen content was greater than that observed for total collagen

content, suggesting there may have been other collagens (containing hydroxyproline) preferentially deposited in the static constructs. Although the amount of collagen formed in our constructs is only 20-25% of native tissue, it is important to note that a significant increase in type II collagen was quantified after only three days of flow and only 17 total days of culture. Compared to previously reported mechanical pre-conditioning studies, this is a relatively short time frame [229]. The observed rapid response to shear stress stimulation, may suggest that longer-term studies will produce levels of type II and total collagen approximating that of native tissue in a shorter culture time than has been previously observed.

While there was a concurrent increase in type II collagen content and mechanical strength in the flow samples, it is unknown to what extent these are related. It is generally accepted that proteoglycans contribute mostly to compressive properties while collagens contribute more to the tensile properties of articular cartilage [231]. It seems intuitive that given collagen's triple helical, rope-like structure, an increase in its content in a tissue would increase the tensile mechanical strength. However, other cross-linking collagens and other accessory proteins (*e.g.*, cartilage oligomatrix protein, link protein) can greatly contribute to the mechanical integrity of articular cartilage [231]. It is not known in our system if any of these proteins were up-regulated, became more organized or cross-linked, but further analyses are to be integrated into future studies.

Despite the encouraging results, the mechanism responsible for increase in type II collagen is unclear. The media flow over the developing tissue has two effects: an increase in mass transport and an application of shear stress. The mass transport through the tissue may serve to enhance nutrient transport/waste removal, or may also induce signaling through effects such as streaming potentials [24]. Fluid-induced shear stress, as discussed previously, also has a stimulatory effect on chondrocytes. Dose-response shear stress experiments are currently being investigated to elucidate the relative contributions of these effects.

In terms of the mechanical properties of our tissue, our results agree well with the tensile Young's modulus found for similar scaffold-free constructs grown under static conditions (4.8 ± 0.1 MPa at eight weeks) [182]. The application of shear increases the average Young's modulus from 1.5-MPa for the static controls to 2.5-MPa, which is on the order of that measured for native tissue albeit ~ 4 -5 times less [52]. This nonetheless indicates a promising advancement in use of bioreactor-mediated mechanical conditioning for tissue-engineered articular cartilage constructs. However, it is important to not overlook the compressive mechanical properties of the tissue. Given the lack of thickness associated with our tissues (~ 250 microns), our initial compressive tests were adversely influenced by artifact. Thin constructs were used for this study in order to conserve cells while establishing feasibility with the bioreactor system, but constructs greater than one millimeter in thickness have been achieved with our culture system, as well as others using scaffold-free techniques [138, 232]. A potential advantage of the dual-chamber design of our bioreactor is that the effects of mass transport limitations associated with producing thicker constructs may be reduced. Future chapters focus on incorporating thicker constructs into bioreactor flow studies similar to that presented here.

This study suggests that flow-induced shear stresses and/or enhanced mass transport associated with the hydrodynamic environment in a parallel-plate bioreactor may be an effective strategy for producing tissue-engineered articular cartilage constructs *in vitro* possessing functional mechanical properties. The ultimate goal of this work is to identify *in vitro* culture conditions that produce tissue-engineered constructs that can withstand *in vivo* loading conditions and functionally integrate with surrounding native tissue.

CHAPTER 4

SHEAR STRESS MAGNITUDE AND DURATION MODULATES MATRIX COMPOSITION AND TENSILE MECHANICAL PROPERTIES IN ENGINEERED CARTILAGE

Introduction

Adult articular cartilage shows little-to-no capacity for self repair and therefore typically requires intervention for restoration of function [4, 19]. If left untreated, both chondral and osteochondral defects may progress to debilitating osteoarthritis [124]. When considering the full spectrum of tissue damage, indications at the far ends of the spectrum currently have treatments with acceptable outcomes: small, superficial asymptomatic lesions are remedied with arthroscopy; at the other end of the spectrum, full-thickness erosion is solved with total joint arthroplasty [106]. However, for many patients in the middle of the spectrum who have defects of approximately 1-2 cm² [111], current treatment options (such as microfracture) only replace the damaged articular cartilage with a fibrocartilage-like tissue [233]. Tissue-engineered articular cartilage is a viable candidate for filling this treatment void, thanks to the promise of a tissue possessing similar matrix composition and mechanical properties as native tissue.

Efforts to engineer a tissue with properties similar to native tissue in an *in vitro* setting have been undertaken using a variety of strategies and met with varying degrees of success. Approaches to engineer a cartilage-like tissue employ permutations of many variables such as cell source (*e.g.*, species [1, 184] and/or anatomical location [234]), cell/tissue maturity [202], construct geometry (*e.g.*, scaffold [1, 194, 210, 211] or scaffold-free [133, 140, 182, 185, 212]), growth factor supplementation [235], and culture configuration (*e.g.*, bioreactor-driven mechanical stimulation [7, 157, 158, 171,

187-192]). Common to virtually all these approaches is that many tissues assume the proper amount of glycosaminoglycan content, but lack the proper organization and amount of type II collagen, the major structural protein in articular cartilage [155, 191, 192, 209].

Type II collagen's importance to the function of articular cartilage cannot be understated; in fact, its presence distinguishes articular (hyaline) cartilage from other cartilage types (*e.g.*, fibrous, elastic) [13] and is most abundant protein in the matrix accounting for between 50-73% of the tissue's dry weight [14] and 80-85% of the total collagen [236]. This protein is known to play a crucial role in determining the tensile, shear and swelling properties of articular cartilage [31]. However, it is not merely the presence of type II collagen, but its complex interactions with the other matrix constituents [19] such as type IX collagen, type XI collagen [236] and cartilage oligomeric matrix protein (COMP) [237] which contributes to the function of articular cartilage. These interactions and the zonal distribution of type II collagen are paramount to the mechanical properties and thus the overall function of articular cartilage [31].

While replicating the amount and organization of type II collagen remains a challenge, one promising approach to modulating the matrix composition and mechanical properties of engineering tissues is through the use of bioreactor-driven mechanical stimulation. It is thought that subjecting an *in vitro* developing tissue to mechanical signals (*e.g.*, compression, shear stress) similar to that witnessed by the *in vivo* native tissue may aid in coaxing the cells and tissue to respond accordingly. Since type II collagen is known to resist shear and tensile forces [31], we hypothesized that imparting a shear stress to an engineered construct would result in the increased deposition of this protein. In previous studies, we developed a novel dual-chamber flow bioreactor to impart fluid-induced shear stress to a scaffold-free cartilage construct of primary bovine chondrocytes origin [183]. We found increases in type II collagen, total collagen and tensile mechanical properties of the shear-exposed cartilage after only three days of flow.

However, the collagen content and mechanical properties were still significantly less than that of native tissue, prompting further exploration of the parameter space.

In the experiments presented here, we hypothesized that extending the duration of flow would further increase collagen deposition and tensile mechanical properties compared to that of static controls. In addition, we included another group of intermediate shear stress magnitude in order to examine any possible dose-dependent effects of flow. Lastly, we analyzed the gene expression over multiple time points in order to better understand the development and response of the tissue to fluid-induced shear stress.

Materials and Methods

Bioreactor

A custom-designed parallel-plate bioreactor was used for culturing and applying a consistent level of fluid-induced shear stress to the engineered tissues. Details of the bioreactor construction and underlying fluid mechanics have previously been described [183]. Briefly, the bioreactor possesses two rectangular chambers (upper and lower) which are bisected by a semi-permeable (0.2- μm diameter pores, ~9% open area, nominally 10- μm thick) polycarbonate membrane (GE Osmonics, Minnetonka, MN), upon which the engineered tissue is grown (Figure 4.1).

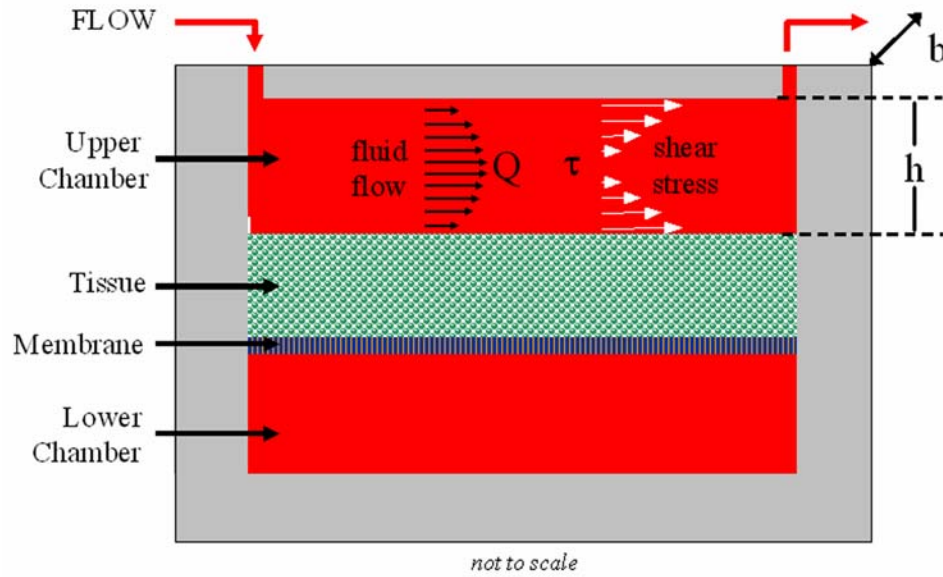


Figure 4.1 – Schematic of Bioreactor (Flow Configuration). The bioreactor consists of an Upper and Lower chamber which contain nutrient media. Nutrient media recirculates through the upper chamber, applying a fluid-induced shear stress to the tissue, which resides on a semi-permeable membrane.

Both chambers are loaded with nutrient media so that the cells residing in the tissue can feed from either chamber. Two culture configurations exist for the bioreactor: static and flow. In the static configuration, the upper chamber is allowed to exchange gases with the incubator via a non-sealing cover, which has an overhanging lip that discourages contaminants. In the flow configuration, a cap is affixed to the bioreactor in order to create a flow chamber of set length (L), width (b) and height (h). The height is altered by adding shims under the cap (outside the fluid flow path). A peristaltic pump recirculates nutrient media through the bioreactor via pump tubing and associated fittings at a constant volumetric flow rate (Q). As media flows through the flow chamber, a shear stress (τ_w) is applied to the top surface of the engineered tissue, and can be estimated using the following equation:

$$\tau_w = \frac{6\mu Q}{bh^2}$$

where τ_w is in Pa, μ is the media viscosity in Pa-sec, Q is the flow rate in mL/sec, b is the flow chamber width in cm and h is the fluid gap height in cm. This wall shear stress is estimated to be valid over more than 95% of the bioreactor length.

Cell Isolation and Seeding

Primary bovine articular chondrocytes were harvested from 2-4 week old calves (Research 87, Boylston, MA), as previously described [183]. Briefly, harvest tissues were minced, incubated for 48 hours in 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) in DMEM (Invitrogen, Carlsbad, CA), washed twice in DMEM containing 10% FBS (Hyclone, Logan, UT), pelleted and frozen in liquid nitrogen until time of seed. Cells were thawed and counted using a haemocytometer, with viability assessed using trypan blue (Invitrogen). Cells were suspended at a concentration of 2.5×10^6 cells/mL in DMEM containing 10% FBS, 50 μ g/mL ascorbic acid-2-phosphate (Sigma, St. Louis, MO), 10 mM non-essential amino acids (Invitrogen) and 10 mg/mL gentamycin (Invitrogen). 10×10^6 cells (in 4-mL media) were loaded into each bioreactor in the static configuration. 24 hours prior to the addition of cells, bioreactors were loaded with media in order to let serum proteins adsorb to the membrane and encourage cell adhesion. Media was exchanged every 48 hours throughout the experiment.

Flow Experiments

All bioreactors remained in the static configuration for two weeks as cells secreted matrix and self-assembled a neo-tissue. After this pre-culture period, bioreactors were converted to the flow configuration and tissues were subjected to one of two estimated shear stress levels: 0.001-Pa (0.1-mL/min) and 0.1-Pa (3.0-mL/min). Compared to our previous studies [183], the additional 0.001-Pa group was added for two reasons: to examine any dose-dependent effect of shear on construct development, and

second, to account for the media volume differences between the flowed (9 mL) and static (6 mL) groups. A subset of bioreactors was kept in the static configuration to remain as controls. In order to keep shear stress and flow rate constant, the flow channel height was increased in 50- μ m increments by adding additional shims during each feed (every 48 hours) to account for tissue growth. Static bioreactors were fed 4-mL media in the upper chamber and approximately 2-mL in the bottom chamber. Flow bioreactors received approximately 7-mL in the flow loop and 2-mL in the bottom chamber. The increase in media volume was due to the additional ports and tubing in the flow configuration and was kept as close to the static condition as possible. Tissues remained in either the static or flow configuration for a period of one week.

At the end of the week, all tissues were removed from the bioreactors and divided up for various biochemical assays and mechanical tests. Prior to division, each tissue was weighed wet and had approximately 2-mm of the perimeter trimmed and discarded in order to minimize edge-effects. Tissue samples for all biochemical assays were frozen at -20°C, except for sections reserved for RNA isolation, which were flash-frozen in liquid nitrogen and stored at -80°C. Mechanical testing was performed immediately (details to follow).

In a separate series of experiments, tissues were harvested at time intervals of 2 hours, 24 hours, 3 days and 7 days specifically for gene expression analysis. These tissues were similarly trimmed, and the entire tissue was frozen immediately in liquid nitrogen and stored at -80°C. No assays other than PCR were performed on these tissues (with the exception of the 7-day flowed tissues) in order to ensure enough RNA for analysis.

Histology

For each bioreactor, a section of tissue was fixed for 24 hours in each 10% neutral buffered formalin and 1% acid alcohol (1% glacial acetic acid in 95% reagent alcohol).

Additionally, a section of native bovine articular cartilage and ligament was taken during harvest and subjected to the same fixation, processing and embedding to serve as a positive and negative tissue control (respectively) for each stain. After fixation, tissues were rinsed in PBS for 2 hours and stored at 4°C in 70% reagent alcohol until time of tissue processing and paraffin embedding. 5 µm sections were cut and mounted on slides: the acid alcohol-fixed sections were dedicated to immunohistochemistry and the formalin-fixed sections to basic histology stains. Each specimen was stained for the presence of specific proteins using antibodies against type I collagen (mouse monoclonal MAB3391, Chemicon Inc, Temecula, CA), type II and type IX collagen (mouse polyclonals II-II6B3 and B3-1 respectively, Developmental Hybridoma Studies Bank, Iowa City, IA), with a biotinylated anti-mouse secondary antibody (BA 2001, Vector Laboratories, Burlingame, CA), as well as type X collagen (rabbit polyclonal, kindly donated by Dr. Gary Gibson, Henry Ford Hospital, Detroit, MI) with a biotinylated goat anti-rabbit IgG (BA-1000, Vector) in conjunction with the Vectastain ABC alkaline phosphatase kit (SK 5100 and AK 5000, Vector). Antigen retrieval was achieved with a 10 minute (60 minutes for type X collagen) digestion using Digest-All 3 (Invitrogen) at 37°C. For each staining session, a secondary-antibody control was included to ensure specificity of the stain.

Basic histological stains included routine hematoxylin and eosin (H&E) for gross morphology, 0.1% safranin-O with 0.02% fast green counterstain for the presence of sulfated glycosaminoglycans, and 0.1% picrosirius red for collagen presence and fiber alignment. As before, native bovine articular cartilage and tendon samples were run alongside experimental samples to ensure proper staining.

Type I collagen presence was quantified using Image Pro software (MediaCybernetics, Inc., Silver Spring, MD) by selecting area stained red by the Vectastain alkaline phosphatase kit. Images at 10X magnification were taken sequentially along the entire length of the tissue (approximately five images per tissue)

and analyzed: an area of interest was manually drawn around the entire tissue, then the software automatically calculated the number of pixels stained red (*i.e.*, type I collagen present) and the total number of pixels in the tissue. Data are expressed at “percent area stained” defined as the number of pixels representing type I collagen divided by the total number of pixels present in the tissue. Type II collagen or safranin-O were not quantified as all tissues were stained throughout; while there were variations in intensity, using intensity as a basis for quantification is variable from sample to sample (due to background, staining technique, *etc.*) and was therefore not used.

Mechanical Testing

Fresh specimens were stamped into the shape of a dogbone (gauge length and width of 20-mm and 5-mm, respectively), loaded into soft-tissue grips of the ELF 3200 mechanical testing system (EnduraTEC / Bose, Eden Prairie, MN), and pulled to failure at a rate of 0.02-mm/sec. Force was recorded using a 5.6-lb load cell (Interface, Scottsdale, AZ) and displacement recorded via computer acquisition interface (WinTest, EnduraTEC). Stress and strain were calculated from the force and displacement data by adjusting for the tissue’s cross-sectional area and gauge length respectively. From the stress-strain plot, the Young’s modulus (linear portion of the curve) and ultimate stress (maximum stress obtained prior to failure) were calculated.

Matrix Composition Assays

For each bioreactor, two sections of approximately 50 mg of wet tissue were weighed, dried overnight (SpeedVac), and then weighed dry in order to calculate the water content. One tissue section was dedicated to proteinase-K digestion for sulfated glycosaminoglycan (sGAG), DNA and total collagen assays; the other to pepsin / elastase digestion for type II collagen (ELISA assay).

Proteinase-K tissue was digested in a 60°C water bath for 18-24 hours using 125 µg of freshly-prepared proteinase K (Worthington) in 1 mL of 100 mM ammonium acetate. Total collagen was determined via hydroxyproline content [221], whereby 100 µL of digest is hydrolyzed in 2-mL 6N HCl at 110°C for 24 hours, re-suspended in 0.25M sodium phosphate buffer, reacted with chloramine T (Mallinckrodt Baker, Phillipsburg, NJ) and p-dimethylaminobenzaldehyde (Mallinckrodt Baker) and read on a PowerWave spectrophotometer (Bio-Tek, Winooski, VT) at an optical density of 557 nm. Experimental samples were compared to standards of known hydroxyproline content, then converted to total collagen by using a conversion of 10:1 hydroxyproline:collagen [238, 239]. Purified type II collagen (Southern Biotech, Birmingham, AL) was hydrolyzed using the previously described process to support use of this ratio. sGAG content was quantified by reacting 50-fold diluted proteinase-K digest with dimethyl-methylene blue [223, 224] (DMMB, Aldrich), read on the PowerWave spectrophotometer at 525 nm and compared to standards of chondroitin sulfate. DNA content was quantified by reacting samples with the Hoechst 33258 dye [222] (Sigma), read on PerkinElmer (Wellesley, MA) HTS 7000 fluorometric plate reader at excitation/emission wavelengths of 360/465 nm and compared to standards of calf thymus DNA in 1X TEN buffer.

Tissues for the capture enzyme-linked immunosorbent assay (ELISA, Chondrex Inc., Redmond, WA) assay were digested at 4°C in pepsin (P7012, Sigma) for 72 hours, followed by a 24 hour digest in pancreatic elastase (LS006365, Worthington Biochemical) in accordance with manufacturer's protocol. Digested samples were compared against type II collagen standards (supplied with kit) and read on the PowerWave spectrophotometer at an optical density of 490 nm.

RNA Isolation, Reverse Transcription and Quantitative PCR

Tissues dedicated for RNA isolation were flash frozen in liquid nitrogen and stored at -80°C. RNA was isolated using the TRIspin method [240] by first grinding the tissue to a fine powder with a mortar and pestle under liberal amounts of liquid nitrogen. Tissues were further homogenized by suspending in 1-mL TRIzol reagent (Invitrogen) and passing 5-10 times through a 1 mL syringe outfitted with a 20-gauge needle. After a 15 minute incubation in TRIzol at room temperature to dissociate nucleoprotein complexes, samples were centrifuged for 10 minutes at 12,000 x g. The supernatant was transferred to a new tube containing 0.2 mL chloroform, vortexed, incubated for 3 minutes, and centrifuged for 15 minutes. The upper, clear, aqueous phase was removed and mixed with 0.5 mL 100% isopropyl alcohol to precipitate the RNA in a new tube. Following a 10 minute incubation and 10 minute centrifugation, the supernatant was discarded while the remaining pellet was resuspended in 350-μL RLT buffer (RNeasy kit; QIAgen, Valencia, CA) plus 3.5 μL β-mercaptoethanol. This lysate was pipetted directly onto a QIAshredder column (QIAgen), centrifuged for 5 min, and then combined with 350 μL 70% ethanol. This supernatant was added to an RNeasy column (QIAgen) and RNA was isolated according to manufacturer's protocol. Total RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE), then frozen at -80°C until time of reverse transcription. Samples with a 260:280 (nm) absorbance ratio less than 2.00 were not used in analysis, as these were considered impure due to the presence of proteins, phenol or other contaminants [241]. cDNA was generated by reacting 4 μL MgCl₂, 2 μL RT 10X buffer, 2 μL dNTPs, 1 μL oligo DTs, 0.5 μL RNasin, and 0.75 μL AMV reverse transcriptase (A3500 Reverse Transcription Kit; Promega, Madison, WI) per sample (1 μg RNA in 9.75 μL nuclease-free water) at 42°C for 60 minutes, at 95°C for 5 minutes, and 4°C for at least 5 minutes. Samples were frozen at -20°C until time of qPCR.

Gene expression was quantified using SYBR Green intercalating dye (Applied Biosystems, Foster City, CA) and the Prism 7770 Sequence Detector (Applied Biosystems; 40 cycles: melting = 15 s at 95°C; annealing and extension = 60 s at 60°C). The cycle number where the measured fluorescence exceeds a threshold (cT) was calculated automatically using the Applied Biosystems software package. Experimental samples, standards and no-template controls were run in duplicate in 50-μL reaction volumes. Standards were generated from bovine chondrocyte cDNA amplicons using primers listed below (Table 1). For each well, 1 μL of cDNA was combined with nuclease-free water (23.75 μL), forward primer (0.125 μL), reverse primer (0.125 μL) and SYBR Green Master Mix (25 μL; Applied Biosystems). Melting curves (data not presented) were run at least once for each primer set and experimental condition to rule out false-positive signals generated by non-specific double-stranded DNA sequence amplification. Primers for each gene are listed below in Table 4.1.

Table 4.1: Primers

Target Gene (all bovine)	Primer Sequences (5'-3')
Aggrecan	Forward: CCT CAG GGT TTC CTG ACA TTA Reverse: TAA GCT CAG TCA CGC CAG ATA
Type I Collagen [76]	Forward: AAG AAC CCA GCT CGC ACA TG Reverse: GGT TAG GGT CAA TCC AGT AGT AAC CA
Type II Collagen [76]	Forward: GCA TTG CCT ACC TGG ACG AA Reverse: CGT TGG AGC CCT GGA TGA
GAPDH	Forward: CCT TCA TTG ACC TTC ACT ACA TGG TCT A Reverse: TGG AAG ATG GTG ATG GCC TTT CCA TTG

Data are expressed normalized by internal cellular housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistics

Samples from three experiments were pooled and analyzed using Minitab software (State College, PA) by ANOVA general linear model and pair-wise comparisons, with statistical significance assigned to $p \leq 0.05$. Data are expressed as average $\pm$ standard error of the mean. Sample size is 14 for each group, unless otherwise noted.

Results

Histology

After 7 days of continuous flow, constructs stained positively throughout for type II collagen and sulfated glycosaminoglycans (Figure 4.2). However, with increasing flow, there appeared to be a thicker band of type I collagen, restricted to the top (flow) surface. Co-localized in this band of type I collagen was an increase in cell density, with most cells assuming a more flattened, fibroblastic morphology. This band appeared to have a concentration of oriented collagen fibers, as witnessed in the picrosirius red stains under birefringent light. The area of intense type I collagen staining seemed to positively correlate with a lack of type IX presence and a decrease in intensity of safranin-O stain as well. While not shown, no tissue stained for type X collagen and all positive controls (all antibodies) were appropriately stained.

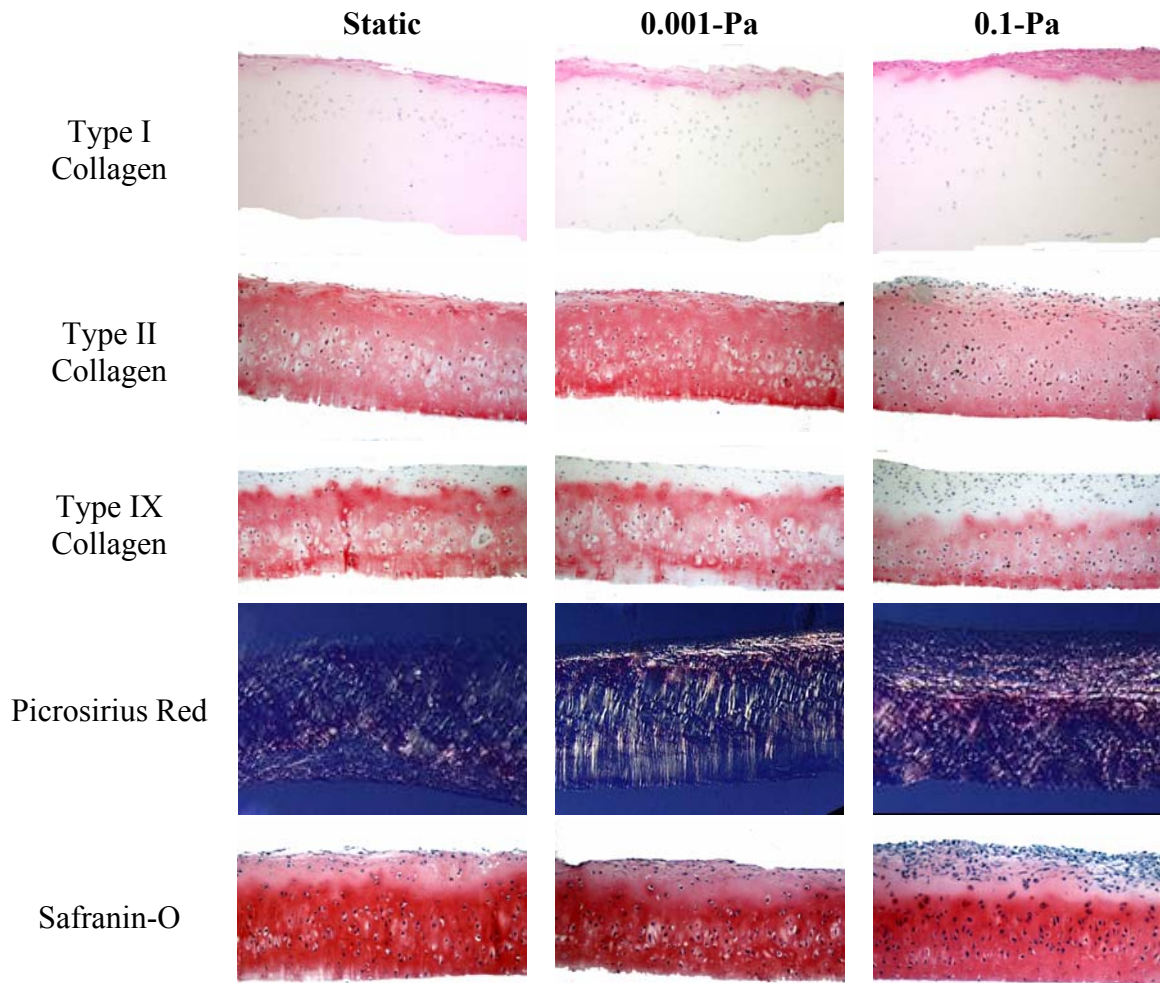
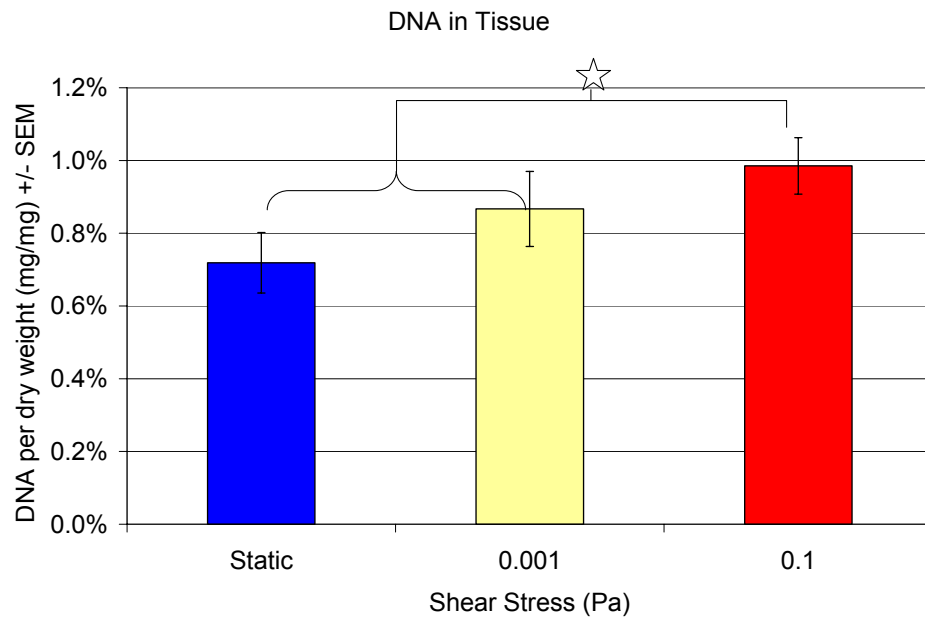


Figure 4.2 – Histology. Representative cross-sections for static (left), flow at 0.001-Pa (middle) and flow at 0.1-Pa (right) when stained for type I, II, and IX collagen, total collagen (picrosirius red, birefringent light) and sulfated glycosaminoglycans (Safranin-O).

