The sensitivity of articular chondrocytes to dynamic mechanical stimulation

By

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Abstract

Chondrocyte sensitization and desensitization to mechanical stimuli are complex phenomena that can limit the effectiveness of mechanical stimulation in cartilage tissue engineering. The studies in this thesis aim to provide a more complete understanding of chondrocyte mechanical sensitivity as well as to develop new methods to aid in the successful use of dynamic compressive stimulation for tissue engineering applications. Through the examination of the durations of dynamic compressive loading, it was observed that a minimum amount of stimulation was required to elicit an anabolic response, but desensitization could quickly be reached with increased loading cycles. These anabolic responses could be predicted through the observation of calcium signaling, where an increase in intracellular calcium signaling levels correlated with an anabolic response. Calcium signaling could also be used to predict the wait time needed between successive applications of stimuli, where a return to baseline signaling levels indicated a full recovery of mechanosensitivity. To maintain mechanosensitivity throughout loading and to mitigate load-induced desensitization, which could negatively impact the anabolic response to mechanical stimuli, stochastic resonance in the form of superimposed random vibrations was investigated. In younger cells, stochastic resonance was able to improve cellular sensitivity and elicit further increases in matrix synthesis. Load-induced desensitization was also limited by stochastic resonance, allowing the cells to remain sensitive to increased loading durations. The recovery rate also appeared to be decreased by stochastic resonance indicating that less time would be required between successive applications of stimuli. In older cells which are normally insensitive to mechanical stimuli, stochastic resonance was able to induce sensitivity resulting in a positive anabolic response. The beneficial effects of stochastic resonance were able to be maintained over long term culture, where matrix accumulation was

enhanced through the increased production of collagen and the mitigation of proteoglycan loss during dynamic compressive loading. Therefore the overall positive effects of stochastic resonance observed in this thesis indicate that it can be a valuable tool for the successful application of mechanical stimuli to engineered cartilage constructs.

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Chapter 1: Introduction

1.1 Research Problem

Osteoarthritis is a degenerative disease that affects articular cartilage as well as the underlying bone and surrounding tissues. Its causes range from progressive aging to acute trauma. The prevalence of symptomatic osteoarthritis has been estimated to be around 10% in the older adult population in the US[1,2]. It is a significant contributor to functional joint impairment causing joint pain and/or limited mobility.

Because articular cartilage does not have a blood supply, healing is difficult since cells and growth factors are unable to reach the damaged area. Several surgical strategies have been developed to address cartilage repair[3–6], however the success of these strategies is limited due to the variability in response from patient to patient. Therefore, many of the recent developments in articular cartilage repair have been focused on the development of tissue engineered cartilage suitable for joint resurfacing.

Dynamic mechanical loading is a treatment often used on engineered cartilage constructs to accelerate tissue growth and improve mechanical properties[7–15]. However, one major problem associated with this method is that the response of the cells can be quite variable, resulting in little consensus between different research studies as to the stimulation parameters or frequency of treatments that should be used to elicit a beneficial response. Another confounding factor is the potential desensitizing response that has been observed in response to prolonged loading[9,16–18].

1.2 Motivation

To successfully use mechanical stimulation treatments for improving tissue engineered cartilage constructs, as close as possible to optimal conditions should be used to mitigate the negative responses observed with cellular insensitivity and desensitization. However, determining optimal conditions for each experiment can be tedious, and given the real possibility of batch to batch variation, finding optimal conditions may be impossible. Therefore, in order to fully utilize the potential of mechanical stimuli for the treatment of tissue engineered cartilage constructs, it is important to explore these sensitization and desensitization phenomena as well as work towards a method to mitigate the negative effects associated with sub-optimal mechanical stimulation.

1.3 Objectives and Hypotheses

The overall objective of this work was to characterize the sensitization and desensitization response of chondrocyte-agarose constructs under various types of dynamic mechanical loading protocols, both compressive and vibrational, in order to determine the most appropriate method of applying mechanical treatments to enhance the tissue's growth response.

The first study of this work examined the temporal aspect of sensitization and desensitization as well as the effects of mechanical stimulation on calcium signaling and whether changes in calcium signaling could be used as a predictor of the biosynthetic response. Cell-seeded agarose gels pre-incubated with an intracellular Ca^{2+} dye (Fluo-4) were subjected to dynamic compressive loading under varying conditions (amplitude and duration). Induced changes in Ca^{2+}

signaling were determined by confocal imaging and matrix biosynthesis by radioisotope incorporation.

Hypothesis 1:

Chondrocyte sensitization and desensitization is dependent on the magnitude and duration of the mechanical stimulation protocol.

Hypothesis 2:

A correlation between calcium signaling and cartilaginous matrix biosynthesis exists which allows for the prediction of a sensitized or desensitized response.

The second study investigated the effects of stochastic resonance on chondrocyte matrix metabolism. Chondrocyte-seeded agarose gels were subjected to dynamic compressive loading, with or without, superimposed vibrations of different amplitudes and frequency bandwidths. Changes in matrix biosynthesis were determined by radioisotope incorporation and subsequent effects on intracellular calcium signaling were evaluated by confocal microscopy.

Hypothesis 3:

Applying noise in the form of superimposed vibrations during mechanical stimulation will increase cell mechanosensitivity and improve tissue growth.

