EFFECTS OF INTERMITTENT STATIC AND DYNAMIC TENSION ON ARTICULAR CHONDROCYTES IN HIGH DENSITY CULTURE

By

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Abstract

Tissue engineering has the potential of becoming an effective approach for the replacement of articular cartilage. However, the problems of small tissue size and inadequate mechanical properties of the tissue have yet to be overcome. Mechanical stimulation of cartilaginous tissues is one method of accelerating chondrocyte proliferation and ECM synthesis. While the effects of compression and shear have been well studied, the effects of tension have received little attention. Based on the findings of previous mechanical stimulation studies and photographic evidence of tension acting in native articular cartilage in its physiological environment, it was hypothesized that intermittent applications of tensile strain can be used to stimulate cellular proliferation and ECM synthesis and thereby improve the size and mechanical properties of cartilaginous tissues.

A loading fixture was constructed to apply biaxial tensile strains (BTS) to cartilaginous tissues grown in vitro. The optimal conditions for stimulating proliferation and ECM synthesis were found to be static tension (as opposed to dynamic tension), 3.8% radial and 2.1% circumferential strain magnitude for a 30 minute duration. Tissues subjected to BTS stimulation for 4 weeks at a frequency of once every 2-3 days had increased thickness, wet weight, and proteoglycan content, but had little effect on tissue mechanical properties. Tissues stimulated at a frequency of once per day over the same period had a negligible effect. A subsequent experiment confirmed that the effects of BTS stimulation on proliferation and ECM synthesis were dependent on load frequency, as well as culture media pH.

The experimental results of this thesis suggest that the physical stretching of chondrocytes may have had more of an impact on stimulating proliferation and ECM synthesis than induced...
interstitial fluid flow. Chondrocytes also require a period of preconditioning before the stimulated effects occur, but too high of a loading frequency can cause possible desensitization and/or a catabolic response. Overall, the experiments were successful in identifying the stimulatory potential of tensile strains. However, further improvements must be made to the long-term effects on tissue mechanical properties before tension can be used as an effective stimulus to produce better quality in engineered cartilaginous tissues.
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Chapter 1
Introduction

1.1 Articular Cartilage Injury and Damage
Articular cartilage is the thin layer of tissue covering the ends of articulating bones. This tissue is not connected to the vascular or nervous systems [1], yet homeostasis is maintained through interactions with the surrounding synovial fluid and the underlying bone [2]. A near frictionless contact surface is provided at the articulating joints by the combination of articular cartilage and synovial fluid. Articular cartilage is structured in a unique pattern that enables it to distribute and absorb energy and to withstand repetitive, physiological loads. In osteoarthritis, where the tissue is worn down by injury, the energy storage capability of articular cartilage is reduced. Fibrillations and fissures gradually form at the tissue surface and ultimately lead to joint dysfunction [3]. Osteoarthritis can also be caused by biochemical imbalances that come with the onset of advanced age, which lead to increased stresses being applied to the tissue and subsequent tissue destruction [4]. Osteoarthritic tissue is also characterized by increased cell death, increased presence of tissue degrading enzymes, changes in cell shape and presence of bony nodules (called osteophytes) on the tissue [5]. Currently 3 million Canadians suffer from osteoarthritis [6]. Complications arising from arthritis include joint pain and tenderness, variable degrees of joint inflammation and limited range of motion [7].

1.2 Articular Cartilage Repair
The repair response of articular cartilage is limited and rarely successful due to its lack of blood supply and its lack of available cells that are able to move to the injured site [2]. Many methods have therefore been developed to alleviate pain and to restore cartilage defects for arthritic patients. Minor procedures aimed at reducing pain in the short term include lavage, or irrigation...
of a joint with sodium chloride or similar solutions [8]. Debridement is another minor corrective procedure in which damaged or diseased cartilage is shaved off, debris in the joint is removed, and osteophytes is excised [9]. Though debridement reduces pain by smoothing out the articular surface, the wounds caused by this procedure can lead to programmed cell death (apoptosis) and premature cell death (necrosis) [10, 11]. Laser chondroplasty is another alternative used to remove damaged cartilage. However, potential complications caused by the use of lasers include thermal damage to the surrounding tissue, air bubble formation, and necrosis [12]. Osteotomy is a more invasive corrective technique in which parts of the bone are cut and shortened or lengthened in order to correct for misalignments. Surfaces are also adjusted during the osteotomy procedure such that the surfaces with cartilage remaining can articulate against each other [13].

Other arthroscopic procedures have also been developed that focused on inducing natural cartilage formation (chondrogenesis). These procedures were designed to penetrate the subchondral bone and thereby induce a spontaneous repair reaction by fibrin clots, infiltration of blood and marrow cells, cytokines and growth factors [14]. The most common of these procedures are abrasion chondroplasty, Pridie drilling, microfractures and spongialization [14]. Abrasion chondroplasty is surgical removal of cartilage to reach bone-marrow spaces [8]. In the microfractures procedure, which is comparable to Pridie drilling at a smaller scale, the subchondral bone is reached by drilled holes 0.5-1.0 mm in diameter which are distributed across the site of abnormality [8]. Spongialization involves removal of the subchondral bone plate such that the underlying cancellous bone is revealed [8]. Repair tissue is then laid down during bleeding and subsequently sets in the blood clot [8]. While these procedures were found to induce natural tissue repair responses, they are also very invasive and the regenerated tissue had variable composition, structure, and durability [8].
Due to limitations in the healing process of cartilage, tissue transplantation of healthy cartilage is an area of research that has gained much attention. In tissue transplant procedures, the affected cartilage is removed and the void is filled with osteochondral grafts which are cylindrical cut-outs of full thickness cartilage attached to the underlying bone [14]. Tissue grafts used in the procedure could be taken from non-weight bearing areas from the same joint of the patient (autografts) or from cadavers (allografts). The use of autografts is burdened with two concerns; the limited sources for harvesting autografts, and the destruction of otherwise healthy joint areas. While allografts do not share these harvest problems, allograft usage can lead to other problems such as an immunological response, possible transmission of diseases, and cell death as a result of prior cryopreservation of the replacement tissue [8]. Transplanted tissue can degrade due to a lack of lateral mechanical support, zones of death created by drilling, and injurious compressions sustained by the cartilage during surgical hammering [8]. Grafts made from the perichondrium (the fibrous membrane surrounding the cartilage of developing bone) are also used in some cases because of its chondrogenetic capabilities. However, the tissue formed usually contained abnormalities in composition and structure [14].

1.3 Tissue Engineering
Cartilaginous tissues that are suitable for transplantation are not readily available. The ideal tissue to be implanted is similar to native tissue in composition, structure and mechanical properties. Tissue engineering is the implementation of disciplines in cellular biology, engineering and materials science to create suitable tissues for implantation [1]. Due to the basic nature of articular cartilage (it is not connected to the vascular or nervous systems), there is much hope that tissue engineering can be a successful method of cartilage repair.
Tissue engineering typically makes use of three components: (i) cells, (ii) scaffolds and (iii) signals. Both chondrocytes (cartilage cells) and mesenchymal stem cells have been used for tissue engineering of cartilage. Chondrocytes are relatively easy to obtain, but they have limited regeneration potential. Mesenchymal stem cells have superior abilities of proliferation and differentiation, thus they are more able to replicate the microarchitecture and biochemistry of normal articular cartilage [13, 14]. However, mesenchymal stem cells are found almost exclusively in bone marrow, and their population ratio to other cells is typically between $1:10^4$ to $1:10^6$ and becomes more scarce with the increasing age of the donor [1, 7]. Scaffolds are biocompatible 3D porous structures used to anchor chondrocytes while permitting solubilized nutrients to flow through. Studies have shown that chondrocytes seeded in 3D scaffolds regenerated tissue that had more resemblance to normal cartilage than the tissue generated by chondrocytes seeded onto 2D surfaces [7, 13]. Scaffolds have been made from natural polymers (e.g. collagen, hyaluronate, fibrin, alginate, chitosan) as well as synthetic polymers (e.g. polyglycolic acid, polylactic acid) [15]. Scaffold-free methods of tissue engineering have also shown promise. Pellet cultures, or micromass cultures, are formed by centrifugation of a high density of chondrocytes. They have been found to synthesize characteristic components of cartilage such as type II collagen and proteoglycans (PGs) [16]. Over time, pellet cultures can achieve similar cellular distribution, matrix composition, density and ultrastructure to that of native cartilage [17]. However, further work is necessary in pellet culture research in order to overcome problems such as small tissue size and molding the tissue to fit defect sites [18]. Signaling molecules are usually used to stimulate differentiation, proliferation, and matrix deposition of chondrocytes. Commonly used molecules are transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1 (IGF-I), and bone morphogenic proteins (BMP) [13].
There are still many problems in tissue engineering that need to be addressed. Histological images of tissue regenerated by transplanted cartilage often showed lack of cellular and collagen architecture as observed in native tissue [7]. In addition, major problems include implant fixation, integration of native and implanted tissues, as well as uniformity in mechanical properties between the two tissues [7]. The implanted tissue must also be able to withstand the mechanical forces of joint motion [19].

1.4 Mechanical Stimulation
One method of improving tissue quality is through mechanical stimulation [1]. Native articular cartilage experiences complex loads throughout its lifetime from natural growth and physical exercise. Hence, mechanical stimulation of chondrocytes has gained much attention in efforts to correlate potential relationships between mechanical forces and chondrocyte processes. Indeed, studies have shown that chondrocytes seeded in 3D cultures responded to mechanical compression, tension and shear by altering cellular proliferation and biosynthesis of cartilaginous tissue constituents [20-23].

1.4.1 Case for Tensile Strain
Tension is not normally associated with articular cartilage in its native environment. However, the presence of tension in native cartilage can be identified both mathematically and experimentally. The natural behavior of materials subjected to compression dictates an increase in width, thus creating a barreling effect termed Poisson’s ratio. Therefore, while cartilage bears weight in the vertical direction, its horizontal plane of stress is actually in a state of tension. Furthermore, when an element is in a state of pure shear, tension is present at planes angled from the plane of shear stress (as demonstrated in Mohr’s circle [24]). In cartilage, shear can occur during joint motion when the tissue articulates against a viscous film of synovial fluid [25, 26].
Narmonova et al. used optical methods to measure cartilage strains due to swelling and found that strains in the superficial and middle zones were tensile but strains in the deep zone were compressive [27]. Fry found that upon complete removal from bone, full thickness cartilage became less convex at the surface over time (Figure 1.1) [28]. It was also noted that cartilage of thinner size deformed more intensely, suggesting tension was more prevalent at the surface of cartilage. Interestingly, cartilage removed from osteoarthritic joints did not change shape as drastically, if at all.

**Figure 1.1: Tension acting in articular cartilage.** Cartilage slices removed from the subchondral bone experienced uncurling effects, but osteoarthritic cartilage remained intact [29].
Similarly, Broom and Myers found strong crimp formations on the surface of excised cartilage [30]. When the tissue was subjected to tensile strains, the noted tissue crimps disappeared (Figure 1.2). Since the surface of native articular cartilage is normally smooth [31], the formation of crimps in removed cartilage further suggested that passive tension was present in the cartilage and it may be related to the curved surface of the underlying bone.
Figure 1.2: Crimps on the surface of removed articular cartilage. Tissue is (a) at rest; (b) in 0.07 strain; (c) in 0.12 strain; and (d) in 0.17 strain. Magnification is 950 times in all cases [30].

If the source of tension in native cartilage is its attachment to the curved underlying bone, then cartilage attached to bone undergoing skeletal development would theoretically experience higher amounts of tension compared to cartilage attached to bone that is skeletally mature.

Coincidentally, cellular proliferation and matrix synthesis rates of immature cartilage have been
found to be higher than that of adult cartilage [32, 33]. Therefore, it is possible that tensile strains stimulate cellular proliferation and biosynthesis, both functions of which are necessary for successful *in vitro* engineering of cartilage.

### 1.5 Research Outline and Objectives

The body of literature regarding mechanical stimulation of tissues is largely focused on compression and shear, with little attention paid to the effects of tension. Previous tensile stimulation studies have usually concentrated on the effects of dynamic tensile strains applied to the tissue constructs; however, the effects dynamic tensile strains have little physiological relevance since the main source of tension in native cartilage is likely static due to the attachment to the underlying bone. Thus, the purpose of this thesis was to determine the potential of mechanical tensile strains (static and dynamic) to accelerate growth of 3D cartilaginous tissues engineered *in vitro*. To achieve this purpose, this thesis was separated into 3 objectives: (i) identification of the optimal conditions of tensile stimulation, (ii) identification of the long-term effects of tensile stimulation, and (iii) exploration of the response of chondrocytes to other related factors, namely tensile strain frequency and tension stimulation-related changes in culture media pH. Therefore, three sets of experiments were conducted to meet these objectives.

In the first set of experiments, short-term intermittent tensile stimulation (static and dynamic) was applied to the chondrocyte cultures. Three different strain amplitudes and durations were applied every day for 3 days. The optimal tensile loading condition(s) was then identified based on the positive changes in proliferation and biosynthesis determined by radioisotope incorporation. The identified condition(s) were then used for future experiments. In the second set of experiments, the effects of long-term stimulation (up to 4 weeks) of the cultures were explored. Long-term stimulated cultures were analyzed in terms of changes in tissue thickness and weight, cellularity
and extracellular matrix (ECM) accumulation (determined by biochemical assays), histological appearance, and changes in mechanical properties. In the last set of experiments, the effects on chondrocyte response to load application frequency and culture media pH were investigated. A time course (1 week) was established for tensile stimulation of chondrocyte cultures at two different frequencies (once per day or once every 2-3 days) to quantify proliferation and ECM synthesis changes as determined by radioisotope incorporation. In separate chondrocyte cultures, the pH of culture media was adjusted to two different values (7.4 as per normal culture media and 6.7 for acidified culture media) and the consequent effects in proliferation and ECM synthesis to tensile strains were measured by radioisotope incorporation.
Chapter 2
Literature Review

2.1 Articular Cartilage Structure
Articular cartilage is the tissue covering the surfaces of contacting joints (Figure 2.1). Healthy cartilage lines the ends of articulating bones with a wear-resistant surface that is low in friction and capable of absorbing loads in multiple directions [2]. Friction within the joint is further reduced by synovial fluid, which is a viscous liquid that fills the joint cavity and contains nutrients to support cartilage homeostasis [2]. The structure of articular cartilage is complex. Its components are integrated with each other to form a network called the extracellular matrix (ECM) which can withstand large compressive forces, as well as shear and tensile forces [26]. Encapsulated within the ECM are chondrocytes, which are cells responsible for cartilage synthesis during growth, and tissue maintenance throughout its lifetime [2].
Figure 2.1: The human knee joint. Multiple articulating surfaces within the joint are covered with articular cartilage [34].

2.1.1 Chondrocytes

Chondrocytes are differentiated from mesenchymal stem cells and are the only cell type responsible for cartilage generation. Chondrocytes take up only 10% of the total tissue volume [2] and are few in number; their density in tissue is in the order of tens of millions per cubic centimeter [1]. Yet they induce cartilage growth and maintain cartilage homeostasis by cellular
proliferation and synthesis of ECM components. In adult cartilage, chondrocytes balance anabolic and catabolic processes to replace dead cells and damaged tissue. An imbalance between these processes would result in disease states, most notably osteoarthritis [5].