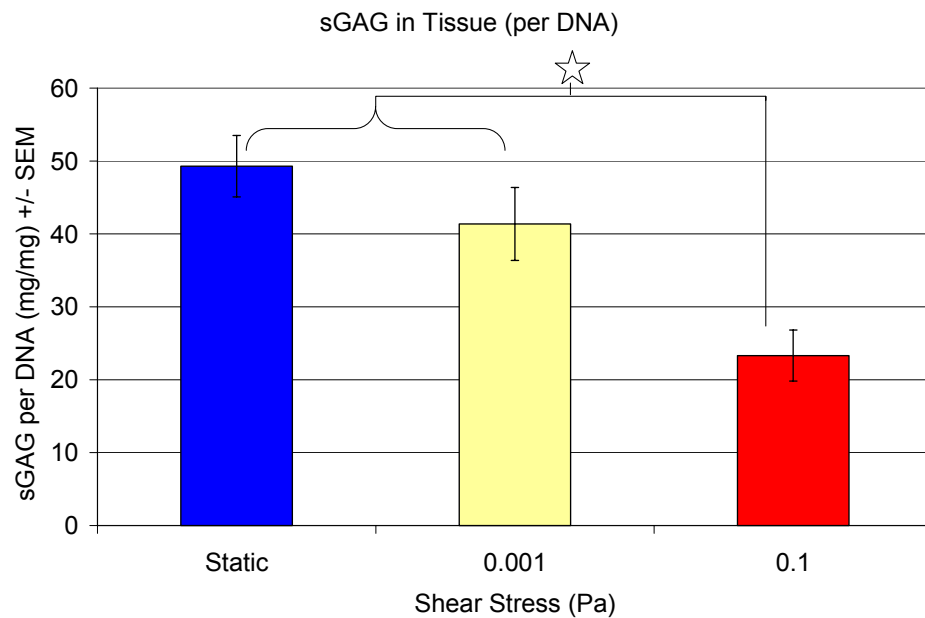
Matrix Composition

There were no significant differences among groups in terms of tissue weight (~270 mg), water content (~87% water) or thickness (~310 μm), although constructs in the 0.1-Pa group attained significantly more cells (DNA) than the 0.001-Pa or static control groups (Figure 4.3, A). Despite this, these constructs possessed a significantly lower amount of sGAG on both a per-dry weight (Figure 4.3, B) and per-cell (Figure 4.3,

C) basis than the other groups. These data are corroborated by both the qualitative assessment of the safranin-O staining seen previously as well as the significant increase in sGAG present in the media (Figure 4.3, D). Not only was the amount of sGAG in the media significantly higher in the 0.1-Pa group at each time point, but the deposition rate increased with time. Interestingly, when adding the sGAG present in the tissues to that collected over the week of flow in the media, the total sGAG was not different among groups.

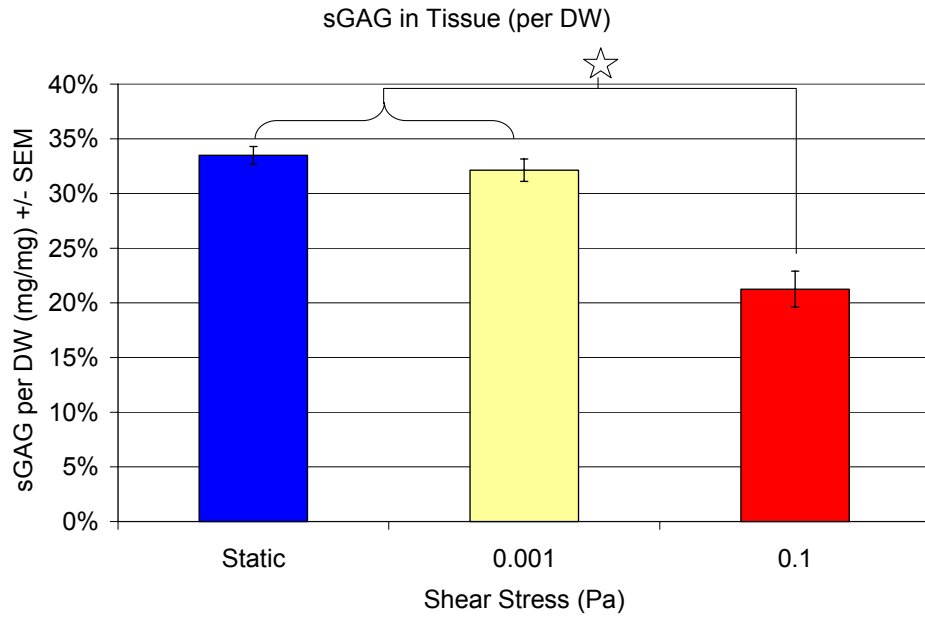


A

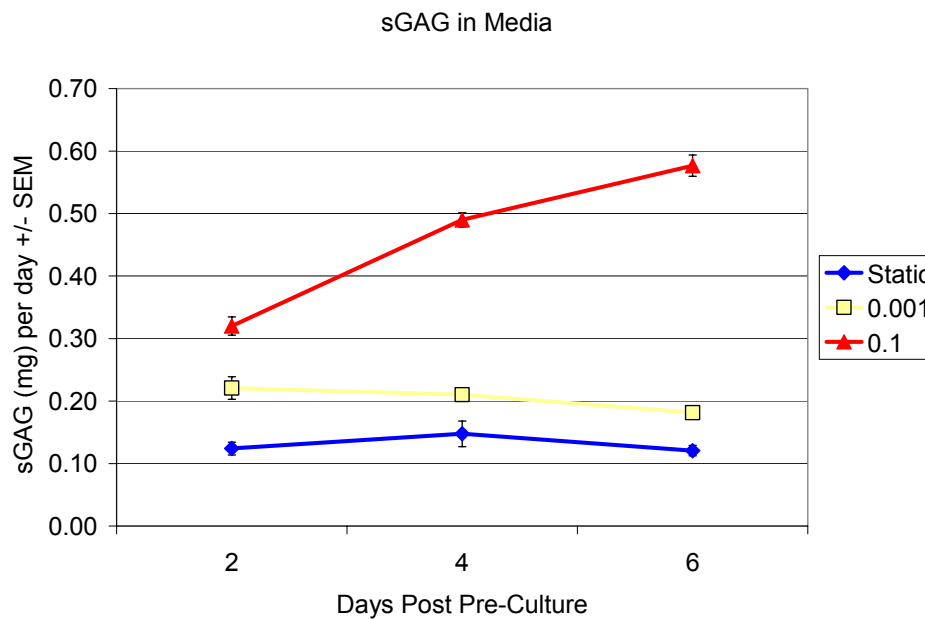


B

Figure 4.3 – Matrix Composition. Tissues flowed at 0.1-Pa possessed a greater number of cells (A), but less sGAG on a per-dry weight (B) and per-cell (C) basis than the other groups. Similarly, more sGAG was present in the media in the 0.1-Pa group, which increased over time (D). However, no difference was found between groups when summing the media and tissue contributions (E). [N=8, 9, 11 for static, 0.001, and 0.1 respectively for A, B and C] Star represents $p \leq 0.05$.

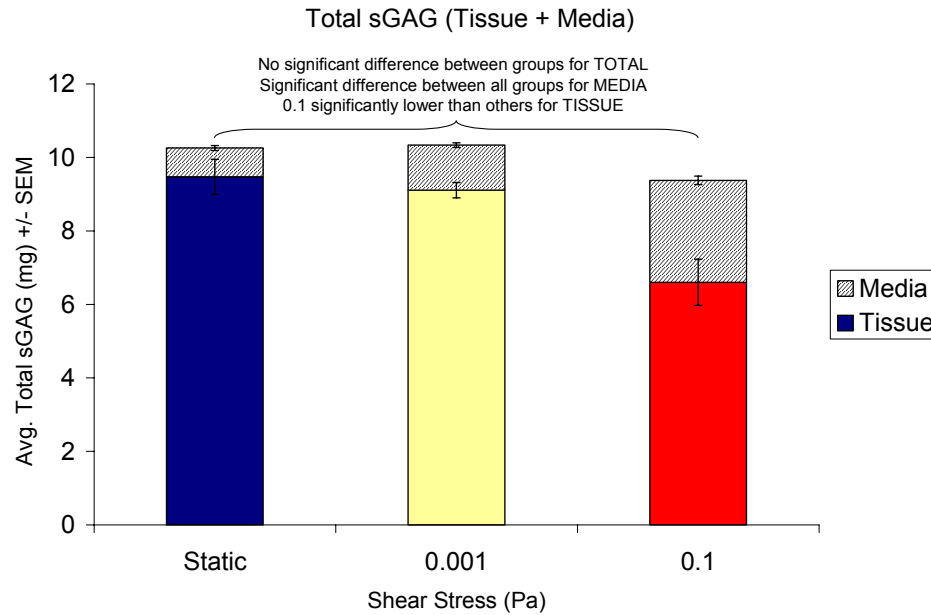


C



D

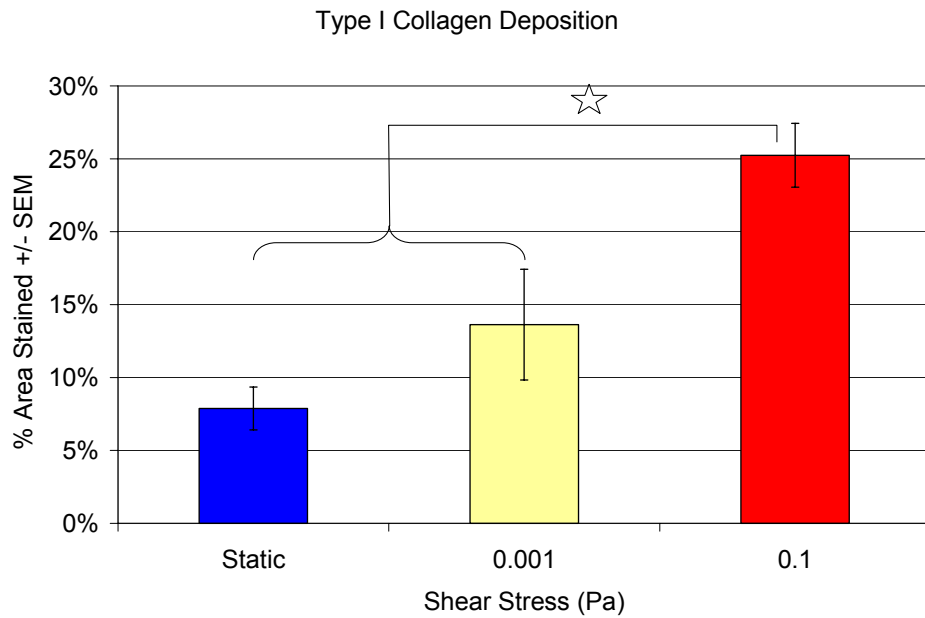
Figure 4.3 continued – Matrix Composition. Tissues flowed at 0.1-Pa possessed a greater number of cells (A), but less sGAG on a per-dry weight (B) and per-cell (C) basis than the other groups. Similarly, more sGAG was present in the media in the 0.1-Pa group, which increased over time (D). However, no difference was found between groups when summing the media and tissue contributions (E). [N=8, 9, 11 for static, 0.001, and 0.1 respectively for A, B and C] Star represents $p \leq 0.05$.



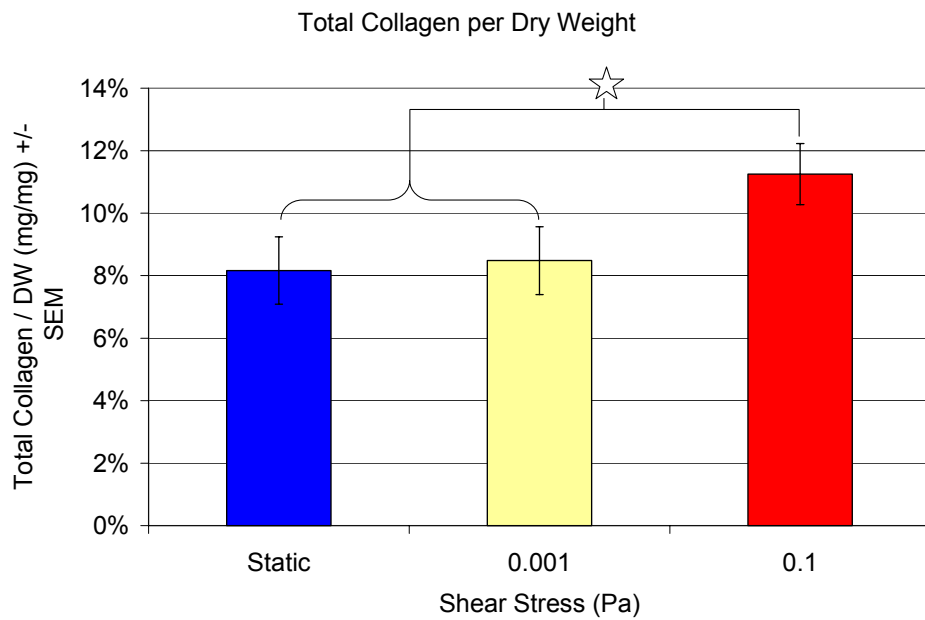
E

Figure 4.3 continued – Matrix Composition. Tissues flowed at 0.1-Pa possessed a greater number of cells (A), but less sGAG on a per-dry weight (B) and per-cell (C) basis than the other groups. Similarly, more sGAG was present in the media in the 0.1-Pa group, which increased over time (D). However, no difference was found between groups when summing the media and tissue contributions (E). [N=8, 9, 11 for static, 0.001, and 0.1 respectively for A, B and C] Star represents $p \leq 0.05$.

In terms of collagen, 0.1-Pa shear exposed tissues had more total collagen on a per-dry weight basis than the other groups (Figure 4.4, A); this was not the case on a per-cell basis (Figure 4.4, B), where the static controls were significantly higher than either of the flowed tissues. According to the histomorphometric analysis, type I collagen was present in a significantly greater percentage area of the tissue in the 0.1-Pa group ($25.2 \pm 2.2\%$) compared to either the 0.001-Pa ($13.6 \pm 3.8\%$) or the static ($7.9\% \pm 1.5\%$) groups (Figure 4.4, C). There was no difference between 0.001-Pa and static groups. These trends repeat when considering the type II collagen content, as assessed by ELISA (Figure 4.4, D), where the 0.1-Pa group possesses more collagen than the other two groups.

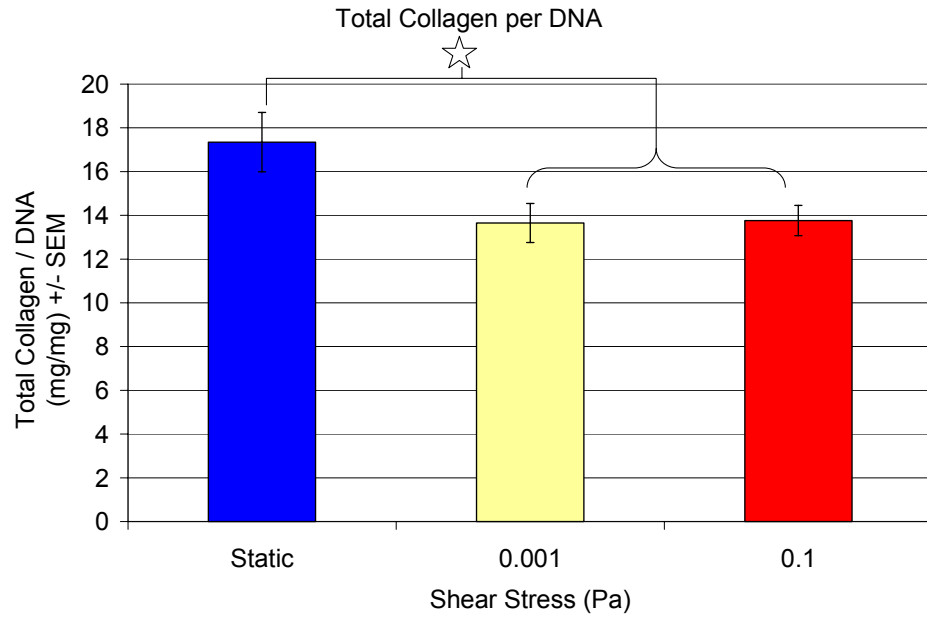


A

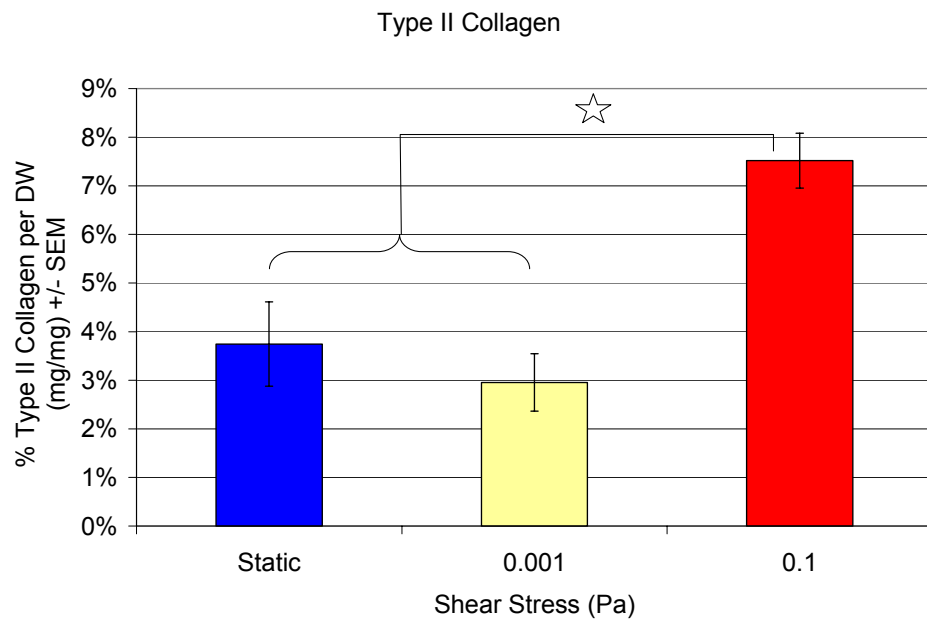


B

Figure 4.4 – Collagen Content. Tissues stained positive for type I collagen over significantly more area in the 0.1-Pa flow group compared to the other two groups (A). This 0.1-Pa flow group also had more total collagen (B) and type II collagen (D) compared to static and 0.001-Pa flow on a dry weight basis, but not a per cell basis (C). Star represents $p \leq 0.05$.



C

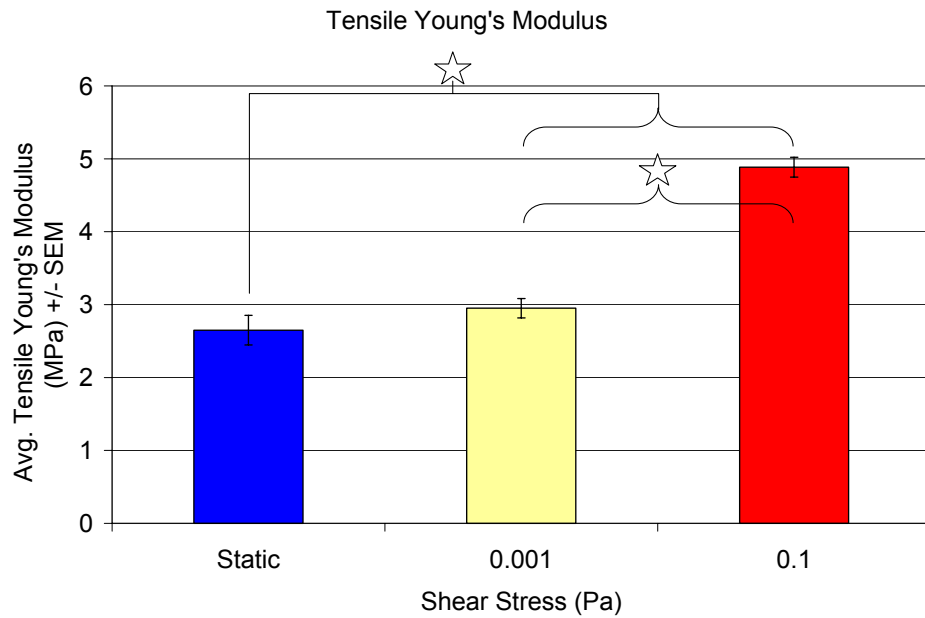


D

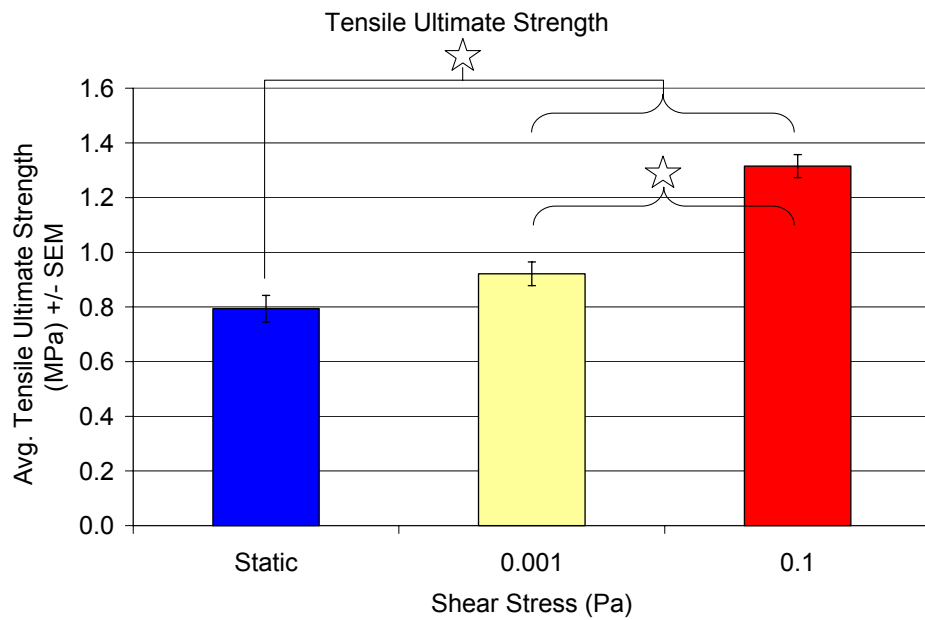
Figure 4.4 continued – Collagen Content. Tissues stained positive for type I collagen over significantly more area in the 0.1-Pa flow group compared to the other two groups (A). This 0.1-Pa flow group also had more total collagen (B) and type II collagen (D) compared to static and 0.001-Pa flow on a dry weight basis, but not a per cell basis (C). Star represents $p \leq 0.05$.

Mechanical Testing

A dose-dependent increase in tensile Young's modulus and ultimate strength was observed with increasing shear stress (Figure 4.5). While both flow groups were significantly higher than the static, the 0.1-Pa group showed the greatest increase up to nearly 5 MPa in modulus and 1.3 MPa in strength.



A



B

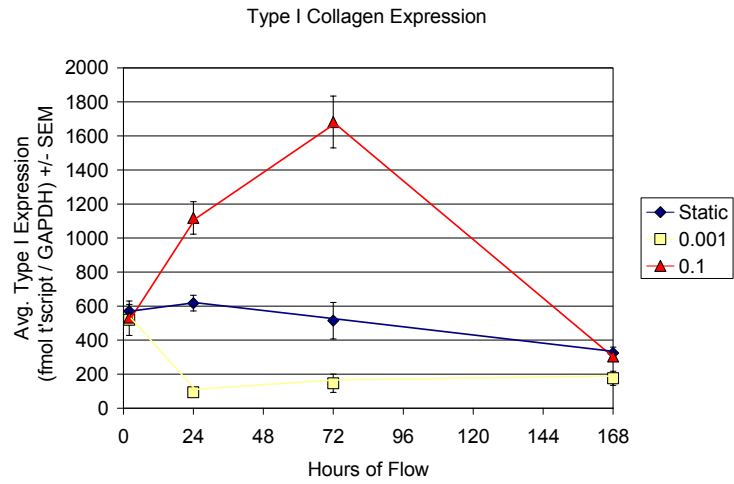
Figure 4.5 – Mechanical Properties. Both flow conditions resulted in higher Young's modulus (A) and ultimate strength (B) than the static condition. The higher flow group (0.1) was also significantly increased compared to the lower flow group (0.001). [N=9, 11 and 14 for static, 0.001, and 0.1 respectively] Star represents $p \leq 0.05$.

Gene Expression

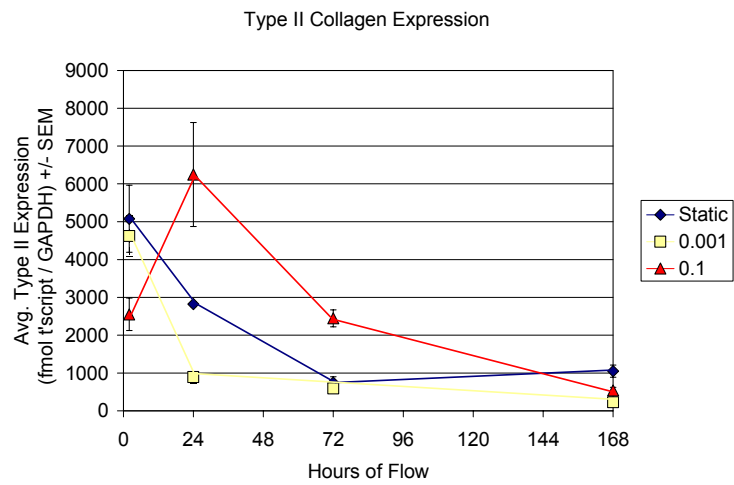
Expression of type I and type II collagen were modulated by the magnitude and duration of shear stress (Figure 4.6). No significant differences were observed for the expression of aggrecan (not shown). While GAPDH levels did vary across time points, no significant difference exists between groups at a given time point. One exception is between the static and 0.001-Pa group at Day 7, however no significant difference exists between these groups for type I or type II collagen expression whether normalized by GAPDH or total RNA. The timecourse of GAPDH expression is shown in Figure 4.6.