Hypothesis 4:

The application of stochastic resonance and resulting increased mechanosensitivity will affect calcium signaling

The third study investigated the effects of age-related cell insensitivity on the effectiveness of stochastic resonance loading protocols. Cells obtained from both younger and older aged animals were used to create the cell-agarose constructs. Dynamic compressive loading with and without superimposed vibrations were used to stimulate the constructs. Radioisotope incorporation was used to measure matrix biosynthesis.

Hypothesis 5:

Stochastic resonance will cause mechanically insensitive cells to be able to anabolically respond to stimulation.

The last study investigated the effects of stochastic resonance on chondrocyte metabolism over long-term culture. Cell-agarose gels were subjected to dynamic compressive loading, with or without, superimposed vibrations every other day (3 times a week) for up to 4 weeks. Accumulation and distribution of matrix components were determined through biochemical assays and histological staining respectively.

Hypothesis 6:

The improved tissue growth observed from a single application of mechanical stimulation with stochastic resonance will continue to be observed over long term culture with multiple treatments.

Chapter 2 – Background and Literature Review

2.1 Articular Cartilage

Articular cartilage is a highly specialized connective tissue which caps the ends of bones to provide the gliding surfaces for articulating joints. It has a highly organized 3D structure which is composed of several types of collagens, a variety of glycosaminoglycans, water, and relatively few cells. These constituents vary in quantity and organization in four distinct layers: the superficial tangential zone, middle zone, deep zone, and calcified zone[19,20]. Articular cartilage does not have a direct blood supply, but is instead maintained by a viscous liquid called synovial fluid. The synovial fluid is contained within the joint capsule and is responsible for nutrient delivery and waste disposal as well as lubrication and shock absorption during movement[19]. Because cartilage relies on diffusion and convection of nutrients through synovial fluid for tissue maintenance and because of the low cell content, lack of progenitor cells, and inability to form fibrin clots as in normal tissue repair, articular cartilage has an extremely poor capacity for self-repair[21].

2.1.1 Articular Cartilage Constituents

2.1.1.1 Cells

Chondrocytes are highly specialized cells derived from the mesenchymal cell lineage. During skeletal maturation, chondrocytes grow and develop the extracellular matrix of the cartilage[19,22]. In mature human tissue, these cells account for less than 10% of the total tissue volume and are responsible for its maintenance. To be able to maintain the tissue, chondrocytes must be sensitive and able to respond to a variety of stimuli[7,19]; however, because articular cartilage is confined in a synovial membrane and lacks nerves, the types of stimuli that are able to reach the cells are limited[19]. The types of stimuli encountered *in vivo* can be separated into two main

categories: chemical and mechanical. Chemicals such as growth factors and pharmaceutical agents must be able to pass through the synovial membrane and diffuse into the tissue in order act on the chondrocytes. Mechanical forces such as compression, shear, and friction affect the cell by inducing a cellular deformation to which the cell can respond[23]. Responses to these stimuli can either be catabolic or anabolic and are typically dose dependent.

2.1.1.2 Collagens

Collagens are large structural proteins which contain a characteristic triple-helical structure. Collagens make up more than 50% of the dry weight of cartilage. About 90 to 95% of this is collagen type II, which equates to approximately 10 to 20% of the wet weight of cartilage[19]. The triple helix of type II collagen generally contains the following amino acids: glycine, proline, and hydroxyproline. These amino acids are sequenced such that the triple helix structure can be formed and stabilized by hydrogen bonds[24]. Type II collagen forms fibrils that are interconnected with each other as well as other types of collagens to create a 3D structure that is relatively insoluble and results in a strong cartilage matrix[19,25].

Type II collagen is widely distributed throughout cartilage, although it has different fibre orientation depending on its location[19]. Other collagens are not so evenly distributed. Type VI collagen is mostly found close to the chondrocytes in their pericellular area. It is thought to be important in anchoring the cells to the matrix and providing a method for cell-matrix interaction[19,26]. Type X collagen is found primarily in the deep and calcified zones of the cartilage and is thought to play a key role in the mineralization of bone just above the subchondral bone[19,27].

Other collagens present include types V, VI, IX, X, and XI. These collagens occur in much smaller weights compared to type II collagen; however they occur in similar molar amounts and likely contribute to the functionality of the cartilage matrix[19,25,26].

2.1.1.3 Proteoglycans and Glycosaminoglycans

Proteoglycans make up about 10% of the cartilage volume[19]. Proteoglycans are large, complex molecules that are formed by glycosaminoglycan chains bonded covalently to a protein core. Glycosaminoglycans are long-chain unbranched molecules made up of 25 to 30 repeating disaccharide units. Chondroitin sulfate, keratan sulfate, and dermatan sulfate are the three main types of glycosaminoglycans that are found in cartilage proteoglycans[19,28]. The amounts of glycosaminoglycans and proteoglycans as well as the length of proteoglycans differ depending on the age and health of the cartilage tissue[19,29]. In addition to age variation, proteoglycan content also varies in the matrix based not only on cellular distance, but also tissue surface distance. In articular cartilage, all glycosaminoglycans have carboxyl and/or sulfate groups which form negatively charged ions in solution. Due to the density of the proteoglycans in the tissue and the requirement of electroneutrality, positive ions in interstitial water give rise to pressure within the tissue[30].