Research has indicated that mechanical loads can alter the orientation and shape of chondrocytes, perhaps due to the reorientation of matrix components [35]. Guilak and Mow suggested that the effective Poisson’s ratio of chondrocytes is likely related to the density of the surrounding ECM [36]. Compressive forces applied to chondrocytes also compressed their respective cell nuclei [36]. Mechanical stimuli may be translated through the cytoplasm by a network of intermediate filaments (e.g. vimentin) that connects the cell surface to the cell nucleus [37]. Mechanical properties of the chondrocyte cytoplasm and the cell nucleus were found to have magnitudes of the same order [38]. Chondrocytes are also known to respond to some soluble proteins (e.g. TGF-β1, IGF-I, and BMP) [13] and hydrostatic pressure changes [2].

2.1.1.1 Chondrocyte Metabolism
Energy production by the chondrocytes is achieved by anaerobic metabolism of glucose, which also leads to the production of lactic acid [2]. However, a buildup of lactic acid would lower extracellular pH and lead to decreases in chondrocyte biosyntheses [39].

2.1.2 Cartilage Biochemistry
The major components of articular cartilage are water, collagen and PGs [2]. Water and dissolved ions make up the interstitial fluid, which serves as both a structural component and a vehicle for nutrient transport within the tissue. Collagen fibres are wound around each other to form a densely interwoven network, providing structural support for the tissue. Large, charged
macromolecules called PGs reside within this network and bind with ions dissolved in the interstitial fluid to form a gel that is capable of resisting tissue compression.

2.1.2.1 Water
Water is the major component of articular cartilage, accounting for 75-80% of the total wet tissue weight [38]. This interstitial fluid plays a major structural role in articular cartilage by filling up the intrafibrillar space within individual collagen fibres, as well as the molecular pore space of the ECM [2]. Frictional resistance against interstitial fluid exudation is very high because the ECM is dense and composed of charged molecules which are attracted to ions dissolved in the fluid. Since water permeability through the tissue is very low, pressures built within the tissue are not easily relieved. The tissue is therefore able to sustain the high physiological loads applied [2]. Water also serves as a vehicle for transporting dissolved ions such as sodium, calcium, chloride and potassium to chondrocytes [2].

2.1.2.2 Collagen
Collagen is a major macromolecule measuring approximately 300 nm in length and 1.5 nm in diameter. It is composed of three polypeptide chains that follow a repeating amino acid sequence of glycine-X-Y where X and Y are most often proline and hydroxyproline respectively [2]. Glycine is the smallest of the amino acids and accounts for 33% of the total residues [2]. Glycine is present in every third position on the amino acid chain and acts to pull the three chains to the interior axis of the triple helical structure [2]. Proline accounts for 25% of the total residues and causes the three polypeptide chains to individually structure themselves in left-handed helical configurations [2]. The three separate left-handed chains assemble together in a right-handed configuration, thus enabling the collagen molecule to withstand large tensile forces. Hydroxyproline helps stabilize the collagen molecule by forming hydrogen bonds within the molecule [2].
Collagen macromolecules are categorized into 28 different types according to the manner in which cross-links are formed between the three polypeptide chains and between multiple triple-helical structures, and also according to their amino acid sequences. Type II collagen is the most prevalent in articular cartilage; it accounts for 90-95% of the total collagen content [2]. Type II collagen typically organizes itself by aligning head to tail, and side by side in a staggered array to form fibrils that contain overlaps and holes. Other collagens present in articular cartilage are types V, VI, IX, X and XI [2]. Covalent cross-links between multiple collagen chains result in the formation of an ECM that is complex, stable and strong [2].

2.1.2.3 Proteoglycans
PGs are large complex macromolecules that have important structural roles in articular cartilage. A PG macromolecule is made up of a protein core covalently bound to multiple polysaccharide chains called glycosaminoglycans (GAGs) [2] (Figure 2.2). The most notable GAG types found in cartilage are chondroitin sulphate, keratan sulphate, dermatan sulphate, and hyaluronate [2]. These chains contain carboxyl and sulphate groups which ionize in solution and attract positive counter ions (Ca$^{2+}$ and Na$^+$) from the interstitial fluid to obtain charge neutrality [2]. Water is recruited in the process due to osmosis and causes the tissue to swell. This is referred to as the Donnan osmotic pressure effect [2]. The large molecular nature of PGs prevents them from escaping the dense collagen network around them, and contributes to high intermolecular friction within the tissue. Water molecules are thus held within the ECM and form a gel with PG macromolecules that increases the strength and stiffness of the ECM. However, collagen fibrils can slide through the PG gel during loading and motion since PGs do not share covalent bonds with collagen fibrils [2].
Figure 2.2: Structure of a PG macromolecule. The PG is composed of multiple GAG units covalently bound to a protein core [40].

The most prominent PG molecule in cartilage is aggrecan; it accounts for 80-90% of PGs in cartilage [2]. Aggrecans can bind up to 100 chondroitin sulphate and 50 keratan sulphate GAG chains [2]. At one end of the molecule, a link protein binds the aggrecan to a hyaluronate [2]. Multiple aggrecans can bind to the hyaluronate backbone to form a PG aggregate (Figure 2.3). Aggregate size decreases with age and cartilage degeneration [2]. Other smaller PG types present in cartilage are biglycan, decorin, and fibromodulin, which are primarily located in the superficial region of the tissue [41, 42].
2.1.3 Zonal Organization

Three distinct layers make up articular cartilage: the superficial zone, the middle zone, and the deep zone [43] (Figure 2.4). Each zone differs from the other zones in biological composition and cellular orientation. The superficial zone makes up the top layer. It extends from the articulating surface to 10-20% tissue depth [44]. Chondrocytes in this zone are usually flattened in shape [35]. Water content is highest in this layer, which helps to lower the friction coefficient on the articulating surface (estimated to be 0.0075) [44]. The collagen fibers present in this zone are oriented parallel to the surface and in the direction of load articulation [45]. PG content is lowest in the superficial zone compared to the other zones [44].
Figure 2.4: Cross-section of articular cartilage. In this general representation of articular cartilage, the tissue is categorized into three zones in depth; superficial zone, middle zone, and deep zone. The regions in between chondrocytes are also outlined by the pericellular region, territorial region, and interterritorial region [43].

The middle zone accounts for 40-60% of the tissue thickness [2]. The chondrocytes in this zone are predominantly spherical in shape [35]. Collagen fibres are randomly distributed here [2]. PG content is highest in the middle zone compared to the other zones and are distributed homogeneously [2, 44].
The deep zone takes up the bottom layer of the tissue and accounts for approximately 30% of the overall tissue thickness [44]. The volumetric fraction of chondrocytes is smallest in this zone compared to the other zones [35]. Chondrocytes here are spherical in shape and are organized in a columnar fashion [2, 35]. The collagen fibres in this zone are oriented perpendicular to the articular surface [2]. There is a moderate PG concentration in this zone, while water content is lowest here compared to the other zones [2]. A zone of calcified cartilage lies between the deep zone and the underlying subchondral bone.

Articular cartilage composition differs not only in depth, but also with distance from the cell [2]. The regions are defined by compositional differences and are most commonly referred to as the pericellular region, territorial region, and interterritorial region [2]. The pericellular region completely surrounds each chondrocyte and contains mainly PGs and other non-collagenous matrix components [2]. The territorial region surrounds the pericellular region and is made up of thin collagen fibrils [2]. The interterritorial region fills the space between territorial regions. Collagen fibrils in the interterritorial region are of larger diameters and are usually oriented parallel to each other. This region contains the majority of PG content in the tissue, and accounts for the bulk of material properties of the tissue [2]. However, PG density is about twice as high in the pericellular region than the interterritorial region [2].

### 2.2 Cartilage Biomechanics

Cartilage is capable of withstanding large, repeating physiological stresses because the ECM is strong and durable. The compressive modulus of human cartilage has been measured to range between 0.51-1.82 MPa [46, 47], and the tensile modulus was measured to range between 0.9-20.7 MPa [48].
2.2.1 Biphasic Nature

Cartilage is often modeled as a biphasic material composed of a solid phase and a liquid phase. The solid phase refers to the permeable and deformable matrix of collagen fibrils and PGs, and the liquid phase refers to the interstitial fluid within the tissue. Even though the solid matrix is usually accepted as elastic material [36, 49], the interstitial fluid provides viscoelastic properties for the overall tissue. Viscoelastic materials are defined by time dependent responses to applied constant load, or applied constant deformation (Figure 2.5).

**Figure 2.5: Creep and stress relaxation response of viscoelastic materials.** Creep occurs when a constant load is applied to the material, and the material deforms increasingly over time until a constant strain is reached (top). Stress relaxation occurs when a material is deformed at a constant strain, and the resultant stress released by the material decreases over time until an equilibrium is reached (bottom) [2].
Intermolecular friction within the solid matrix actually causes the matrix to have some degree of viscoelasticity [50]. However, the bulk of viscoelastic properties of cartilage are attributed to the interstitial fluid. As physical stress is applied to the tissue, there is an initial pressure build up within the tissue. The high pressure causes interstitial fluid to overcome friction against the small pores of the solid matrix, and exude to low pressure regions [36]. Over time, the applied stress is redistributed from the liquid phase to the solid phase of the tissue [36]. The strain rate dependency of the elastic modulus of cartilage is therefore attributed to the changing water content, as well as the PGs present which attract the water molecules [3].

2.2.2 Tissue Charge Density

Soluble cations and anions within the interstitial fluid make up what is sometimes referred to as a third phase of cartilage [49]. Ionic concentrations within the tissue has been shown to have significant influence on cartilage behavior under unconfined compression loading [51]. In the uncharged tissue, the pressure buildup induced by tissue compression is at a maximum at the tissue centre. Over time, the pressure gradient within the uncharged tissue disappears. This is because the load is initially supported by the fluid within the tissue, then transferred to the solid ECM as fluid escaped, as explained earlier [36]. In the charged tissue, the load at equilibrium was supported not only by the ECM, but also by osmotic pressure and hydraulic pressure within the tissue [51]. Osmotic pressure built up within the tissue due to dissolved ions having integrated into the tissue. Hydraulic pressure built up within the tissue due to an inability of the fluid to escape the denser ECM. It was found that osmotic pressure within tissue can support up to 13-22% of the total load [51]. Using finite-element numerical modeling, hypothetical cartilage free of electric charges recoiled faster from step compressions than charged tissues [51].
2.3 Mechanical Stimulation

In an effort to identify stimulatory conditions for cartilage growth, numerous studies have been conducted to replicate the mechanical forces experienced by articular cartilage in its physiological environment. Researchers found that mechanical forces applied to cartilaginous tissues alter the osmolarity and electrical potential gradients around chondrocytes [49], and have the capability of changing the shape and volume of chondrocytes and their nuclei [38, 52, 53]. Further, mechanical stimuli have been found to cause changes in the rates of cellular proliferation and biosynthesis of ECM molecules. Described below is a summary of multiple types of mechanical stimulation studies (static compression, dynamic compression, shear, and tension) on cartilaginous tissues.

2.3.1 Static Compression

Since the main function of articular cartilage is to support compressive loads, the effects of static compression of articular cartilage were amongst the first stimuli to be studied. Chondrocytes subjected to static compression for long durations tend to inhibit biosynthesis regardless of how they were cultured. For example, cartilage explants (full thickness sections of excised tissue) compressed statically (by 17-52% thickness) for durations between 5 to 48 hours were found to have lower levels of cellular proliferation and ECM synthesis [21, 54-58]. Inhibition of biosynthesis was found to increase with the magnitude of static compression [55, 57, 59]. Chondrocytes seeded in 3D constructs of both agarose gels or on cartilage disks also displayed inhibitory effects on biosynthesis in response to static compression [20, 54].

There are probably multiple causes that contribute to the negative effects of static compression on chondrocyte metabolism. Buschmann et al. found that the inhibition as a result of static loading increased as the ECM content increased, suggesting that the load-induced inhibition may be
mediated by cell-matrix interactions, such as activation of receptors on the cell membrane [20]. Compaction of the ECM would also decrease tissue water content and hence reduce the availability of solubilized nutrients to the chondrocytes.

### 2.3.2 Dynamic Compression

The dynamic compressive loads applied to cartilage physiologically during daily activities (e.g. gait) have led researchers to study the effects of dynamic compression on chondrocytes. Dynamic compressions (3-6.8% strain amplitude, at 0.01-0.1 Hz frequency, for 4-48 hours) applied to cartilage explants were found to stimulate ECM synthesis and deposition in the surrounding matrix [21, 60, 61]. Chondrocytes seeded in agarose gels increased cellular proliferation and PG synthesis in response to the applied dynamic compressions (5-15% strain amplitude at 0.05-3 Hz frequency applied for 2-48 hours) [62, 63]. Interestingly, the effects of dynamic compressions on chondrocytes are frequency dependent, and the frequencies that stimulate proliferation may not stimulate biosynthesis [62] suggesting that proliferation and biosynthesis likely involve different signal transduction mechanisms.

Intermittent applications of dynamic compression to chondrocytes seeded on calcium polyphosphate substrates, or within foams or gels (polybutylene terephthalate scaffolds, agarose, peptide hydrogels, poly(ethylene glycol) hydrogels, fibrin gels) were also found to stimulate ECM synthesis [64-69]. In general, dynamic compressions that were applied intermittently elicited greater biosynthesis rates than dynamic compressions that were applied continuously [67]. The benefits of intermittently applied dynamic compressions are also reflected by increased tissue thickness and improved mechanical properties (modulus and strength) [64-67]. The resting period in between load applications may help reverse desensitization of chondrocytes, as has been observed after 60 minutes of continuous dynamic compression [70].
Since dynamic compression induces fluid flow in both the tissue interior and exterior whereas static compression does not, fluid flow is presumably a major cause for the positive effects in proliferation and biosynthesis. Kim et al. applied dynamic compression to cartilage explants and found that biosynthesis was stimulated greater in the peripheral regions than in the centre of the tissue [61]. This suggested that the stimulatory response of chondrocytes has more to do with fluid flow than changes in hydrostatic pressure. Likewise, Bonassar et al. found that the effect of insulin-like growth factor-I (IGF-I) on explant cultures were accelerated and augmented by dynamic compression [60]. According to Bonassar et al., the phenomenon was probably due to faster transport of the IGF-I via fluid flow by dynamic compression [60]. The stimulatory response elicited by dynamic compression probably also involves cell-matrix interactions. Multiple studies have found greater stimulation of ECM biosynthesis after a period of growth or when an increased amount of matrix has been synthesized [20, 67, 68].

2.3.3 Shear
Shear stress is also thought to be important during cartilage development since physiological shear is applied to the surface of articulating joints during joint motion. Researchers have therefore applied both mechanical shear (by lateral movement of clamped surfaces) and fluid-induced shear to chondrocytes and cartilaginous tissues. Cartilage explants responded to mechanically applied dynamic shear (1-3% shear strain amplitude, at 0.01-1 Hz frequency for 24 hours) by stimulating ECM synthesis [22]. Interestingly, the collagen synthesis increase caused by shear was two-folds greater than the increase in PG synthesis [22]. Mechanical shear applied to chondrocyte-seeded scaffolds produced similar effects. Waldman et al. found that mechanical shear applied intermittently (2-12% amplitude, at 1 Hz frequency, for 400 cycles) to chondrocytes seeded on calcium polyphosphate substrates increased matrix synthesis at low shear amplitudes,
but large shear amplitudes suppressed matrix synthesis [71]. Tissues that were exposed to intermittently applied shear over a 4 week period were found to be thicker and also contained more ECM [71]. Their mechanical properties were also improved; they had higher load bearing capacities and higher stiffness [71]. However, cellularity of the tissue was not influenced by the applied shear [71].