Type II collagen expression in the 0.1-Pa group was significantly decreased relative to the 0.001-Pa and static groups initially at the 2-hour time point, but rebounded to a significantly increased level (relative to the same groups) at the 24-hour time point. No differences between groups were observed by 7 days. Type I collagen followed similar trends in that it was significantly up-regulated at 24-hours and 3-days compared to the other groups. At the 2-hour and 7-day time points, no difference was observed for the 0.1-Pa group compared to the others. These trends are well corroborated by quantitative protein data (total collagen, ELISA and histomorphometry) and immunohistochemical observations.

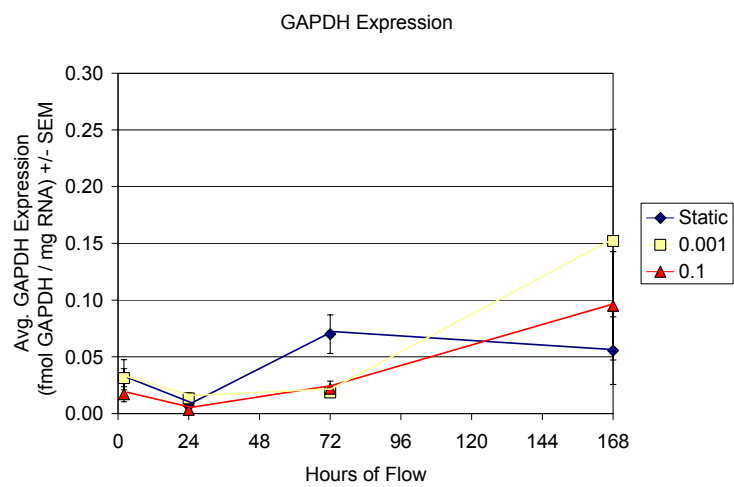
Gene expression trends were sensitive to shear stress magnitude, as the 0.001-Pa sheared tissues did not follow similar trends as the 0.1-Pa samples. The 0.001-Pa group maintained relatively low gene expression levels over time, with the exception of the 2-hour time point. Interestingly, at the time points where type I and type II collagen were most significantly up-regulated relative to static groups (*e.g.*, the 24-hour and 3-day time points) in the 0.1-Pa group, this corresponded to the greatest decrease observed in the 0.001-Pa group. One potential reason for this discrepancy is that there is a media volume difference between shear-exposed and static cultures.



A



B



C

Figure 4.6 – Gene Expression. Type I and type II collagen are modulated by shear stress magnitude and duration of application. [N=3-4 for 2, 24, 72h; N=7-9 for 168h]

Discussion

While many strategies aim to increase similarity of engineered tissue to that of native tissue, none have achieved this goal. In particular, the amount and organization (*e.g.*, fiber orientation, interplay with other matrix molecules) of type II collagen in engineered tissues is consistently inferior to that in native tissue [155, 191, 192, 209]. This crucial protein has direct effects on the unique mechanical properties of articular cartilage [31]. Type II collagen is most responsible for trapping the proteoglycans in the fibrillar matrix [27], determining the tensile mechanical properties and resisting shearing deformations [31] and has even been shown to have a positive correlation with the compressive properties [54]. Given this inherently crucial role of type II collagen – combined with the deficiency in most tissue-engineered constructs – we sought to increase deposition of this protein by using a novel flow bioreactor.

Recently, we developed a bioreactor that was shown to increase the type II collagen content and tensile mechanical properties after three days of flow [183]. Similar to previous work however, this study – while having shown promising results – still does not reproduce the matrix composition or mechanical properties of native tissue. In order to expand and improve upon these results, in this study, we extended the duration of flow to seven days in the hopes of further increasing type II collagen content and mechanical properties. Indeed, extending the duration of exposure to hydrodynamic shear stress further increased the total and type II collagen content compared to controls, but at the expense of an increase in type I collagen presence and decrease of sGAG in the tissue.

Other hydrodynamic bioreactors have been shown to modulate the response of chondrocytes using fluid-induced shear stress in both monolayer [9, 91, 213] and tissue-engineered systems [7, 157, 158, 187-192]. One drawback to many hydrodynamic systems used in tissue engineering is the heterogeneous field of shear stress applied to the cells and tissues. This heterogeneity arises due to the turbulent flows generated by the bioreactor and tissue (*e.g.*, spinner flask [2, 7]) or the perfusion of media through a

complex three-dimensional geometry of a scaffold [1]. To circumvent this problem, we apply shear stress using a parallel-plate bioreactor (founded on Poiseuille fluid mechanics [219]) in conjunction with a scaffold-free tissue. Limited success was shown in our previous work, which showed a specific increase in type II collagen as well as tensile mechanical properties after only three days of continuous flow. The disparity in these parameters between our tissue and native tissue prompted the exploration of longer applications of shear stress and at different magnitudes to better understand the response of the cells and tissue to this stimulus.

After seven days of flow, type II collagen, total collagen as well as the presence of type I collagen were increased in the 0.1-Pa group compared to the other two groups. The 0.001-Pa group was not different from the static controls for any of these metrics. Thus, while the shear stress-mediated increase in type II collagen evident after three days of flow was indeed extended to seven days of flow, there was an emergence of type I collagen. While type I collagen was present in all tissues, it was significantly increased in the 0.1-Pa group where more than 25% of the tissue's total area was stained positively for type I collagen. Deposition of this protein was relegated only to the flow-exposed surface of the tissue along with flattened, fibroblast-like cells. This fibrous capsule is consistent with other studies using dynamic fluid flow [7, 187]. Type I collagen presence in an articular cartilage construct is detrimental and renders the tissue more like fibrocartilage, which is an inferior tissue for bearing load [242]. However, our results are not necessarily unexpected as long-term cultures (67 days) have been known to possess thick bands of type I collagen co-localized with flattened, more fibroblastic cells on the outer surface of the construct [156]. In light of the qPCR data, which showed significant increases in types I and II collagen expression in the 0.1-Pa group over the others at days 1 and 3, a concomitant increase in these proteins' deposition was expected.

Another ramification of the extended duration of flow was the lack of sGAG in the tissue and the concurrent increase in sGAG present in the media in the 0.1-Pa group

compared to the other groups. It is unknown whether or not this sGAG in the media was extracted from the tissue or merely released (and not incorporated) by the cells. sGAG content retained in the matrix did appear to be sensitive to fluid-flow as shown here and by others who found that constructs grown in static flasks, mixed flasks (turbulent shear) and rotating vessels (laminar shear) all obtained significantly different amounts of sGAG [7]. Furthermore, sGAG leaching into the media from engineered constructs has been shown to be affected by fluid flow magnitude (intensity) [211], suggesting that the increase in sGAG in the media was probably lost from the matrix *and* released from the cells (never incorporated).

Consistent with the increase in total collagen, the 0.1-Pa tissues exhibited a greater tensile modulus and ultimate strength compared to the other two groups. Despite not showing any significant increase in total collagen, type I or type II collagen, the 0.001-Pa group narrowly showed an increase in mechanical properties over static controls as well. This may be due to an increase in organization, which could be discerned from the picrosirius red staining which shows a denser band of collagen fibers on the top surface. The modulus of 4.89 ± 0.14 MPa attained by the 0.1-Pa group agrees well with that found by others using scaffold-free constructs after eight weeks of culture (4.8 ± 0.1 MPa) [182]. However, all groups were still significantly less than that measured for calf bovine articular cartilage in terms of both the modulus (~ 12 MPa) and ultimate strength (~ 5 MPa) [52]. The biochemical basis for the increase in mechanical properties is unknown, but can reasonably be assigned to the increase in collagen content, given its role in the native matrix and the parallel increases between collagen and mechanical properties in our system. Whether this can be attributable to the increase in type I or type II collagen is debatable and may have been related, in part, to increased organization. The picrosirius red stains, which under birefringent light shows collagen fiber orientation (does not distinguish collagen type), shows a compaction of stratified collagen with the application of flow. Nothing is known about degree of cross-linking within the collagen

network, which also may have affected the mechanical properties. Interestingly, these increases in mechanical properties come despite a decrease in sGAG retained in the matrix in the 0.1-Pa group. This is reminiscent of others' observations that collagen (compared to proteoglycans) is most responsible for determining the tensile mechanical properties [31].

We can also reasonably conclude that the effects seen in this system were due to shear stress and removal of the static boundary layer and not an increase in flux through the tissue. Mathematical models of a porous medium (cartilage) subject to Poiseuille flow using bioreactor dimensions and parameters similar to ours suggest that there is no fluid flux through the tissue [243]. In fact, intra-tissue flux would only occur if the tissue permeability was approximately eight orders of magnitude lower. We independently calculated the permeability in several of our specimens based on empirical data (not shown), and believe that no flux is occurring through our tissue. While the shear effects visually appear to “penetrate” through the matrix (as evidenced by the thick type I collagen band), this was probably less due to penetration and more likely due to a surface-relegated effect which builds over time.

Certainly, one limitation of the assessment of the mechanical properties was the lack of compressive data. Because of the thickness of these tissues ($\sim 310\ \mu\text{m}$), it was found to be difficult to get reliable, repeatable data for the compressive properties due to the influence of artifact. We have engineered thicker ($\sim 1\ \text{mm}$) constructs, used in the bioreactor system in the next series of experiments.

This study showed that shear stress magnitude and duration modulate the matrix composition and tensile mechanical properties of engineered cartilage. While type II collagen and the tensile mechanical properties were increased with the highest level of shear stress, this came at the expense of an increase in type I collagen presence and depletion of sGAG from the matrix. Fluid induced shear stress is certainly a potent modulator of tissue development and requires greater understanding if it is to be

employed to generate a functional articular cartilage construct. Intermittent or oscillatory flows may be necessary to preserve the pro-hyaline effects (increase in type II collagen, tensile mechanical properties) but discourage the pro-fibrocartilaginous effects (type I collagen). Further exploration of the parameter space in the bioreactor will aid in attaining our goal of understanding the effects of fluid flow on engineered cartilage.

CHAPTER 5

EFFECT OF SEEDING DENSITY AND TIME IN CULTURE ON MATRIX COMPOSITION AND COMPRESSIVE MECHANICAL PROPERTIES ON ENGINEERED CARTILAGE

Introduction

In order to restore function to damaged articular cartilage, the implanted tissue may need to closely replicate the matrix composition, organization and mechanical properties of native tissue [188, 192]. One of many challenges to attaining this goal is the generation of a tissue of clinically-relevant size. The average thickness of human articular cartilage of the knee is 1.69 mm, ranging from 0.22 mm and 3.65 mm [23]. However, engineered cartilage and other tissues are at risk of developing poorly distributed cells and matrix due to mass transport limitations in static or free-swelling culture [210]. For this reason, many have undertaken the use of bioreactors to improve the movement of nutrients and waste products into and out of engineered tissues. Bioreactors, either by increasing the mass transport or applying mechanical stimuli, have been shown to modulate chondrocyte metabolism [7, 157, 158, 171, 187-192], thus enabling them to sustain thicker, more realistic tissues.

One approach to immediately augmenting the thickness of a tissue-engineered construct is through the use of a scaffold material capable of supporting the growth and chondrogenesis of the cellular component. But, when these cell-seeded scaffolds are placed in a perfusion bioreactor system, the fluid flowing through the pores of the scaffold creates a widely heterogeneous shear stress distribution [1, 2, 195]. This complex shear field – estimated only through the use of complex numerical algorithms [1, 2, 195] – makes interpretation of results based on the applied shear stress difficult. This problem is circumvented by using a scaffold-free approach, which involves plating

cells at high densities so that they are literally piled on top of each other, enough for maintenance of the rounded, chondrocytic phenotype and functional production of cartilage matrix components such as collagen and proteoglycans [181, 182, 184, 244]. Scaffold-free techniques have produced robust tissues even in static culture using a variety of cell sources (*e.g.*, human [244], porcine [184], bovine [181], rabbit [182] and goat [245]). Another advantage of the simplicity inherent in the scaffold-free approach is that analysis of data, whether it is biochemical or mechanical, does not require (potentially confounding) subtraction of the scaffold's influence. While the facility of the scaffold-free approach is advantageous in many respects, it does have the challenge of producing thicker tissues. Combining the diffusion limitations inherent to a thick construct with the lack of a vascular or porous internal architecture, scaffold-free constructs suffer to attain a clinically relevant thickness [181, 182].

We have shown success in improving the matrix composition and mechanical properties of a scaffold-free slab of engineered cartilage in our novel dual-chambered flow bioreactor [183]. Similar to others, a major limitation of this study was the lack of thickness of these tissues (~300 μm) and the subsequent inability to reliably test these tissues in compression. Since one of the major forces that articular cartilage undergoes *in situ* is compression, measuring the compressive mechanical properties of the engineered tissues is a vital assessment of the tissue's potential success *in vivo*. A variety of compressive testing methods exist, including dynamic and equilibrium confined and unconfined compression. Unconfined compression does not use a confining chamber to restrain the specimen while being tested. The confining chamber used in confined compression prevents the lateral expansion of the compressed tissue and therefore does not take into account the tensile strains in the lateral direction. Unconfined compression allows for contributions of the proteoglycans, collagens and interstitial fluid flow. Equilibrium testing is appealing because the analysis is simplified by assuming the articular cartilage is an isotropic elastic material at mechanical equilibrium [246].

Consequently, measurements are recorded after the tissue has reached equilibrium and interstitial water has stopped flowing [42]. However, this disregards an important phenomenon, since the drag exerted on the matrix by the interstitial water flow is known to play a role in determining the compressive mechanical properties [31]. The use of dynamic testing also allows for a range of frequencies to be imparted onto the specimen.

The objective of this study was to engineer scaffold-free tissues of “clinically-relevant” thickness (*i.e.*, >1mm), track their development over three weeks and examine their matrix composition and mechanical properties in dynamic unconfined compression. We hypothesized that increasing the cell seeding density will produce tissues thicker than 1 mm and that these tissues will show an effect of seeding density and time in culture on the dynamic compressive modulus. As this experiment was performed under static (no dynamic fluid flow) conditions and is inherently subject to mass transport limitations, we expected a disproportional relationship between increases in seeding density and resultant tissue thickness. Lastly, we translated the results obtained in the static, free-swelling series of experiments into the bioreactor setting in order to establish feasibility for further tests.

Materials and Methods

Cell Isolation and Seeding

Harvest

Full thickness cores of articular cartilage were harvested under aseptic conditions from the femoral condyles and femoropatellar groove of 2-4 week old bovine stifle joints (Research 87, Boylston, MA). Cores were minced in PBS supplemented with 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA) down to pieces 1-3 mm², then transferred to vented T75 flasks (2.0-2.5 g of tissue / flask) loaded with 30 mL of 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) in DMEM (high glucose,

Invitrogen) containing 0.5% neomycin (Sigma-Aldrich, St. Louis, MO), 1% kanamycin (Sigma) and 1% antibiotic/antimycotic. Tissues were incubated at 37°C, 95% relative humidity and 5% CO₂ for 48 hours under constant agitation on a rocker plate in order to isolate a single-cell suspension. Digest solutions were run through a 70 µm strainer and pelleted after 10 min centrifugation at 1100 x g. Pellets were resuspended in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT), 1% non-essential amino acids (Invitrogen), 0.25% gentamycin (Invitrogen) and 50 µg/mL ascorbic acid-2-phosphate (Sigma), centrifuged and resuspended twice in order to wash the cells. For this experiment, four stifle joints (unmatched) were used with cells pooled and seeded fresh.

Cell Culture Insert Study

Cells were counted in triplicate using the Vi-CELL automated cell viability counter (Becton-Coulter, Fullerton, CA) according to manufacturer's protocols, with viability >85%. The cell suspension was brought to a concentration of 20×10^6 cells/mL; from this, 1.3X, 2X and 8X dilutions were made, resulting in concentrations of 15, 10 and 2.5×10^6 cells/mL. 400 µL of each cell suspension was pipetted into a Millicell PCF cell culture insert (Millipore, Billerica, MA) housed in either a 24-, 12-, or 6-well non-tissue culture treated plate, depending on cell density used as listed below in Table 5.1.

Table 5.1: Experimental Design

Group Name	Cells per Insert	Cell Density	Media Volume	Size Plate
1M	1×10^6	2.5×10^6 cells/mL	1 mL	24 well
4M	4×10^6	10×10^6 cells/mL	4 mL	12 well
6M	6×10^6	15×10^6 cells/mL	6 mL	12 well
8M	8×10^6	20×10^6 cells/mL	8 mL	6 well

24 hours prior to seeding with cells, cell culture inserts were incubated in media in order to let serum proteins adsorb and encourage cellular attachment. Inserts are a 12 mm diameter x 10.5 mm high polystyrene cylinder with a polycarbonate membrane affixed to the bottom of the cylinder (0.6 cm^2 membrane area, pore diameter of $0.4 \mu\text{m}$; see Figure 5.1). The cylinder has small feet so that the culture vessel stands off the bottom of the culture plate; combined with the semi-permeable nature of the membrane, this allows for cells to feed from the media contained above the cells within the cylinder housing and from the media below the cells (in the plate, through the membrane).

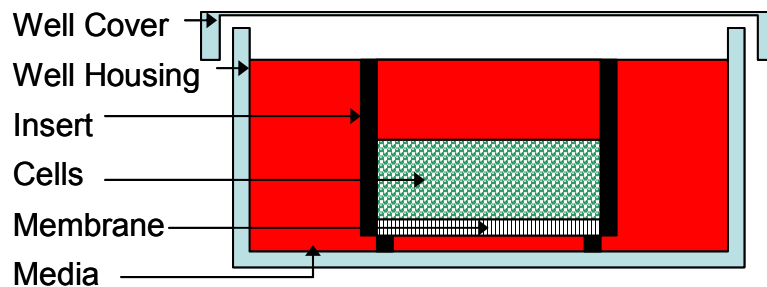


Figure 5.1 – Schematic of the Cell Culture Insert. Cells/tissues adhered to membrane are submerged in media; allowed to mix gasses with incubator air via non-sealing cover.

Constructs were cultured for three weeks with full media replacements every 48 hours and harvested at weekly intervals. Constructs for each density were harvested at each time point and assessed for matrix composition and mechanical properties. Tissues

designated for histology were fixed immediately, while those for biochemical assay were frozen at -20°C and those for mechanical testing were punched to 6 mm diameter and frozen in 1 mL PBS with 1X protease inhibitor (PBS+PI) cocktail set III (Calbiochem / EMD Biosciences, San Diego, CA) at -20°C.

Bioreactor Study

The overall goal of the cell culture insert study was to find a cell density which can produce an articular cartilage-like tissue with functional mechanical properties and clinically-relevant thickness that can be translated into use into our bioreactor system. Details of the bioreactor are detailed previously [183]; briefly, the bioreactor in the static (no dynamic fluid flow) configuration is a rectangular polycarbonate device with media above and below the cells, which are grown on a semi-permeable polycarbonate membrane, analogous to the cell culture insert. The bioreactor growth chamber has dimensions of 4.6 cm x 1.3 cm x 1.3 cm (length x width x height), an area of 6 cm², exactly 10X that of the cell culture insert. Thus, before undertaking full-scale bioreactor flow studies, the same cell densities were seeded into the bioreactor, but with different pore diameter sized membranes. The end point of this study was gross appearance only and did not employ any quantitative assays.

Matrix Composition Assays

Histology

Each sample was assessed for the presence of type I collagen, type II collagen, type IX collagen, type X collagen, sulfated glycosaminoglycans (sGAG) and general morphology. Tissues were fixed for 24 hours in either 10% neutral buffered formalin or 1% acid alcohol (1% glacial acetic acid in 70% reagent alcohol), then washed for 2 hours in PBS and stored in 70% reagent alcohol at 4°C until time of processing. Samples were

processed, embedded in paraffin, sectioned at 5 μ m and mounted on slides. Those tissues fixed in formalin were stained for sGAG using 0.1% safranin-O (0.02% fast green counterstain) and general morphology using hematoxylin and eosin (H&E). Tissues fixed in acid alcohol were used in immunohistochemical (IHC) staining for collagen types using specific antibodies against the following: type I collagen (mouse monoclonal MAB3391, Chemicon Inc, Temecula, CA), type II and type IX collagen (mouse polyclonals II-II6B3 and B3-1, respectively, Developmental Hybridoma Studies Bank, Iowa City, IA), and type X collagen (rabbit polyclonal, kindly donated by Dr. Gary Gibson, Henry Ford Hospital, Detroit, MI). Antibodies raised in mouse were reacted with a biotinylated anti-mouse secondary antibody (BA 2001, Vector Laboratories, Burlingame, CA) while those raised in rabbit with a biotinylated goat anti-rabbit IgG (BA-1000, Vector). Color development was precipitated by the use of the Vecastain ABC alkaline phosphatase kits (SK 5100 substrate and AK 5000 complex, Vector Labs, Burlingame, CA). Antigens were retrieved by a 10 minute digestion (60 minutes for type X collagen) using Digest-All 3 (Invitrogen) at 37°C. For each staining session, a positive (bovine articular cartilage), negative (bovine tendon) and secondary-antibody control were included to ensure specificity of the stain.

Quantitative Biochemistry

Each tissue was subject to quantitative biochemical assays, including the Hoeschst 33258 (Sigma) dye binding assay for DNA (cell) content [222], dimethyl-methylene blue [223, 224] (DMMB, Aldrich) dye binding assay for sGAG, and the hydroxyproline assay [221] for total collagen content. Approximately 50 mg of wet tissue was digested in 125 μ g of freshly prepared proteinase K (Worthington) in 1 mL of

100 mM ammonium acetate for 18-24 hours in a 60°C water bath. Total collagen was quantified by first hydrolyzing 100 μ L of digest solution in 2 mL of 6N HCl overnight at 110-115°C, then re-suspending in 0.25M sodium phosphate buffer, reacting 50 μ L of suspension (in triplicate) with 50 μ L chloramine T (Mallinckrodt Baker, Phillipsburg, NJ) for 20 min at room temperature and 50 μ L p-dimethylaminobenzaldehyde (Mallinckrodt Baker) for 60 min at 60°C, and finally read on a PowerWave spectrophotometer (Bio-Tek, Winooski, VT) at an optical density of 557 nm. Experimental samples were compared to hydroxyproline standards, then converted to total collagen by using a conversion of 10:1 hydroxyproline:collagen [7, 247]. Purified type II collagen (Southern Biotech, Birmingham, AL) was hydrolyzed using the previously described process to confirm this ratio. sGAG was quantified by reacting 20 μ L of 50-fold diluted experimental digest with 200 μ L DMMB (pH 3.0), read on the PowerWave spectrophotometer at 525 nm and compared to standards of chondroitin sulfate (Sigma), all in triplicate. DNA was quantified by incubating 10 μ L of undiluted experimental digest with 200 μ L Hoechst dye in 1X TEN buffer at a concentration of 0.5 μ g/mL for 10-15 min (in triplicate), reading on a PerkinElmer (Wellesley, MA) HTS 7000 fluorometric plate reader at excitation/emission wavelengths of 360/465 nm (manual gain = 60) and compared to standards of calf thymus DNA (Sigma). Thickness of each sample was calculate during the mechanical testing assay by taking the average of three measurements taken at different point on the construct using a contact-sensing micrometer.

Mechanical Testing

Specimens from each tissue were tested in dynamic unconfined compression in order to elucidate a dynamic modulus over a range of frequencies. Samples were thawed and equilibrated in PBS+PI at room temperature for one hour. Immediately prior to testing, samples were punched to a diameter of 5 mm and measured in three places using

a contact-sensing micrometer and averaged to determine thickness. Punched discs were then loaded into an ElectroForce 3200 Series uniaxial system outfitted with a custom-designed compression chamber (machined stainless steel surfaced contained in a polycarbonate cylinder) filled with 5-7 mL PBS+PI connected to a 1.1-lb load cell (Interface, Scottsdale, AZ) on the immobilized (bottom) platform and a 9.5 mm diameter stainless steel indenter affixed to the uniaxial mover arm (resolution of 0.1 μm). The indenter was slowly lowered until a stable force of 10-20 mN was registered, indicating contact. A frequency sweep was then initiated under displacement control using the Dynamic Mechanical Analysis (DMA) sub-package within the WinTest software used to control the system. The sweep began after an hour equilibration at a 15% offset (ramp to offset at 0.1 mm/sec) and cycled through frequencies of 0.01, 0.1, 1 and 10 Hz at a dynamic amplitude of 1.5%. The number of sinusoids generated and recorded was optimized within the DMA software. As construct dimensions were input into the DMA prior to each test, the software, via FFT analysis, converted stiffness properties into material properties and calculated the tangent of the phase angle, complex modulus (E^*), storage modulus (E') and loss modulus (E''). Displacement traces for each sample were visually inspected for plateaus at the sinusoid peaks to ensure the loading platen and sample never lost contact.

Statistics

Statistical analysis was performed with Minitab (State College, PA) software using the ANOVA general linear model with pairwise comparisons. Significance was assigned to $p \leq 0.05$. Sample size is 3 for each cell density and time point for mechanical testing, N=4 for all quantitative biochemical assays, N=2 for histology.

Results

General Observations

Cells produced cartilage tissue which could be easily manipulated with laboratory forceps after only 1 week of culture for all seeding densities. The most notable observation is that after only 1 week, the 8M condition formed blebs and wrinkles in the tissue. This mottled tissue was extremely easy to pull off the membrane as time in culture progressed, and upon closer inspection, it was noted that the bumps on the tissue were from a lack of adherence to the membrane. For this reason, it was unfair to measure a thickness or mechanically test these constructs. Constructs were kept in culture for the full 3 week duration and assayed for quantitative biochemistry, but it must be stressed that these were grossly inferior tissues.

Histology

All constructs accumulated matrix over the 3 week experiment and stained positively throughout the construct for types II and IX collagen, with the vast majority of cells assuming the rounded, chondrocytic morphology. Only at the three week time point did type I collagen become present. Observed for each density, a thin band of type I collagen coincided with the appearance of flattened, fibroblast-like cells. Also observed in each tissue was a decrease in staining intensity for type II and type IX collagen as time progressed. This may be due to a loss of the protein, or more likely, an increase in overall matrix density, reducing the availability or prominence of the epitope presenting to the antibody. Shown below is the time course of histology for each protein for the 1M seeding density (Figure 5.2). The trends seen here are representative of the other seeding densities (not shown for the sake of brevity). Not shown is a representative picture for the type X stain, as no presence was detected in any sample; all positive tissue, negative tissue and secondary antibody controls were appropriately stained.

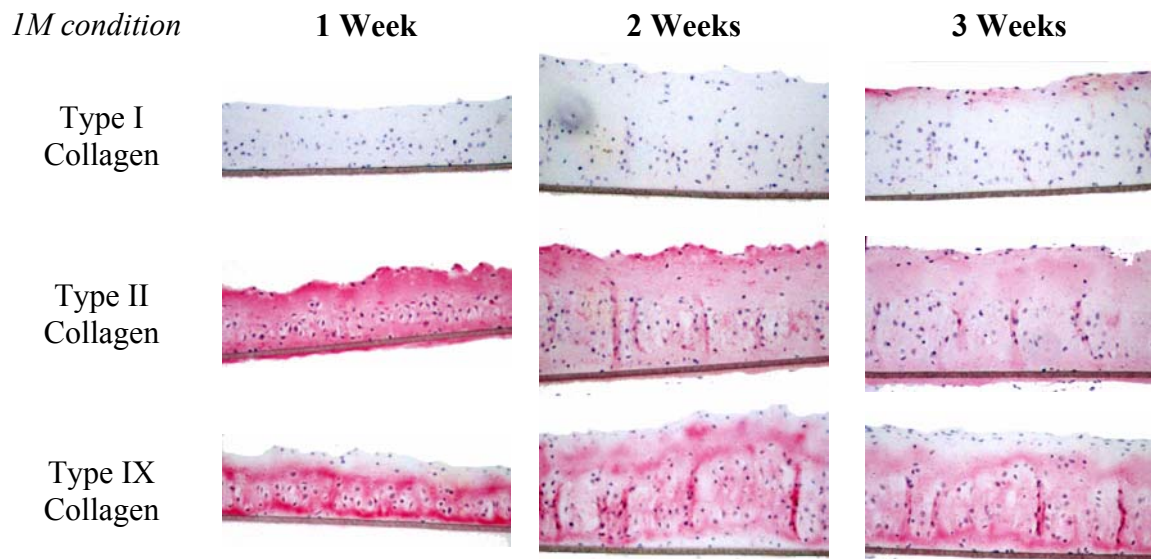


Figure 5.2 – Time Course Histology. 1M condition stained for types I, II and IX collagen at weekly time points. Original magnification = 10X. The dark line under the tissues is the membrane.