Aggrecan makes up the majority of the proteoglycans in cartilage[19,31]. Aggrecan consists of about 150 proteoglycan molecules which are linked to a long-chain glycosaminoglycan, hyaluronan, by a binding domain which is stabilized with a link protein. Several aggrecan molecules group together to form aggregates which effectively immobilize the proteoglycans within the collagen network. Many other types of proteoglycans are present in cartilage. These

molecules are much smaller than aggrecan and their roles in cartilage tissue development and maintenance are not as well defined. Decorin, fibromodulin, and collagen type IX are proteoglycans that are found in the vicinity of collagen fibres[19]. These proteoglycans are thought to control the formation of collagen fibrils and regulate the diameter of the fibrils[29]. Glypican and syndecans are cell surface proteoglycans that are more abundant in newly forming cartilage and are hypothesized to facilitate cell-matrix and cell-cell interactions to induce tissue growth[29]. Finally, biglycan and perlecan are two other proteoglycans found in cartilage, though their location and function are poorly understood. It has been indicated that these proteoglycans regulate cellular differentiation, with biglycan being a regulator of bone formation and perlecan being a regulator of the chondrocyte phenotype[29].

2.1.1.4 Water

Water is the biggest component of articular cartilage, contributing to between 65% and 80% of the wet weight[32]. While water at the surface of the tissue can aid in joint lubrication during movement, the majority of the water is contained within the matrix of the tissue as opposed to being found intracellularly. The extracellular water is either located within the pores of the matrix or in the intrafibrillar spaces of the collagen[32]. Cartilage tissue hydration depends on two main and opposing factors, the proteoglycan's hydrophilicity, involving both the tendency for proteoglycan swelling in solution and Donnan osmotic pressure[33], and the strong collagen network which constrains the tissue swelling[30].

Flow of water in the extracellular matrix can be induced by applying mechanical forces to the tissue (i.e. convection). This will cause the water to not only move within the tissue, but also exude at the surface. Both these results have functional implications.

2.1.2 Organization

Cartilage is a complexly organized tissue with its constituent distribution and orientation varying depending on the region of tissue examined[19,20,34]. Figure 2.1 depicts the cellular and collagen fibril components as they are in a section of native cartilage as well as the delineation of the organizational zones based on depth. As can be seen in the figure, in the deep zone, the both the collagen fibrils and chondrocytes are organized in columns which run perpendicular to the articular surface. In the superficial-tangential zone, both the chondrocytes and collagen fibrils are generally oriented parallel with the articular surface, with the chondrocytes taking on a more elongated form. In the middle zone, the arrangement of collagen and cells are more disorganized than either the deep or superficial-tangential zone. The collagen fibrils are randomly oriented while the cells are more sparsely distributed and vary in shape from spherical (like in the deep zone) to somewhat elongated (transitioning to the superficial zone). Both water and proteoglycans are zone-dependent. Proteoglycan content is higher further away from the surface[19] and water following the opposite trend, with the surface of the tissue being more hydrated.

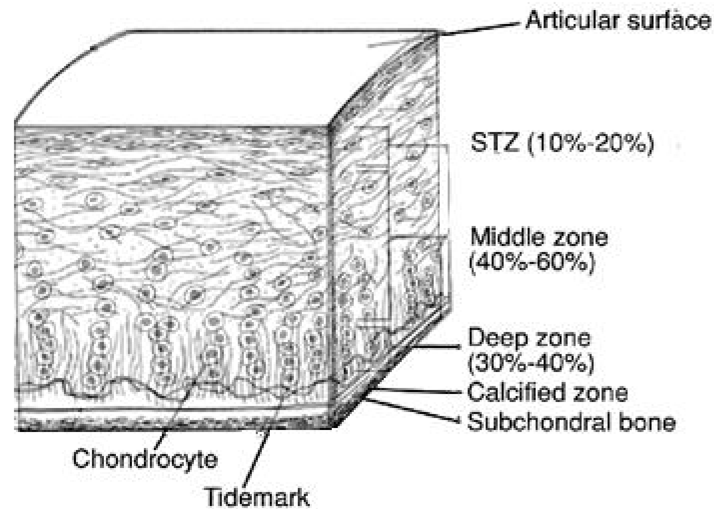

Figure 2.1: Cross-section of articular cartilage (adapted from Mow, et al., 1989[34])

Figure 2.2 depicts the molecular organization of the cartilage matrix which is divided into three zones based on distance from the cell[19,35]. The pericellular matrix is the area immediately surrounding the cell. In this zone, molecules are close enough to the cell that they are able to interact with cell surface receptors such as integrins. The pericellular zone also has a much higher proteoglycan concentration than either the territorial or interterritorial zones. Immediately outside is the territorial zone. This region is generally defined by a fine fibrillar collagen network made up of collagen type VI. Most distant is the interterritorial zone. This zone is most noted for its coarse type II collagen fibers which are organized in parallel as opposed to in a network. The territorial and interterritorial zones have about the same proteoglycan content which is approximately half the amount that is in the pericellular matrix zone.