Fluid-induced shear was also found to be beneficial for cartilaginous tissues. Raimondi et al. found that dynamic fluid shear caused higher structural integrity in chondrocyte-seeded hyaluronate fibre scaffolds [72]. The chondrocytes exposed to fluid shear also did not have the membrane vesicles (typical of apoptosis) present on chondrocytes cultured in control conditions [72]. Chondrocytes grown in monolayer (2D) responded to fluid shear stress by elongating their shapes in the direction parallel to fluid flow and increasing PG synthesis [73]. The synthesized PGs were of increased length and greater hydrodynamic size [73].

In shear stimulation, the deformation of chondrocytes at low amplitudes is the more likely transduction mechanism for stimulating biosynthesis, as opposed to fluid flow. Unlike mechanical compression (static and dynamic), pure shear has the unique ability of keeping tissue volume constant so that no pressure gradient is formed, and as a result interstitial fluid flow is absent [2, 22].

2.3.4 Tension
As explained earlier (§1.4.1), there are reasons to assume that tension acts on cartilage in vivo and that application of mechanical tension to chondrocytes may stimulate growth. However, only one study relating to statically applied tension (16% strain amplitude, for 24 hours duration) has been conducted so far, and reported no significant effects on biosynthesis of chondrocytes [74]. All
other tensile stimulation studies focused on the effects of dynamic application of tension.
Research groups have mainly used either of two methods to apply dynamic tensile strains to
chondrocytic tissues grown in vitro: Flexercell™ vacuum pump to stretch monolayer (2D)
cultures of chondrocytes, and variations of clamps to physically stretch 3D chondrocyte-seeded
scaffolds.

Chondrocytes maintained in 3D culture responded to dynamic tensile strains by altering cellular
proliferation and ECM biosynthesis. The magnitude of proliferation and biosynthetic changes
was dependent on the duration and amplitude of tensile loading. Short applications (3 hours) of
dynamic tension upregulated (increased) the gene expressions of collagen (types I, II and X),
superficial zone protein, and inhibitors to matrix degradation (TIMP-2), but the expression of
bone markers was also upregulated (Cbfa1) [74, 75]. For longer applications (24-68 hours), small
strain amplitudes (5%) did not affect ECM biosynthesis [76] while large strain amplitudes (10-
20%) increased tissue cellularity [23], but inhibited ECM biosynthesis [23, 76]. However,
intermittent application of dynamic tensile strains for long durations (12 hours) increased PG
synthesis and accumulation [77], which further confirms that intermittent application of loads is
more beneficial than continuous applications. Tensile strains may also change the function of
chondrocytes. Vanderploeg et al. found that the application of tension caused deep zone
chondrocytes to synthesize more small sized PGs and large proteins (likely collagen molecules),
which are both characteristics of the superficial zone of cartilage [77]. The superficial zone is
known to have superior tensile properties due to the high amount of collagen present [47, 78].

Chondrocytes cultured in 3D scaffolds also responded to dynamic tensile strains by elongating
their shapes in the direction of load [74]. Interestingly, one research group found that a
subpopulation of chondrocytes changed shape to a three-dimensional stellate morphology (non-physiological) after tensile stretch was applied (Figure 2.6) [23]. The projections were determined to be comprised of F-actin filaments, vimentin, and vinculin with no preferential direction reported [23].

![Figure 2.6: Stellate morphology of chondrocytes seeded in 3D construct.](image)

Chondrocytes in 3D control constructs retained a rounded morphology up to 14 days in culture (A,D). Dynamic tension caused a subpopulation of chondrocytes to take on a 3D stellate morphology (B,C,E,F). Scale bars measure 100 μm [23].

Monolayer chondrocyte cultures responded to dynamic tensile stretch in a similar manner to 3D cultures; stimulation of cellular proliferation and biosynthesis were dependent on tensile strain duration and amplitude. Dynamic tensile strain applied for short durations (20-180 minutes) upregulated the expression of ECM genes (aggrecan and type II collagen), but longer durations (12 hours) of tensile strain caused either no effect or down-regulation [79, 80]. Proliferation was stimulated after 24 hours of tensile strain application [81] but inhibited after 48 hours [82]. Dynamic tensile strains (at 0-0.5 Hz frequency) of low amplitudes (2 kPa) were found to stimulate biosynthesis [81], but higher amplitudes (5-17 kPa) of tensile strains were inhibitory.
Dynamic tensile strains caused chondrocytes in monolayers to increase their expression of both anabolic and catabolic genes; growth factors (TGF-β1), inhibitors to matrix degradation (TIMP-1), proinflammatory cytokines (IL-1β, TNF-α), and mediators (NO and PGE₂) [79, 83]. Morphological changes in cell shape were also observed in response to dynamic tensile strains, with chondrocytes obtaining spindle-like morphologies (Figure 2.7) [83].

![Figure 2.7: Spindle-like morphology of chondrocytes in monolayer culture.](image)

Dynamic tension caused chondrocytes in monolayer cultures to transform into a spindle-like morphology (A), while chondrocytes in control cultures remained round (B). Scale bar measure 50 μm [83].

It is not clear how tensile strain elicits stimulatory proliferation and biosynthesis responses in chondrocytes. Dynamic tensile strains can similarly induce interstitial fluid flow, which has been shown by mechanical compression studies to be stimulatory. From a numerical model of cartilage in tension however, Park et al. noted that fluid flow is not the principal means of energy dissipation by cartilage under tensile loads, but rather energy is mainly dissipated by deformation of the solid ECM [84]. Cell-matrix interactions and cellular deformation may therefore be an
important gateway for mechanical signals to stimulate cellular responses, as suggested by mechanical and fluid shear studies.

Static application of tensile strain holds untapped potential in stimulating growth in cartilaginous tissues. It has the ability to hold chondrocytes in stretched positions for an extended period of time similar to their physiological states. This thesis explored the effects of statically applied tension on cartilaginous tissues, as well as the effects of dynamically applied tension for comparison. Tensile strains were applied intermittently as other studies have demonstrated that intermittent loading yielded more favorable results than continuously applied loads. Tensile strains were applied for short durations at low amplitudes according to the positive results of dynamic tension studies.
Chapter 3
Tension Apparatus

3.1 Loading Fixture Design

Loading fixtures were custom designed specifically for applying biaxial tensile strain (BTS) (radial and circumferential) stimulation to cartilaginous tissues grown in vitro (Figure 3.1). Engineered tissues were developed using a 3D culture system grown off the filter membrane surface of Millicell™ filter units (Millipore, Billerica, MA, USA) as described in §4.2. BTS was applied to the tissue by pushing the filter unit against a dome-shaped surface of the loading fixture, thereby biaxially stretching the pliable filter (Figure 3.2). Different magnitudes of BTS were achieved by pushing the filter unit to different depths against the fixture. Strains were relieved by allowing the filter unit to return to its resting position by a helical compression spring surrounding the dome-shaped protrusion. To ensure that air would not be trapped underneath the fixture, a 2.0 mm hole was placed on the side surface of the dome. A seat on the top of the helical compression spring retained the filter unit such that the central axis of the filter unit was aligned with the central axis of the dome, and the bottom surface of the filter unit was touching the apex of the dome. The loading fixtures were produced by a rapid prototyper (Stratasys, Eden Prairie, MN, USA) with 0.254 mm accuracy [85] and using acrylonitrile butadiene styrene (ABS) copolymer.
Figure 3.1: Loading fixture for tensile strain application. Design features of the loading fixture included a seat that aligned the filter unit in the correct loading position, and a helical spring that returned the filter unit to the unloaded position during rest, as shown in the isometric view (A). A cavity underneath the loading fixture led to a hole on the side, which allowed air bubbles trapped underneath the fixture to escape, as shown in the bottom view (B).

Figure 3.2: Resting position of Millicell™ filter unit on loading fixture. No BTS was applied to the tissue grown on the filter membrane at this resting position. BTS was applied by pushing down the filter unit on the loading fixture.

3.1.1 Validation of Loading Fixture

To determine the maximum allowable tension in the filter membrane, the integrity of the filter material was first investigated. Empty filter units were placed on top of different loading fixtures and the assemblies were compressed using a Mach-1 mechanical tester (Biosyntech, Laval,
Québec). Filter-fixture assemblies were compressed by increments of 200 μm at a rate of 20 μm/s, allowed to rest for 60 s, and then released at the same rate. By visual inspection, it was found that the filter membrane sustained permanent deformation when the assembly was compressed by 1.4 mm or more. Therefore, filter-fixture assembly compression was limited to a maximum of 1.4 mm.

The spring constant of the loading fixture was measured to determine how much force would be required to compress the filter-fixture assembly to a specified depth. To isolate testing to the spring on the loading fixture, filter membranes were removed from the filter units before the test was initiated. Spring constants were determined by using a Mach-1 mechanical tester (Biosyntech) to compress the filter-fixture assembly at a rate of 25 μm/s to a depth of 2 mm. The resistive compressive force throughout the test was measured using a 1-kg (9.81-N) load cell and the data was collected at a frequency of 10 Hz using Mach-1 Motion data acquisition software (Biosyntech). The spring constant (N/mm) was then plotted as a function of the loading fixture compression (Figure 3.3). It was found that the spring constant initially increased with increasing loading fixture compression up to 0.4 mm compression, and then remained constant up to 1.7 mm compression. Upon further compression beyond 1.7 mm, the spring constant increased dramatically. This dramatic increase correlated with the loading fixture approaching full spring compression, as observed by visual inspection. The spring constant curves were similar for loading and unloading, suggesting that damage was not sustained by the loading fixtures as a result of compression. Since it was previously determined that the filter unit could withstand a maximum compression of 1.4 mm, the spring constant of the assembly was determined by averaging the spring constant data collected between 0.4-1.4 mm filter-fixture assembly compression. The average spring constant of the assembly in compression was determined to be
$0.339 \pm 0.009 \text{ N/mm (n=7, mean \pm SEM)}$. The spring constant was then used to calculate the amount of force required to compress the filter-fixture assemblies, as outlined in §3.3 and §3.4.

![Graph showing spring constant profile](image)

**Figure 3.3: Spring constant profile of a filter-fixture assembly.** Spring constant increased from 0-0.4 mm compression, remained constant from 0.4-1.7 mm compression, and increased dramatically beyond 1.7 mm. Spring constant curves for loading and unloading were similar.

The next series of tests evaluated the durability of the loading fixtures. To isolate testing to the spring on the loading fixture (which was the smallest component of the fixture and experienced the most stress), filter membranes were removed from the filter units before the tests were initiated. The filter-fixture assemblies were then subjected to either static or dynamic compression using a Mach-1 mechanical tester (Biosyntech). Static compressions of 2 mm were applied at a rate of 25 $\mu$m/s, held for 60 seconds and then released. Similarly, dynamic sinusoidal compressions were applied with an amplitude of 2 mm at a frequency of 1 Hz for 10 cycles. All tested loading fixtures were visually inspected and determined to be intact and free from fatigue.
cracks. The seat on top of the spring returned to its original position: on a plane aligned to the apex of the dome.

3.2 Strain Profile Determination

BTS profiles were generated using ANSYS (version 11.0) finite element software (ANSYS, Canonsburg, PA, USA). Only the filter membrane and loading fixture dome were modeled, the cartilaginous tissue was not modeled due to the variability in physical and mechanical properties between the cultured samples. As such, strain data collected from the surface elements of the filter membrane were used to represent strains acting on the tissues grown on the membrane surface. Tensile loading was simulated using contact analysis routines in ANSYS to stretch the filter membrane against the surface of the dome. Strain results were collected from each element on the surface of the filter and BTS profiles were then plotted.

3.2.1 Finite Element Model

Representation of the filter-fixture interface was simplified to a 2D axisymmetric model to reduce computation costs. An axisymmetric model is the simplification of a symmetric 3D model to a representative 2D model on one side of the axis of symmetry, which in this case was the vertical axis of the dome. Planar elements (PLANE42) were used to model both the dome and the filter membrane. The elements were capable of plasticity, creep, stress stiffening, large deflection, large strain, and axisymmetric modeling [86]. The filter membrane was subdivided into 2,280 elements with an average width of 7 μm and an average height of 5 μm. To reduce computation costs, only regions of contact on the dome and the filter membrane were lined with contact elements (TARGE169 and CONTA171, respectively), which were capable of detecting contact and preventing overlaps between the two surfaces. The outer ring of the filter membrane did not come into contact with the dome in either the strained or unstrained positions and did not require contact elements (Figure 3.4).
Figure 3.4: Cross-sectional plane of filter-fixture assembly under tensile strain. The centre region of the filter membrane was in contact with the loading fixture dome, while the outer region of the filter membrane was not in contact.

Mechanical properties assigned to the loading fixture dome were based on published values for ABS plastic: friction coefficient of 0.5 [87], an elastic modulus of 1,627 MPa [88], and a Poisson’s ratio of 0.35 [89]. The filter membrane dimensions were measured by calipers and were 8.5 mm in diameter and 20 μm thick under wet conditions. Made of hydrophilic poly(tetrafluoroethylene) (PTFE) [90], the membrane was assigned a friction coefficient of 0.02 based on published values for PTFE [91]. The actual elastic modulus and Poisson’s ratio of the filter membrane were not available due to proprietary reasons, so the effective elastic modulus (E*) and effective Poisson’s ratio (μ*) used in the model were estimated based on mechanical properties of 100% dense PTFE and recalculated for its 80% porosity [92] using equations derived by Ramakrishnan and Arunachalam [93]:

\[
E^* = \frac{E(1-\theta)^2}{(1+b_\theta \theta)} \tag{3.1}
\]

\[
\mu^* = \frac{(1/4)(4\mu + 3\theta - 7\mu\theta)}{(1 + 2\theta - 3\mu\theta)} \tag{3.2}
\]
where $E$ and $\mu$ are the elastic modulus and Poisson’s ratio with no porosity, $\theta$ is the porosity and $b_\theta = (11-19\mu)/(4+4\mu)$. The nominal mechanical properties of 100% dense PTFE and the calculated effective mechanical properties of porous PTFE are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Mechanical Property of Membrane</th>
<th>Nominal Value</th>
<th>Effective Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastic Modulus</td>
<td>393 MPa [91]</td>
<td>12 MPa</td>
</tr>
<tr>
<td>Poisson’s Ratio</td>
<td>0.46 [94]</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Table 3.1: Mechanical properties of filter membrane. The effective values were calculated based on 100% dense PTFE and corrected for 80% porosity using equations derived by Ramakrishnan and Arunachalam [93].

The boundary conditions used in the model were chosen to reflect the experimental conditions as well as to guide loading of the filter membrane. Elements on the edge of the filter membrane were bound to zero movement in the horizontal and vertical directions as the membrane was bound to the filter unit. Elements on the bottom surface of the loading fixture dome were bound to zero horizontal movement. The centre elements of both the membrane and dome were bound in the horizontal direction as this was the axis of symmetry for the axisymmetric model.

3.2.2 Loading of Finite Element Model

To reduce complexity in the model for BTS application, the actual downward translation of the filter membrane against the loading fixture dome was instead modeled as an upward translation of the dome against the membrane. The results generated by this loading configuration should ideally be the same as those generated by a downward translation of the membrane since the geometry and the magnitude of the force applied are the same in both configurations. To restrict dome travel to distances equal to the compression depths applied in the actual experiments, the
maximum vertical translation of the elements on the bottom surface of the dome were adjusted for each compression depth simulated. Other boundary conditions were also set to accommodate for the upward translation of the dome (§3.2.1) which was achieved by applying an arbitrary upward force of 0.5 N to elements on the bottom surface of the dome (Figure 3.5).