Constructs attained greater thickness with increasing cell density, but not in a linear relationship. This is apparent through both the one week stains (show below for type II collagen, Figure 5.3) and the thickness measurements in Figure 5.4.

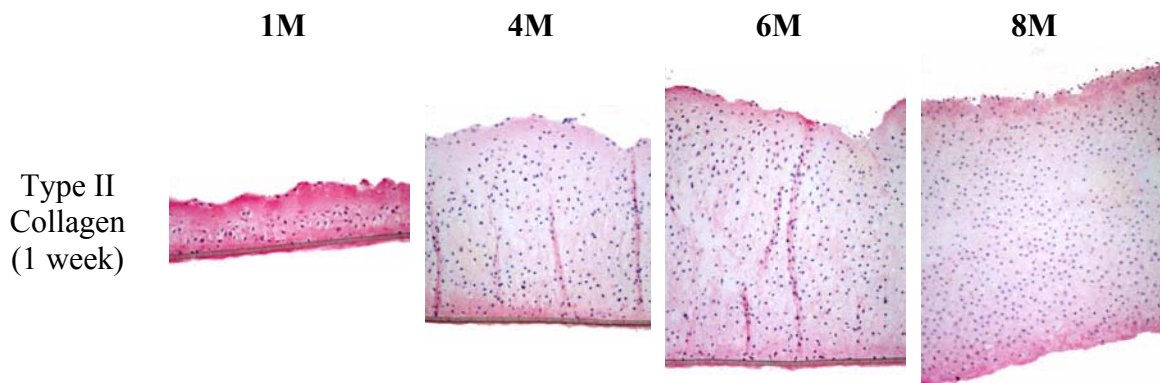


Figure 5.3 – Cell Density Histology. 1M, 4M, 6M and 8M conditions at one week stained for type II collagen. Original magnification = 10X.

Matrix Composition

As detailed below in Figure 5.4, construct average thickness and associated rate of increase showed a seeding density-dependent effect, but at a disproportional ratio. Linear curves fit to the points below show rates of growth of 0.05, 0.15, and 0.25 mm/week for the 1M, 4M and 6M groups, respectively (not shown). However, while thicker constructs were generated using higher seeding densities, the fold-increase in thickness was less than that of the fold-increase in seeding density. The greatest thickness was 1.16 ± 0.03 mm, reached by the 6M group at the 3 week time point.

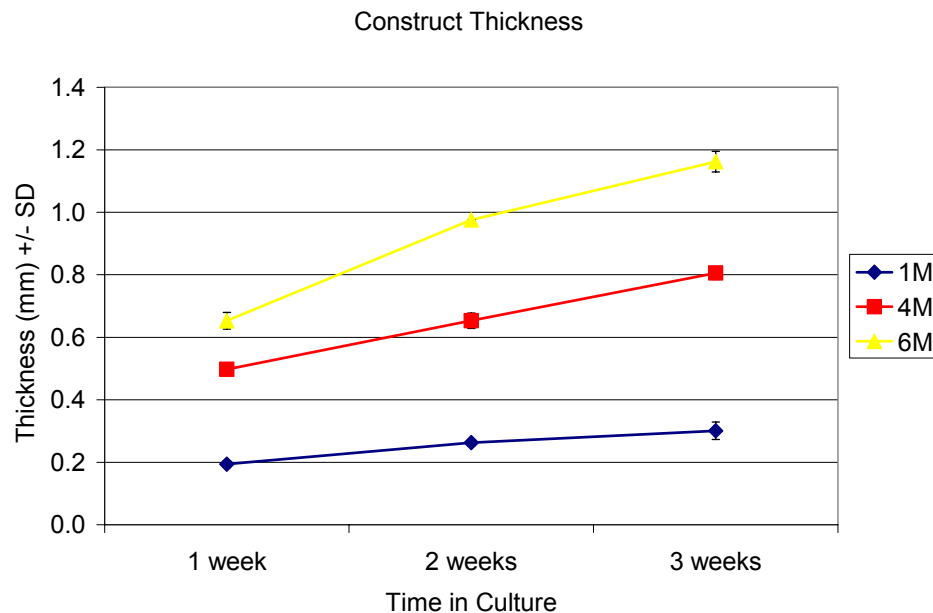


Figure 5.4 – Construct Thickness. The average thickness measured during mechanical testing for each cell density over three weeks. Points represent averages $\pm$ standards deviation.

No significant proliferation was seen in any group over time, with DNA (cell) amounts remaining nearly constant (Figure 5.5 A), but significance was achieved at each

time point between each seeding density group. The exception was the 8M group which showed a significant decrease between weeks 2 and 3, but as previously mentioned, these tissues were separating off the membrane at this point and behaving unlike the other tissues. When normalized by the 1M group average at the same time point, no group exhibits the same fold increase in DNA content as the original seeding density (Figure 5.5 B). In fact, the efficiency decreases with increasing seeding density and worsens over time.

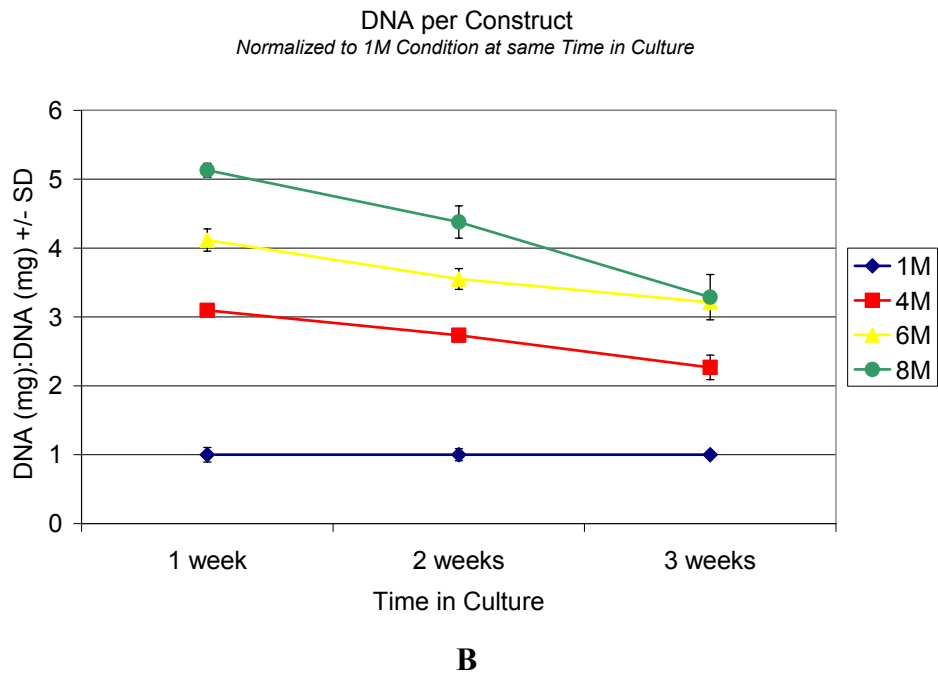
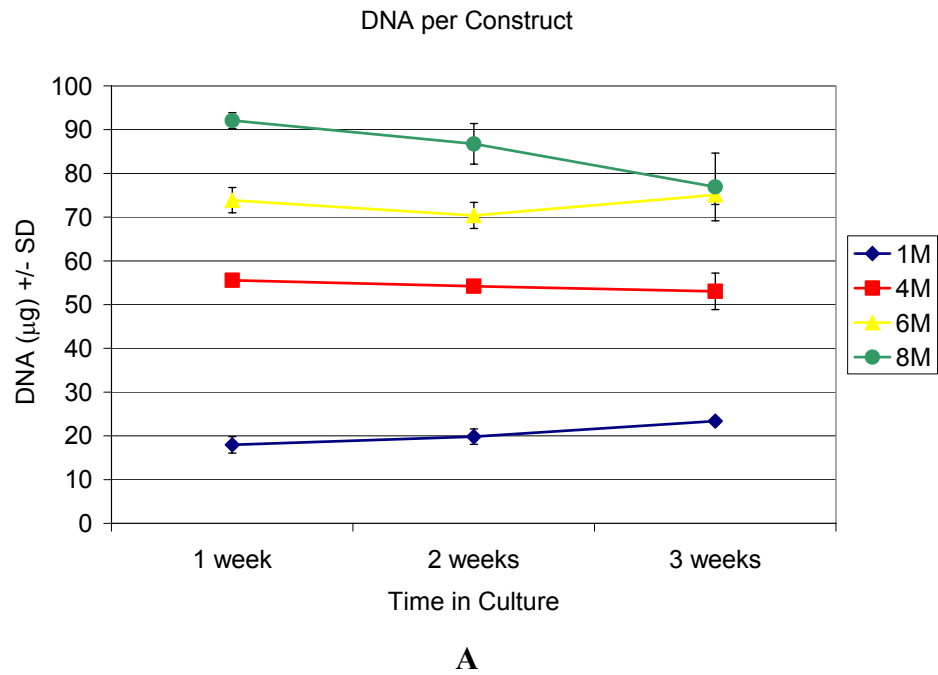


Figure 5.5 – DNA per Construct. Total DNA per construct (A) and normalized to the 1M condition (B) for each density over three weeks. Points represent averages $\pm$ standard deviation.

Total collagen (hydroxyproline) accumulation increased over time in all seeding density conditions, with the most collagen being deposited in the 4M and 6M constructs (Figure 5.6 A), which were both significantly higher than that the 1M or 8M groups. The 8M constructs possessed collagen content in between these groups and the 1M group. However, on a per-cell basis, the amount of total collagen per cell decreased with seeding density, significant at 2 and 3 weeks; *i.e.*, the most efficient cells in producing collagen were from the 1M group (Figure 5.6 B).

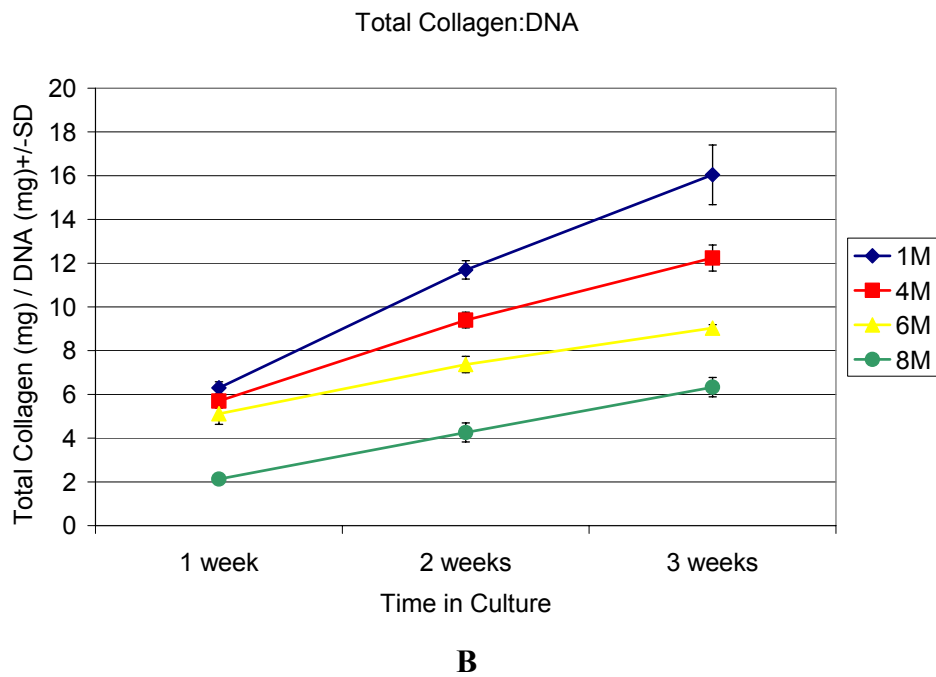
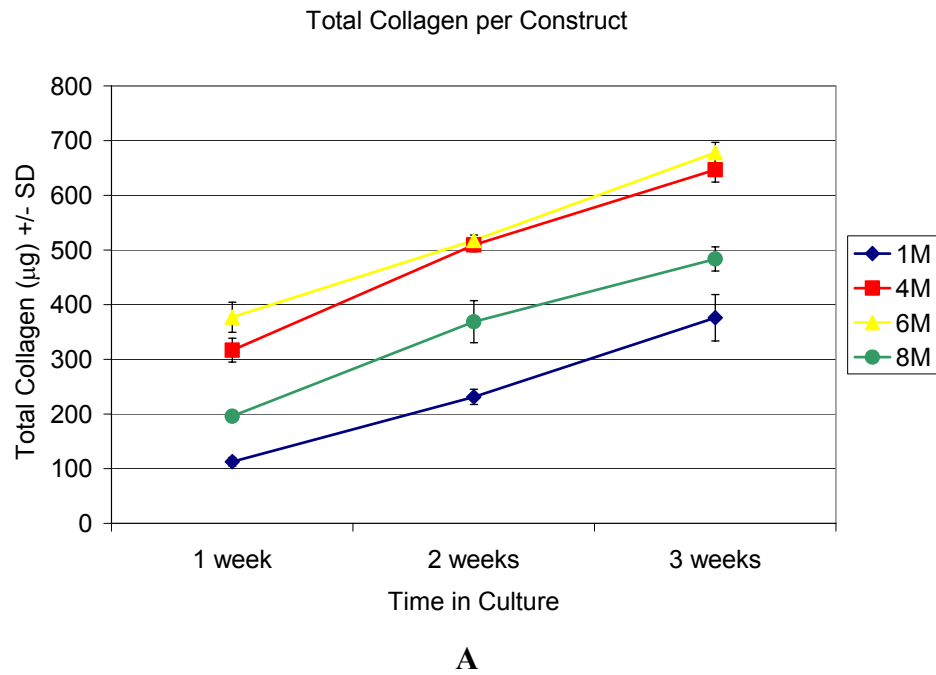
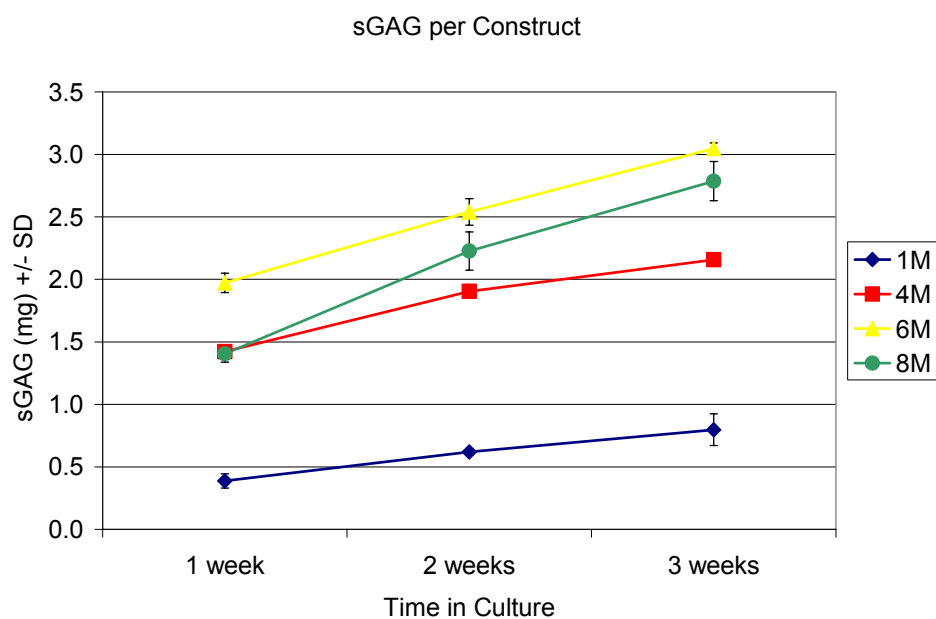
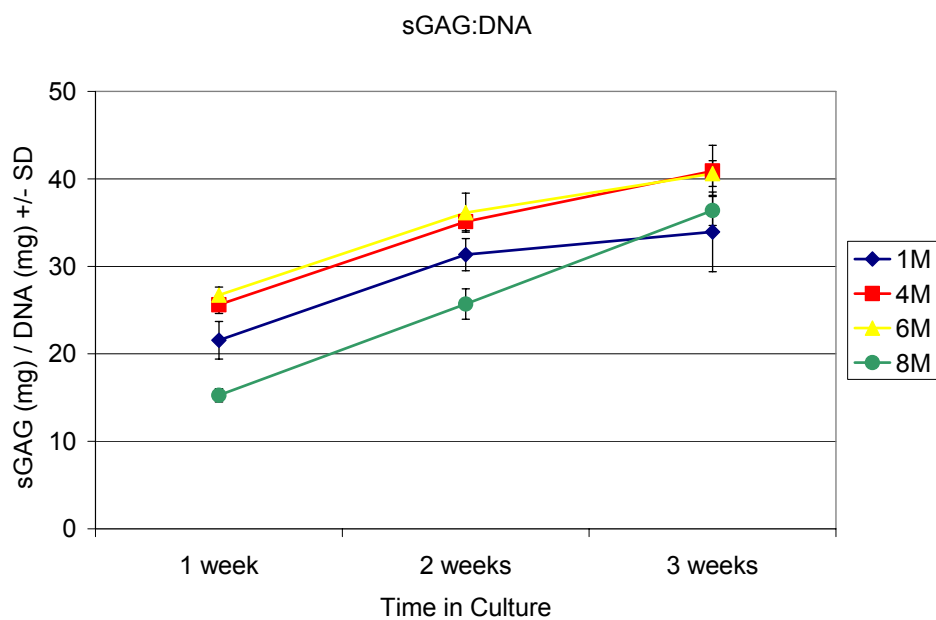


Figure 5.6 – Total Collagen Content. Total collagen per construct (A) and total collagen normalized by construct DNA (B) for each density over three weeks. Points represent averages $\pm$ standard deviation.

In terms of total sGAG in the matrix, the 6M group outperformed all others, attaining a significantly greater deposition of sGAG than all other groups at all time points, save the 8M group at 3 weeks. A nearly dose-dependent effect on sGAG accumulation was seen with seeding density, with the exception of the 8M group (Figure 5.7 A). When analyzing the data on a “per cell”-basis, no significant differences were observed at 1 week and 2 weeks between the 1M, 4M, and 6M groups. The 8M group was significantly lower than these groups at these time points. At three weeks, the 1M group was significantly less than the 4M and 6M groups (Figure 5.7 B)



A



B

Figure 5.7 – Sulfated Glycosaminoglycan Content. Total construct sGAG (A) and normalized by DNA per construct (B) for each cell density over three weeks. Points represent averages $\pm$ standard deviation.

Taken together, a greater amount of matrix is generally obtained with increasing the cell density, provided the tissues stay attached to the membrane (as was not the case in the 8M group). However, the efficiency of the cells to produce matrix is reduced with increasing cell density, so a tradeoff exists between seeding density and matrix production efficiency.

Mechanical Testing

The dynamic modulus varied with cell density, time in culture and applied frequency at certain points. Shown below in Figure 5.8 A is the frequency sweep for the three time points for the 6M group. While the average modulus generally increased with frequency, a significant difference existed only between the 10Hz frequency and the other frequencies, at the same week. At each frequency, the modulus at 3 weeks was significantly higher than at 1 week; 2 weeks was significantly higher than 1 week at all frequencies except 0.01 Hz. No differences existed between weeks 2 and 3 at any given frequency. Other cell densities showed similar trends at different magnitudes, but no significant differences were observed in these groups.

The dynamic moduli for the three tested densities (1M, 4M, 6M) at the three time points for 1Hz is shown in Figure 5.8 B. Both the 4M and 6M tissues possessed significantly higher moduli than the 1M group at all three time points, with the 4M group outperforming 6M only at 1 week. The same was true for the 0.01 and 0.1 Hz frequencies (not shown); at 10 Hz across all time point, 1M was significantly less than 4M and 6M, while 6M outperformed 4M.

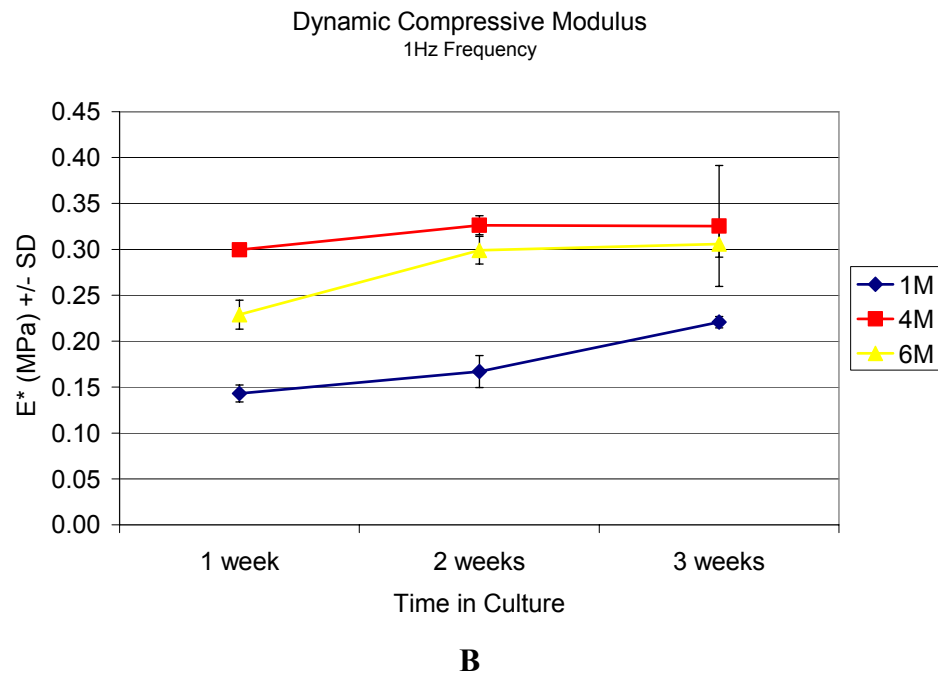
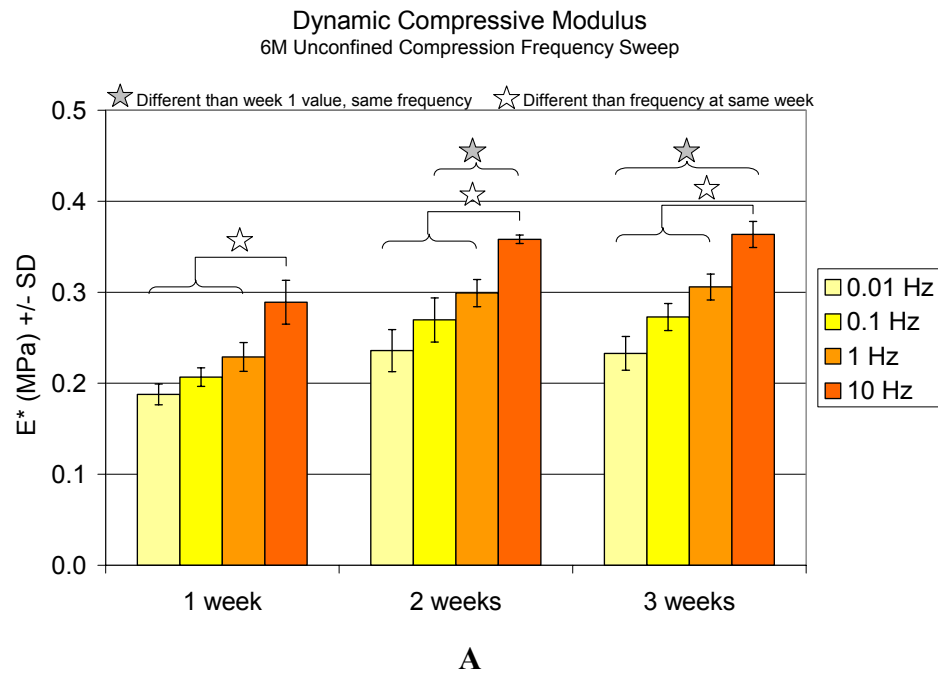


Figure 5.8 – Compressive Mechanical Properties. Dynamic modulus for the 6M group over a 4-decade frequency sweep for each week (A) and the dynamic modulus for each cell density at each week for the 1 Hz frequency (B). Points represent averages $\pm$ standard deviation. Not shown is 8M, which due to wrinkling was unfit to test.

Bioreactor Study

Since it appeared that there was a maximum density of cells that could form acceptable (flat sheets) of tissue from the cell culture insert studies, a similar experiment was carried out in the bioreactor. Different pore sized membranes were used in the bioreactor; membranes were all made from polycarbonate, with pore diameters of 0.2, 0.4, 0.6 and 0.8 μm . Cells were isolated and seeded as described above, at a concentration of 15×10^6 cells/mL for a total of 60×10^6 cells/bioreactor (corresponds to the 6M condition from the insert study). Constructs were maintained for two weeks and removed from the bioreactor and assessed for gross morphology only. As is shown in Figure 5.9 below, it was apparent that the membrane pore diameter plays a significant role in the development of the tissues. Tissues grown in the 0.2 and 0.4 μm membranes were visibly wrinkled and mottled. The 0.2 μm tissue was wrinkled throughout, whereas the 0.4 μm tissue had both mottled and smooth areas. However, those in the 0.6 and 0.8 μm membranes were smooth, flat and well-adhered to the membrane, as it took more force (qualitatively) to remove these tissues from the membrane as opposed to the 0.2 and 0.4 μm tissues. Going forward, it is apparent that the membrane pore size must be adjusted based on the cell density to be used. For constructs aiming to grow at least 1 mm, the cell density must be at least 15×10^6 cells/mL (result from the insert study), and must be seeded on 0.6 μm membranes in the bioreactor.

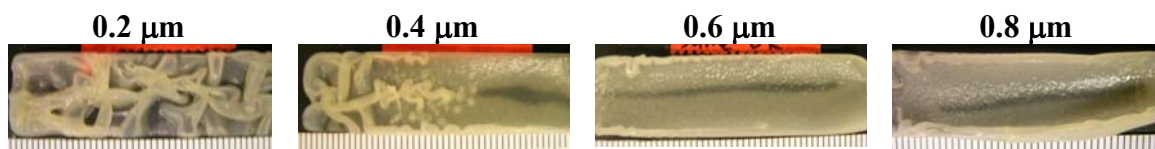


Figure 5.9 – Gross Morphology of Bioreactor-Grown Tissues. Column headings represent pore diameter size of membrane. Constructs grown for 14 days, originally seeded with 15×10^6 cells/mL (corresponding to 6M condition in the insert study).

Discussion

The objective of this study was to examine the effect of seeding density on the ability to engineer scaffold-free tissues of clinically relevant thickness in static culture. A secondary aim was to translate these results into the bioreactor setting in order to establish feasibility for future experiments. In a recent study, we introduced a novel bioreactor system which applies a fluid-induced shear stress to a flat slab of scaffold-free tissue. Bioreactor-stimulated tissues were found to have improved matrix composition and tensile mechanical properties compared to static controls [183]. While promising, a major limitation of this study was that the tissues were approximately 300 μm thick, which is on the low end of that seen in native tissue and far from the average thickness [23]. Engineering thicker tissues for use in the bioreactor system is necessary for this to become a clinically relevant solution. In this study, we statically culture tissues for three weeks that eventually exceeded 1 mm in thickness (in the highest density) and achieved a dynamic compressive modulus over 300 kPa. However, when translating these constructs into the bioreactor, the tissues wrinkled and peeled off the bioreactor membrane. Increasing the pore diameter of the membrane to 0.6 μm was found to alleviate this problem.