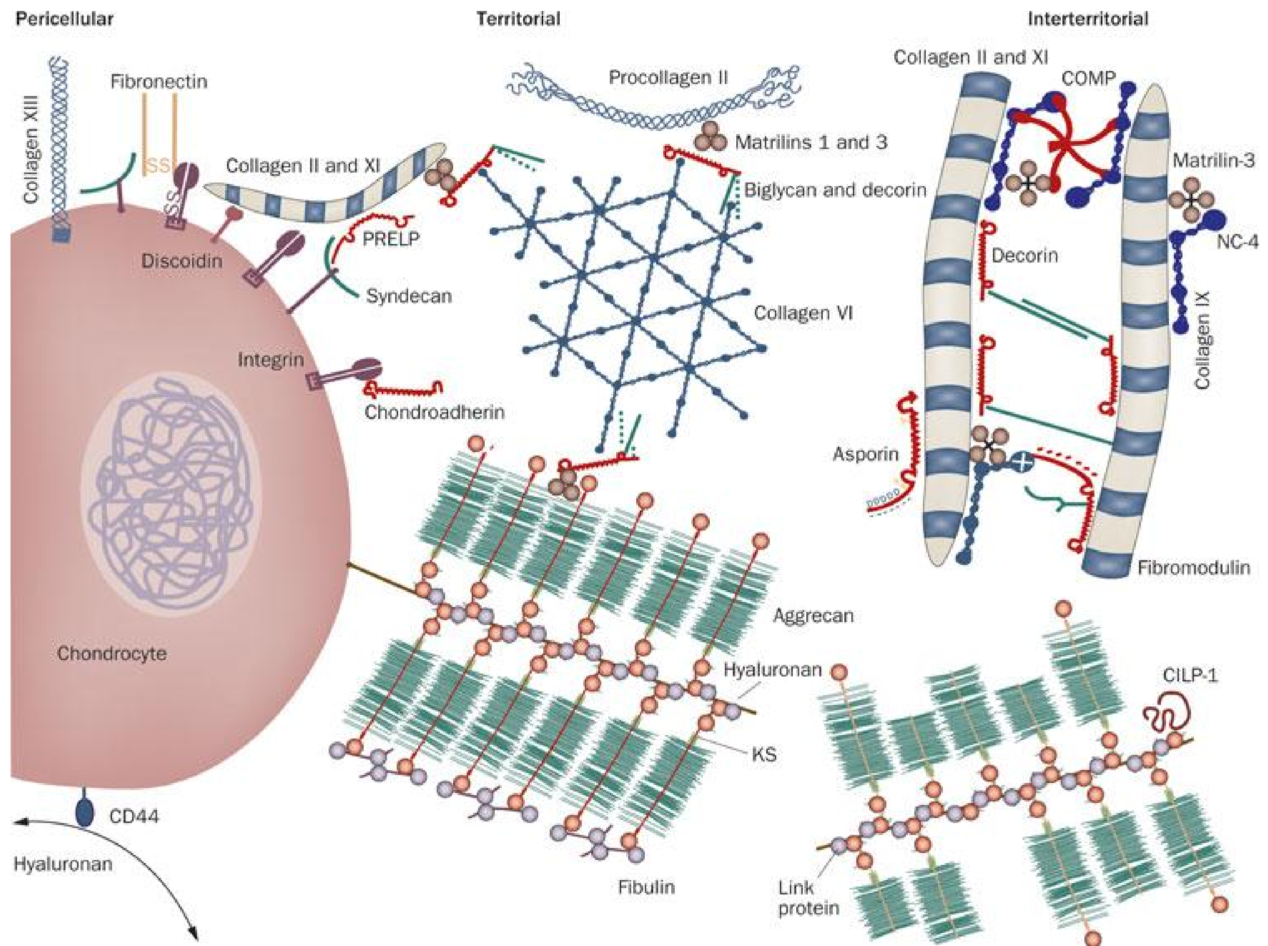

Figure 2.2: The molecular organization of articular cartilage [35]

2.1.3 Mechanical Properties

Cartilage is classified as a viscoelastic material and it therefore displays the three distinct time-dependent characteristics of viscoelastic materials: hysteresis, creep, and stress relaxation. The mechanical response of cartilage to applied forces also depends on the force or deformation applied.

Hysteresis is the phenomenon where the previous loading history of the material has an effect on the current behaviour of the tissue. Because of hysteresis, loading and unloading curves on a

stress-strain curve do not overlap. Similarly, subsequent loading and unloading curves of the same material do not follow the previous loading and unloading curves. The energy lost during one cycle can be calculated from the area of the loop of a loading and unloading cycle. Creep describes the tendency of a viscoelastic material under constant stress to slowly strain to an equilibrium value over time. Stress relaxation, on the other hand, is the tendency for a viscoelastic material under constant strain to slowly decrease in stress until equilibrium is reached. Although creep and stress relaxation seem to be related at first, the response of one cannot yet be mathematically used to predict the response of the other.

Cartilage viscoelasticity is said to be dependent on two separate phenomena: flow-dependent mechanisms and flow-independent mechanisms. The flow dependency is based on friction between the water and matrix macromolecules as well as the pressure developed by the affinity of proteoglycans for water[36]. The flow-independency is based on the friction that is present between the macromolecules of the extracellular matrix as the matrix is deformed under load[37].

2.1.4 Mechanotransduction

In chondrocytes, the process by which mechanical signals are transduced into cellular responses (mechanotransduction) has not been fully elucidated and several different signaling pathways have been identified (e.g. ECM receptors, mechanically sensitive ion channels, autocrine/paracrine signaling, etc.)[14,38]. Although quite distinct from one another, each of these pathways typically results in intracellular calcium signaling[39]; either directly from the influx through mechanically sensitive ion channels, or indirectly, as a secondary messenger system resulting from receptor binding.

While several mechanotransduction pathways are possible in chondrocytes[40–42], previous studies have shown that mechanical stimulation can be transduced into matrix synthesis by way of the purinergic receptor pathway[16,40,43]. Figure 2.3 is a schematic showing how mechanical stimulation results in calcium ion signalling through the purinergic receptor pathway and ATP signaling. Mechanical stimulation causes the extracellular release of ATP by matrix vesicles. The ATP then binds to the purinergic receptors, predominantly the $P2Y_2$ for chondrocytes, present on the cell membrane. This initiates an intracellular signalling cascade involving calcium ion signalling which causes an upregulation of extracellular matrix gene expression and ultimately results in synthesis of matrix proteins [44].