Figure 3.5: Axisymmetric finite element model of the filter-fixture interface. Triangles denote the boundary conditions applied to the model, and arrows denote an arbitrary force distributed equally along the bottom surface of the dome. Surface protruding lines denote contact elements which lined the contact surfaces. Shadows on this image of the dome were formed by the condensation of lines outlining each element, but the model is actually in 2D. A higher number of elements were used to model the contact regions than the non-contact regions.
3.2.3 Results
The radial and circumferential strains applied to the filter membrane were determined from strain results of the finite element model returned by ANSYS. Strains in the $x$ and $z$ directions of all surface elements of the filter membrane were recorded as radial and circumferential strains, respectively, and plotted as functions of radial distance from the centre of the filter. The plotted strain profile due to 1.1 mm spring compression showed equibiaxial strain (equal radial and circumferential strains) acting at the centre of the membrane (Figure 3.6). The radial strains remained fairly constant from the centre to the edge of contact between the membrane and the dome. At the edge of contact, the radial strains dropped sharply and then gradually decreased towards the filter unit wall. Compressive radial strains were observed to develop at the membrane perimeter. This was likely due to folding effects on the membrane perimeter caused by attachment of the membrane perimeter to the filter unit wall and lifting of the membrane at its centre. The circumferential strains decreased dramatically from the centre of the membrane to the edge of contact and then gradually decreased to zero at the membrane perimeter. Both radial and circumferential strains displayed unexpected, small fluctuations between 0-0.03% which were present even in duplicated models with a higher number of elements. These fluctuations may have been due to small strain calculation errors by ANSYS; however, the small magnitude of the fluctuations and the continuity of the radial and circumferential strains (except at the edge of contact) suggested that the filter-fixture interface was modeled with enough elements, and that the returned values were reliable.
Figure 3.6: Biaxial tensile strain profile of filter membrane. This strain profile was representative of a 1.1 mm compression of the loading fixture spring. The profile showed an equibiaxial strain state at the centre of the membrane (0.0 mm), and a discontinuity at the edge of contact between the membrane and dome (1.91 mm).

Three different magnitudes of spring compression were modeled: 0.4 mm (small), 0.7 mm (medium), and 1.1 mm (large). Strain results from the three magnitudes of spring compression yielded comparable profiles and are presented in Appendix A. Representative strain values from the three different magnitudes of spring compression are listed in Table 3.2. The average radial and circumferential strains across the entire filter membrane at the three different magnitudes of spring compression are presented in Table 3.3. Frictional effects in the simulation were studied by comparing the strain results of models with defined friction coefficients (§3.2.1) to the strain results of models with zero friction coefficients for both surfaces. The largest difference between elements of the same radial positions in the two models was 0.06% for radial strain and 0.03% for circumferential strain.
### Table 3.2: Descriptive strain values of filter membrane.
Increasing amplitudes of spring compressions yielded increasing radii of contact and increasing amplitudes of radial and circumferential strains.

<table>
<thead>
<tr>
<th>Spring Compression [mm]</th>
<th>Contact Radius [mm]</th>
<th>Radial Strain [%]</th>
<th>Circumferential Strain [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Centre</td>
<td>Edge of Contact</td>
</tr>
<tr>
<td>0.4 (Small)</td>
<td>0.78</td>
<td>0.85</td>
<td>0.62 – 0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.7 (Medium)</td>
<td>1.27</td>
<td>1.98</td>
<td>1.69 – 1.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 (Large)</td>
<td>1.91</td>
<td>4.28</td>
<td>3.93 – 4.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3.3: Average strain values of filter membrane.
Increasing amplitudes of spring compression yielded increasing average radial strain and increasing average circumferential strain.

<table>
<thead>
<tr>
<th>Spring Compression [mm]</th>
<th>Average Radial Strain [%]</th>
<th>Average Circumferential Strain [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 (Small)</td>
<td>0.51</td>
<td>0.23</td>
</tr>
<tr>
<td>0.7 (Medium)</td>
<td>1.54</td>
<td>0.74</td>
</tr>
<tr>
<td>1.1 (Large)</td>
<td>3.79</td>
<td>2.13</td>
</tr>
</tbody>
</table>

### 3.3 Static Tensile Stimulation
To apply static BTS to the tissues grown on the surface of the filter membranes, rapid prototyped spacers (Stratasys) of defined thicknesses were placed between the lid of the culture plate and the filter-fixture assembly. To compensate for the height difference between the filter-fixture assembly and the culture plate well (in which the filter-fixture assembly was maintained), spacers were constructed 1.6 mm thicker than the desired spring compression required to achieve the intended BTS in the filter membrane (Figure 3.7).
Figure 3.7: Spacer used for static BTS application. Spacers were designed to fit within the wells of a culture plate, and compensate for the height difference between the well and the filter-fixture assembly. This particular spacer (2.3 mm thick) was designed for 0.7 mm compression of the loading fixture spring.

Weights of 3.90-4.90 N were then placed on top of the culture lid to compress the filter-fixture assembly to specific depths. To ensure the weights were adequate for overcoming the combined force exerted by the springs of loading fixtures, no more than eight filter-fixture assemblies were placed in each 24-well plate. Eight springs with an average spring constant of 0.339 N/mm (§3.1.1) placed in parallel and compressed by 1.1 mm (the maximum compression applied in experiments) would exert a force of 2.99 N according to Hooke’s law:

\[ F = (n \times k) \times h \]  

where \( F \) is the exerted force, \( n \) is the number of springs, \( k \) is the spring constant, and \( h \) is spring compression.

3.4 Dynamic Tensile Stimulation

A loading jig was designed for use with the Mach-1 mechanical tester (Biosyntech) in conjunction with the filter-fixture assemblies to apply oscillatory BTS to the cartilaginous tissues.
grown on the surface of the filter membranes (Figure 3.8). Instead of spacers, plungers were placed on top of filter-fixture assemblies and secured to a sleeve which was then attached to the Mach-1 actuator (Biosyntech). No more than six filter-fixture assemblies were compressed at one time by the Mach-1 actuator (Biosyntech), which was capable of applying 49.1 N in compression [95]. Sinusoidal compression was applied at a frequency of 1 Hz with the amplitude corresponding to the desired spring compression required to achieve the intended BTS in the filter membrane.

**Figure 3.8: Exploded view of dynamic tensile strain rig.** Filter unit-loading fixture assemblies were compressed by plungers which were secured to a sleeve by screws. The sleeve itself was secured to the Mach-1 mechanical tester by another screw. A setter was used for vertical alignment, and a custom plate lid ensured sterility during testing.
Chapter 4
Materials and Methods

4.1 Bovine Chondrocytes Isolation

The articular chondrocytes used to grow cartilaginous tissues were obtained from the metacarpal-phalangeal joints of 12- to 18-month-old calves provided by Brian Quinn’s Meats (Yarker, ON). In a laminar flow hood sterilized by UV light, cartilage sections were aseptically extracted from the metacarpal condyles of the joint. The cartilage sections were placed into a Petri dish and digested in 0.5\% protease (w/v) (Sigma-Aldrich, Oakville, ON) in 24.5 mL of Ham’s F12 media (HyClone, Logan, UT, USA) supplemented with 25 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) (Sigma-Aldrich) and 0.5 mL of an antibiotic solution containing: 100 Units/mL penicillin, 100 $\mu$g/mL streptomycin and 0.25 $\mu$g/mL amphotericin B (Sigma-Aldrich) [96]. The Petri dish was placed in an incubator kept at 37°C with 95\% relative humidity and 5\% CO$_2$. After 90 minutes of incubation, the media was replaced with 20 mL of 0.15\% collagenase A (w/v) (Roche Diagnostics Canada, Laval, QC) dissolved in Ham’s F12 media and incubated for an additional 18 hours. The digested tissue mixture was passed through a 200 mesh filter (Sigma-Aldrich) to remove undigested tissue and bone fragments. The isolated chondrocytes were centrifuged at 600 RCF for 7 minutes, and the resulting cell pellet was washed with Ham’s F12 media three times. Viability of the isolated cells was then determined using the Trypan Blue (Sigma-Aldrich) dye exclusion assay [97].

4.2 Tissue Culture

Viable, isolated chondrocytes were seeded in high density, 3D culture on the surface of Millicell™ filter units (Millipore) in order to synthesize cartilaginous tissue. To prepare the filter units for chondrocyte-seeding, sterile filter units (Millipore) were placed into 24-well culture
plates and subsequently coated with 100 μL of 0.5 mg/mL type II collagen (from chicken sterna) (Sigma-Aldrich) dissolved in 0.1 N acetic acid and then dried overnight. Prior to use, filter units were sterilized by UV light in a laminar flow hood for 30 minutes and washed two times with Ham’s F12 culture media supplemented with 5% fetal bovine serum (FBS) (v/v) (Sigma-Aldrich). Chondrocytes were then seeded onto the filters at a density of 35,000 cells/mm² and maintained in 1 mL of 5% FBS (v/v) in Ham’s F12 media. Culture media was replaced every two days and the FBS concentration was increased to 20% (v/v) on the fifth day of culture. Similarly, the media was further supplemented with 100 μg/mL ascorbic acid (Sigma-Aldrich) beginning on the seventh day of culture. Culture media was then replaced every 2-3 days for the duration of the culture period. After a two-week culture period, filter units were placed on the loading fixtures described in §3.1 in preparation for BTS stimulation. Media volume in each well was increased from 1 mL to 1.5 mL to compensate for the increased height of the filter unit due to the loading fixture, thereby ensuring the cartilaginous tissue culture remained submerged in media.

4.3 Biaxial Tensile Strain Stimulation

Biaxial tensile strain (BTS) stimulation was applied to cartilaginous tissue cultures after a two-week culture period using the tension apparatus described in Chapter 3. No more than eight filter-fixture assemblies were placed in each 24-well plate and BTS was applied inside an incubator kept at 37°C. Filter units were kept on top of the loading fixtures during rest periods as were the control (unstimulated) cultures. BTS stimulation was applied to the tissue cultures in three different experiments: (i) determination of optimal mode, amplitude and duration, (ii) the effects of long-term stimulation, and (iii) the effects of loading frequency.
4.3.1 Optimal Mode, Amplitude and Duration

This short-term experiment was conducted to find the optimal mode (static versus dynamic), amplitude and duration of BTS for stimulating cellular proliferation and ECM biosynthesis (PG and collagen synthesis) in the tissues. After two weeks of growth, tissue cultures were either subjected to intermittent BTS or maintained unstimulated (control) under identical conditions. Intermittent BTS was applied either statically or dynamically at one of three amplitudes (small, medium or large, as defined earlier in §3.2.3) for a duration of 15, 30 or 60 minutes. For those samples exposed to dynamic tension, BTS was applied using a 1 Hz sinusoidal waveform. BTS was applied once every 24 hours for three consecutive days. Immediately after the third application of BTS stimulation, cellular proliferation, PG and collagen synthesis were measured using radioisotope incorporation (§4.6.4). Proliferation and biosynthesis results from each condition were compared statistically (§4.8) to determine the optimal tensile strain mode, amplitude and duration.

4.3.2 Effects of Long-Term Stimulation

The cumulative effects of BTS on the physical, biochemical and mechanical properties of stimulated tissues were measured in this long-term experiment. The optimal tensile strain mode, amplitude and duration as determined from the previous experiment (§4.3.1) were applied to two-week-old tissue cultures under two different loading frequencies: (i) once every 24 hours and (ii) only on the days when the culture media was replaced, which occurred once every 2-3 days. Control tissue cultures were maintained under identical conditions, but without BTS stimulation. Under either loading frequency, tissues were harvested after 2 and 4 weeks of long-term BTS stimulation for evaluation. Harvested tissues were measured to determine resultant tissue thickness (§4.4) followed by mechanical property assessment (§4.5). Immediately following mechanical property assessment, tissues cultures were removed from the filter units (§4.6.1), and measured for tissue wet and dry weight (§4.6.2). Tissues were then digested by papain (§4.6.3)
and aliquots of the digest were assayed for DNA, PG and collagen contents (§4.6.5). All results were compared to control cultures that were harvested at the same time as the stimulated cultures. Additional tissue culture samples from each group (stimulated and unstimulated) that were not subjected to thickness or mechanical measurements were also collected for histological and immunohistochemical assessment (§4.7).

4.3.3 Effects of Loading Frequency

To gain insight into the physical and biochemical differences between the tissues stimulated under the two loading frequencies in the previous experiment, a separate experiment was conducted in which the anabolic effects of BTS stimulation were measured. BTS under the optimal mode, amplitude and duration as previously determined (§4.3.1) were applied to two-week-old tissue cultures for a period of one week. Tissue cultures were stimulated at either of the two previously applied loading frequencies (once per 24 hours or once every 2-3 days). Age-matched control tissue cultures were maintained under identical conditions, but without BTS stimulation. Cellular proliferation and PG synthesis were measured every day during the week of BTS application using radioisotope incorporation (§4.6.4). Collagen synthesis was not determined in this experiment due to the minimal effect observed during the previous short-term experiment (§5.1).

To determine the potential effect of conditioned culture media pH on cellular proliferation and PG synthesis elicited by BTS stimulation, the pH of the conditioned culture media of tissue cultures stimulated under both loading frequencies (once per 24 hours or once every 2-3 days) was measured at both 24 and 48 hours after BTS stimulation. Media pH was measured using an Accumet model AP71 pH probe (Fisher Scientific, Pittsburgh, PA, USA). A separate set of two-week-old tissues were stimulated by BTS under the optimal conditions once every 24 hours for
three consecutive days. On the third day before BTS stimulation, the culture media was replaced with fresh media adjusted to a lower pH level that corresponded to the pH of the conditioned tissue culture media 48 hours after BTS stimulation as measured earlier. The tissue cultures were then stimulated for a third time, after which cellular proliferation and PG synthesis were measured using radioisotope incorporation (§4.6.4). The results were compared to tissue cultures that were stimulated for three consecutive days with normal culture media replacement on the third day.

4.4 Tissue Thickness Measurements

Tissue thickness was measured using a needle probe method reported by Hoch et al. [98]. Filter units were placed on a stainless steel stage with a flat surface such that the bottom surface of the filter membrane rested on the surface of the stage. A 25Ga needle (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was attached to a 1-kg (9.81-N) load cell of a Mach-1 mechanical tester (Biosyntech) which was then displaced towards the filter membrane at a rate of $5 \mu m/s$. Resistive compressive forces (measured by the load cell) and needle displacement were recorded at a frequency of 10 Hz using the Mach-1 Motion data acquisition software (Biosyntech). On a plot of resistive compressive force as a function of needle displacement, changes in force were interpreted as either the initial contact between the needle and the test material, or contact with the underlying stainless steel stage such that the test material was fully penetrated by the needle. Three bare filters of 20 $\mu m$ thickness (as determined using digital calipers) were first tested which yielded similar results. Before the needle contacted the filter, there was little change in the measured force. However, upon initial contact between the needle and the filter, there was an observed inflection in the force versus displacement plot, followed by an appreciable increase in force with increasing needle displacement (Figure 4.1). The force was observed to decrease slightly after an additional 15-20 $\mu m$ from the initial contact point which
corresponded to the thickness of the filter. Beyond this point, the measured force increased again with increasing needle displacement. Thus, thickness of the material could be measured from determining the displacement between the first force inflection point (initial contact with the material) and the slight drop in force (initial contact with the support stage).