Scaffold-free tissue-engineered articular cartilage constructs have enjoyed success in terms of producing a viable, mechanically functional matrix [182, 234] and having been implanted into various animal models *in vivo* [185, 245, 248]. However, due to the lack of a scaffolding material, these self-assembling tissues lack the thickness of native human cartilage of the knee [190, 191], unless cultured for significant periods of time [234]. The thickest scaffold-free construct produced to date was 2.2 mm thick, in a bioreactor system using porcine chondrocytes [184]. Approaches to increase the thickness of scaffold-free tissues include simply increasing the cell density [181, 234] or imparting mechanical conditioning (in order to encourage additional matrix production)

[190, 191, 249]. In our bovine model, where the availability of cells is not a constraint, we attempted to engineer thicker tissues simply by increasing the cell density. Historically in our system, a cell density of 2.5×10^6 cells/mL produced constructs approximately 300 μm thick; increasing the cell density 4X, 6X and 8X should have theoretically produced constructs with a thickness of approximately 1.2, 1.8 and 2.4 mm, respectively. In this experiment though, the thickest construct was in the 6M group, 1.16 ± 0.03 thick, obtained after 3 weeks. The thickness was still increasing at this point, although the rate had slowed from the previous week. Across the different seeding densities, the thickness did not scale over time: on average, the 4M and 6M tissues were only 2.6X and 3.6X thicker than the 1M condition, indicating a significant loss of efficiency. Whether or not this was due to initial seeding efficiency (*e.g.*, Day 0 attachment) is unknown.

Analysis of the matrix composition added insight into the reason for the lack of scaling in thickness with cell density. While the seeding efficiency is unknown, it is certainly clear by 1 week that the seeding density ratio was no longer preserved and was in fact decreasing with time. At 1 week, the number of cells (DNA) per construct in the 4M, 6M and 8M groups was 3.1X, 4.1X, and 5.1X that of the 1M group. It is unlikely that oxygenation played a role in such a thin tissue's development over the first week. Similarly, in terms of feed volume, constructs at a rate of 2 million cells per mL media per day; this rate was conserved over each seeding density and was kept constant over time. Similar rates have been used with success in other tissue engineering systems [7, 154, 155, 158], but deviating substantially from that rate (~ 3.5 million cells per mL per day) resulted in loss of efficient matrix maturation [198]. Thus, it does not appear that media volume or absolute nutrient availability was a factor. Examining the amount of matrix (sGAG and collagen) on a per cell basis elucidated some reason for the inefficiency: while the per-cell rates of sGAG production were nearly equal across all groups, the collagen production was inversely proportional to the seeding density. No

difference, in terms of sGAG amount per cell, existed between the 4M and 6M group, although both were significantly higher than the 1M group – only narrowly however. Thus, the cells in the different seeding density groups are nearly equal in terms of producing sGAG, but this is not the case in terms of total collagen. The amount of total collagen per cell was significantly greatest in the 1M group, with less collagen being produced per cell as the seeding density increased. What aspect of the thicker, higher seeding density constructs that promoted a decrease in total collagen production is unknown. However, the combination of the original cell number and lack of collagen production hindered the attainment of clinical thickness.

Another variable which hindered the growth of the constructs was the pore diameter of the membrane. Since the tissue of the 8M group was observed to wrinkle off the membrane, it was thought that the membrane characteristics were influential in promoting adhesion. We hypothesized that tissue adhesion to the membrane was facilitated by larger pore sizes which allowed more matrix to be secreted through the membrane and act as an anchor. Formulation of this hypothesis was aided by the observation that matrix components and cells were on the underside of the membrane (Figure 5.2). Therefore, membranes of increasing pore diameter (0.2, 0.4, 0.6 and 0.8 μm) were placed into the bioreactor and seeded with 15×10^6 cells/mL (corresponding to the 6M condition in the insert study) and maintained for two weeks. Within days, it was grossly apparent that the smallest pore size membrane could not sustain the development of tissue; blebs and bumps were clearly evident. As time in culture progressed, these bumps turned into large wrinkles of tissue that had delaminated from the membrane. A similar trend was seen in the 0.4 μm group, but to a lesser extent; the 0.6 and 0.8 μm membranes allowed for a flat, smooth tissue to develop. Others who use a filter substrate for their scaffold free constructs have never reported such a finding, but have not used this high of seeding densities [181].

In terms of the mechanical properties, the 6M condition produced dynamic moduli 233-364 kPa at three weeks over a 4-decade range of applied frequencies. Dynamic moduli for young bovine knee articular cartilage ranged between 14.6 and 61.7 MPa when tested between 0.1 and 10 Hz under load control with a 0.4 N tare load and 40 N amplitude [56]. The modulus measured for our construct agreed well with other free-swelling tissue-engineered constructs: agarose-gel based tissues cultured for 56 days measured approximately 250 kPa at 0.1 and 1.0 Hz [154]. Also similar to our results, there appeared to be a significant increase in dynamic modulus at these frequencies between the low (10×10^6 cells/mL, or 2.2×10^6 cells/cm²) and high (60×10^6 cells/mL, or 13.2×10^6 cells/cm²) seeding densities using 10% FBS [154]. Going forward in translating these constructs into our mechanical conditioning bioreactor, promising results have been shown by others who found a significant increase in dynamic modulus in mechanically conditioned (compression) constructs over free-swelling controls [154].

It is not clear that we should have expected to see any difference in dynamic modulus, given the relatively small differences in sGAG and collagen content between groups. While proteoglycans are generally accepted as the predominant contributor to compressive properties [31], it is the interstitial fluid flow (governed by proteoglycans) which plays a more important role in the dynamic response [31, 56]. Even still, the collagen network of the matrix does in fact influence the dynamic response, even in compression [250]. Indeed, in our results, the total collagen qualitatively correlated well with the mechanical properties with respect to seeding density; *i.e.*, the 4M and 6M groups possess the most collagen and the highest moduli compared to the 1M group. Furthermore, the 4M and 6M total collagen amount were nearly identical, as were their moduli.

This study showed that scaffold-free tissues grown in static culture reach a maximum matrix-producing efficiency at approximately 2.5×10^6 cells/mL. Production of a clinically-relevant thick tissue in a short amount of time may need to be cultured in conjunction with a stimulatory influence, such as bioreactor-mediated mechanical conditioning. In studies to be discussed next, we employed our novel flow bioreactor system to impart shear stresses to thick, scaffold-free tissues with the goal of improving not only tissue thickness, but matrix composition and mechanical properties.

CHAPTER 6

EFFECT OF INTERMITTENT AND STEADY SHEAR STRESS ON MATRIX COMPOSITION AND MECHANICAL PROPERTIES OF ENGINEERED CARTILAGE

Introduction

Many current tissue engineering strategies aim to restore function to sites of damaged articular cartilage by creating a thick, cellularized plug of tissue that approximates the matrix composition and mechanical properties of native tissue. Such a construct has yet to be created and achieve success in a load bearing *in vivo* environment. A possible reason for these shortcomings is that constructs may fall short in their mechanical properties or matrix composition in order to withstand the complex and demanding loads seen *in vivo*. One strategy to increase the mechanical properties and matrix composition in terms of similarity to native is through the use of mechanical stimulation and physical forces to condition the tissue while developing *in vitro*. In order to deliver these physiologically-inspired mechanical loads (*e.g.*, compression, shear), bioreactors have been developed to stimulate engineered cartilage to become more like native tissue.

Bioreactors used in applying mechanical stimuli to engineered cartilage have ranged from the simplistic spinner flask [7, 193] to complex 3D systems mimicking joint kinematics by applying a concurrent compression and rotation [251]. Not only do bioreactor systems differ in design, but in the applied physical stimulus, including compression [75, 80, 171, 197], fluid-induced and tissue shear [158, 183, 190, 194], hydrostatic pressure [164]. Take together, bioreactor systems and physical stimuli such as these have been shown to be able to modulate chondrocyte metabolism, spatial and

temporal deposition of proteins, depending on stimulus magnitude, duration and waveform.

Perhaps not surprisingly, the effects of bioreactor-mediated mechanical conditioning resemble that of the *in vivo* situation: positive effects are produced with dynamic loading within a normal physiologic range whereas negative effects are born of influences outside this range. *In vivo*, biomechanical factors outside the normal physiologic range (*e.g.*, trauma, obesity, and disuse) negatively affect the integrity of articular cartilage [59] on multiple levels including cellular apoptosis and death, matrix degradation (loss of proteoglycans, increase in enzymatic breakdown) and decrease in quality of mechanical properties (compressive and tensile) [60-63]. *In vitro* engineered tissues undergoing static compression (analogous to disuse and obesity) show reduced expression of type I and II collagen [76] as well as decreased synthesis rates of proteoglycans and proteins [75, 76, 197]. In contrast to the negative effects of static compression, Mauck *et. al* showed a six-fold increase in equilibrium aggregate modulus, as well as significant increases in proteoglycans and collagen of dynamically compressed alginate disk cultures [171]. Similar beneficial effects of dynamic loading have been seen by others, even with different offset compression and applied frequencies [75, 80, 197]. Yet again, these *in vitro* results parallel that of the *in vivo* case: moderate degrees of exercise have been shown to positively influence (maintain or improve) articular cartilage integrity [64, 65]. Thus, effects of static versus dynamic loading are parallel in both *in vivo* and *in vitro* systems.

Despite the promising results of dynamic mechanical conditioning, the mechanical properties and type II collagen content and organization of engineered tissues have yet to reproduce that of native tissue [155, 191, 192, 209]. Given type II collagen's abundance and critical role in determining cartilage's mechanical properties, this protein has been the focus of earlier mechanical conditioning studies [31]. Furthermore, as collagen's rope-like structure is well suited to resist shear forces and is also known to

play the most important role in influencing the tensile mechanical properties of cartilage [31], shear appears to be a prime candidate for up-regulating deposition of this critical protein. Application of fluid induced shear stress is usually reserved for scaffold-based perfusion systems, but the level and distribution of shear stresses are unknown and widely heterogeneous in these systems [1, 2, 189, 195]. To alleviate such issues, some have moved to a scaffold-free engineered tissue, which has been shown to produce a mechanically functional tissue and amenable to bioreactor conditioning [181-183, 190]. Shear stress has been shown to regulate the matrix composition and mechanical properties of scaffold-free tissues with modest success [183, 191].

In this study, we examined the effect of intermittent (dynamic) and steady shear stress on matrix composition and mechanical properties of scaffold-free engineered cartilage. Steady application of shear stress has shown both beneficial (*e.g.*, increase in type II collagen deposition) and detrimental (*e.g.*, increase in type I collagen deposition, loss of sGAG) effects depending on magnitude and duration on thin ($\sim 300\ \mu\text{m}$) tissues in our previous studies. Here, we transitioned to thicker, more clinically-relevant sized constructs ($\sim 1\ \text{mm}$) and test steady and intermittent flow. We hypothesized that intermittent application of shear stress will retain the beneficial effects of flow while discouraging the detrimental effects on construct matrix organization and mechanical properties.

Materials and Methods

Cell Isolation and Seeding

Bioreactor

A novel parallel-plate bioreactor was used for culturing developing tissues while applying a consistent level of fluid-induced shear stress, as previously described [183]. Briefly, cells are seeded directly onto a semi-permeable polycarbonate membrane (GE

Osmonics, Minnetonka, MN), which is epoxied onto a stainless steel frame to keep the membrane flat and taught. Above and below the tissue are rectangular media chambers, loaded with nutrient media and outfitted with ports allowing for dynamic flow. In between each bioreactor part are sealing silicone gaskets, clamped between parts by stainless steel screws. All bioreactor parts are machined out of polycarbonate. The bioreactor growth chamber has dimensions of 4.6 cm x 1.3 cm x 1.3 cm (length x width x height), an area of 6 cm².

There are two culture configurations for the bioreactor: static and flow. In the static configuration, the upper chamber is allowed to exchange gases with the incubator via a non-sealing cover, which has an overhanging lip that discourages contaminants. For dynamic flow of media in the upper chamber, “the flow configuration,” a cap is affixed to upper chamber in order to create a flow chamber of set length (L), width (b) and height (h). The height is altered by adding shims in between the upper chamber and the cap (outside the fluid flow path). Nutrient media is driven in a re-circulatory fashion through the bioreactor via pump tubing and associated fittings. The flow is programmed into the pump for a constant volumetric flow rate (Q). Consequently, a constant shear stress (τ_w) is applied to the top surface of the engineered tissue, and can be estimated using the following equation:

$$\tau_w = \frac{6\mu Q}{bh^2}$$

where τ_w is in dynes/cm², μ is the media viscosity in dyne-sec/cm², Q is the flow rate in mL/sec, b is the flow chamber width in cm and h is the fluid gap height in cm. This wall shear stress is estimated to be valid over more than 95% of the bioreactor length.

Harvest

Articular cartilage from the femoral condyles and femoropatellar groove of a 2-4 week old bovine stifle joint (Research 87, Boylston, MA) were harvested under aseptic

conditions. Tissue was minced in phosphate buffered saline (PBS) supplemented with 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA) and digested for 48h at 37°C under constant agitation in 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) in DMEM (high glucose, Invitrogen) containing 0.5% neomycin (Sigma-Aldrich, St. Louis, MO), 1% kanamycin (Sigma) and 1% antibiotic/antimycotic. Final digest was passed through a 70 µm strainer and pelleted after 10 min centrifugation at 1100 x g. Pellets were suspended in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT), 1% non-essential amino acids (Invitrogen), 0.25% gentamycin (Invitrogen) and 50 µg/mL ascorbic acid-2-phosphate (Sigma), centrifuged and resuspended twice in order to wash the cells. Cells were counted in triplicate using the Vi-CELL automated cell viability counter (Becton-Coulter, Fullerton, CA) according to manufacturer's protocols, with viability >85%. For each experiment, five stifle joints (two matched pairs, one unmatched) were used, cells pooled and seeded fresh into the bioreactors. Additionally, a section of native bovine articular cartilage and ligament was taken during harvest and fixed (details to follow) to serve as a positive and negative tissue control (respectively) for each histological stain. Sections of native articular cartilage were frozen at -20°C to serve as positive controls for biochemical assays.

Experimental Design

Flow Experiments

Each bioreactor piece was sterilized via autoclave individually, except the membrane and frame composite piece, which is soaked overnight in 70% ethanol. Bioreactors are assembled into the static configuration, primed with media, and placed in an incubator the day prior to seeding. Primary bovine articular chondrocytes, fresh from harvest (described above), are seeded directly into the bioreactor in 5-mL media at 12×10^6 cells/mL, for a total of 60×10^6 cells/bioreactor. Cell-seeded bioreactors are

incubated overnight, after which the media is removed and then the bioreactors are converted into the flow configuration with fresh media on Day 1. For a two-week “pre-culture” period, each bioreactor was subject to flow at 0.1 mL/min, a shear stress less than 0.001-Pa, with 24 mL media exposed to the upper chamber (re-circulatory flow loop) and 2 mL in the lower chamber. During this pre-culture period, cells formed neo-tissues of approximately 0.9 mm thickness. It should be noted that each bioreactor device was shimmed to a height of 1.8 mm so that the tissue thickness (~0.9 mm) is in an approximate 1:1 ratio with the flow channel height. This ratio has been found to be a critical parameter in fluid flux through the tissue in a parallel-plate geometry [243] and is the tissue thickness-to-gap height ratio used in previous experiments [183].

Following the two week pre-culture, bioreactors were subjected to the experimental flow condition (discussed below). In order to keep shear stress and flow rate constant, the flow channel height was increased 50 μ m increments by adding additional shims during each feed (every 48 hours) to account for tissue growth. In this manuscript, two studies are presented:

Study 1: To examine the effects of shear stress magnitude and duration on the thicker, more clinically relevant constructs, bioreactors were flowed at either 0.001-Pa or 0.1-Pa for a period of 3 or 7 days and harvested for assay (discussed below). These flow rates / shear stresses and time points correlate to those used in previous studies of our system [183]. This study was performed in duplicate with no less than three bioreactors used for each time point and shear stress.

Study 2: In order to investigate the effects of intermittent (dynamic) shear stress on tissue development, bioreactors were flowed for 7 days at one of the following protocols:

- Steady: 0.001-Pa (control, same as Study 1)
- Steady: 0.1-Pa (control, same as Study 1)
- Steady: 0.01-Pa

- Intermittent: 0.001 for 3 hours, stepped to 0.01 for 1 hour and repeat cycle [labeled “0.001 (3) / 0.01 (1)” in graphs]
- Intermittent: 0.01 for 3 hours, stepped to 0.1 for 1 hour and repeat cycle [“low duty cycle”, labeled “0.01 (3) / 0.1 (1)” in graphs]
- Intermittent: 0.01 for 1 hour, stepped to 0.1 for 1 hour and repeat cycle [“high duty cycle”, labeled “0.01 (1) / 0.1 (1)” in graphs]

Intermittent flows were controlled by a custom-written program coded in LabVIEW (National Instruments, Austin, TX) which sends an analog output to the Masteflex pump (Cole-Parmer, Vernon Hills, IL) via the PCI-6229 data acquisition card installed on a PC. The analog (mV) output controlled the RPM and thus flow rate of the pumps, which were manually calibrated for flow rate prior to each experiment.

At the end of the week, all tissues were removed from the bioreactors and divided up for various biochemical assays and mechanical tests. Prior to division, each tissue was weighed wet and had approximately 2 mm of the perimeter trimmed and discarded in order to minimize edge-effects. Tissue for all biochemical assays were frozen at -20°C, except for sections reserved for RNA isolation, which were flash-frozen in liquid nitrogen and stored at -80°C. Tensile mechanical testing was performed immediately, whereas specimens for compressive mechanical testing were punched to 6 mm diameter and frozen in 1 mL PBS with 1X protease inhibitor (PBS+PI) cocktail set III (Calbiochem / EMD Biosciences, San Diego, CA) at -20°C.

Matrix Composition Assays

Histology and Histomorphometry

A section of tissue spanning the full width of the device and approximately 0.5 cm in length was fixed for 48 hours in each 10% neutral buffered formalin and 1% acid alcohol (1% glacial acetic acid in 95% reagent alcohol). Following fixation, tissues were

rinsed in PBS for 2 h and stored at 4°C in 70% reagent alcohol until time of processing and paraffin embedding. 5 µm sections were cut and mounted on slides: the acid alcohol-fixed sections were dedicated to immunohistochemistry (IHC) and the formalin-fixed sections to basic histology stains. IHC staining revealed the presence of proteins using antibodies against type I collagen (mouse monoclonal MAB3391, Chemicon Inc, Temecula, CA), type II and type IX collagen (mouse polyclonals II-II6B3 and B3-1 respectively, Developmental Hybridoma Studies Bank, Iowa City, IA), with a biotinylated anti-mouse secondary antibody (BA 2001, Vector Laboratories, Burlingame, CA). Type X collagen (rabbit polyclonal, kindly donated by Dr. Gary Gibson, Henry Ford Hospital, Detroit, MI) was used in conjunction with a biotinylated goat anti-rabbit IgG (BA-1000, Vector). Color was developed with the Vecastain ABC alkaline phosphatase kit (SK 5100 and AK 5000, Vector). Antigens were retrieved by a 10 minute digest (60 minutes for type X collagen) with Digest-All 3 (Invitrogen) at 37°C. For each staining session, a positive tissue, negative tissue and secondary-antibody control were included to ensure specificity of the stain.

Each formalin-fixed specimen was subject to a basic set of histological stains: routine hematoxylin and eosin (H&E) for gross morphology, 0.1% safranin-O with 0.02% fast green counterstain for the presence of sulfated glycosaminoglycans, and 0.1% picrosirius red for collagen presence and fiber alignment. Native tissues were run alongside experimental samples as controls.

Type I collagen presence was quantified using Image Pro software (MediaCybernetics, Inc., Silver Spring, MD) by selecting area stained red in the slides stained for type I collagen. The entire length of tissue was digitally photographed at 4X magnification and analyzed: an area of interest was manually drawn around the entire tissue, then the software automatically calculated the number of pixels stained red (*i.e.*, type I collagen present) as well as the total number of pixels in the tissue. Data is

expressed at “percent area stained” and is defined as the number of pixels representing type I collagen (*i.e.*, red color) divided by the total number of pixels present in the tissue.

Prior to insertion into the manuscript, representative photos were selected, cropped and background deleted using Adobe Photoshop (Adobe, San Jose, CA). Colors or features were not altered.

Quantitative Biochemistry

Each tissue was subject to quantitative biochemical assays, including the Hoeschst 33258 (Sigma) dye binding assay for DNA (cell) content [222], dimethyl-methylene blue [223, 224] (DMMB, Aldrich) dye binding assay for sGAG, and the hydroxyproline assay [221] for total collagen content. Approximately 50 mg of wet tissue was digested in 125 µg of freshly prepared proteinase K (Worthington) in 1 mL of 100 mM ammonium acetate for 18-24 hours in a 60°C water bath. Total collagen was quantified by first hydrolyzing 100 µL of digest solution in 2 mL of 6N HCl overnight at 110-115°C, then re-suspending in 0.25M sodium phosphate buffer, reacting 50 µL of suspension (in triplicate) with 50 µL chloramine T (Mallinckrodt Baker, Phillipsburg, NJ) for 20 min at room temperature and 50 µL p-dimethylaminobenzaldehyde (Mallinckrodt Baker) for 60 min at 60°C, and finally read on a PowerWave spectrophotometer (Bio-Tek, Winooski, VT) at an optical density of 557 nm. Experimental samples were compared to hydroxyproline standards, then converted to total collagen by using a conversion of 10:1 hydroxyproline:collagen [7, 247]. Purified type II collagen (Southern Biotech, Birmingham, AL) was hydrolyzed using the previously described process to confirm this ratio. sGAG was quantified by reacting 20 µL of 50-fold diluted experimental digest with 200 µL DMMB (pH 3.0), read on the PowerWave spectrophotometer at 525 nm and compared to standards of chondroitin sulfate (Sigma), all in triplicate. DNA was quantified by incubating 10 µL of undiluted experimental digest with 200 µL Hoechst dye in 1X TEN buffer at a concentration of 0.5 µg/mL for

10-15 min (in triplicate), reading on a PerkinElmer (Wellesley, MA) HTS 7000 fluorometric plate reader at excitation/emission wavelengths of 360/465 nm (manual gain = 60) and compared to standards of calf thymus DNA (Sigma). Thickness of each sample was calculate during the mechanical testing assay by taking the average of three measurements taken at different point on the construct, using a contact-sensing micrometer.

RNA Isolation, Reverse Transcription and Quantitative PCR (qPCR)

Tissues dedicated for RNA isolation were flash frozen in liquid nitrogen and stored at -80°C. RNA was isolated using the TRIspin method [240] by first grinding the tissue to a fine powder with a mortar and pestle under liberal amounts of liquid nitrogen. Tissues were further homogenized by suspending in 1-mL TRIzol reagent (Invitrogen) and passing 5-10 times through a 1 mL syringe outfitted with a 20-gauge needle. After a 15 minute incubation in TRIzol at room temperature to dissociate nucleoprotein complexes, samples were centrifuged for 10 minutes at 12,000 x g. The supernatant was transferred to a new tube containing 0.2 mL chloroform, vortexed, incubated for 3 minutes, and centrifuged for 15 minutes. The upper, clear, aqueous phase was removed and mixed with 0.5 mL 100% isopropyl alcohol to precipitate the RNA in a new tube. Following a 10 minute incubation and 10 minute centrifugation, the supernatant was discarded while the remaining pellet was resuspended in 350-μL RLT buffer (RNeasy kit; QIAgen, Valencia, CA) plus 3.5 μL β-mercaptoethanol. This lysate was pipetted directly onto a QIAshredder column (QIAgen), centrifuged for 5 min, and then combined with 350 μL 70% ethanol. This supernatant was added to an RNeasy column (QIAgen) and RNA was isolated according to manufacturer's protocol. Total RNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE), then frozen at -80°C until time of reverse transcription. Samples with

a 260:280 (nm) absorbance ratio less than 2.00 were not used in analysis, as these were considered impure due to the presence of proteins, phenol or other contaminants [241]. cDNA was generated by reacting 4 μL MgCl_2 , 2 μL RT 10X buffer, 2 μL dNTPs, 1 μL oligo DTs, 0.5 μL RNasin, and 0.75 μL AMV reverse transcriptase (A3500 Reverse Transcription Kit; Promega, Madison, WI) per sample (1 μg RNA in 9.75 μL nuclease-free water) at 42°C for 60 minutes, at 95°C for 5 minutes, and 4°C for at least 5 minutes. Samples were frozen at -20°C until time of qPCR.

Gene expression was quantified using SYBR Green intercalating dye (Applied Biosystems, Foster City, CA) and the Prism 7770 Sequence Detector (Applied Biosystems; 40 cycles: melting = 15 s at 95°C; annealing and extension = 60 s at 60°C). The cycle number where the measured fluorescence exceeds a threshold (c_T) was calculated automatically using the Applied Biosystems software package. Experimental samples, standards and no-template controls were run in duplicate in 50- μL reaction volumes. Standards were generated from bovine chondrocyte cDNA amplicons using published primers listed below (Table 1). For each well, 1 μL of cDNA was combined with nuclease-free water (23.75 μL), forward primer (0.125 μL), reverse primer (0.125 μL) and SYBR Green Master Mix (25 μL ; Applied Biosystems). Melting curves (data not presented) were run at least once for each primer set and experimental condition to rule out false-positive signals generated by non-specific double-stranded DNA sequence amplification. Primers for each gene are listed below in Table 6.1.

Table 6.1: Primers

Target Gene (all bovine)	Primer Sequences (5'-3')
Aggrecan	Forward: CCT CAG GGT TTC CTG ACA TTA Reverse: TAA GCT CAG TCA CGC CAG ATA
Type I Collagen [76]	Forward: AAG AAC CCA GCT CGC ACA TG Reverse: GGT TAG GGT CAA TCC AGT AGT AAC CA
Type II Collagen [76]	Forward: GCA TTG CCT ACC TGG ACG AA Reverse: CGT TGG AGC CCT GGA TGA
GAPDH	Forward: CCT TCA TTG ACC TTC ACT ACA TGG TCT A Reverse: TGG AAG ATG GTG ATG GCC TTT CCA TTG

Data is expressed normalized by internal cellular housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Mechanical Testing

Tension

Tissues were stamped into the shape of a dogbone (gauge length and width of 15-mm and 3-mm, respectively), loaded into soft-tissue grips of the ELF 3200 mechanical testing system (EnduraTEC / Bose, Eden Prairie, MN). As tissues were pulled to failure at a rate of 0.02-mm/sec, force was recorded using a 5.6-lb load cell (Interface, Scottsdale, AZ) and displacement recorded via computer acquisition interface (WinTest, EnduraTEC). Stress and strain were calculated from the force and displacement data by adjusting for the tissue's cross-sectional area and gauge length respectively. From the stress-strain plot, the Young's modulus (linear portion of the curve) was calculated. Due

to premature failure (away from the center of the specimen), the ultimate strength was not calculated.

To determine if the application of flow had an effect of matrix organization (*e.g.*, collagen fiber alignment), a dogbone specimen was cut in both the parallel and perpendicular direction (with respect to flow) and tested in tension (Study 1, only).

Compression

Specimens from each tissue were tested in dynamic unconfined compression in order to elucidate a dynamic modulus over a range of frequencies. Samples were thawed and equilibrated in PBS+PI at room temperature for one hour. Immediately prior to testing, samples were punched to a diameter of 5 mm and measured in three places using a contact-sensing micrometer and averaged to determine thickness. Punched discs were then loaded into an ElectroForce 3200 Series uniaxial system outfitted with a custom-designed compression chamber (machined stainless steel surfaced contained in a polycarbonate cylinder) filled with 5-7 mL PBS+PI connected to a 5 N load cell (Interface, Scottsdale, AZ) on the immobilized (bottom) platform and a 9.5 mm diameter stainless steel indenter affixed to the uniaxial mover arm (resolution of 0.1 μm). The indenter was slowly lowered until a stable force of 10-20 mN was registered, indicating contact. A frequency sweep was then initiated under displacement control using the Dynamic Mechanical Analysis (DMA) sub-package within the WinTest software used to control the system. The sweep began after an hour equilibration at a 15% offset (ramp to offset at 0.100 mm/sec) and cycled through frequencies of 0.01, 0.1, 1 and 10 Hz at a dynamic amplitude of 1.5%. The number of sinusoids generated and recorded was optimized within the DMA software. As construct dimensions were input into the DMA prior to each test, the software, via FFT analysis, converted stiffness properties into material properties and calculated the tangent of the phase angle, complex modulus (E^*), storage modulus (E') and loss modulus (E''). Displacement traces for each sample were

visually inspected for plateaus at the sinusoid peaks to ensure the loading platen and sample never lost contact.