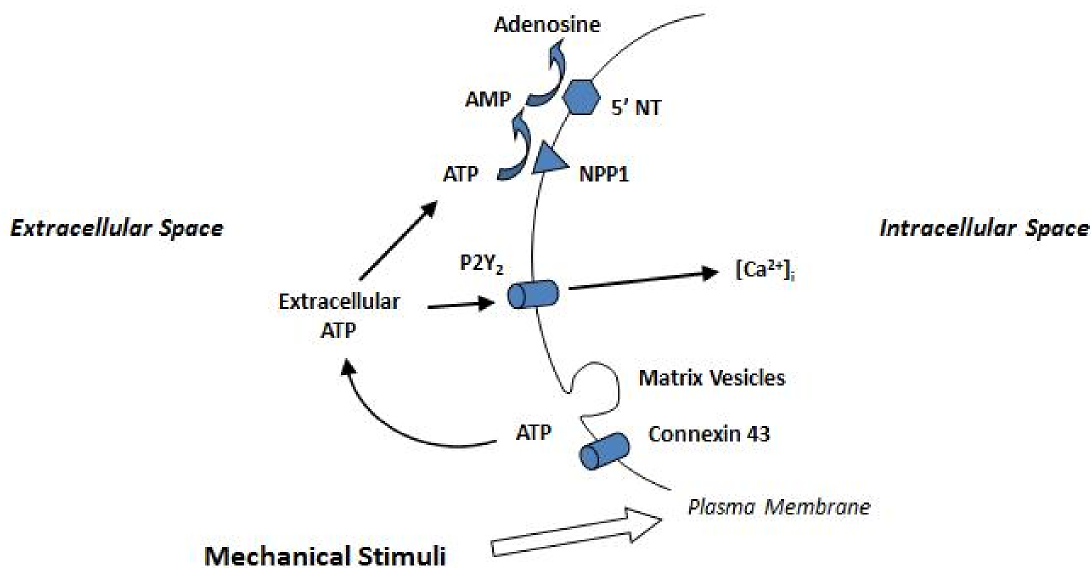

Figure 2.3: ATP-purinergic receptor mechanotransduction pathway

Calcium signaling is a ubiquitous second messenger involved in several different intracellular signaling processes and is responsible for the initiation of gene transcription[45,46]. In

chondrocytes, the effect of mechanical loading on intracellular calcium signaling has been extensively studied[43,47–56]. Through the use of calcium sensitive dyes, the number of chondrocytes experiencing calcium sparks (or transients) tends to increase as a result of dynamic mechanical stimulation[43,56]. Although it is generally accepted that increased calcium signaling in response to mechanical loading leads to increased matrix biosynthesis, the exact relationship between calcium signaling and biosynthesis has not yet been established.

2.2 Cartilage Tissue Engineering

Although not yet used clinically, tissue engineering is currently under investigation as a new approach to cartilage repair. The expectation is that constructs can be developed which will be used as replacement tissue in the damaged area of the joint surface[57–59]. Generally, these constructs are 3-dimensional and can involve a combination of cells, scaffolds, and/or chondrogenic stimuli.

While articular chondrocytes present an obvious cell source for cartilage tissue engineering, the availability of healthy donor tissue is rare[60]. For this reason, multipotent mesenchymal stem cells, derived from bone marrow or adipose tissue, have been investigated as a possible cell source[61,62]. Because mesenchymal stem cells are relatively abundant and have the potential to differentiate into chondrocytes, they are a lucrative cell source.

When chondrocytes are cultured in a 2D environment, they tend to dedifferentiate into fibroblast-like cells with a characteristic spindle shape and an upregulation of collagen type I[63]. Therefore,

a 3D environment is necessary for chondrocytes to maintain their phenotype and produce cartilage-related extracellular matrix molecules. Both natural and synthetic biomaterials have been used for cartilage tissue engineering. Natural based materials include: the protein based collagen[64,65] and fibrin[66], scaffolds; and the polysaccharide based alginate[67], cellulose[68], chitosan[69], hyaluronic acid[70], and agarose[58] gels. Synthetic based materials include: PLGA[71], PLA[72], and PEG[73]. Many of these materials are commercially produced and have been tested in their chondrogenic capacity for both chondrocytes and mesenchymal stem cells. Choice of scaffold strongly depends on its intended use. A simple scaffold that does not interfere with subsequent analysis techniques is useful for *in vitro* studies, while more complex scaffolds are better for *in vivo* constructs because they can be tailored to interact with the surrounding tissue.

Biochemical stimuli are often used to improve cartilage constructs. For mesenchymal stem cells the biochemical stimulus can be in the form of differentiating media. Otherwise, the classical chemical stimuli are growth factors. Commonly investigated growth factors include[74,75]: transforming growth factor (TGF) 1, bone morphogenic proteins (BMP), insulin-like growth factor (IGF) 1, and fibroblast growth factor (FGF) 2. Stimuli are not just limited to biochemical agents; many cell types have mechanical receptors thus mechanical stimulation can be used to affect construct behaviour.

2.2.1 Mechanical Stimulation

Under physiologic conditions, cartilage is subjected to a variety of mechanical stimuli. Peak stresses of 10 to 20 MPa and peak deformations of 15% to 45%[7] can occur under dynamic and static physiologic loading conditions. Chondrocytes metabolism is influenced by these

mechanical environments and therefore the condition of cartilage tissue as a whole depends on the mechanical stimuli experienced. Based on these observations, researchers have been attempting to use mechanical stimuli to enhance tissue formation.