![Figure 4.1: Resistive force response of needle on a bare filter membrane.](image)

Thickness of the bare filter membrane was measured as the displacement between the first force inflection point (initial filter contact) and the slight drop in force (filter punctuation).

With the presence of a tissue layer on the filter material, resistive needle forces increased more gradually (Figure 4.2). Upon initial force inflection, forces increased at a much slower rate compared to the rates of force increase measured for bare filter membranes. Resistive forces increased steadily over a considerable displacement (27-251 μm), after which the forces increased at a much higher rate (0.10 N/μm) similar to the rate at which forces were measured for bare filter
membranes. Thus, the initial point of contact with the filter membrane was determined to be the point at which the slope of the curve reached 0.10 N/μm. Tissue thickness was therefore identified as the displacement between the initial inflection point and the point at which the slope of the curve reached 0.10 N/μm. The thickness of each tissue sample was measured at two different locations within the tissue and the average value was recorded. All thickness measurements were conducted in culture media at 37°C.

Figure 4.2: Resistive force response of needle on a tissue-filter assembly. Thickness of the tissue culture sample was measured as the displacement between the first force inflection point (initial tissue contact) and point of considerable force increase (initial filter contact).
4.5 Indentation Tests

Mechanical strength and stiffness of the cartilaginous tissues were determined using indentation tests. After tissue thickness was measured, tissue cultures still within their filter units were placed flush on the surface of a porous steel stage. A plane-ended indenter with a diameter of 2.10 mm was attached to the 1-kg (9.81-N) load cell fitted to the Mach-1 mechanical tester (Biosyntech) which was used to indent the tissue samples. Resistive compressive force and indenter position data were recorded at a frequency of 10 Hz using Mach-1 Motion data acquisition software (Biosyntech). Contact between the indenter and the tissue was first achieved by preloading the tissue to 0.005 N which was then defined as the zero-strain state. Compression was applied in incremental steps of 2% strain to a maximum of 20% strain, which was calculated using tissue thickness measurements (§4.4). Each compressive step was held until equilibrium was reached, which was defined as a change in force of less than 0.002 N/min. To account for noise, the equilibrium force of each step was averaged from the last 3 seconds of recorded forces at each step. The equilibrium force recorded at 20% strain was used to calculate the equilibrium stress (equilibrium force normalized to the cross-sectional area of the indenter) and the equilibrium elastic modulus using the expression derived by Hayes et al. for indentation testing of cartilage [99]:

\[
E = \frac{P(1-v^2)}{2awK}
\]  

(4.1)

where \(P\) is equilibrium load, \(v\) is Poisson’s ratio (\(v = 0.4\) for bovine articular cartilage [2]), \(a\) is indenter radius, \(w\) is displacement, and \(K\) is a correction factor based on \(v\) and the aspect ratio of \(a\) to tissue thickness \(h\). The \(K\) value for each tissue culture sample was interpolated from values
provided by Hayes et al. (Table 4.1) [99]. Equation 4.1 was derived based on a model of articular cartilage and the underlying subchondral bone as an infinite elastic layer bonded to a rigid half space (everything underneath the elastic layer). After indentation, cultures were allowed to relax for 30 minutes at 37°C. The data of a sample indentation test is provided in Appendix B.

**Table 4.1: Values used to interpolate for correction factor K.** These values were provided by Hayes et al. for indentation of cartilage by a plane-ended cylindrical indenter and a Poisson’s ratio of 0.4 for the material [99].

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### 4.6 Tissue Biochemistry

#### 4.6.1 Tissue Harvest

Cartilaginous tissues (along with the attached filter membrane) were cut out from the filter units using a No. 11 scalpel blade. Samples were rinsed three times in phosphate buffered saline (PBS) at pH 7.4 and blotted dry with weighing paper, before being placed into 1.5-mL microcentrifuge tubes.
4.6.2 Wet and Dry Weight Measurements
The mass and water retaining ability of the developed tissues were quantified by measuring the wet and dry weights of the tissues using a model P-114 balance (Denver Instrument, Denver, CO, USA). Since the tissues were still attached to filter membranes, wet tissue weight was determined by subtracting the wet weight of filter membranes from the total measurement. The tissues were then lyophilized (-45°C under vacuum) overnight, and dry tissue weights were measured the next day. Dry tissue weight was determined by subtracting the dry weight of filter membranes from the total measurement. To determine the wet and dry weights of the filter membrane alone, filters were removed from unused filter units using a No. 11 scalpel blade. After their dry weights were measured, the filters were soaked in water for 10 seconds, blotted dry with weighing paper, and measured for their wet weights. Three bare filter membranes were found to have an average dry mass of 1.3 mg and an average wet mass of 1.6 mg.

4.6.3 Tissue Digestion
To measure the mass of accumulated biochemical constituents in the developed tissues, tissue cultures were first digested by papain (40 μg/mL) (Sigma-Aldrich) in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid, and 2 mM dithiothreitol (Sigma-Aldrich) for 72 hours at 65°C. Digested tissues were stored at -20°C until needed for analysis.

4.6.4 Cellular Proliferation, Proteoglycan and Collagen Synthesis
Proliferation and ECM synthesis rates were measured to quantify the anabolic response of the cells to mechanical stimuli. Incorporation of [³H] thymidine has been shown to correlate with cellular proliferation [100] and incorporations of [³⁵S] sulphate and [³H] proline have been shown to correlate with the synthesis of PGs [101] and collagen [102], respectively. Thus, cellular proliferation and ECM synthesis were measured by supplementing the culture media with the radioisotopes (obtained from PerkinElmer, Waltham, MA, USA) at a concentration of 5 μCi per
isotope per culture. Cultures were incubated with the radioisotopes for a period of 24 hours, after which the unincorporated radioisotopes were removed by rinsing the samples three times in PBS after tissue harvest (§4.6.1). Tissues were then digested with papain (§4.6.3) and radioisotope incorporation was determined from aliquots of the tissue digest measured by a β-liquid scintillation counter (Beckman Coulter, Mississauga, ON). PG and collagen syntheses were normalized to the measured DNA content of the tissue (§4.6.5).

4.6.5 DNA, Proteoglycan and Collagen Accumulation
Accumulated DNA, PG and collagen content of the developed tissues were measured from aliquots of the papain digest (§4.6.3) using established biochemical assays. DNA content was measured using the Hoechst 33258 (Sigma-Aldrich) dye binding assay and fluorometry as described by Kim et al. [103]. Tissue digest aliquots were diluted with PBS, pH 7.4 immediately prior to performing the assay. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Standard curves were generated using calf thymus DNA (Sigma-Aldrich) and used to correlate fluorescence measurements to DNA content. This assay was performed in 96-well fluorescence plates (VWR International, Mississauga, ON). All standards and samples were measured in triplicate.

PG content of the tissue was estimated by measuring the sulphated GAG content using the dimethylmethylene blue dye (Polysciences, Warrington, PA, USA) binding assay and spectrophotometry, as described by Farndale et al. [104] and later improved by Goldberg et al. [105]. Tissue digest aliquots were diluted with 1% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS, pH 7.4 immediately prior to performing the assay. Absorbance was measured at a wavelength of 525 nm. Standard curves were generated using bovine cartilage chondroitin sulphate A (Sigma-Aldrich) and used to correlate absorbance to GAG content. This assay was
performed in standard 96-well assay plates (VWR International). All standards and samples were measured in triplicate.

To quantify collagen content in tissues, hydroxyproline content was measured and assumed to account for 10% of the total collagen mass [106]. Hydroxyproline content was measured using the chloramine-T/Ehrlich’s reagent assay and spectrophotometry described by Woessner et al. [107]. Aliquots of the papain digest were hydrolyzed in 6 N HCl for 18 hours on a heating block kept at 110°C. Samples were neutralized with 5.7 N NaOH and diluted with distilled water. Aliquots of the hydrolysate were then reacted with chloramine-T and Ehrlich’s reagent (Sigma-Aldrich) and then the absorbance of the sample was measured at a wavelength of 560 nm. Standard curves were generated using L-hydroxyproline (Sigma-Aldrich) and used to correlate absorbance to hydroxyproline content. This assay was performed in standard 96-well assay plates (VWR International). All standards and samples were measured in triplicate.

### 4.7 Histological and Immunohistochemical Evaluation

Histological and immunohistochemical analyses were performed to provide a visual depiction of the developed tissues. Immediately after harvest, tissue samples attached to the filter membranes were rinsed three times in PBS at pH 7.4, fixed in 4% paraformaldehyde overnight at 4°C, and stored in 70% ethanol until further processing as ethanol helps to reduce solubility of GAG side chains [108]. Fixed tissues were embedded in paraffin blocks, cut into 5 μm sections and bound to glass slides, all of which were performed by Mr. John DaCosta in the Department of Pathology and Laboratory Medicine, Queen’s University, Kingston, ON.

For histology staining, tissue sections were first immersed in toluene to remove the paraffin followed by immersion in a series of graded alcohol and water solutions to rehydrate the tissue
sample. Tissue sections were stained with either Gill’s hematoxylin and eosin (general connective tissue stain used to stain nuclei dark blue and proteins pink, respectively) or toluidine blue (to stain sulphated PGs blue). After staining, the tissues were dehydrated by sequential immersion in alcohol and mounted with PRO-TEXX mounting medium (Fisher Scientific) and examined by light microscopy.

Immunohistochemical methods were used to identify type I collagen and type II collagen in the developed tissues. Using a procedure similar to the one mentioned above, the paraffin in the sections was first removed and then tissue sections were dehydrated by immersion in alcohol and re-hydrated by immersion in water for a short duration. Prior to antigen staining, tissue sections were subjected to enzymatic pretreatment in order to facilitate primary antibody binding. For the sections stained for type II collagen, sections were first digested in 0.25 % trypsin (w/v) (Sigma-Aldrich) in Tris-buffered saline (50 mM Tris with 150 mM NaCl), pH 7.6, for 30 minutes at room temperature. All sections (for type II and I collagen) were then treated with 0.5 units/mL chondroitinase ABC (Sigma-Aldrich) in Tris-acetate buffer (40 mM Tris acetate with 1 mM EDTA), pH 8.5 for one hour at 37 ºC, followed by treatment with 2.5 % hyaluronidase (w/v) (Sigma-Aldrich) in PBS, pH 7.4 for 30 minutes at 37 ºC. Sections were then immersed in 0.3 % H₂O₂ (v/v) for 30 minutes. To reduce non-specific protein binding, type I collagen sections were blocked with 20% goat serum (v/v) (Vector Laboratories, Burlington, ON) in PBS, pH 7.4, with 0.1 % Triton X-100 (v/v) for 30 minutes at room temperature, whereas type II collagen sections were blocked with 20% horse serum (v/v) (Vector Laboratories) in PBS, pH 7.4, with 0.1 % Triton X-100 (v/v) for 30 minutes at room temperature. Type I collagen sections were incubated with rabbit polyclonal type I primary antibody (Biodesign International, Saco, ME, USA) at a 1:200 dilution in 10% goat serum (v/v) in PBS, pH 7.4, with 0.1 % Triton X-100 (v/v) overnight at 4 ºC. Type II collagen sections were incubated with mouse monoclonal type II primary
antibody (Iowa Hybridoma Bank, IA, USA) at a 1:10 dilution in 10% horse serum (v/v) in PBS, pH 7.4, with 0.1 % Triton X-100 (v/v) overnight at 4 ºC. Sections were rinsed in PBS, pH 7.4, and incubated with biotinylated secondary antibody using the Vectastain® Elite ABC kit (Vector Laboratories) with 10 % goat serum (v/v) in PBS with 0.1% Triton X-100 (v/v) for type I collagen, or 10% horse serum (v/v) in PBS with 0.1% Triton X-100 (v/v) for type II collagen. Immunodetection was performed according to instructions provided by the kit manufacturer, using 3,3′-diaminobenzidine tablets (Sigma-Aldrich) for colour development. To ensure there was no background staining, the primary antibody was omitted from selected slides for negative control samples. Sections were counterstained with 1 % methyl green (w/v) (Sigma-Aldrich) for two minutes. Finally, sections were cleared and dehydrated by immersion in water and alcohol, and mounted with PRO-TEXX mounting medium (Fisher Scientific). This protocol was adapted from Chevrier et al. [109] and performed by Ms. Aasma Khan in the Department of Chemical Engineering, Queen’s University, Kingston, ON. Sections were examined with light microscopy.

4.8 Statistical Analyses

Variability within each experiment was minimized by two means. First, bovine articular chondrocytes were obtained from a pooled source of at least two dissected legs for all experiments. Second, each experiment was performed at least two times with chondrocytes obtained from different animals, and the combined data were used for analysis. In total, each experimental group had a sample size of at least four.

All numerical results were normalized to age-matched controls for each experiment, and expressed as the mean ± SEM. Data in the optimal mode, amplitude and duration experiment (§4.3.1) were analyzed separately between static and dynamic modes. For each mode, data were compared between each experimental group using a two-way analysis of variance (ANOVA) to
simultaneously determine the effects of tensile strain amplitude and duration. Statistical
significance was determined using Fisher’s least significant difference (LSD) post-hoc test (SPSS
16.0 for Windows, SPSS Inc., Chicago, IL, USA). Data from the long-term stimulation
experiment (§4.3.2) and the loading frequency experiment (§4.3.3) were compared between
experimental groups using a one-way ANOVA, and statistical significance was determined using
Fisher’s LSD post-hoc test. In the effect of extracellular pH experiment (§4.3.3), data of each
experimental group were compared using Student’s t-test and statistical significance was
determined using the non-parametric Mann-Whitney U-test. Significant differences in all
experiments were associated with $p$ values of less than 0.05 and trends were associated with $p$
values between 0.05 and 0.1.
Chapter 5
Results

5.1 Optimal Mode, Amplitude and Duration

The optimal mode, amplitude and duration of biaxial tensile strain (BTS) stimulation on cartilaginous tissues were identified based on the subsequent effect on cellular proliferation and ECM synthesis. Two-week-old cartilaginous tissues were intermittently stimulated in different modes (static and dynamic), amplitudes (small, medium and large) and durations (15, 30 and 60 minutes) with cellular proliferation and ECM synthesis determined by radioisotope incorporation.

Cellular proliferation was not significantly affected by BTS amplitude when applied in either static or dynamic modes (p>0.10) (Figure 5.1). However, proliferation was significantly affected by the duration of BTS for both static and dynamic modes (Figure 5.2). Intermittent static BTS applied for short durations of 15 and 30 minutes increased proliferation by 24% (p=0.012 and p=0.013 respectively); whereas 60 minutes of intermittent static BTS did not elicit any significant response (p=0.427). In response to dynamic BTS, 15 minutes of stimulation had no effect (p=0.936), 30 minutes increased proliferation by 20% (p=0.001) and 60 minutes of stimulation resulted in an inhibition in proliferation (9%, p=0.094).
Figure 5.1: Effect of short-term intermittent BTS amplitude on proliferation. None of the groups were statistically different from control (p>0.10). Data presented as mean ± SEM, n=17-18 samples per group.
PG synthesis was significantly affected by both the amplitude and duration of intermittent static BTS, while the effects of amplitude and duration of dynamic BTS were less apparent.