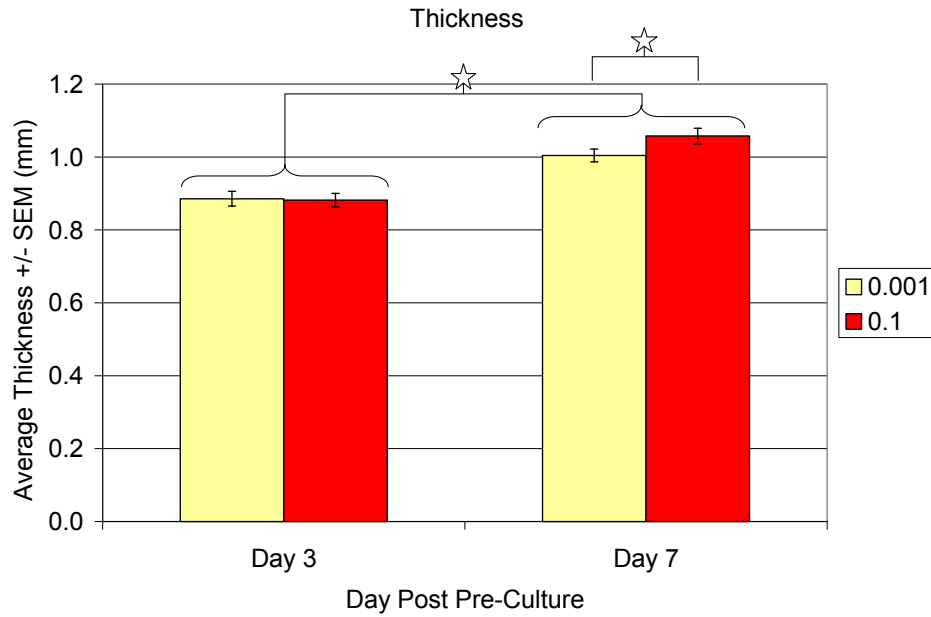
Statistics

Statistical analysis was performed with Minitab (State College, PA) software using the ANOVA general linear model with pair-wise comparisons. Significance was assigned to $p \leq 0.05$ unless otherwise noted. For Study 1, N=6 and 8 for 0.001 at Day 3 and 7, respectively; N=11 and 10 for 0.1 at Day 3 and 7, respectively. In Study 2, N=5 for all groups, except 0.01 and 0.1 where N=8.

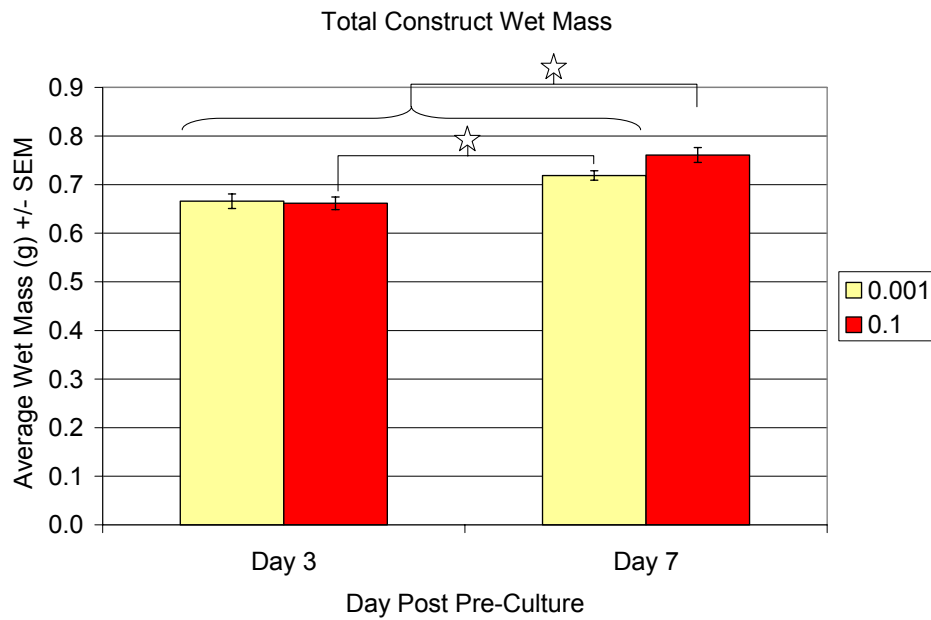
Results of Study 1: 3&7 Day Steady Shear Stress

General Observations

Tissues grew to more than 1 mm thickness by the third week of culture (one week of experimental flow) and attained a mass of approximately 700 mg (Figure 6.1). Both the 0.001 and 0.1 groups were significantly thicker at Day 7 compared to Day 3; the 0.1 group was greater than the 0.01 group on Day 7 only. Similarly, the 0.1 group at Day 7 attained a significantly greater mass than all other flow groups and days in culture. Each tissue handled well with surgical forceps and was easily manipulated.



A



B

Figure 6.1 – Construct Thickness and Mass. Average construct thickness (A) and mass (B) for shear stresses 0.001- and 0.1-Pa at 3 and 7 days. Star denotes $p \leq 0.05$.

Histology

Tissues for each time point and shear stress stained diffusely for type II collagen and sGAG, with a high cell density throughout the thickness (Figure 6.2). With both time in culture and shear stress, the appearance of a type I collagen band became more apparent; this band was relegated to the top (shear-exposed) surface of the tissue which co-localized to regions of increased fast green staining (depletion of sGAG) and lack of type IX collagen intensity. Also in this region, cellularity appeared greater with more fibroblast-like, flattened cells. Some sections of the tissues appeared torn, but this was most likely artifact due to the peeling of the tissue off the membrane surface; for this reason, qualitative comments on tissue thickness are avoided. The presence of type X collagen was not noted in any tissues; positive and negative tissue and secondary antibody controls all stained appropriately (not shown).

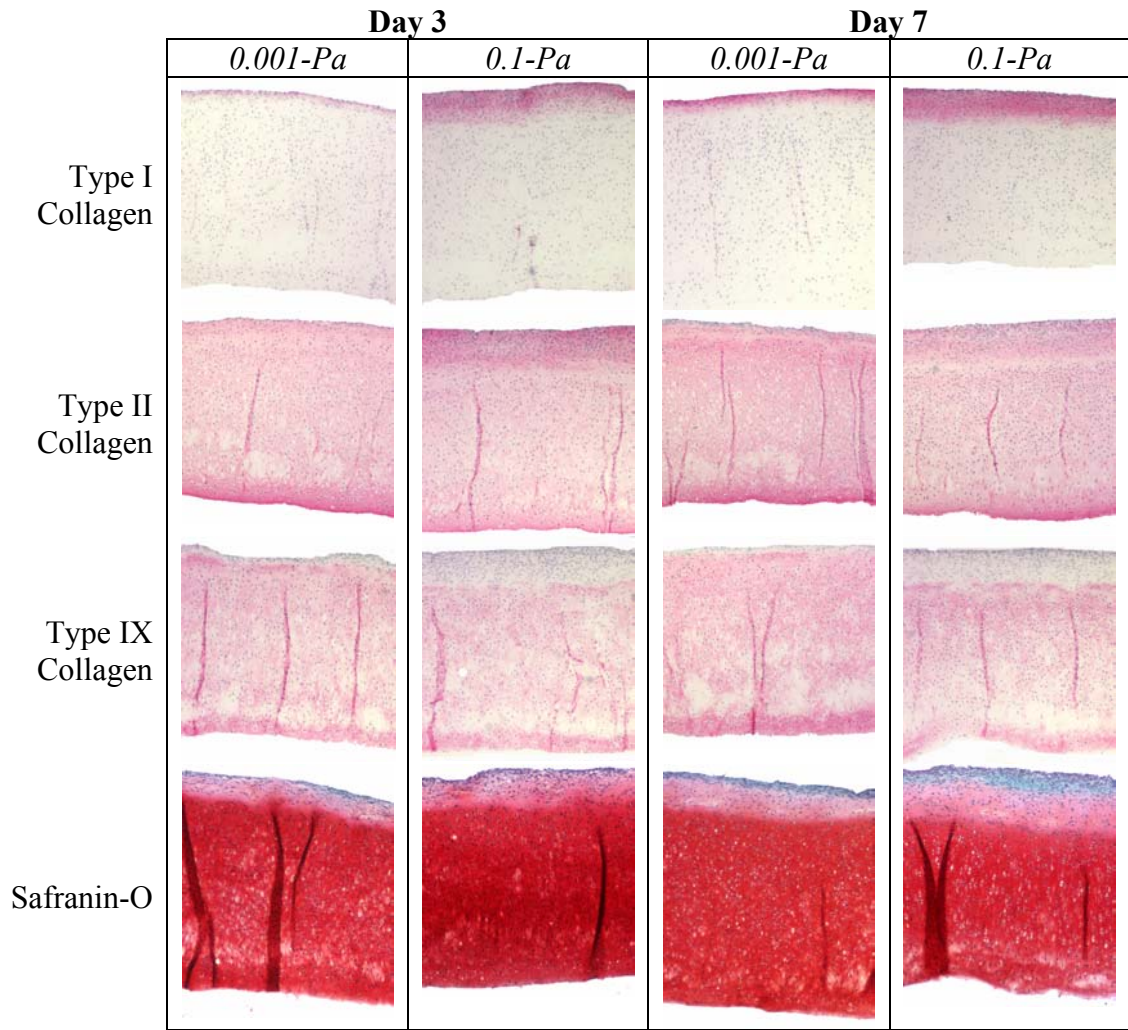
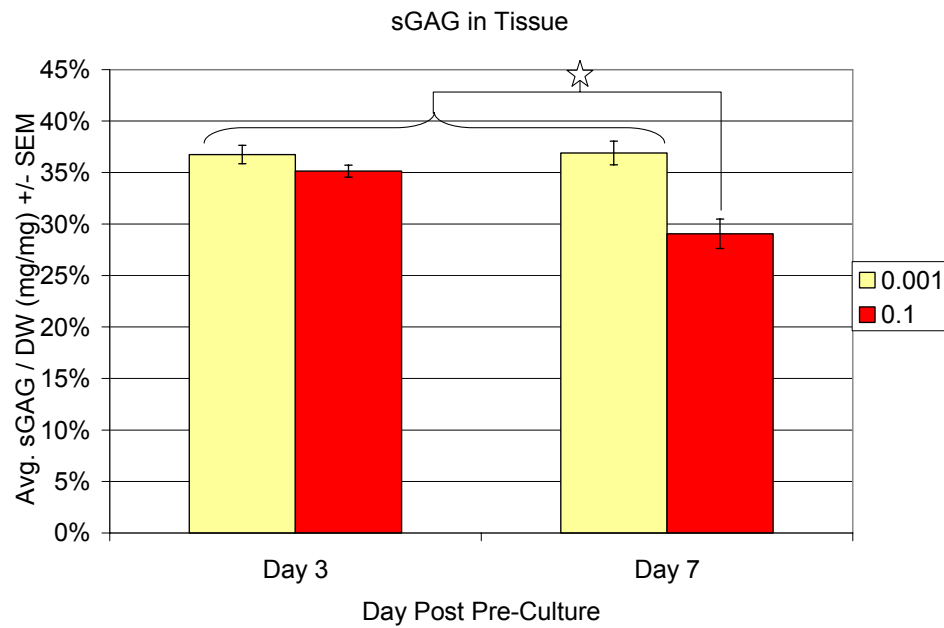


Figure 6.2 – Histology. Representative histological stains (rows) for each shear stress and time point condition (columns). Original magnification 4X.

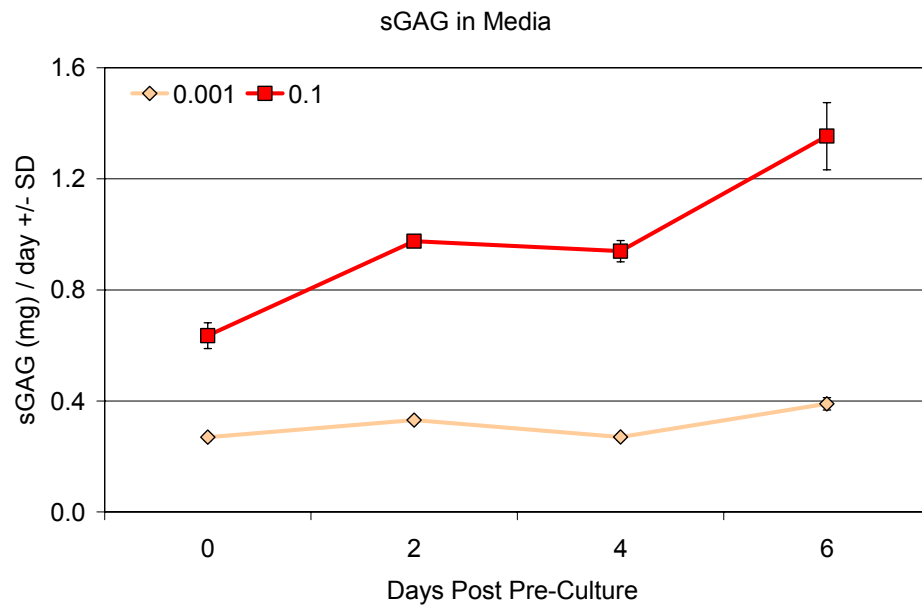
Matrix Composition

At Day 7, the 0.1-Pa exposed group had a significantly lower amount of sGAG per unit dry weight than the other groups and time points (Figure 6.3). This is evidenced by the qualitative support of the safranin-O stains previously discussed as well as the significant increase in sGAG amount and rate of presence increase in the media for the

0.1-Pa group. Whether this sGAG was released into the media directly or withdrawn from the tissue is unknown.



A



B

Figure 6.3 – sGAG in Tissue and Media. Average sGAG per unit dry weight in the tissue (A), sGAG in the media per day (B), and combined contributions of media- and tissue-present sGAG (C) for 0.001- and 0.1-Pa exposed tissues for 3 and 7 Day time points. Star denotes $p \leq 0.05$.

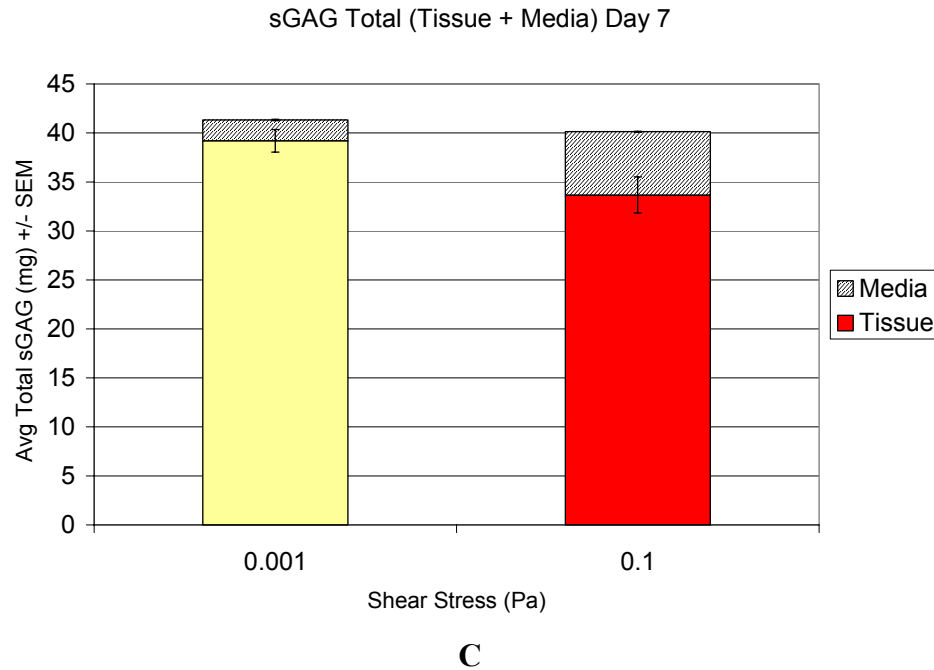
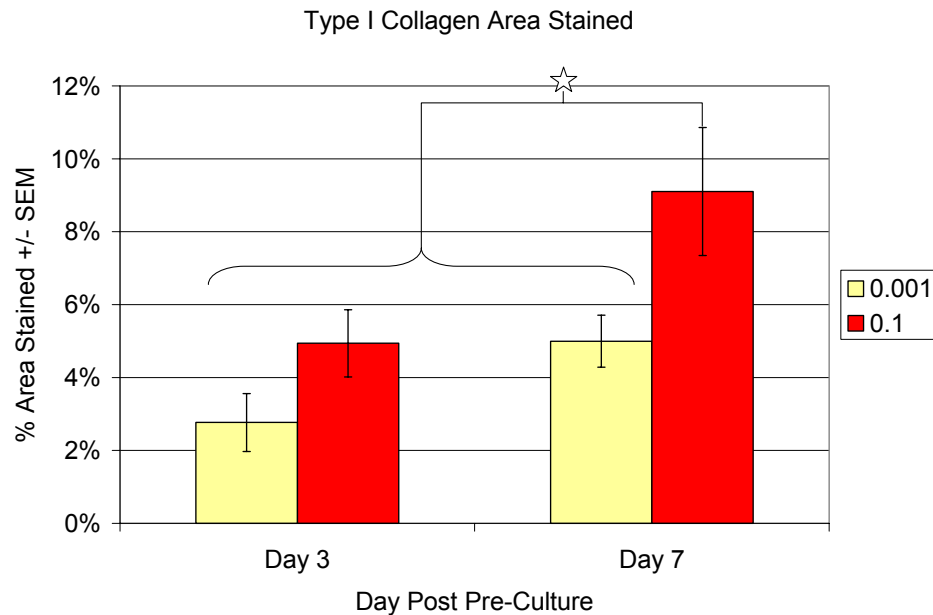


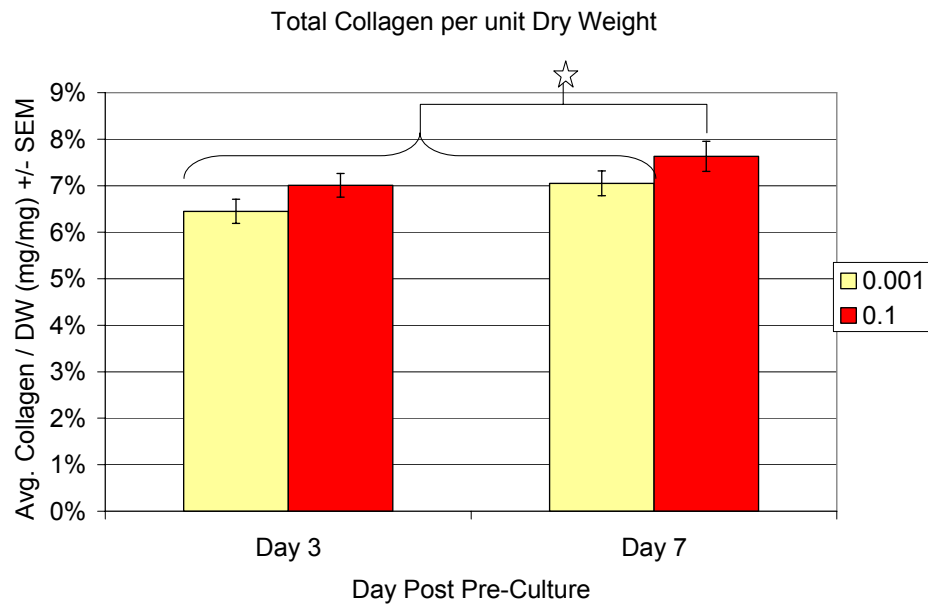
Figure 6.3 continued – sGAG in Tissue and Media. Average sGAG per unit dry weight in the tissue (A), sGAG in the media per day (B), and combined contributions of media- and tissue-present sGAG (C) for 0.001- and 0.1-Pa exposed tissues for 3 and 7 Day time points. Star denotes $p \leq 0.05$.

Type I collagen was present in a greater percentage of the tissue at Day 7 for 0.1-Pa group compared to others; no differences were seen at Day 3 (Figure 6.4). This is corroborated by the total collagen content; while the hydroxyproline assay does not distinguish between collagen types, it can be assumed that the increase was in type I collagen as no difference was seen among shear stress groups or time points for type II collagen, as determined by ELISA (not shown).

In terms of gene expression, type I collagen was up-regulated with 0.1-Pa at Day 7 compared to all other time points, whereas type II collagen was up-regulated only at Day 3 for 0.1-Pa with respect to 0.001-Pa. No differences in aggrecan or GAPDH gene expression was seen between shear stress and duration of flow (Figure 6.4)

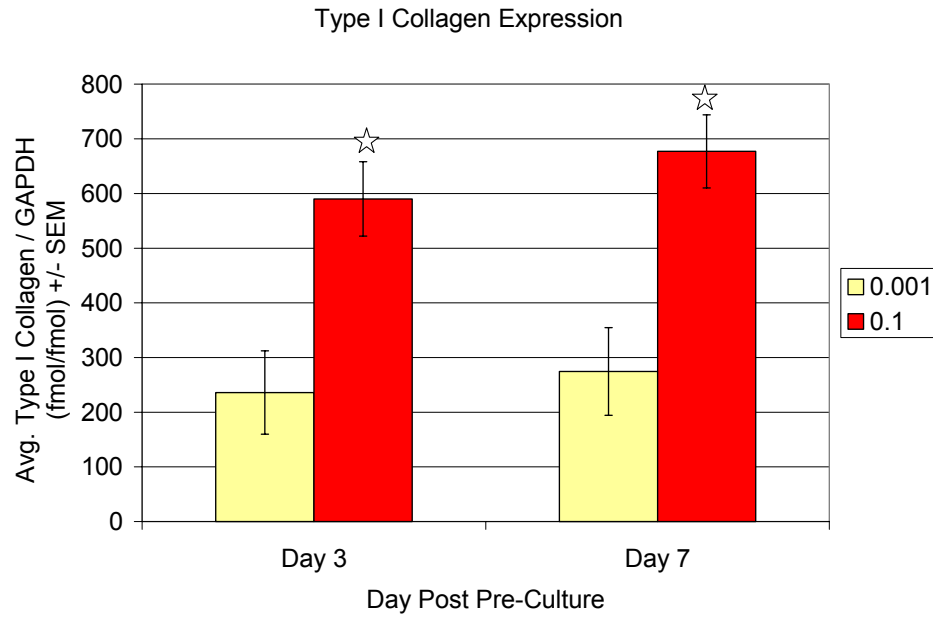


A

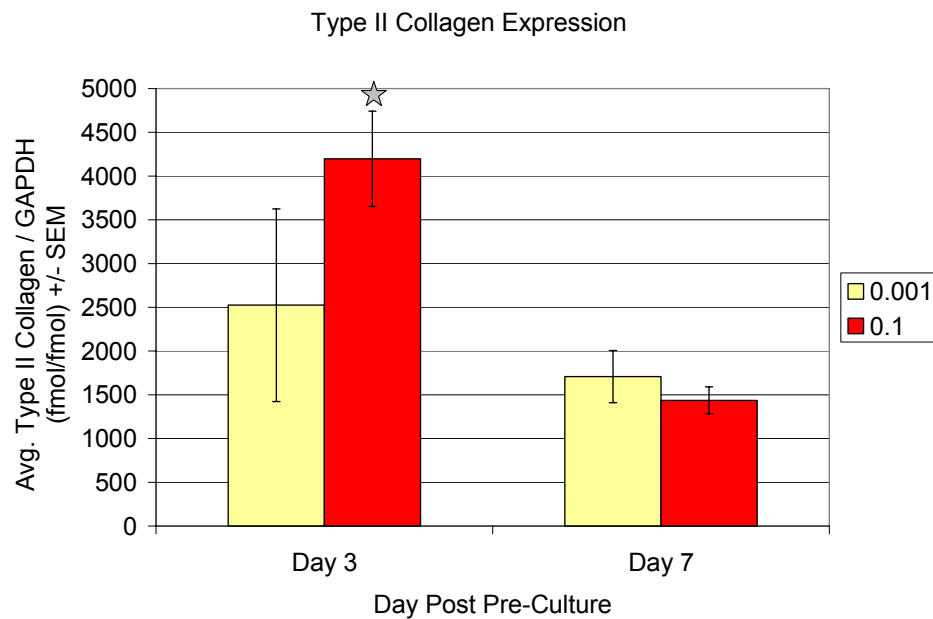


B

Figure 6.4 – Collagen Deposition and Expression. Type I collagen presence as quantified by histomorphometry (A) and total collagen content via hydroxyproline (B). qPCR-determined average type I collagen (C) and type II collagen (D) expression normalized by GAPDH for 0.001- and 0.1-Pa exposed tissues for 3 and 7 days. Open star denotes $p \leq 0.05$ compared to 0.001-Pa for both time points. Shaded star denotes $p \leq 0.05$ compared to 0.001-Pa for same time point.



C

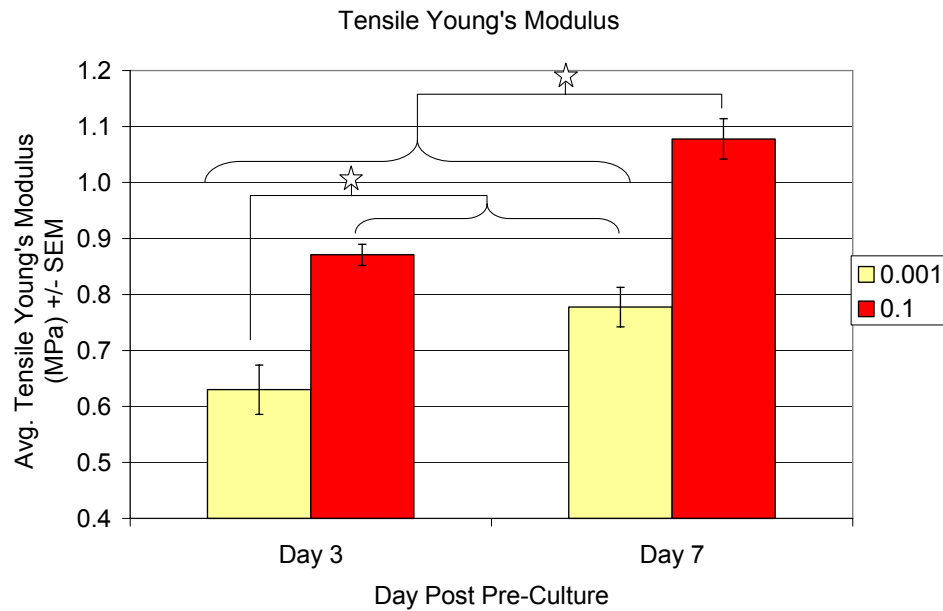


D

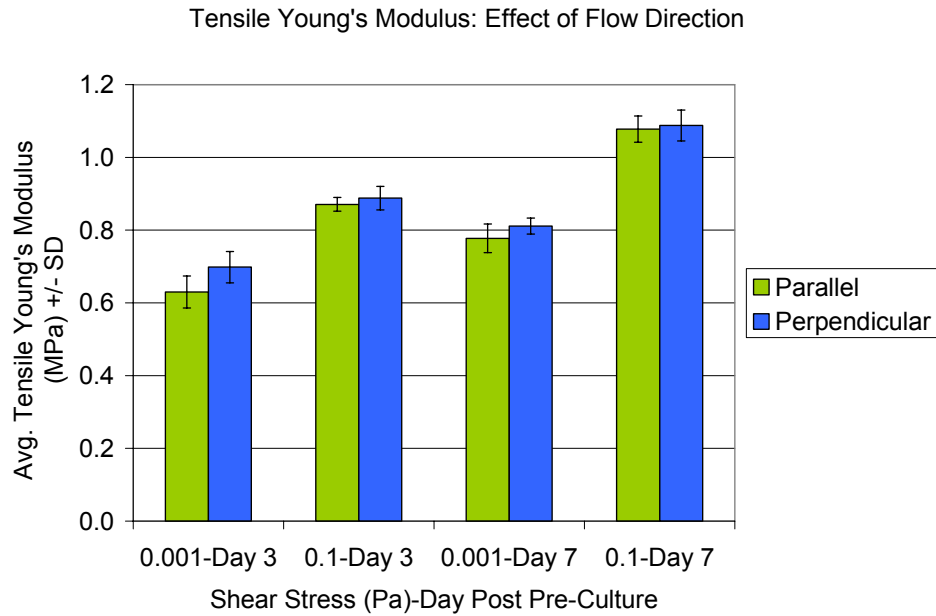
Figure 6.4 continued – Collagen Deposition and Expression. Type I collagen presence as quantified by histomorphometry (A) and total collagen content via hydroxyproline (B). qPCR-determined average type I collagen (C) and type II collagen (D) expression normalized by GAPDH for 0.001- and 0.1-Pa exposed tissues for 3 and 7 days. Open star denotes $p \leq 0.05$ compared to 0.001-Pa for both time points. Shaded star denotes $p \leq 0.05$ compared to 0.001-Pa for same time point.