Chondrocytes can react to mechanical stimulation in a variety of manners. Responses that have been observed include: maintaining or changing cell phenotype[76], increasing or supressing cell proliferation[15,77], inducing matrix macromolecule synthesis or catabolism[77–83], and affecting matrix macromolecule organization[82,84,85]. Because the mechanical properties of cartilage tissue primarily depend on the content and organization of the extracellular matrix, it is important for researchers to examine the various responses of chondrocytes to a variety of mechanical environments.

Numerous mechanical stimulation methods have been examined with respect to improving tissue engineered cartilage constructs. These stimuli include: static compression[15], dynamic compression[15,76,77,79–81,85–87], shear[82], hydrostatic pressure[78,83,84], ultrasound[88,89], and vibration[65]. The focus of these stimuli has been either to increase cellular proliferation or to increase extracellular matrix macromolecule production in order to achieve a more robust construct more efficiently.

2.3 Desensitization of Chondrocytes

While little research has been conducted on chondrocyte desensitization, many other cells experience this phenomenon and have been better documented. Although the stimulus is

different for these other cells, the mechanisms of their desensitization may be useful in elucidating the mechanism of chondrocyte desensitization to mechanical stimulation.

Desensitization is most readily observed in sensory cells. The rods and cones in the eye which are responsible for differentiating light and dark as well as colour can both undergo receptor desensitization that can affect the way a person sees something. Two common examples of colour desensitization are the Troxler effect[90], where a faint colour seen somewhat in the periphery is sometimes seen as its complementary colour after a period of time; the second is the afterimage that can be seen when a vivid image is suddenly replaced by a solid white backdrop, the image appears to exist on the white backdrop in faint complementary colours to the original image. Desensitization of the rod cells[91] can be observed when a person who has been exposed to an extremely bright environment suddenly enters a dark environment, such as coming inside from a bright summer day, and is temporarily unable to see because the rod cells have been oversaturated by being exposed to the intense light and require time to recover in the darker environment before a person can see properly.

Electrical desensitization is most easily seen in the control of muscles[92]. Muscles are moved by the body by sending an electrical signal down a nerve to a motor neuron which innervates a muscle fibre bundle. The fibre bundle contracts as a result of this electrical pulse. These electrical pulses can be observed by attaching electrodes to a subject performing muscle movements. Long term stimulation of the muscle to repeat a specific contraction can cause the electrical communication to decrease, which is not only measureable with electrodes but also

measureable by the reduced muscle contraction force. These reductions are due to polarizing and depolarizing ions which are unable to get sequestered into their unfired state quickly enough for the next contraction, causing fewer signals to be conducted to the motor neurons and therefore fewer muscle fibres contracting to perform the desired muscle movement.

The response to mechanical stimulation is especially important to investigate for cells and tissues in the body which are subjected to regular mechanical loading in physiological conditions (i.e. bone, tendon and ligament, muscle, and cartilage). Much work has been done on structural tissues such as bone, muscle, and cartilage. Exercise studies on bone[93] and muscle[94] show that given the same loading protocols (i.e. weights and repetitions), once the body has accommodated that particular loading scenario, further development does not occur. Several molecular pathways have been identified as possible mechanisms for bone mechanotransduction. These pathways include: membrane-bound ion channels and ATP purinergic signalling[93]. For cartilage studies, encapsulated cells were subjected to the same frequency and amplitude of stimulation, but different durations. As the duration of stimulation increased, the response, as measured by matrix molecule production, increased until it reached a maximum at which point the response plateaued. Continuous loading past a certain time point did not measurably increase matrix molecule production[9,17,65,95]. This type of behaviour indicates that the cells were able to respond to the mechanical stimulus by producing matrix, however; the cells were desensitized at some point and further matrix production was halted.

2.3.1 Possible Mechanisms of Mechanical Desensitization

Desensitization of the purinergic receptor pathway may occur at several points. An increase in extracellular ATP concentration is naturally mitigated by membrane-bound ATPases (e.g. NPP1, 5' NT)[40]. In other cell types, the expression of these enzymes has been shown to increase in response to an increase in ATP[96]. It is possible that an increase in ATP caused by mechanical stimulation could be negated by increased expression of these membrane enzymes and their enzymatic activity which would mean fewer signals to the ATP receptors responsible for initiating the calcium signalling and therefore matrix synthesis.

Desensitization of this purinergic receptor pathway could also occur when the $P2Y_2$ receptor is phosphorylated by protein kinase C (PKC) and then internalized by the regulating protein arrestin[97]. Without ATP receptors present on the cell membrane, downstream signalling cannot be initiated. The $P2Y_2$ receptors have several possible locations for phosphorylation. Artificial truncation of the receptor's structure can eliminate some of the phosphorylation sites. When stimulated with UTP, the number of receptors on the cell surface is reduced, but the calcium signalling in the cells with the truncated receptors remains higher than those with the unchanged receptors[98].

Finally, desensitization may also occur at the calcium signalling level. The calcium signalling caused in this pathway[99] can be observed as transient flashes of light when observed using intracellular calcium ion dyes such as Fluo-3[48], Fluo-4[43,54], indo-1[50], and Fura-2[99]. The calcium ions must first be sequestered into endoplasmic reticulum stores before being released to cause a spike in intracellular cytosolic calcium ions. It is hypothesized that the sequestration of the

calcium ions becomes less efficient over time when cells are mechanically stimulated causing fewer calcium ions to be available for release.