Intermittent static BTS of medium and large magnitudes increased PG synthesis by 35% (p=0.016) and 57% (p=0.001), respectively (Figure 5.3). Intermittent static BTS applied for short durations of 15 and 30 minutes also increased PG synthesis by 43% (p=0.003) and 59% (p=0.001), respectively (Figure 5.4). In contrast, dynamic BTS applied at the medium amplitude slightly increased PG synthesis (9%, p=0.062) (Figure 5.3) and dynamic BTS applied for 30 minutes also slightly increased PG synthesis (9%, p=0.075) (Figure 5.4).
Figure 5.3: Effect of short-term intermittent BTS amplitudes on PG synthesis.
*Significantly different from control (p=0.016), **trend with respect to control (p=0.062). Data presented as mean ± SEM, n=17-18 samples per group.
Amplitude and duration of BTS stimulation had relatively little effect on collagen synthesis. Intermittent static BTS applied only at the medium magnitude increased collagen synthesis by 16% (p=0.014); whereas none of the amplitudes of dynamic BTS stimulation resulted in a significant response (Figure 5.5). However, intermittent static BTS stimulation duration did not have a measurable effect on collagen synthesis (p=0.335) (Figure 5.6). Similarly, dynamic BTS stimulation applied only for 15 minutes induced a slight increase in collagen synthesis (9%, p=0.071) (Figure 5.6).
Figure 5.5: Effect of short-term intermittent BTS amplitudes on collagen synthesis.
*Significantly different from control (p=0.014). Data presented as mean ± SEM, n=18 samples per group.
Since intermittent BTS stimulation had relatively little effect on collagen synthesis, the focus in determining optimal BTS conditions was narrowed to the stimulated effects on cellular proliferation and PG synthesis. The optimal mode of intermittent BTS stimulation was determined to be statically applied strains based on the significant increases observed in both cellular proliferation and PG synthesis, whereas dynamic BTS resulted in relatively few positive effects. The optimal amplitude of BTS stimulation was determined to be the large amplitude (3.79% average radial strain, 2.13% average circumferential strain) based on the peak increase in stimulated PG synthesis, whereas cellular proliferation was not affected by BTS amplitude. Similarly, the optimal duration of BTS stimulation was determined to be 30 minutes based on peak increases in stimulated proliferation and stimulated PG synthesis.

**Figure 5.6: Effect of short-term intermittent BTS durations on collagen synthesis.** **Trend with respect to control (p=0.071). Data presented as mean ± SEM, n=18 samples per group.**
5.2 Effects of Long-Term Stimulation

The effect of long-term BTS stimulation on cartilaginous tissue constructs were analyzed in this experiment. Two-week-old tissues were stimulated by BTS at the optimal conditions (identified in §5.1) applied under two loading frequencies: (i) stimulated every day or (ii) stimulated every 2-3 days. After 2 and 4 weeks of BTS stimulation, tissue constructs were harvested and analyzed for changes in physical, biochemical and mechanical properties.

Cartilaginous tissues stimulated at a frequency of once every 2-3 days were significantly thicker than both unstimulated tissues (control) and the tissues that were stimulated every day for the same period (Figure 5.7). After 2 and 4 weeks of BTS stimulation, there were significant differences between the thicknesses of tissues stimulated under the two loading frequencies (p=0.002). However, when tissue thickness was compared to unstimulated age-matched controls, these differences were not statistically significant until after 4 weeks of stimulation. After 4 weeks of long-term stimulation, the thickness of tissues stimulated once every 2-3 days increased by 35% (p=0.009) relative to the unstimulated tissues.
Figure 5.7: Effect of long-term BTS on tissue thickness. *Significantly different from age-matched control (p=0.009), **trend with respect to the age-matched control (p=0.080). †Significantly different between stimulation frequencies (p=0.002). Data presented as mean ± SEM, n=4-7 samples per group.

Long-term BTS stimulation also elicited an increase in tissue wet weight but not tissue dry weight. The wet weight of tissues stimulated for 2 weeks by either loading frequency was unaffected (Table 5.1); whereas after 4 weeks of stimulation, tissues stimulated once every 2-3 days had a 21% (p=0.004) increase in wet weight compared to the age-matched controls. Tissues stimulated every day for the same period displayed a trend towards decreased wet weight (15%, p=0.059). The dry weight of tissues stimulated at either frequency for 2 and 4 weeks was unaffected (p=0.434) (Table 5.1).

While long-term BTS stimulation did not affect tissue cellularity (p=0.315) (Table 5.1), long-term stimulated tissue significantly accumulated more ECM. Tissues stimulated daily did not show
any changes in PG content after 2 and 4 weeks of stimulation (p=0.453) (Table 5.1). However, tissues that were stimulated once every 2-3 days for 4 weeks contained 22% more (p=0.001) PGs compared to the age-matched control tissues. Interestingly, when normalized to DNA, PG accumulation in the long-term simulated tissues was unchanged (p=0.175). The collagen content of tissues stimulated once every 2-3 days for 4 weeks was also significantly higher than that of tissues stimulated every day for the same period (p=0.025) (Table 5.1). However, when the stimulated tissues were compared to age-matched controls, no significant effect on collagen content was observed (p=0.131). Similarly, collagen content relative to DNA was also unaffected by BTS stimulation applied at either loading frequency (p=0.145).

Table 5.1: Effect of long-term BTS on the physical and biochemical properties of tissues.
*Significantly different from age-matched control (p=0.004), **trend with respect to age-matched control (p=0.086). †Significantly different between stimulation frequencies (p=0.025). Data presented as mean ± SEM, n=4-7 samples per group.

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</tr>
<tr>
<td>Wet Weight</td>
<td>1.04 ± 0.07</td>
<td>0.85 ± 0.04**†</td>
</tr>
<tr>
<td>Dry Weight</td>
<td>0.92 ± 0.06</td>
<td>1.07 ± 0.05</td>
</tr>
<tr>
<td>DNA</td>
<td>0.94 ± 0.07</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>PG/Construct</td>
<td>0.99 ± 0.05</td>
<td>0.95 ± 0.06†</td>
</tr>
<tr>
<td>PG/DNA</td>
<td>1.07 ± 0.06</td>
<td>1.11 ± 0.21</td>
</tr>
<tr>
<td>Collagen/Construct</td>
<td>1.17 ± 0.10**</td>
<td>0.89 ± 0.08†</td>
</tr>
<tr>
<td>Collagen/DNA</td>
<td>1.24 ± 0.06</td>
<td>1.02 ± 0.21</td>
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BTS stimulation did not significantly affect either tissue strength (equilibrium stress) or tissue stiffness (equilibrium modulus). A trend of increased average tissue strength was observed

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between the tissues stimulated every day for 2 weeks compared to tissues stimulated once every 2-3 days in the same period (Table 5.2). However, this effect was lost after 4 weeks of BTS stimulation. Similarly, tissue stiffness was not affected by BTS stimulation applied at either loading frequency for all periods of time investigated.

Table 5.2: Effect of long-term BTS on the mechanical properties of tissues. ††Trend with respect to stimulation frequency (p=0.072). Data presented as mean ± SEM, n=6-7 samples per group.

<table>
<thead>
<tr>
<th></th>
<th>Stimulated Every Day</th>
<th>Stimulated Every 2-3 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stimulated for 2 Weeks</td>
<td>Stimulated for 4 Weeks</td>
</tr>
<tr>
<td>Equilibrium Stress</td>
<td>1.26 ± 0.32 ††</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>Equilibrium Modulus</td>
<td>0.84 ± 0.15</td>
<td>0.82 ± 0.13</td>
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5.2.1 Histological Analyses of Long-Term Stimulated Cartilaginous Tissues

Representative long-term stimulated tissues were also prepared for histological assessment. Since the tissues tested at the two loading frequencies were cultured from different pools of chondrocytes, separate control (unstimulated) tissue samples for each loading frequency were also prepared in the same fashion for comparison with the stimulated tissues.

Tissue sections stained with hematoxylin and eosin displayed a balanced distribution of general connective tissue proteins throughout the tissues (Figure 5.8). Tissues stimulated once every 2-3 days for 4 weeks appeared to have slightly higher stain intensity than tissues stimulated daily. Tissues stimulated once every 2-3 days were also visibly thicker than control samples especially at the tissue edge, whereas the tissues stimulated every day did not appear to increase in
thickness. Tissues stimulated at either loading frequency for 2 weeks did not visibly differ from control tissue samples (images not shown).
Figure 5.8: Hematoxylin and eosin stained tissue samples. Tissue samples were stimulated either once per day or once every 2-3 days for 4 weeks, or maintained as their respective age-matched controls. Scale bar: 100 μm.
Separate tissue sections were stained with toluidine blue for visualization of sulphated PGs (Figure 5.9). All cartilaginous tissue samples stained positive for sulfated PGs. Tissues stimulated once every 2-3 days for 4 weeks appeared to stain darker than their respective control samples especially in the bottom regions of the tissues, while the tissues that were stimulated once per day did not display much differences from their control samples. The cells located at the top surface of both stimulated and unstimulated samples exhibited a flattened morphology while cells in the remainder of the tissue were generally round in appearance. There were no visible differences between tissue samples stimulated for 2 weeks and control tissue samples (images not shown).
Figure 5.9: Toluidine blue stained tissue samples. Tissue samples were stimulated either once per day or once every 2-3 days for 4 weeks, or maintained as their respective age-matched controls. Scale bar: 100 μm.
5.2.2 Immunohistochemical Analyses of Long-Term Stimulated Cartilaginous Tissues

Representative long-term stimulated tissues were also prepared for immunohistochemical assessment to determine the presence of both type I collagen (negative indicator of articular cartilage) and type II collagen (positive indicator of articular cartilage). Control (unstimulated) tissue samples were cultured from the same pools of chondrocytes as the stimulated tissues and were prepared in a similar fashion.

Type I collagen staining was evident in the sections of both unstimulated tissues and tissues that were stimulated for 4 weeks (Figure 5.10). All tissue sections appeared to stain positive for type I collagen and to a similar stain intensity. All tissue sections also appeared to have stained more intensely for type II collagen than type I collagen after 4 weeks of stimulation (Figure 5.11). Type II collagen was present evenly throughout the tissue depth and between the tissue center and tissue edge. Tissues stimulated once per day displayed no detectable differences in type II collagen staining compared to control samples. In contrast, the tissues that were stimulated once every 2-3 days appeared to stain more intensely for type II collagen compared to the controls. There were no detectable differences in immunostaining for either type I or type II collagen between the control samples and samples that were only stimulated for 2 weeks (images not shown).
Figure 5.10: Type I collagen stained tissue samples. Tissue samples were stimulated either once per day or once every 2-3 days for 4 weeks, or maintained as their respective age-matched controls. Scale bar: 100 μm.
Figure 5.11: Type II collagen stained tissue samples. Tissue samples were stimulated either once per day or once every 2-3 days for 4 weeks, or maintained as their respective age-matched controls. Scale bar: 100 μm.
5.3 Effects of Loading Frequency

The effects of BTS stimulation frequency on the anabolic response of chondrocytes were studied in this experiment. Similar to the previous experiment, tissue constructs were stimulated for one week with BTS applied under the optimal conditions (as determined in §5.1) at a frequency of either once per day or once every 2-3 days. However, in this experiment, changes in cellular proliferation and PG synthesis were measured daily by radioisotope incorporation.

Tissues stimulated once every 2-3 days displayed significantly higher proliferation rates compared to both the control samples and those tissues that were stimulated once per day for the same period (Figure 5.12). Proliferation rate in the tissues stimulated once every 2-3 days was increased by 23% on day 4 (p=0.018) and by 25% on day 5 (p=0.028) compared to the tissues that were stimulated daily. While the proliferative response of the tissues stimulated by BTS once per day did not reach statistical significance, there were trends of increased proliferation on day 3 (14%, p=0.083) and day 6 (47%, p=0.096). On day 4 and day 7, proliferation decreased from the elevated levels back to control levels (p=0.145). Alternatively, the tissue samples stimulated once every 2-3 days did not display any significant increases in proliferation until day 5. Proliferation of these tissues was increased by 29% (p=0.017) on day 5 and 73% (p=0.021) on day 6 compared to the age-matched controls. Proliferation rate dropped back to control levels by day 7. The proliferation of control samples was relatively low on day 5 (Appendix C). Otherwise, no distinctive pattern was observed in the proliferation of control samples.
Figure 5.12: Daily changes in cellular proliferation as a result of BTS stimulation.
*Significantly different from control (p=0.021), **trend with respect to control (p=0.096).
†Significantly different between stimulation frequencies (p=0.028). Tissues stimulated once every 2-3 days were stimulated on days 1, 3 and 6 (S) after culture media replacement. Data presented as mean ± SEM, n=6-8 samples per group.

BTS stimulated increases in PG synthesis occurred quicker, and with greater magnitude, in the tissues that were stimulated daily as opposed to the tissues that were stimulated once every 2-3 days (Figure 5.13). PG synthesis in the tissues stimulated once per day was increased by 43% on day 3 (p=0.001) and 33% on day 7 (p=0.036) compared to the tissues that were stimulated every 2-3 days. Tissues stimulated once per day displayed significantly increased PG synthesis on day 3 by 50% (p=0.001) then returned to control levels on day 4 and day 5 (p>0.16). Synthesis was significantly increased again on day 6 by 57% (p=0.024) and day 7 by 60% (p=0.001). In contrast, significant increases in PG synthesis as a result of BTS stimulation once every 2-3 days did not occur until day 6 when PG synthesis was increased by 77% (p=0.007) relative to control levels. Trends of increased PG synthesis were also measured on day 5 by 36% (p=0.071) and day
7 by 27% (p=0.098). PG synthesis of control samples decreased drastically from day 4 to day 5, and stayed at the low PG synthesis levels through to day 7 (Appendix C).

Figure 5.13: Daily changes in PG synthesis as a result of BTS stimulation. *Significantly different from control (p=0.024), **trend with respect to control (p=0.098). †Significantly different between stimulation frequencies (p=0.036). Tissues stimulated once every 2-3 days were stimulated on days 1, 3 and 6 (S) after culture media replacement. Data presented as mean ± SEM, n=6-8 samples per group.

The results of this experiment suggested there may be a possible correlation between culture media renewal and BTS-stimulated increases in cellular proliferation and PG synthesis. Tissue culture samples that were stimulated every day displayed increased proliferation and PG synthesis rates relative to control on day 3 and day 6, both of which were days when the culture media was replaced (Figure 5.12 and Figure 5.13). With the exception of the day 7 results, the tissues appeared to have the same proliferation and PG synthesis rates as control on days when culture
media was not replaced. Similarly, the tissue samples that were stimulated once every 2-3 days displayed the highest increases in proliferation and PG synthesis rates relative to control on day 6, which was a day when the culture media was replaced. Furthermore, proliferation and PG synthesis results of control samples reveal low cellular activity on day 5, which was 48 hours after the last culture media replacement.