Mechanical Testing

The tensile Young's modulus was significantly modulated by duration of flow and shear stress magnitude; E_Y was higher in the 0.1-Pa group at Day 7 than all other groups and time points as 0.001-Pa at Day 3 was lowest of all others (see Figure 6.5). No differences were seen between parallel and perpendicular cut specimens indicating that collagen fiber or other matrix molecule alignment with flow did not affect tensile mechanical properties. No differences were seen between any applied shear stresses, duration of flow or applied frequency for dynamic compressive modulus. Not shown are the 10 Hz, as the generated force sinusoid showed significant distortion. Generally however, an increase in modulus was observed with increasing frequency which may have been significant with a greater sample size. Also noted was a curling effect as samples were thawed; this may have skewed data if residual stresses were built up in the tissue.

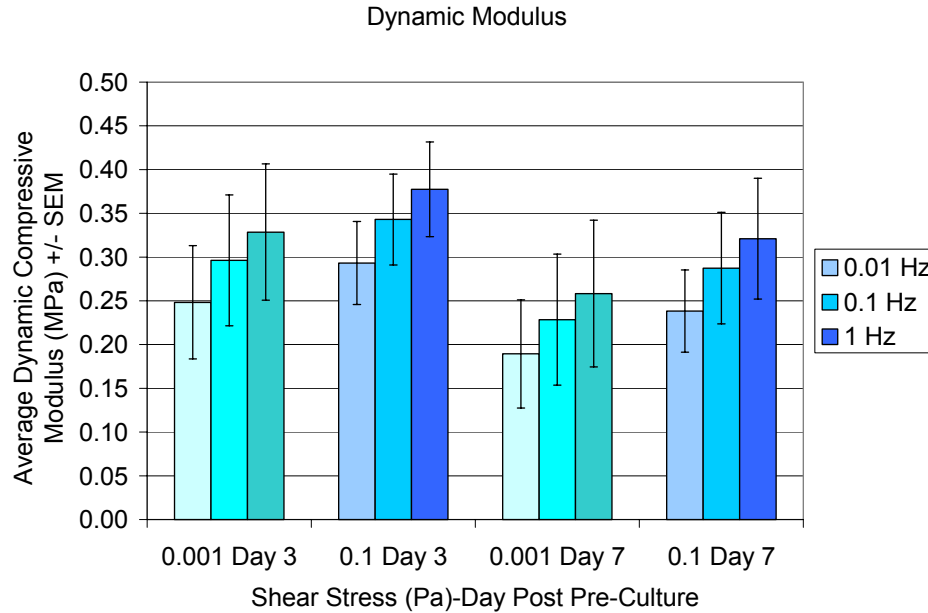


A



B

Figure 6.5 – Mechanical Properties. Tensile Young's modulus (E_Y) for 0.001- and 0.1-Pa groups at Day 3 and 7 (A) and comparison of parallel- and perpendicular-cut specimens (B) all failed at a constant strain. Compressive dynamic modulus (E^*) in dynamic unconfined compression over a frequency sweep (C). Star denotes $p \leq 0.05$.



C

Figure 6.5 continued – Mechanical Properties. Tensile Young’s modulus (E_Y) for 0.001- and 0.1-Pa groups at Day 3 and 7 (A) and comparison of parallel- and perpendicular-cut specimens (B) all failed at a constant strain. Compressive dynamic modulus (E^*) in dynamic unconfined compression over a frequency sweep (C). Star denotes $p \leq 0.05$.

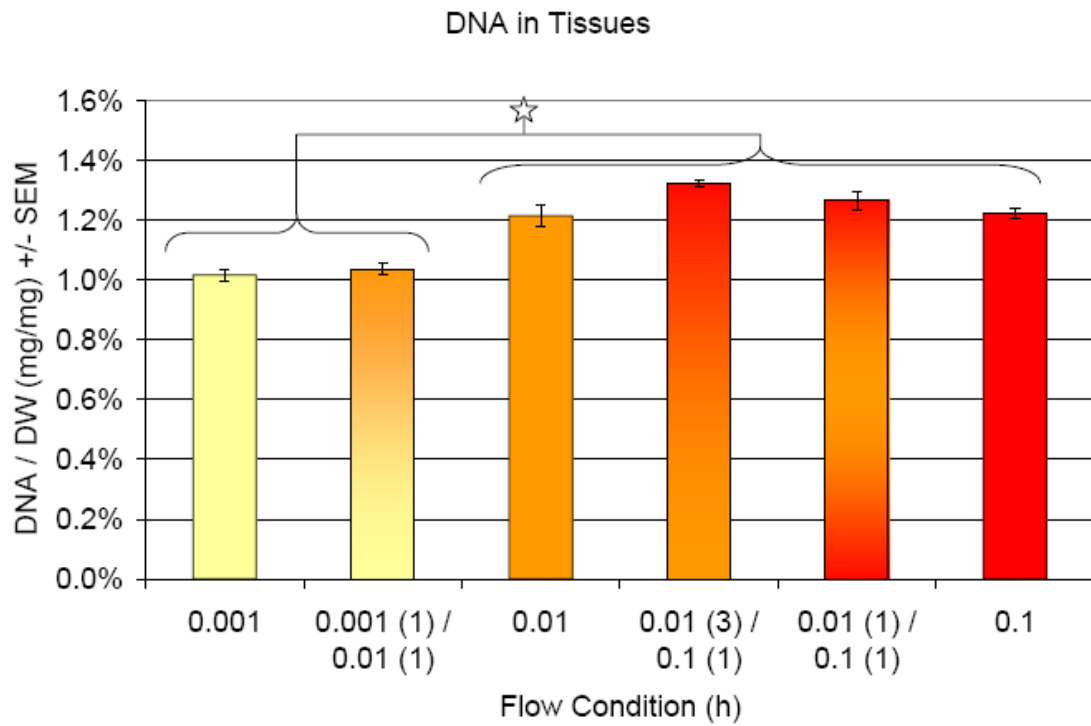
Results of Study 2: Day 7 Steady and Intermittent Shear Stress

General Observations

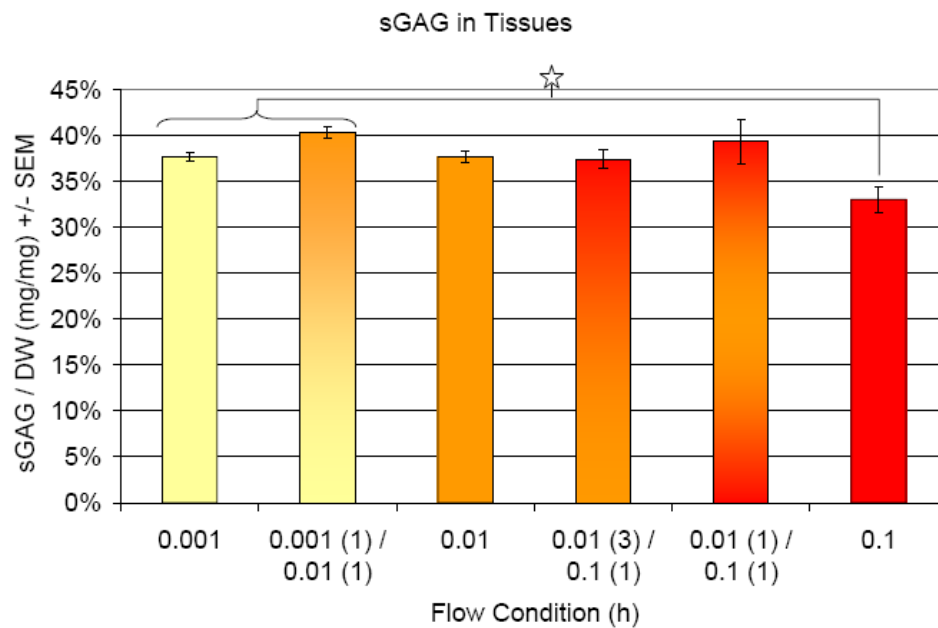
No differences were observed between groups in terms of tissue thickness or total mass. Similar to Study 1, tissues stained diffusely for types II and IX collagen as well as sGAG, with no staining for type X collagen. Type I collagen bands were evident in all, again, co-localized with flattened chondrocytes and deposited only on the top surface; the relative amounts of type I collagen are best described with the quantitative histomorphometry (discussed later).

Matrix Composition

Tissues exposed to steady 0.001-Pa or intermittent 0.001/0.01 shear possess a significantly lower amount of cells (DNA) than all the other groups; no differences existed between those other groups (Figure 6.6). Interestingly, these same groups (steady 0.001-Pa and intermittent 0.001/0.01) had a higher sGAG content than (only) the 0.1-Pa group. This is evidenced by analyzing the sGAG in the media, which shows nearly a dose-dependent effect of shear stress.

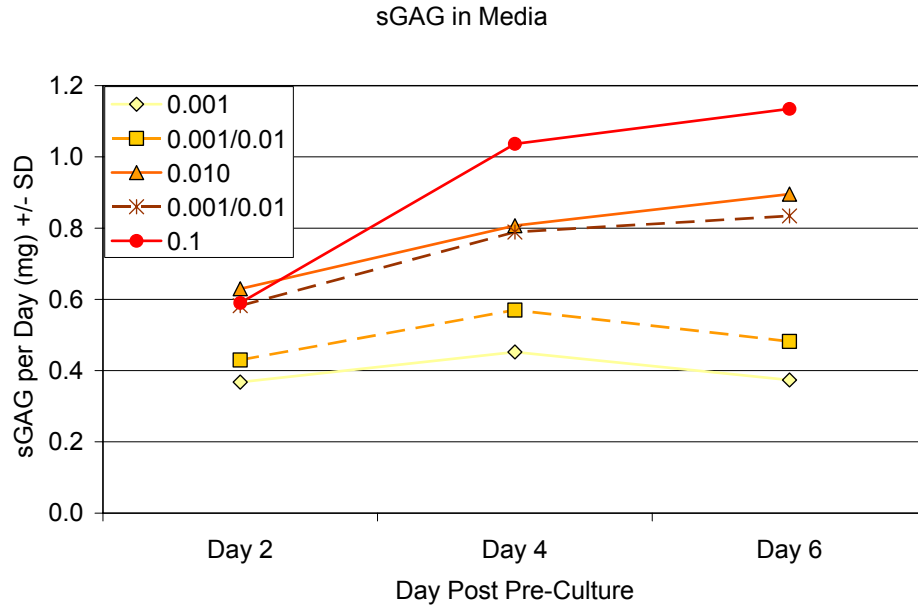


A



B

Figure 6.6 – Matrix Composition. DNA (A) and sGAG (B) content in the tissues per unit dry weight. sGAG per day present in the media (C). The hour (h) figure in parentheses represents the time in hours spent at the preceding shear stress. Star denotes $p < 0.05$.



C

Figure 6.6 continued – Matrix Composition. DNA (A) and sGAG (B) content in the tissues per unit dry weight. sGAG per day present in the media (C). The hour (h) figure in parentheses represents the time in hours spent at the preceding shear stress. Star denotes $p < 0.05$.

In terms of collagen content, the steady 0.001-Pa and intermittent 0.01/0.01 shear groups had less total collagen than all of the other groups, between which no significant differences existed (Figure 6.7, A). Type I collagen presence was significantly lower in the 0.001-Pa group compared to all others (B). This gene was up-regulated most notably in the 0.1-Pa group, which closely parallels results seen in previous studies.

Interestingly, the steady 0.01-Pa shear also showed an elevated expression, even over 0.001/0.01 intermittent flow. While not shown, no differences were observed in type II collagen content (not shown) or gene expression (D). Generally, a dose-dependent effect was observed, although significance was not achieved. While not shown, there were no differences among groups for GAPDH or aggrecan expression.

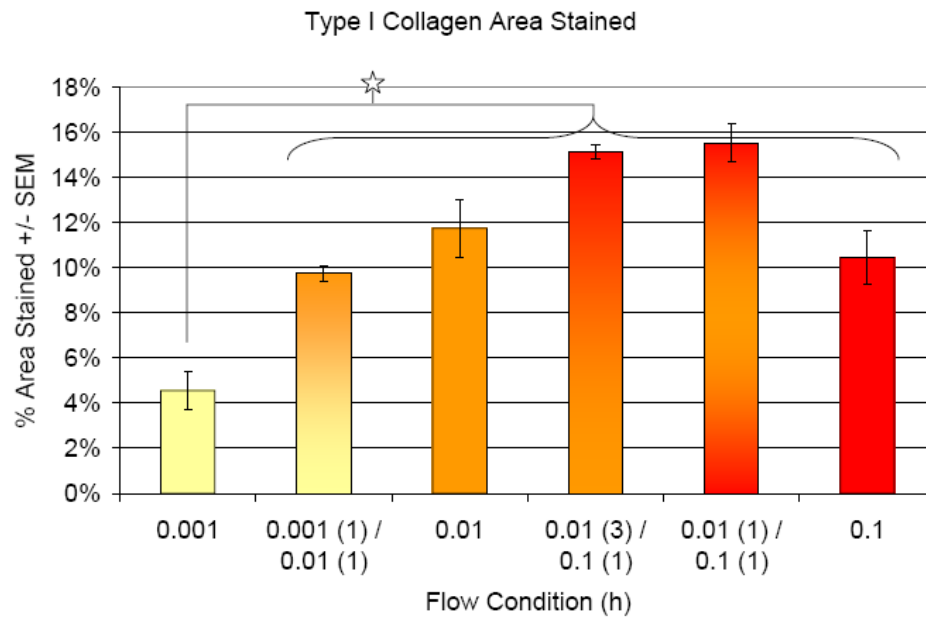
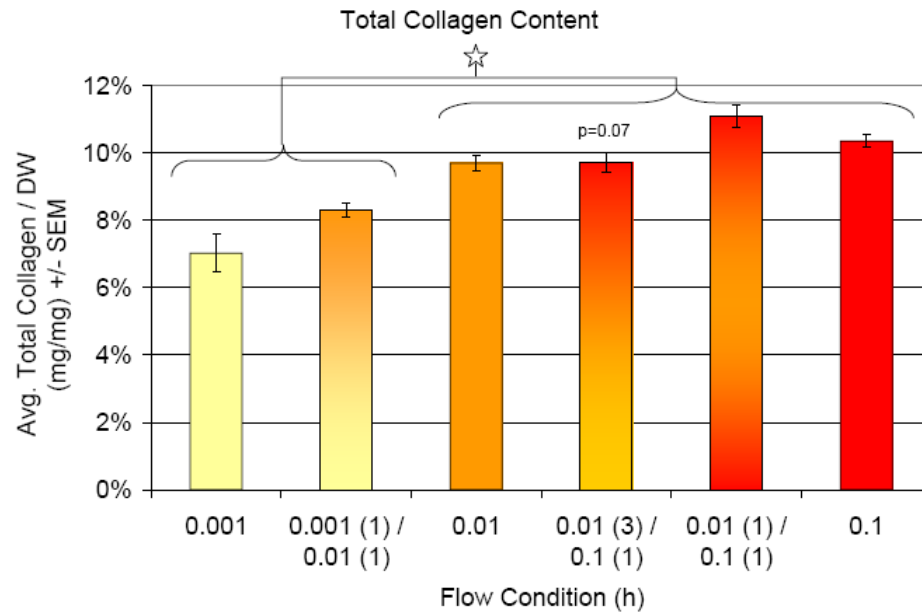


Figure 6.7 – Collagen Content and Expression. Total collagen (hydroxyproline) per unit dry weight (A) and type I collagen presence (histomorphometry, B). Type I (C) and II (D) collagen gene expression. The hour (h) figure in parentheses represents the time in hours spent at the preceding shear stress. Star denotes $p < 0.05$.

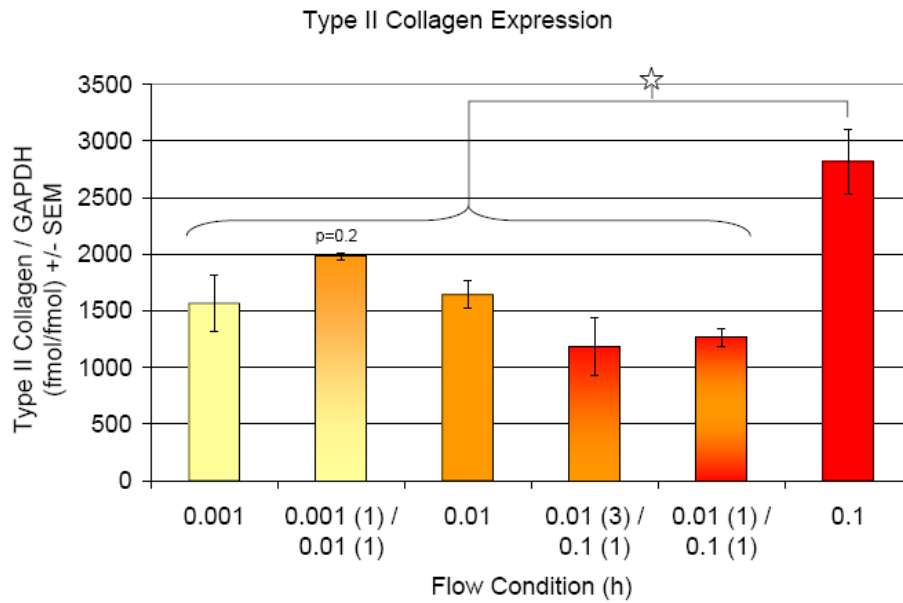
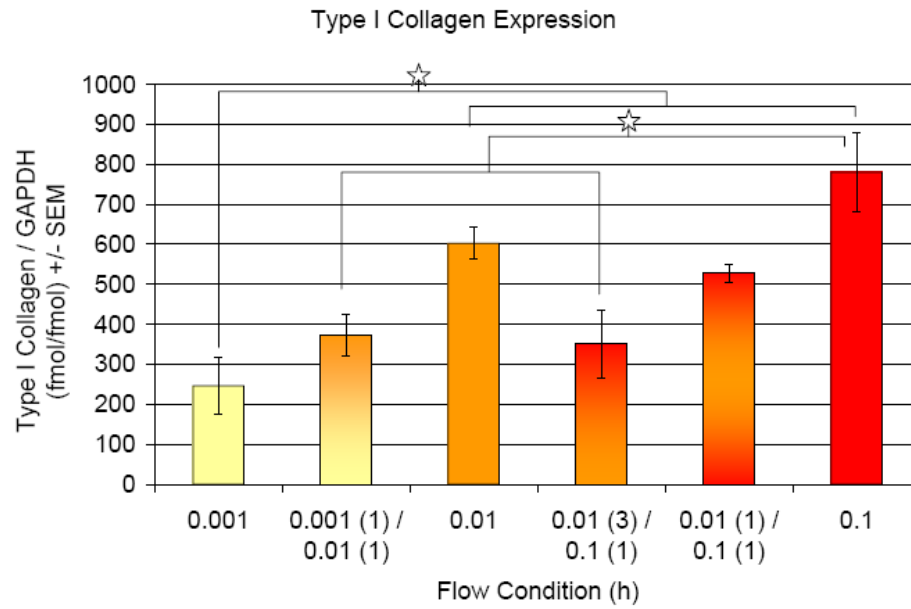
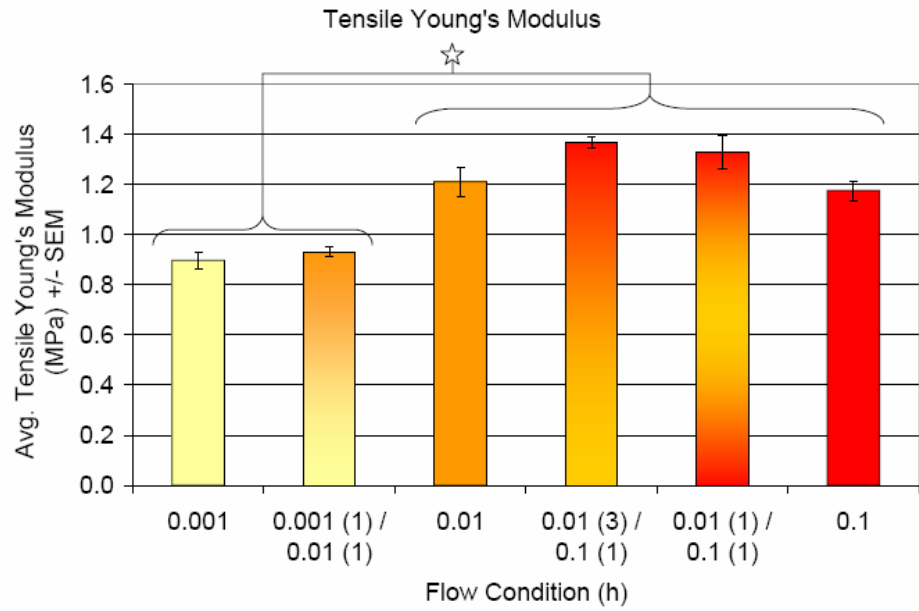


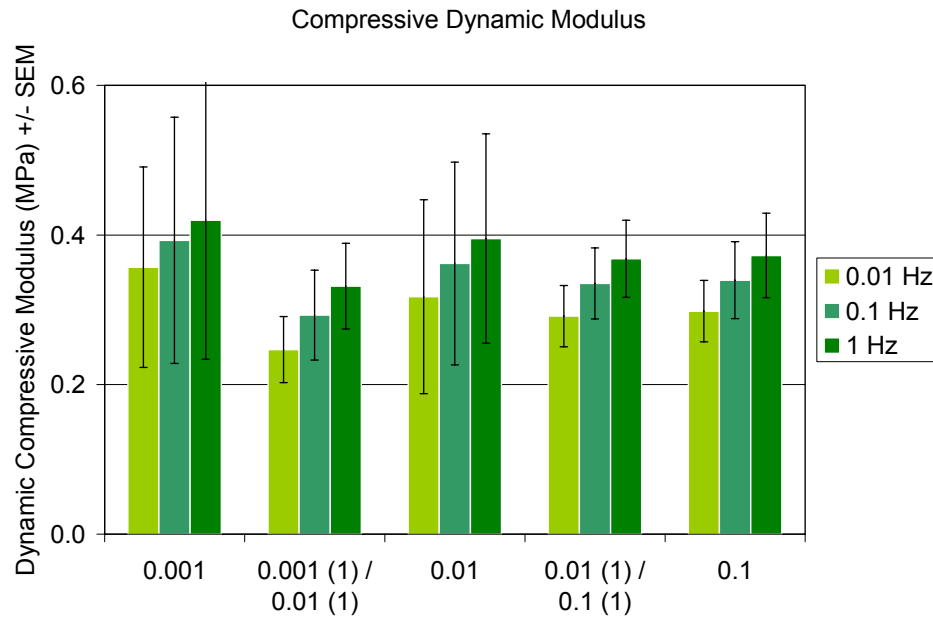
Figure 6.7 continued – Collagen Content and Expression. Total collagen (hydroxyproline) per unit dry weight (A) and type I collagen presence (histomorphometry, B). Type I (C) and II (D) collagen gene expression. The hour (h) figure in parentheses represents the time in hours spent at the preceding shear stress. Star denotes $p < 0.05$.

Mechanical Testing

Akin to previous studies, the tensile modulus of the steady 0.1-Pa shear group was higher than the steady 0.001-Pa group; in fact the steady 0.001-Pa and intermittent 0.001/0.01 group displayed lower moduli than all the other groups in nearly a dose-wise manner (Figure 6.8 A). No differences were observed in the compressive modulus for any group or applied frequency (B). Not shown is the 10 Hz frequency, which was influenced by distortion artifact and thus disregarded.



A



B

Figure 6.8 – Mechanical Properties. Average tensile Young's modulus (E_Y , A) and compressive dynamic modulus (E^* , B). The hour (h) figure in parentheses represents the time in hours spent at the preceding shear stress. Star denotes $p \leq 0.05$.

Discussion

Static culture conditions produce tissue-engineered cartilage with inferior matrix composition and mechanical properties [14, 157, 183]. Bioreactors that increase mass transport of nutrients and waste while providing a mechanical stimulus have been shown to greatly modulate chondrocyte metabolism and biosynthetic activity [7, 157, 158, 171, 187-192]. The physical stimuli applied by these bioreactors have ranged widely from compression [75, 80, 171, 197], to fluid-induced and tissue shear [158, 183, 190, 194] to hydrostatic pressure [164]. Shear stress is particularly attractive since it has been shown to modulate collagen expression and production [183, 191, 249, 252]. This is crucial in articular cartilage tissue engineering as engineered cartilage tissues are generally deficient in type II collagen and lack the native tissue's zonal organization [155, 191, 192, 209]. Here, we applied fluid-induced shear stress to scaffold-free engineered cartilage using multiple shear stress magnitudes, durations and waveforms. We hypothesized that shear stress would increase type II and total collagen production while concurrently improving the tensile and compressive mechanical properties in thick, scaffold-free tissue-engineered cartilage.

While bioreactor-mediated shear stress can improve the development of engineered cartilage, the nature (*i.e.*, magnitude, duration, and flow regime) in which the shear is applied is tremendously important [7]. For example, Vunjak-Novakovic *et. al* showed that cell-seeded PGA scaffolds cultured in the turbulent regime of spinner flasks generated constructs with a fibrous outer capsule [7]. On the contrary, tissues cultured in the laminar regime of rotating flasks possessed improved matrix composition, mechanical properties and morphology relative to static and spinner flask constructs [7, 193]. In addition to flow regime, shear stress magnitude differentially alters chondrocyte response in a time-sensitive manner. Fluid-induced shear stress at 16-dyne/cm² applied to monolayer chondrocytes increased expression of the pro-apoptotic gene *bcl-2* and increased NO release on a time- and magnitude-dependent manner [96]. However, there

was no increase in these markers at 1.6 dynes/cm² [96]. Other studies support the notion that high shear magnitudes (~20 dyne/cm²) are detrimental (*i.e.*, apoptosis-gene promoting, reduction in anti-oxidative capacity) to chondrocytes, while lower shear magnitudes (~5 dyne/cm²) do not elicit these negative responses and can even exhibit protective effects [253]. Thus, optimal fluid regimes for beneficial modulation of chondrocyte metabolism exist, but are highly dependent on the magnitude, duration and characteristic fluid dynamics of the shear stress environment.