2.3.2 Refractory Period

The refractory period is the amount of time required for a mechanically desensitized cell to become resensitized to the same type of stimulus to which it was originally desensitized. While refractory periods for the use of mechanically stimulating tissue engineered cellular constructs have not been specifically examined for chondrocytes, studies on bone has shown that recovery periods are beneficial[100,101]. Researchers have noted that chondrocyte constructs which undergo bouts of intermittent dynamic stimulation perform better, in terms of matrix molecule synthesis, compared to constructs which receive the same duration of dynamic stimulation continuously[86]. Experiments on bone where the protocol includes periods of rest resulted in up to twice the increase in the anabolic response to mechanical loading as measured by resultant changes in the cross-sectional moment of inertia of the stimulated bone[100].

2.4 Stochastic Resonance

Stochastic resonance is observed in non-linear systems and characterized by an increase in sensitivity to a weak input signal due to the presence of noise[102]. This phenomenon has been observed in a multitude of biological systems from the molecular level systems of DNA transcription in gene expression[103] up to the whole body level control systems involved with maintaining homeostasis of blood pressure[104], blood oxygenation[105,106], and balance[107] where stochastic resonance devices have been developed for regular use[105]. In addition, previous studies on bone (both whole bone *in vivo*[108] and isolated osteoblasts *in vitro*[109]) have also attempted to

harness this effect by using loading protocols superimposed with broad frequency (0 - 50 Hz) mechanical vibrations[108,109]

Chapter 3 – Study 1: Sensitization and Desensitization to Dynamic Mechanical Loading

The work from this chapter was published in Biomechanics and Modeling in Mechanobiology with co-author Stephen D Waldman[110].

3.0 Introduction

Dynamic physiologic loading is essential for the development of healthy articular cartilage *in vivo*[111]. Similarly, both exercise and immobilization studies have shown that chondrocyte homeostasis, specifically in terms of extracellular matrix (ECM) synthesis, can be controlled by mechanical loading[112–116]. For these reasons, dynamic mechanical loading has been routinely used as a means in cartilage tissue engineering to improve the mechanical and biochemical properties of the developed tissue constructs[7–10].

Although there have been numerous studies of the effect of mechanical stimuli on chondrocyte metabolism[7,11], the optimal stimulation conditions for a particular experimental model (cells, constructs, tissues, *etc.*) are generally determined empirically[12–14,79]. With the possible exception of stimulation frequency (the optimal frequency being around 1 Hz[14,15]), there is little consensus as to the amplitude or number of cycles that should be administered to elicit an anabolic effect, nor is there agreement on how often the stimulus should be administered. In addition, as some studies have observed a potential desensitization response to prolonged loading[9,16–18], the determination of optimal stimulation conditions is important for a sustained anabolic effect to influence tissue formation and properties over the long-term. Given the potential benefits of dynamic mechanical stimulation, it is of interest to understand the chondrocyte response to

dynamic mechanical loading in order to optimize the growth of tissue engineered cartilage constructs.

In chondrocytes, the process by which mechanical signals are transduced into cellular responses (termed mechanotransduction) has not been fully elucidated and several different signaling pathways have been identified (e.g. ECM receptors, mechanically sensitive ion channels, autocrine/paracrine signaling, etc.)[14,38]. Although quite distinct from one another, each of these pathways typically results in intracellular calcium signaling[39]; either directly from the influx through mechanically sensitive ion channels, or indirectly, as a secondary messenger system resulting from receptor binding. Calcium signaling is a ubiquitous second messenger involved in several different intracellular signaling processes and is responsible for the initiation of gene transcription[45,46]. In chondrocytes, the effect of mechanical loading on intracellular calcium signaling has been extensively studied[43,47–56]. Through the use of calcium sensitive dyes, the number of chondrocytes experiencing calcium sparks (or transients) tends to increase as a result of dynamic mechanical stimulation[43,56]. Although it is generally accepted that increased calcium signaling in response to mechanical loading leads to increased matrix biosynthesis, the exact relationship between calcium signaling and biosynthesis has not yet been established. However, with such a correlation, it may be possible to not only quickly determine the effectiveness of a particular loading protocol, but also determine specifically how much loading is required to elicit an optimal response. Similarly, this would also greatly reduce the empirical effort needed to determine optimal recovery periods between repeated applications of mechanical stimuli.

3.1 Specific Aim

The specific objectives for this study were to: (i) investigate the short-term temporal response (sensitization and desensitization) on matrix biosynthesis by chondrocytes subjected to dynamic mechanical loading, (ii) correlate calcium signaling and cartilaginous matrix biosynthesis in response to loading, and (iii) investigate whether calcium signaling could be used to predict an optimal rest period between repeated applications of mechanical stimuli.