A separate test investigated the potential relationship between decreased anabolic response and the decline in extracellular pH associated with repeated stimulation on days when the culture media was not replaced. Tissues were stimulated by BTS under either loading frequency (as noted before) and the extracellular pH of the culture media was measured on days 3, 4 and 5. Extracellular pH of all cultures (stimulated and unstimulated) dropped from 7.44 on day 3 (after culture media replacement) to 6.95-6.96 on day 4 and further dropped to 6.70-6.75 on day 5 (Figure 5.14). There were no statistical differences between the extracellular pH values of any group on days 3, 4 or 5 (p=0.173).
To determine the potential effect of acidic extracellular pH on the BTS-stimulated increases in cellular proliferation and PG synthesis, tissue constructs were stimulated once per day for 3 days under the optimal conditions. On the third day, the culture media was replaced with fresh media adjusted to pH 6.70 (representative of 48 hours in culture) or fresh media without pH adjustment (pH 7.40). Proliferation and PG synthesis were then measured by radioisotope incorporation. Tissues cultured in normal (pH 7.40) media displayed increased proliferation by 20% (p=0.038) in response to BTS stimulation, while the tissues cultured in media adjusted to pH 6.70 were not statistically different than the unstimulated control samples (p=0.661) (Table 5.3). The proliferation rate of all tissue samples cultured in pH 6.70 media was significantly decreased to 12% (p=0.001) of the control tissues cultured in pH 7.40 media.
Table 5.3: Proliferation of tissues in normal and acidified culture media. *Significantly different from unstimulated controls (p=0.038). †Significantly different from normal culture media (p=0.001). Data presented as mean ± SEM relative to control samples in normal media, n=6-13 samples per group.

<table>
<thead>
<tr>
<th>Day 3 Culture Media pH</th>
<th>Control Tissues</th>
<th>Stimulated Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal – 7.40</td>
<td>1.00 ± 0.07</td>
<td>1.20 ± 0.05*</td>
</tr>
<tr>
<td>Acidified – 6.70</td>
<td>0.12 ± 0.01 †</td>
<td>0.12 ± 0.01 †</td>
</tr>
</tbody>
</table>

Culture media adjusted to pH 6.70 also eliminated the previously observed BTS-stimulated effect on PG synthesis (Table 5.4). BTS stimulation increased PG synthesis by 26% (p=0.011) in tissues cultured in normal (pH 7.40) media, while BTS stimulation failed to elicit a significant response in the tissues cultured in media adjusted to pH 6.70 (p=0.808). In comparison, the PG syntheses of all tissues cultured in pH 6.70 media were significantly reduced to 13-14% (p=0.001) of the control tissues cultured in pH 7.40 media.

Table 5.4: PG synthesis of tissues in normal and acidified culture media. *Significantly different from unstimulated controls (p=0.011). †Significantly different from normal culture media (p=0.001). Data presented as mean ± SEM relative to control samples in normal media, n=9-16 samples per group.

<table>
<thead>
<tr>
<th>Day 3 Culture Media pH</th>
<th>Control Tissues</th>
<th>Stimulated Tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal – 7.40</td>
<td>1.00 ± 0.06</td>
<td>1.26 ± 0.07*</td>
</tr>
<tr>
<td>Acidified – 6.70</td>
<td>0.13 ± 0.03 †</td>
<td>0.14 ± 0.03 †</td>
</tr>
</tbody>
</table>
Chapter 6
Discussion

The objective of this thesis was to explore the effects of tensile stimulation on the growth of engineered cartilaginous tissue in vitro. It was hypothesized that tensile stimulation would improve the quantity and quality of engineered tissue by stimulating chondrocyte proliferation and biosynthetic activity. This hypothesis was based on previous research which indicates the presence of tensile forces acting on articular cartilage in vivo [27, 28, 30] as well as the numerous studies that have reported increases in chondrocyte biosynthesis as a result of mechanical stimulation by other loading types, such as compression [20, 21, 60, 61, 65] and shear [22, 71]. Other research groups have found that small amplitude dynamic tensile strains applied for short durations can stimulate proliferation and biosynthesis of 2D chondrocytic cultures [79-81]. There are only two studies (conducted by the same research group) that found dynamic tensile strains stimulated proliferation [23] and PG synthesis [77] of 3D chondrocytic cultures. However, the long term effect (greater than one week) of tensile stimulation on cartilaginous tissues has yet to be studied.

The present investigation was separated into three parts, each of which used a custom loading fixture designed specifically for applying biaxial tensile strains (BTS) to engineered cartilaginous tissues. It should be noted that although BTS was applied to the bottom surface of the tissues, complex strains involving compression and shear may have been present throughout the tissue and may have contributed to the measured effects. Part one of the experiments identified the optimal mode (static versus dynamic), amplitude and duration for which intermittent BTS stimulation increased cellular proliferation and extracellular matrix (ECM) (PG and collagen) synthesis. Part two investigated the effect of long-term (up to 4 weeks) tensile stimulation on the
physical, biochemical and mechanical properties of the engineered tissues. Part three investigated the effect of loading frequency on the proliferation and ECM synthesis of chondrocytes over the course of one week to explain the observed differences in the previous long-term stimulation experiment.

6.1 Optimal Mode, Amplitude and Duration

This short-term experiment was conducted to determine the optimal mode (static and dynamic), amplitude and duration for stimulating chondrocyte proliferation and ECM synthesis. The results indicated that intermittent static BTS significantly stimulated chondrocyte proliferation and biosynthesis, while the effects of intermittent dynamic BTS were less noticeable. The findings of other researchers have generally indicated that dynamic tensile strains induce inhibitory responses in chondrocyte biosynthesis (PG and collagen) [23, 76]. Vanderploeg et al. found intermittent dynamic tensile strains stimulated the biosynthesis of chondrocytes that were isolated from the superficial zone, but the biosynthesis of chondrocytes isolated from the middle and deep zones were either unchanged or inhibited [77]. Therefore, it was not surprising to find in the present study that dynamic BTS had little effect, since the chondrocytes used were isolated from full thickness cartilage samples (i.e. all zones).

Alternatively, intermittent static BTS stimulation significantly increased chondrocyte proliferation and biosynthesis. The only other research group known to have studied the effects of static tension found no changes in chondrocyte proliferation or ECM gene expression as a result of the stimulus [74]. It is presently unknown why static BTS stimulated greater chondrocyte proliferation and biosynthesis compared to dynamic BTS. A plausible explanation is that chondrocyte proliferation and biosynthesis are stimulated more by the physical stretching of chondrocytes than the interstitial fluid flow presumed to be induced by dynamic tensile strains.
and cited by other researchers in dynamic compressive studies [60, 61]. Vanderploeg et al. also acknowledged that fluid flow was likely not the only mechanism responsible for the stimulated biosynthesis induced by dynamic tensile strains in their study [77]. The chondrocytes in static BTS-stimulated tissues were probably stretched for a longer period of time than the chondrocytes in dynamic BTS-stimulated tissues, since dynamic BTS-stimulated tissues relax at the end of each sinusoidal cycle. To investigate potential mechanotransduction pathways, Guilak demonstrated that the shape of chondrocyte nuclei can change as a result of tissue deformation [38]. Therefore, it is possible that stretching of the tissues caused the chondrocyte nucleus (and potentially other intracellular organelles) to change shape, thereby stimulating cellular proliferation and ECM biosynthesis. Furthermore, chondrocyte proliferation and biosynthesis involve cellular mechanisms that are activated by physical stretch [80, 110]. For example, it was found that stretch-activated ion channels are involved in both the tension-stimulated cellular proliferation response of 3D (embryonic) chondrocyte cultures [110] and the upregulation of aggrecan gene expression in monolayer (adult) chondrocyte cultures [80].

Short durations (15 and 30 minutes) of BTS stimulation increased chondrocyte proliferation and biosynthesis, but longer durations (60 minutes) of BTS stimulation did not elicit any measurable effect. It has been previously demonstrated that other types of mechanical loading (compression, shear and high-frequency vibrations) also required only short durations (6-30 minutes) of stimulation to elicit increased responses in proliferation and biosynthesis [64, 65, 71, 111], whereas longer durations (greater than 30 minutes) of loading resulted in smaller stimulatory responses or inhibitory responses [65, 71, 111]. Similarly, other researchers found dynamic tensile strains applied for 48-68 hours caused inhibitory responses in chondrocytes maintained in 3D culture [23, 76]. Therefore, it is probable that the cells became desensitized to continuous mechanical loading (greater than 30 minutes) regardless of the type of loading. Although the
specific events that occur during desensitization are presently unclear, it may involve the reduction of extracellular ATP. Graff et al. found dynamic compressive loading stimulated ATP release from chondrocyte pellet cultures by 5-12 fold within 5-15 minutes, which then declined to preloading conditions within 30-60 minutes [70].

Small amplitude BTS did not elicit either a proliferative or biosynthetic response, whereas BTS of medium and large amplitudes elicited stimulatory responses. Similarly, other tensile studies that have reported cellular responses were based on strains of at least 5.5% uniaxial strain amplitude [23, 74-77, 112], which is similar to the large strain amplitude used in this experiment (3.79% average radial strain, 2.13% average circumferential strain). Inhibitory responses may occur at higher amplitudes of tensile strains (10% and above), as shown by other researchers [23, 76]. It is unclear what specific mechanisms are responsible for the different responses to the varying strain amplitudes. One reason could be that the ECM provided a shielding effect to the chondrocytes. Since chondrocytes are embedded in the interwoven fiber network of the ECM, it is possible that strain amplitudes must be large enough to overcome a certain amount of laxity in the ECM before chondrocytes experience the applied strains. Even if the chondrocytes do experience strains, they may require some strain amplitude threshold to be reached before cellular mechanisms are activated. Guilak et al. found folds within the cellular membrane of chondrocytes by scanning electron microscopy techniques [113]. On average, stimulated swelling caused the surface area of the cellular membrane to increase by a factor of 2.34 before rupture [113] and numerous cellular components (integrins, ion channels, guanine nucleotide-binding proteins, etc.) that may play a role in controlling cellular proliferation and biosynthesis are located on the cellular membrane [114].
6.2 Effects of Long-Term Stimulation

This experiment was conducted to determine the effects of long-term (4 weeks) intermittent BTS stimulation on the physical, biochemical, and mechanical properties of engineered cartilaginous tissues. BTS stimulation was applied at either of two frequencies (once per day and once every 2-3 days) at the optimal conditions for stimulating proliferation and biosynthesis as determined from part one of the present study. While previous studies have demonstrated that tensile forces on chondrocytes can stimulate PG synthesis in the short term (3 days or shorter) [77, 81], there has been no published report of the long-term effect of tensile stimulation on tissue engineered cartilage.

Tissues that were stimulated by BTS daily displayed slight decreases in tissue thickness and wet weight, but the dry weight of the tissue was unaffected. Similarly, tissues that were stimulated by BTS once every 2-3 days displayed increases in thickness and wet weight, but tissue dry weight remained unaffected. Tissues stimulated once every 2-3 days also displayed an increase in total PG content. The parallel increases in tissue thickness, wet weight, and total PG content were not surprising since water molecules bind to PGs within the ECM and are the primary means by which the tissue becomes hydrated [2]. Interestingly, tissues stimulated once every 2-3 days did not accumulate more PGs (or display increases in thickness or wet weight) until after 4 weeks of BTS stimulation. The results of the loading frequency experiment (§5.3) further suggested that chondrocytes have a delayed response to BTS stimulation. Researchers who studied the effects of dynamic compression on chondrocyte constructs also found that PG synthesis was greater after a culture period in the range of 2-3 weeks [20, 67, 68]. It is possible that PGs synthesized by undeveloped tissues escaped through the porous ECM to the surrounding medium, while PGs synthesized by more mature tissues with higher ECM intricacy were trapped within the ECM network.
While tissues stimulated at a frequency of once every 2-3 days displayed a higher PG content relative to control tissues, the total cellularity and the ratio of PGs to DNA of the stimulated tissues were unaffected by BTS stimulation. Therefore, the increased PG content was probably not the result of more cells having synthesized PGs. Rather it is likely that only a small portion of chondrocytes within the stimulated tissues were strained at high enough amplitudes to increase PG synthesis. The loading fixture used for BTS stimulation applied BTS of higher amplitudes to the tissue center and BTS of lower amplitudes to the tissue perimeter. From part one of this study, it was found that BTS applied at low amplitudes did not elicit any biosynthetic response (§5.1). Therefore, when the total PG content was normalized to total tissue cellularity (of which a large portion may have been unaffected by the BTS stimulation), the stimulated effect could have been decreased to a level that was considered statistically insignificant. Furthermore, histological images of tissues stimulated once every 2-3 days appeared to stain more intensely for PGs in the bottom layer of the tissue compared to the top layer, whereas control tissues were stained more evenly throughout. In theory, the bottom layer of the tissue would have experienced strains of higher amplitudes than the top layer because it is closer to the stretched surface, which further supports the notion that only regions of high amplitude BTS stimulation responded by increasing PG synthesis.

Hydroxyproline assays revealed that tissues stimulated once every 2-3 days for 4 weeks contained a higher collagen content than tissues stimulated once per day, but were not statistically different from the collagen content of control tissues. Histological images appeared to confirm that there was a difference in protein content between the tissues stimulated at the two frequencies. However, immunohistochemical images revealed that the stimulated protein increase was
comprised of both type I collagen (partly) and type II collagen (majority). Normal articular cartilage has a collagen content that is composed of 90-95% type II collagen with no type I collagen [2]. Development of type I collagen was probably not a result of BTS stimulation since unstimulated control samples also stained positive for type I collagen to the same degree. Although it is not certain what caused the development of type I collagen in the developed tissues, it may be an effect caused by the culturing method and further study is necessary to confirm this assertion.

BTS stimulation at a frequency of once per day slightly improved tissues strength (equilibrium stress) after 2 weeks, while tissue stiffness (equilibrium modulus) was unaffected. However, the effect disappeared after 4 weeks of stimulation. BTS stimulation at a frequency of once every 2-3 days did not improve either tissue strength or tissue stiffness despite increases in total PG content after 4 weeks. In theory, increases in ECM macromolecules should lead to increases in tissue strength and stiffness since the ECM provides structural rigidity to the tissue. However, the 22% increase in PG content combined with the lack of significant increase in collagen content was probably not enough to significantly increase the mechanical properties of the stimulated tissues. The interterritorial matrix provides the bulk of material properties of articular cartilage and is composed largely of collagen fibres and PGs [2]. Therefore an increase in PG content alone would most likely not lead to significant effects on the compressive strength and stiffness of the tissue. Alternatively, tissues stimulated once per day had a slight (17%) increase in collagen content which correlated with a slight improvement in tissue strength after 2 weeks. Other studies that found increases in tissue strength or tissue stiffness due to mechanical stimuli (compression or shear) also reported increases in PG and collagen contents of at least 30% [65, 66, 71]. Therefore, the results of the long-term stimulation study suggest that tensile stimulation cannot as yet be considered superior to mechanical stimulation by compression or shear.
Histological and immunohistochemical analysis of the cultures showed the chondrocytes resident in the tissues were slightly flattened at the top layer but generally rounded in appearance. There was no evidence of tensile strains having caused the cells to change shape, as reported by Vanderploeg et al. (3D culture) and Honda et al. (monolayer culture) [23, 83]. The specific reasons for the morphological changes observed in these studies are currently unknown.

Vanderploeg et al. conducted a similar tensile study later which failed to elicit the same changes in cell morphology and suggested that the changes in cell shape earlier may have been caused by inter-animal variability [76]. Honda et al. speculated that the changes in cell shape may have been caused by fluid flow induced by dynamic tensile strains [83].

### 6.3 Effects of Loading Frequency

This experiment was conducted to examine the effects of BTS stimulation loading frequency (once per day and once every 2-3 days) on proliferation and biosynthesis of the chondrocyte tissues. This experiment was prompted by the observed differences in the physical and biochemical properties between tissues stimulated at different frequencies in the long-term experiment (§5.2).