In applying different levels of shear stress over multiple durations, we found both beneficial and detrimental effects on matrix composition and mechanical properties. After three days of exposure to 0.1-Pa or 0.001-Pa shear stress, no differences were found between these groups for tissue thickness, cell number, construct mass, sGAG content, or collagen production. However, the tensile Young's modulus was increased in the 0.1-Pa group (0.82±0.02 MPa) relative to the 0.001-Pa group (0.63±0.04 MPa). Without any significant increase in collagen (although a strong trend was noted for type I collagen) or proteoglycans, this suggests that there may have been an increase in organization or interactions in the matrix. These moduli on the order of MPa are somewhat surprising given the relatively low collagen content (~7-8% per dry weight), but is on par with other scaffold-free tissues cultured for similar durations. Fedewa *et. al* produced tissues with a tensile modulus of 0.9±0.2 MPa after 4 weeks of culture and 4.8±0.1 MPa after 8 weeks [182]. These moduli are still significantly lower than that measured for native young bovine tissue (~12 MPa) [52]. Also interesting after three days of flow was that there was a significant increase in type II collagen expression in the 0.1-Pa group, yet no subsequent increase in type II collagen protein deposition at either Day 3 or Day 7.

When extending the application of these shear stress levels to seven days, the 0.1-Pa shear stress produced constructs with more cells, mass, thickness and a greater tensile Young's modulus compared to the 0.001-Pa group. These beneficial effects were concurrent with several detrimental effects however. Relative to the 0.001-Pa group, the

longer exposure of 0.1-Pa shear also produced a significant increase in type I collagen deposition, decrease in type II collagen expression, loss of sGAG from the matrix and an increase in sGAG in the media. The rate and amount of sGAG in the media was exacerbated with extended duration of shear. This latter effect of GAG leaching into the media is not unexpected given our previous experiences with thin constructs and others' observations using dynamic fluid flow [7, 156, 187, 194]. Saini *et. al* found that between 40% and 60% of the total sGAG produced by their bovine chondrocyte seeded-PLLA scaffolds exposed to fluid-induced shear stress was released into the media in a shear stress magnitude-dependent manner [194]. These studies thus re-affirm the notion that while shear stress can have beneficial effects on the modulation of tissue engineered cartilage, sub-optimal regimes exist which can produce detrimental effects. In our case, the extended duration of 0.1-Pa shear stress to seven days was sub-optimal for the purposes of articular cartilage tissue engineering due to the sGAG leaching and type I collagen deposition.

Some of the effects of shear stress magnitude and duration in these thick constructs were somewhat surprising given our previous studies with thin tissues (Chapters 3 and 4). In both thick and thin tissues, type II collagen expression was increased at Day 3 for 0.1-Pa exposed tissues compared to 0.001-Pa controls; there was no difference in type II expression levels between these two shear groups at Day 7. In the thin tissues, this increase in type II gene expression was translated into an increase in type II collagen (protein) deposition at both three and seven days. However, no increase in type II collagen deposition was seen at either of these time points in thicker constructs. This suggests that the protein deposition or translation in the thick constructs is either delayed or masked, as the resemblance in expression data between thin and thick constructs suggests that these constructs respond similarly to shear. On the other hand, parallel effects between thin and thick constructs were observed in terms of tensile mechanical properties. An increase in tensile Young's modulus was measured for both

increasing shear stress magnitude and duration of exposure in thick and thin tissues. The underlying reason for these increases in tensile modulus is less clear since collagen content was modulated differently in these tissues. For example, when comparing 0.1-Pa tissues to 0.001-Pa tissues after three days of shear, thin tissues showed an increase in total and type II collagen – no such effect was seen in the thick tissues even though the tensile modulus was increased. In comparing the same groups at seven days, thin tissues had an increase in both type I and II collagen; thick tissues showed an increase in only type I collagen, not type II. Thus, the increase in tensile modulus can generally be correlated to collagen content and perhaps an increase in interactions or organization (*e.g.*, alignment of matrix molecules in the direction of applied shear). The latter suggestion was ruled out since parallel or perpendicular-cut (with respect to flow direction) specimens resulted in no difference in terms of tensile Young's modulus. Also similar between thick and thin constructs was the result that the Day 7, 0.1-Pa group achieved the highest modulus despite having a significant decrease in sGAG per unit dry weight compared to all other groups. This is perhaps not surprising given that proteoglycans more influence compressive rather than tensile properties [31], and that this group possessed the highest collagen content per unit dry weight. Lastly, it should be noted that the moduli for the thicker tissues were considerably lower than those found for the thin constructs, but this due to the slower strain rate used for the thick constructs, which was decreased to minimize the viscoelastic response of the tissue. Thus, while the thin and thick tissues respond similarly in many ways to shear stress, disparate results are shown for some metrics, emphasizing that these are different model systems and should be analyzed as such.

In an effort to build upon the beneficial effects exhibited by shear stress while discouraging the negative effects, intermittent waveforms which alternated between different shear magnitudes were applied to our thick tissue engineered constructs. Waveforms stepped between the two shear stress magnitudes previously used (0.001- and

0.1-Pa) as well as a newly implemented intermediate level of 0.01-Pa. This intermediate group was added to better understand the dose-dependent effect of shear on construct development. With literally an infinite number of possibilities of timing to be used for the waveform, inspiration for construction of the waveform was gleaned from other studies examining the time-dependent response of chondrocytes to fluid-induced shear stress. In a study previously discussed, chondrocytes exhibited increased levels of NO synthase expression and NO production at 2, 6, and 24 h when exposed to 16-dyne/cm² [96]. Expression of signaling molecules, such as JNK2 and PI3-K, involved in regulating Phase 2 and anti-oxidative proteins were increased in as little as 1.5 h. Taken together, these studies suggest that fluid-induced shear stresses effect chondrocyte gene expression on the order of hours. However, our data also suggest that elevated gene expression can be lost when exposed to shear for extended durations (*i.e.*, decrease in type II collagen expression at Day 7 relative to Day 3). This suggests that mechano-responsive cells can be saturated with a mechanical signal and lose the beneficial effect. This is supported by *in vivo* bone loading studies where Robling *et. al.* found that a greater osteogenic response was elicited when mechanical loading was divided into discrete bouts as opposed to constant loading [254]. Introduction of a “resting period” may have allowed for the cells to regain their mechanosensitivity after a period of 2-3 hours [254]. Consequently, we constructed our intermittent waveform of applied shear stress to account for time required for stimulus (as little as one hour) and time suggested for recovery of mechanosensitivity (as many as three hours).

In some cases, intermittent flows indeed retained the beneficial effects of shear stress while discouraging the negative effects. The intermittent flows alternating between 0.01-Pa and 0.1-Pa (along with their steady flow counterparts) resulted in significantly increased tensile Young’s modulus, cell number and total collagen per dry weight compared to the low steady 0.001-Pa and intermittent 0.001/0.01-Pa sheared groups. Furthermore, these intermittent groups (0.01-/0.1-Pa) retained more sGAG in the matrix

relative to the 0.1-Pa group (which was significantly reduced compared to all other groups). The intermittent groups did not alleviate all of the detrimental effects of high shear stress however, as there was a significant increase in type I collagen deposition (as indicated by histomorphometry) and no increase in type II collagen content or expression. Interestingly, the low duty cycle intermittent shear (0.01-Pa for 3h and 0.1-Pa for 1h) did show a significantly lower type I collagen expression compared to the higher duty cycle intermittent flow and 0.1-Pa steady flow. This is somewhat confounding since this group also showed an increase in type I collagen deposition. This underscores the importance of realizing that qPCR is only a snapshot in time and is highly dependent on the point in the waveform when the tissue is harvested (all were harvested at the end of the high-level shear step); there may have been a transient decrease in type I collagen expression in response to the one hour of 0.1-Pa shear. Thus, the intermittent flows show promise in terms of retaining positive and discouraging negative effects, although type I collagen production is still significant in these tissues.

No differences were seen in any group or time point (Study 1 or Study 2) for compressive mechanical properties. This is expected given the similarity of sGAG content in the tissue across all shear stress magnitudes and time points. The lone exception is the 0.1-Pa steady shear group at Day 7, which did show a significant decrease in sGAG content per unit dry weight. One may expect a reduction in compressive properties in this group, but since there was also a significant increase in total collagen content, this may have offset any effect. Collagen has been shown to positively correlate with compressive modulus [87] and plays an even more important role in unconfined compression where lateral expansion of the tissue is allowed. Compared to native tissue, the 300-400 kPa dynamic compressive moduli of our engineered tissues are more than an order of magnitude lower [56]. As previously mentioned, as samples were thawed, the punched discs of tissues curled with time. This may have skewed data if residual stresses were built up in the tissue. The origin of this

curling effect was unknown, but could have been due to release of the matrix off the membrane, a higher swelling pressure due to changes in ionic concentration [255], or due strain built up in the collagen layer at the top surface. The latter is most likely as this curling phenomenon was not seen in static-grown tissues tested in compression (Chapter 5). Compression testing in these static constructs also produced data sets with smaller standard deviations across the population, suggesting that the curling did adversely affect the quality of the data collected (although the static constructs were a smaller, more homogenous population).

It should also be noted that our system applies a *constant shear stress*, even when alternating between different levels of shear. While this could be regarded as a type of static mechanical signal, it is unclear to what extent the pulsatile nature of the fluid flow influences the cells and tissue. This could very well be a factor, as it is known that cells are sensitive to flow frequency and degree of pulsatility [90]. We also feel comfortable ruling out increased mass transport (flux) through the tissue as a possible effector of matrix composition and mechanical properties. Published mathematical models [243] show that cartilage-like tissues subject to Poiseuille flow do not allow for flux through the tissue. We independently verified these calculations with empirically-derived parameters for our tissue and found that flux would occur through the tissue only when the permeability was increased eight orders of magnitude. Consequently, the applied stimulus can reasonably be attributed to fluid-induced shear stress and removal of the static boundary layer.

In conclusion, these studies show that shear stress magnitude, duration and waveform can modulate the matrix composition and mechanical properties of clinically-relevant sized engineered cartilage over time. Intermittent applications of shear stress can preserve some beneficial effects (*e.g.*, increase in tensile Young's modulus) while discouraging some negative effects (*e.g.*, sGAG in matrix preserved), but have yet to be deemed as optimal for articular cartilage tissue engineering due to the increase in type I

collagen deposition. Taken together, the bioreactor system presented here indicates a robust and precise model system which can be used at relatively high resolution for the study of fluid induced shear stress on the growth and development of tissue-engineered articular cartilage.

CHAPTER 7

CONCLUSIONS AND FUTURE CONSIDERATIONS

Synthesis of Thesis

Presented in this thesis is a novel dual-chamber flow bioreactor for the application of fluid-induced shear stress to three-dimensional slabs of engineered tissue. Our central hypothesis was that exposure of these cartilage constructs to shear stress would enhance cartilage matrix deposition, leading to more robust mechanical properties. The overall objective of this project was to employ a novel flow bioreactor to improve matrix composition and mechanical properties of cartilage constructs for load-bearing applications. The central hypothesis was proven in that shear stress potently modulated the matrix composition, gene expression and mechanical properties of both thick and thin engineered cartilage. Modulation was found to be highly dependent on shear stress magnitude, duration, and waveform and affected different matrix constituents and mechanical properties in disparate ways. Our overall objective was satisfied on the basis that the bioreactor created stronger engineered tissues, but with the caveat that the tissues may not be ideal for articular cartilage engineering due to the presence of type I collagen.

In this thesis, two models of engineered cartilage were explored: thin (~300 μm) and thick (~1 mm) scaffold-free constructs. The thin constructs were used in Specific Aims 1 and 2 to demonstrate feasibility with the bioreactor and gain an understanding of shear stress on neo-tissues. Thick constructs were the focus for Specific Aim 3 in order to bring a more clinically-relevant focus to the work as well as allow for compressive mechanical testing. These different models shared some similarities in cellular response to shear stress in the bioreactor, but not in all cases. In both models, rounded, viable chondrocytes were present throughout the matrix, with the exception of some cells on the tissue surface which were more flattened. Tissues stained diffusely for makers of

differentiated chondrocytes, such as type II and IX collagen, as well as sGAG. In tissues exposed to high shear levels and/or extended culture times, the region adjacent to the flow resulted in depletion of sGAG and type IX collagen, concurrent with an increase in type I collagen presence. High levels of shear stress (0.1-Pa) increased both type I and type II collagen gene expression after three days (compared to low shear controls at 0.001-Pa), but dropped to basal levels after one week of steady flow in both models. In thin (~300 μ m) scaffold-free tissues, this translated to an increase in both of these proteins' deposition at three and seven days. This was not the case in thick tissues, where there was no difference in type II collagen deposition at either of these time points. Since the type II expression is similar between the thin and thick tissues, this suggests that protein translation, deposition or incorporation is delayed or masked in the thick tissues. Supporting this suggestion, type I and total collagen were increased at Day 7 for both thick and thin tissues in a near dose-dependent manner with shear stress – however, only the thin tissues showed an increase in total collagen at Day 3 (no type I collagen was noted at this time point). Thus, while generally responding to shear stress in a similar manner, thick tissues may be less sensitive to mechanical stimulation or more difficult to assay differences when compared to the thin tissues.

In assessing the global effect of shear stress on tissue development in our system, the increase in type I collagen expression and deposition was perhaps the strongest and most consistent effect with shear stress magnitude and duration of flow. Concurrent with the increase in type I collagen, which was relegated to the upper (shear-exposed) surface of the tissue, there was an increase in flattened, fibroblast-like cells. This was evidenced by an increase in cell number at the seven day time point in high shear-exposed tissues. This effect overshadowed many of the other benefits of the bioreactor culture environment. While in native articular cartilage, there are flattened chondrocytes at the surface, these cells produce high concentrations of type II collagen, not type I collagen, as was the case in our system. Such an effect would be undesirable for articular cartilage

engineered tissues, but could be very beneficial in fibrocartilaginous tissues such as that found in the temporomandibular joint.

Shear stress also had a profound effect on sGAG in the matrix and in the media. Presence of sGAG in the media showed a dose-dependent response to shear stress and increased in rate with duration of shear exposure. In only the most extreme case (longest duration, highest shear magnitude), was sGAG content different in the tissue: 0.1-Pa shear exposed tissues for one week possessed less sGAG on a dry weight and per cell basis than all other groups. Interestingly, when an intermittent waveform of shear stress (cycling steps between two shear stress levels) was used, the sGAG was retained in the matrix while attaining similar levels of improved mechanical properties.

Tensile mechanical properties were improved with the application of shear over time. This consistently paralleled increases in total collagen, but not sGAG. There was no effect of orientation of matrix molecules in the direction of flow, however: when specimens were tested in tension parallel or perpendicular with respect to flow direction, there was no difference between groups. A dense concentration of collagen strata were noted on the top, shear-exposed surface of tissues which increased with shear magnitude and duration. Whether or not the increase in tensile mechanical properties was due to an increase in collagen content or interactions (*e.g.*, cross-linking) is unknown.

Compressive mechanical properties were unaffected by shear stress or time in culture in bioreactor studies. Testing shear-exposed tissues in compression was confounded by a “curling phenomenon,” where upon thawing, the disc-shaped specimens started to curl upon themselves. Consequently, specimens were allowed to equilibrate in between testing platens to prevent curling and remain flat while testing, but residual strains were built in the tissue. The basis of this curling is unknown, but perhaps due to either changes in ionic bath concentration, relief of stress upon liberation from the membrane or the build up of collagen fibers of the surface of the tissue. While this confounded the compressive testing, it should perhaps not be surprising that there was no

difference in the dynamic modulus for any of these groups. No differences were observed among nearly every group in terms of sGAG content; the lone exception was the 0.1-Pa group at Day 7, which did possess less sGAG per dry weight, but also an increase in collagen content. Both sGAG and collagen play a role in determining the compressive properties, especially in unconfined compression.

The underlying mechanism responsible for fluid-flow mediated cellular effects is likely due to shear stress and not an increase in mass transport. Published mathematical models of Poiseuille flow over cartilage-like materials [243], as well as models created in our lab, suggest that there is minimal fluid flux through the tissue. Intra-tissue flux would occur only with an eight order of magnitude increase in tissue permeability. Thus, the fluid flow applies a shear stress to the tissue which directly contacts the cells on the surface of the tissue as well as a mechanical deformation (albeit minimal) to the bulk tissue. The signaling mechanism in our system – and the response of chondrocytes to fluid-induced shear stress in general – is unclear, but may be mediated in part by G-proteins [95, 256].

The completed work is significant because this represents the first analysis of scaffold-free engineered cartilage in response to multiple shear stress magnitudes, durations and waveforms. While this work did not produce an engineered articular cartilage tissue with matrix composition and mechanical properties similar to that of native tissue, the body of work focusing on cellular modulation in response to shear stress provides valuable direction in future efforts of cartilage tissue engineering. The bioreactor system also provides a flexible platform technology for the study of three-dimensional engineered tissues, not just articular cartilage. Our approach was innovative given the use of a novel dual chambered flow bioreactor that provided controlled, repeatable stimuli and will continue to serve as a valuable testing and production platform technology.

Alternate Strategies for Cartilage Tissue Engineering in the Bioreactor

While this thesis work demonstrated the effects of fluid flow and shear stress on engineered cartilage, a tissue-engineered construct approximating the matrix composition and/or mechanical properties of native tissue has yet to be created. The bioreactor system remains a fertile ground for engineering these tissues, but there exist many alternate strategies yet to be undertaken that could accelerate production of a realistic engineered tissue.

The strategies discussed here are based on the goal of creating an engineered construct which approximates the matrix composition and mechanical properties of native articular cartilage. This goal is founded on the assumption that implantation of such a “mature” construct would (theoretically) allow the patient to resume normal load-bearing activities sooner since the tissue inherently possesses robust architecture and mechanical properties. However, we have no proof that a mature a constructs engineered in our system will integrate properly into the surrounding matrix *in vivo*, but success with procedures like mosaicplasty and other scaffold-free tissues imply that mature tissues can functionally integrate [185]. Even still, it should be noted that other very different strategies exist: evidence suggests that immature constructs or even enzymatic treatment (*e.g.*, trypsin) of surrounding tissue may be more advantageous than implanting a mature construct [202].

The first category of strategies to improve our constructs centers around the mechanical environment put forth by the bioreactor system. To date, only fluid-induced shear stress has been examined, in both steady and intermittent nature with limited success. Certainly, additional shear stress magnitudes, intermittent waveforms and oscillatory patterns could be tried. However, given the current data gleaned from exploration of this parameter space would suggest that additional studies in this realm, keeping other variables constant, would result in minimal changes. Furthermore, type I collagen deposition appears to be the strongest effect elicited by shear stress in the

magnitudes and durations studied. That being said, the bioreactor is amenable to other types of mechanical signals such as compression, hydrostatic pressure and interstitial fluid flows. As discussed at length in the Literature Review, these physical forces have been shown to affect chondrocyte metabolism. These signals would require additional - but minimal - modifications be made to the bioreactor system. Compression could be driven in a direct manner by moving the cap uni-axially normal to the tissue. Hydrostatic pressurization could be achieved by placing a pinch valve over the effluent tubing line and transiently cutting off flow, resulting in pressurization of the system. Interstitial fluid flows could be induced through the tissue by opening the port(s) on the bottom chamber and promoting a cross-current flow. These mechanical signals, along with shear stress, may produce a synergistic effect in our bioreactor system if combined with a growth factor such as TGF- β 1 or IGF-1. The media used in these studies represents a standard-but-minimally-supplemented media; addition of the afore mentioned growth factors could result in a synergistic effect when combined with mechanical stimuli, as has been shown elsewhere [199]. Lastly, while the constructs discussed in this thesis fall short of native tissue in terms of matrix composition and mechanical properties, it must be stressed that the longest time point is three weeks. Many other studies culture constructs on the order of months, which may be required to produce a native-like tissue, but would ideally be avoided if this technology were to ever realize clinical or commercial potential.

The second category of strategies centers on different approaches to creating the engineered tissue. This thesis has focused on a scaffold-free cartilage construct, but in no way has this approach been proven to be the optimal strategy. For example, one major hurdle in implanting any tissue into a defect site is the integration of the tissue - both radially/laterally with adjacent cartilage and with the underlying bone. A relatively new strategy to increase the probability of successful integration is the creation of osteochondral constructs. As the name suggests, these tissues possess a bone-like and cartilage-like region, mimicking the native bone-to-cartilage gradient. More importantly,

adding a bone-like region to the tissue – one that can integrate into the sub-chondral and marrow cavity – may allow the construct to take advantage of the highly vascular and metabolically active region. While scaffolds have not been used here, the bioreactor is amenable to scaffold based constructs. Besides just simply allowing for scaffolds to be placed into the bioreactor, the ports in the lower chamber of the bioreactor conveniently present an opportunity to perfuse the scaffold. Scaffolds may not be required to fabricate an osteochondral construct however; the dual chamber nature of the bioreactor allows for biochemical induction of a cartilage to bone gradient. For example, chondrogenic factors could be used in the upper chamber (*e.g.*, TGF-B1, IGF-1) and osteogenic factors in the bottom chamber (*e.g.*, dexamethasone) in order to biochemically induced a cartilage-to-bone gradient. This could be performed in conjunction with or separate from mechanical conditioning.

Lastly, selection of an optimal cell source remains a constantly evolving field in academia and industry that has not been directly addressed in this thesis research. Primary bovine chondrocytes were used here for their ability to generate cartilage as well as the access and availability to large quantities of cells. Bovine chondrocytes have been shown to respond similarly to human cells, further supporting the use of this model [8]. Obviously, primary bovine chondrocytes are not clinically relevant due to their xenogeneic nature, and a truly “clinically-relevant” project would transition to human cells. Whether those cells are primary (autologous), allogeneic, differentiated or undifferentiated remains to be seen and will undoubtedly be tremendously influenced by business practicality and advancement of the field in years to come.

Alternate Applications for the Bioreactor

The bioreactor system has academic and industrial potential outside the field of cartilage tissue engineering. Indeed, the bioreactor can be used as a tissue-production vessel or as a tool for studying effects of growth factors and/or mechanical signals on

tissue development. For that matter, the bioreactor is not limited to just cartilage; virtually any attachment-dependent cell type can be used in the bioreactor, provided it is amenable to a slab / block geometry (*i.e.*, engineered vessels would not work without major modifications, but monolayers of endothelial cells co-cultured with smooth muscle cells would). In fact, the bioreactor as it is currently used appears well-suited to produce fibrocartilaginous tissues with increased mechanical properties. As examined, the effects of flow are not immediately amenable to articular cartilage, but more fibrocartilaginous tissues such as that found in the temporomandibular joint (TMJ) would welcome the tissues produced by our device. Another such application, which was discussed in depth for this system with industrial partners, was for the “re-vitalization” of cryopreserved tissues post-thaw. We suggested that the beneficial effects of mechanical conditioning on tissue-engineered constructs could be extended to cryopreserved tissues, which are currently clinically relevant and available. In terms of using the bioreactor as a testing tool, industries exploring therapies for osteoarthritis may find the system attractive. Testing a new drug on a sample of engineered cartilage would provide a simple, low-cost yet effective first-screen for pharmaceutical companies. The bioreactor can also create a tissue with features reminiscent of early-stage osteoarthritis; academic and industrial pursuits could test effects of drugs, mechanical stimulation, *etc.* on reversal or acceleration of the arthritic condition.

General Comments on Cartilage Regenerative Medicine and Tissue-Engineering

As alluded to earlier, the tissue engineering community – in particular that of cartilage tissue engineering – faces a significant uphill battle building a sustainable industry. No true tissue-engineered articular cartilage construct exists in the market today, with the closest product being Genzyme’s Carticel™ procedure. This procedure is traditionally very expensive and requires patients to re-visit the surgeon after their autologous cells have been expanded *in vitro* by Genzyme for the implantation.

Consequently, this is an inefficient business model, as evidenced by Carticel's lack of financial success. Indeed, financial success has been limited in tissue engineering / regenerative medicine with both Organogenesis Inc. and Advanced Tissue Sciences filing for bankruptcy some years ago (although Organogenesis Inc. has emerged from Chapter 11 and is now profitable).

A common demise of engineered tissues is their expense. Between operating clean rooms, procuring viable cells, supporting lengthy clinical trials and obtaining insurance reimbursement, tissue engineering companies have an unusual set of cost drivers inhibiting financial success. For cartilage constructs, a practical cell source must be found; autologous chondrocytes from adult donors which must be expanded prior to implantation is a costly and ineffective strategy. An ample cell source that is immediately available must be found – such as allogeneic progenitor cells – in order for cartilage tissue engineering to make business sense. While companies like Genzyme have limited success in this field, they deserve credit for being pioneers and paving the road to clinical and business entrenchment. Genzyme has established a network of orthopaedic surgeons, insurance reimbursement and regulatory approval strategies. These three facets of the tissue engineering business are an absolute necessity for financial stability.

That being said, the future is bright for tissue engineering and biotechnology. In 2004, revenues for S&P listed biotechnology companies rose 14% [257], a far cry from the “dot-com” collapse in 2001 that swept away most of the biotech investments as collateral damage. Specifically for orthopaedic tissue engineering interests, the immediate market opportunity lies in the growing knee segment which was estimated to be \$3-\$4 Billion in 2004 in the U.S. alone. Not only is this a large market, but it also possesses an annual, healthy growth rate of 3-5%. Thus, cartilage tissue engineering (and tissue engineering in general) remains poised to make a strong re-entry into the market.

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VITA

CHRISTOPHER V. GEMMITI

Chris Gemmiti was born in the sleepy town of Granby, CT to Janet and Vin Gemmiti and older brother, Nate, on July 1st, 1975 at 9:18 AM. He is the proud father of a son, Nolan, born February 19, 2006, with his beautiful wife, Amy.

Chris attended Avon Old Farms High School in Avon, CT and graduated in 1993, second in his class. He was a three-sport Varsity athlete in soccer, hockey and baseball. In high school, his passion for sports, biology, physiology, physics, and engineering were born. In 1993, Chris enrolled in the Johns Hopkins University (Baltimore, MD, where he later graduated in 1997 with a BS in both Biomedical Engineering and Engineering Mechanics. In his time at JHU, he played three years of Varsity baseball, capped by a senior year trip to the College World Series. Also in his time in Baltimore, he obtained his first “real” job selling programs at Camden Yards and even served as part-inspiration for a baseball book *Columbus Slaughters Braves* by Mark Friedman, a JHU alumnus.

After college, Chris and his wife Amy lived and worked in Boston where their love between each other and the Boston Red Sox grew. They went to as many games as possible, even sneaking into one game via the handicap entrance on Yawkey Way. This, on top of Chris proposing to Amy in Boston’s Public Garden, prompted many to confuse him as the character portrayed by Jimmy Fallon in *Fever Pitch*. In 2004, while living in Georgia, the night the Sox completed the improbably comeback against the Yankees, Chris and Amy bought plane tickets to Boston so that they could revel in the World Series. They watched Game 1 from Boston Beer Works, his favorite bar and restaurant.

In 2001, they moved to Georgia so that Chris could enroll in the BME program at Georgia Tech. He became more known for his role as Tour Director at Atlanta Brewing Company however. They had an eventful time in GA, buying a house next to a former chop shop, smashing a car, seeing real racism, learning everything there is to know about re-zoning and what an easement is, indulging in a common love for Dairy Queen and the Dillard House, suing a neighbor, finding NASCAR, completing a PhD (Chris) and an MSW (Amy), rescuing (in more ways than one) their dog, Fenway, and having a beautiful son, Nolan. With the PhD complete, they plan on re-locating back to the Boston area so that they can watch Red Sox games on cable without having to spend the extra money on a satellite package.

Chris enjoys baseball, golfing in the morning before school (easier to putt on wet greens), good coffee with an “everything” bagel with onion and chive cream cheese, landscape photography, beer homebrewing and (more importantly) consuming. Chris also loves to cook – especially grill – and has an appreciation for good food and libation. Being raised in an Italian-biased household (his father was born in Italy and both his parents are amazing cooks), Chris begins every dish with olive oil, garlic, salt and pepper and ends with cheese (and more salt) and a good glass of wine. He recently completed three sprint triathlons and hopes to one day run a complete Olympic triathlon. His dream job would be to call the play-by-play for a minor league baseball team (the same team for which his son plays, of course).