Tissues stimulated daily first showed a chondrocyte proliferation response as well as a PG synthesis response on day 3. Tissues stimulated once every 2-3 days first showed a proliferation response and a PG synthesis response on day 5, which was 48 hours after the second dose of BTS stimulation was applied. These results suggest that preconditioning of chondrocytes to the BTS stimulation was important in eliciting the initial responses in proliferation and PG synthesis. The specific events that occur during the preconditioning period are unclear. It is possible that preconditioning involves the restructuring of the ECM and the subsequent repositioning of
chondrocytes embedded in the tissue. Sasazaki et al. used scanning electron microscopy (and other microscopy techniques) to find tensile strains caused the collagen fibers of native cartilage explants to realign in the direction of the tensile strains [115]. Preconditioning may also involve transportation of nutrients to the local areas around chondrocytes by stimulating interstitial fluid flow. Fluid flow is usually cited as the cause for biosynthesis stimulation in dynamically compressed tissues, whereas static compression inhibited biosynthesis [60, 61].

The chondrocyte proliferation response of tissues stimulated at a frequency of once per day was lower than that of tissues stimulated once every 2-3 days on day 4 and day 5, but the PG synthesis response of tissues stimulated once per day was higher than that of tissues stimulated once every 2-3 days on day 3 and day 7. Although BTS stimulation at the faster frequency appeared to elicit a higher PG synthesis response based on same-day comparisons, results of the long-term experiment revealed a higher accumulation of PGs in tissues stimulated at the slower frequency (§5.2). In this experiment, the results over the entire test period (seven days) suggest the delay in response by chondrocytes was more prominent in tissues stimulated at the slower frequency. Altogether, these results suggest that the chondrocytes in tissues stimulated once per day became less responsive to BTS stimulation compared to the chondrocytes in tissues stimulated once every 2-3 days. It is possible that chondrocytes became desensitized to intermittent BTS stimulation, which is probably different from the short-term desensitization to continuous BTS stimulation as described earlier (§6.1). The sensitivity of chondrocytes to mechanical loads may involve multiple time-dependent factors. Kaupp and Waldman found that chondrocytes became desensitized to mechanical vibrations after longer durations of the preculture period, and postulated that the accumulation of ECM shielded chondrocytes from the vibrations [111]. Yamazaki et al. found monolayer chondrocytic cultures responded to cyclic tensile stretch by depolymerization of hyaluronan (a component of PGs) [116]. Thus, it is possible that
chondrocytes experience less of the applied loads over time as the ECM restructures itself. Furthermore, Yamamoto et al. found the adhesive forces exerted by chondrocytes to specific surfaces were dependent on the amount of time chondrocytes spent in culture [117]. Therefore, it is possible that some of the newly proliferated chondrocytes were not securely attached to the ECM, and became dislodged as a result of frequent BTS stimulation.

The results of this experiment indicate cellular proliferation and PG synthesis responded differently to BTS stimulation. For example, tissues stimulated at a frequency of once per day appeared to have a more positive effect on PG synthesis than on proliferation. However, these results do not account for different time durations required by the two cellular functions since both proliferation and PG synthesis were measured over the same time period (24-hours). The time required for PG synthesis by bovine articular chondrocytes is unknown, however studies of other animals (rat, pig) suggest that PG synthesis normally requires approximately 25-100 minutes [118, 119]. While the time required for cellular proliferation of bovine chondrocytes is also unknown, other authors have found the chondrocyte division times of other animals (mouse, human) range from 16-96 hours [120, 121]. As chondrocyte cell division is a significantly longer process than PG synthesis, this would account for the early (day 3) significant PG synthesis response of tissues stimulated daily whereas the proliferation response was more subtle.

The stimulated increases in cellular proliferation that were measured in this experiment were not reflected in the total cellularity of long-term stimulated cultures. Tissues stimulated by both frequencies (once per day and once every 2-3 days) in this experiment also displayed increases in PG synthesis, whereas only tissues stimulated once every 2-3 days in the long-term experiment displayed an increase in total PG content. There could be multiple factors that contributed to the
apparent discrepancies between results of the loading frequency experiment and the long-term experiment. Firstly, it is probable that only sections of the stimulated tissues responded to BTS stimulation. As described earlier, the loading fixture applied BTS stimulation of different amplitudes to the tissues (§3.2.3), and small amplitude BTS stimulation did not cause any observable effects in terms of cellular proliferation or biosynthesis (§5.1). If the stimulated increases in proliferation or biosynthesis were small relative to the total tissue cellularity or PG content, accumulation measurements of the tissue contents might not have reflected the changes. Secondly, BTS stimulation may have caused both anabolic and catabolic responses by the cells. Other researchers found dynamic tensile strains caused chondrocytes in monolayer cultures to upregulate both anabolic (growth factor (TGF-β1), inhibitor to matrix degradation (TIMP-1)) and catabolic (proinflammatory cytokines (IL-1β, TNF-α) gene expression [79, 83]. Similarly, DeCroos et al. found dynamic compressions caused chondrocytes in 3D cultures to upregulate the expression of both anabolic (aggrecan, type II collagen) and catabolic (MMP-3, MMP-13) matrix genes [122].

Tissues stimulated at both frequencies displayed higher proliferation rates on the days of culture media replacement compared to the other days once the initial effect occurred (day 3 for tissues stimulated once per day, day 5 for tissues stimulated once every 2-3 days). PG synthesis of the stimulated tissues also displayed high PG synthesis rates on the days of culture media renewal. However, tissue cultures that were stimulated once per day displayed a high rate of PG synthesis on day 7 (in the absence of culture media replacement), which was contrary to the observed pattern. It was hypothesized that culture media replacement adjusted the media pH to a stimulatory level and/or increased the availability of nutrients to the tissue cultures, thereby eliciting higher proliferation and PG synthesis rate on the days when culture media was replaced. The day 7 effect (high PG synthesis by tissues stimulated once per day on a day when culture
media was not replaced) could be a delayed response caused by the previously described preconditioning effect induced by BTS stimulation.

A separate test was conducted to investigate a potential relationship between decreased anabolic response and the decline in extracellular pH associated with repeated stimulation on days when the culture media was not replaced. The culture media pH of both stimulated (by frequencies of once per day and once every 2-3 days) and unstimulated cultures dropped from 7.44 after culture media replacement to 6.70-6.75 after 48 hours. There was no statistical difference between the culture media pH of stimulated and unstimulated tissues. The drop in culture media pH was likely due to anaerobic metabolism of the cells. Chondrocytes primarily metabolize glucose anaerobically which results in the production of lactic acid [2]. Once the accumulation of lactic acid exceeds the buffering capacity of the culture media, the culture media pH would begin to drop [123]. Media pH of the stimulated and unstimulated tissues were measured to be the same despite their differences in cellular activity. This was likely due to the large ratio of culture media volume (1.5 mL) to tissue volume (~0.006 cm³).

BTS stimulation caused the tissues cultured in normal (pH 7.40) culture media to increase chondrocyte proliferation and PG synthesis as expected, while BTS stimulation did not elicit any effect in tissues cultured in acidified (pH 6.70) culture media. Tissues cultured in acidified culture media also displayed significantly less proliferation and PG synthesis than the unstimulated tissues cultured in normal media. Similarly, other researchers found chondrocyte matrix synthesis was maximized when the culture media pH was between 7.1 and 7.4 [124], but decreased appreciably when culture media pH was below 7.1 [39, 124]. It is unclear what progression of cellular events is caused by acidification of the extracellular environment that
would reduce the stimulatory effect of mechanical loading. Disturbance of intracellular pH may be involved in a reduction of proliferation or cell death. Wilkins and Hall found that when extracellular pH decreased to below 7.1, the intracellular pH of chondrocytes also decreased [124]. Intracellular pH plays a role in the control of cellular proliferation and apoptosis [125]. ECM synthesis may also be stunted by the low pH environment. Bonassar et al. found acidified (pH 6.4) media reduced the ability of articular cartilage explants to respond to insulin-like growth factor I [55], a naturally occurring polypeptide that stimulates proliferation and ECM synthesis [1, 2, 126]. These results do not necessarily explain the observed increase in PG synthesis when the tissues were stimulated on day 7 (non media replacement day). Therefore, a delayed response caused by preconditioning by the BTS was probably large enough to overcome any negative effects caused by acidification of the culture media.
Chapter 7
Conclusions and Recommendations

7.1 Conclusions

The purpose of this thesis was to determine the stimulatory potential of mechanical tensile strains to engineered cartilaginous tissues. Three objectives were set in this thesis: (i) identify the optimal conditions of tensile stimulation, (ii) identify the long-term effects of tensile stimulation, and (iii) explore the response of chondrocytes to tensile stimulation loading frequency.

In the first experiment, three doses of intermittently applied biaxial tensile strains (BTS) were found to stimulate both chondrocyte proliferation and ECM synthesis (PG and collagen) of high density, 3D cartilaginous tissues, although the effect on collagen synthesis was marginal. The optimal conditions of BTS stimulation for stimulating proliferation and ECM synthesis were found to be static tension with a large strain magnitude (3.79% average radial strain, 2.13% average circumferential strain) for a 30 minute duration. The static and dynamic stimulation results suggest that the physical stretching of chondrocytes had a larger impact on the stimulation of proliferation and ECM synthesis than interstitial fluid flow. The amplitude results suggest mechanical stimulation must be applied at amplitudes large enough to surpass a certain threshold in order to elicit proliferation and ECM synthetic responses. The duration results suggest that chondrocytes are susceptible to desensitization to continuous mechanical loading. The desensitization effect has also been found in other studies involving other types of mechanical stimulation.
In the second experiment, intermittent BTS stimulation over a four week period was found to increase the thickness and wet weight of the tissues, but tissue dry weight was unaffected. The biochemical content of the stimulated tissues contained higher amounts of PGs and slightly higher amounts of collagen, but tissue cellularity remained unchanged. The slight increase in collagen content was identified by immunohistochemical analysis to be mostly type II collagen, although type I collagen was also present. The synthesis of type I collagen was probably not the result of BTS stimulation however, as type I collagen was also detected in unstimulated controls. Despite the increases in ECM macromolecules, the stimulated tissues did not display improved mechanical properties after the four weeks of stimulation. The tissues stimulated at a frequency of once every 2-3 days were found to have better physical (thickness, wet weight) and biochemical (PG content, collagen content) properties than the tissues stimulated at a frequency of once per day.

In the third experiment, the BTS-stimulated chondrocyte proliferation response and the BTS-stimulated PG synthesis response of chondrocytes were found to be dependent on loading frequency. BTS stimulation at a frequency of once per day appeared to stimulate higher PG synthesis rates than BTS stimulation at the frequency of once every 2-3 days, while the opposite was true for the stimulated proliferation response. Delays in the initial proliferation and PG synthesis responses suggested that the chondrocyte cultures needed to be preconditioned by mechanical stimulation before the effect could be observed. The different responses between tissues stimulated at the two frequencies, combined with the results of the long-term experiment, suggest that the cells are susceptible to desensitization to intermittent mechanical loading. Therefore, a delicate balance must be reached between load periods and rest periods in order for BTS stimulation to elicit sustained stimulatory effects on chondrocytes. Tissues stimulated once per day displayed increases in proliferation and PG synthesis, but the total tissue cellularity and
PG content were unchanged after long-term stimulation. It was hypothesized that only small portions of the stimulated tissues were affected by the BTS applied by the loading fixtures, and that BTS stimulation probably initiated catabolic processes which were counterproductive to the previously observed anabolic effect. It was also found that the culture media pH became acidified after 48 hours, such that BTS stimulation was rendered ineffective in eliciting chondrocyte proliferation and PG synthesis.

7.2 Recommendations

Further work must be carried out to gain a deeper understanding of how tensile strains affect chondrocytes in order to maximize its stimulatory potential. Even though the long-term stimulated tissues in this study had improved physical and biochemical properties, a stronger increase in tissue quality must be made before tensile stimulation can be considered to be a viable tool for engineering tissues in vitro. Specifically, experiments that could add to the findings of this thesis are described below.

The catabolic effects caused by BTS stimulation need to be further explored. For example, chondrocyte viability can be measured using cell stain techniques that allow live cells to be distinguished from dead cells when viewed under a confocal microscope. Additionally, biochemical analyses could be conducted on the culture media of stimulated tissue cultures to determine whether ECM components were degraded and released from the tissue as a result of BTS stimulation.

Mechanical BTS stimulation triggers increased rates of chondrocyte proliferation and ECM synthesis by first activating signaling pathways which convert mechanical stimuli into biochemical signaling molecules. It is as yet unclear which signaling pathways are involved in
the stimulated effects. The individual signaling pathways that are activated by tensile strains can be identified by using biochemical inhibitors known to deactivate specific cellular components (e.g. stretch-activated ion channels, \( \text{Ca}^{2+} \) pumps on endoplasmic reticulum). Of particular interest are stretch-activated ion channels because of their inherent strain-induced function.

The sensitivity of chondrocytes to BTS stimulation was reduced as culture media pH decreased. The use of bioreactors could serve to maintain media pH and the combination of bioreactors and mechanical stimulation in tissue engineering can potentially prolong stimulated responses in chondrocyte proliferation and ECM synthesis.

The convex surface of the loading fixture used to apply BTS to tissues led to different sections of the tissue being stretched to different amplitudes; the tissue centre was stretched to a higher amplitude than the tissue perimeter. The effects of BTS stimulation could potentially be amplified if the loading fixture applied BTS of the same amplitude across the entire surface of the tissue culture. Uniform strain amplitudes could be achieved by optimizing the shape of the loading fixture.
References


[16] Tare, R. S., Howard, D., Pound, J. C., 2005, "Tissue Engineering Strategies for Cartilage Generation--Micromass and Three Dimensional Cultures using Human Chondrocytes and
a Continuous Cell Line," Biochemical and Biophysical Research Communications, 333(2) pp. 609-621.


Appendix A: Biaxial Tensile Strain Profiles of Filter Membrane
Biaxial Tensile Strain Profile of Filter Membrane at 0.4 mm Spring Compression
Biaxial Tensile Strain Profile of Filter Membrane at 0.7 mm Spring Compression

![Graph showing biaxial tensile strain profile](image-url)
Biaxial Tensile Strain Profile of Filter Membrane at 1.1 mm Spring Compression

![Graph showing biaxial tensile strain profile with radial and circumferential strain plotted against radial position.]
Appendix B: Sample Indentation Test Data
Indentation Test Data of Tissue Culture Sample
Long Term Stimulation; Strained 4 Weeks, Every 2-3 Day Frequency

- Force (mN)
- Time (s)

Unrecorded Data
Equilibrium Data
Appendix C: Radioisotope Incorporation of Unstimulated Cultures in the Effects of Loading Frequency Experiment
Daily Changes in Cellular Proliferation of Unstimulated Cultures

[Graph showing daily changes in cellular proliferation with labeled axes and data points]

- Control Cultures Used in the Daily Stimulation Set
- Control Cultures Used in the Every 2-3 Days Stimulation Set

- [H] Thymidine Incorporation [CPM]
- Day of Strain Period (S=Strain)
Daily Changes in Proteoglycan Synthesis of Unstimulated Cultures

$[^35]S$ Sulphate Incorporation Relative to DNA [CPM/μg]

Control Cultures Used in the Daily Stimulation Set
Control Cultures Used in the Every 2-3 Days Stimulation Set

Day of Strain Period (S=Strain)

1 (S) 2 3 (S) 4 5 6 (S) 7