3.2 Methods

3.2.1 Preparation of cell-seeded agarose constructs

Articular cartilage was harvested from the knees and shoulders of 12 to 16-week-old female New Zealand White rabbits (Queen's Animal Care protocol number Waldman-2012-028). Cells were obtained through sequential enzymatic digestion with 0.5% w/v protease (Sigma Aldrich Ltd., Oakville, ON, Canada) for 1 hour and 0.15% w/v collagenase A (Sigma Aldrich) for 18 hours. Enzymes were prepared in Ham's F-12 (Thermo Fisher Scientific, Mississauga, ON, Canada) with 25 mM HEPES (Bioshop Canada, Burlington, ON, Canada). The cell digest was filtered through a 200-mesh filter (Sigma Aldrich) then washed and resuspended at a concentration of 20 x 10^6 cells/mL in Ham's F-12 with 25 mM HEPES. A 4% w/v solution of type VII low melt agarose (Sigma Aldrich) was prepared in phosphate buffered saline (PBS, pH 7.4) and mixed slowly (to avoid the formation of bubbles) in equal parts with the cell suspension to form 2% agarose gels with a concentration of 10 x 10^6 cells/mL. The gel constructs were cast in custom-made Teflon™ cylindrical moulds, 3 mm in diameter by 3 mm in height containing approximately $2x10^5$ cells. Following gelation at room temperature, cell-seeded gels were levelled with a scalpel, then removed from the moulds and placed in a petri dish containing complete media (Ham's F12 media supplemented with 20% v/v fetal bovine serum (FBS, Sigma

Aldrich), 20 mM HEPES, 100 µg/mL ascorbic acid (Sigma Aldrich), and 2X antibiotics/antimycotics (Sigma Aldrich)). Cell-seeded gels were then allowed to incubate for 24 hours at 37°C and 5% CO_2 prior to the application of mechanical stimulation.

3.2.2 Application of dynamic compressive loading

A Mach-1 Micromechanical Testing System (Biomomentum, Laval, PQ, Canada; displacement-control resolution of 0.5 µm or a strain resolution of 0.017% for a 3 mm tall construct) with a custom-built loading rig [95] with individually adjustable loading platens was used to stimulate the cell-seeded gels housed in 24 well culture plates (Figure 3.1a) in unconfined compression. Rapid prototyped plastic (ABS) retaining rings were used to keep the gels stationary within the wells during stimulation. Prior to stimulation, constructs were transferred from the petri dishes to the 24 well plates and supplied with 400 µL of fresh complete media immediately prior to stimulation. To determine the sensitivity to dynamic compressive loading, cell-seeded gels were stimulated under a range of durations (0 to 90 minutes) at three different strain amplitudes (2.5 ± 0.07%, 5 ± 0.1%, and 10 ± 0.3%), each at a rate of 1 Hz. Unloaded control constructs were similarly transferred to 24 well plates with retaining rings, but did not undergo dynamic compression.

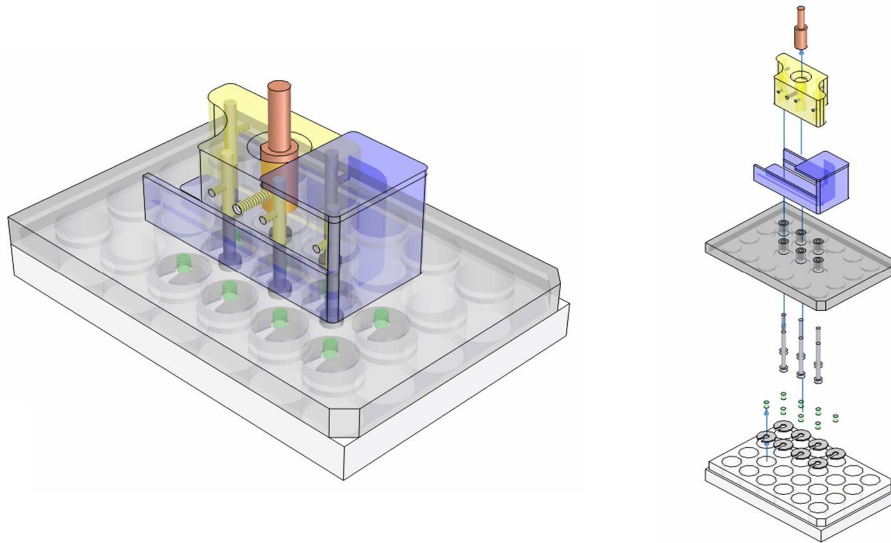

Figure 3.1: (a) 3D representation of the custom-designed unconfined compression system used in conjunction with 24 well culture plates[117]. (b) Experimental timelines of studies related to: determination Ca^{2+} signaling (top) and (c) determination of optimal rest periods (bottom)

Immediately after stimulation, cell-seeded gels were incubated in the presence of both [^{35}S]SO_4 (5 μCi/culture) to label proteoglycans and [^{3}H]proline (5 μCi/culture) to label collagen for a period of 24 hours. Although proline can be incorporated into different proteins, in chondrocyte cultures approximately 90% of proline becomes incorporated into collagen [118,119]. Constructs were washed thoroughly with PBS during harvesting to remove any unincorporated isotope and

then digested in 40 µg/mL papain (Sigma Aldrich), 1 mM ethylenediaminetetraacetic acid (Sigma Aldrich), and 2 mM dithiothreitol (Sigma Aldrich) at 65°C for 72 hours. The accumulation of newly synthesized proteoglycans and collagen was then estimated by quantifying radioisotope incorporation from aliquots of the papain digest using a LS6500 β-liquid scintillation counter (Beckman Coulter, Mississauga, ON, Canada). Proteoglycan and collagen synthesis were calculated relative to the DNA content of the tissue, determined from aliquots of the papain digest using the PicoGreen® dsDNA dye (Life Technologies, Burlington, ON, Canada) assay [120] and expressed as a percentage of the unstimulated controls.

3.2.3 Measurement of intracellular Ca^{2+} signaling

Using separate samples, intracellular Ca^{2+} signaling was measured for each mechanical stimulation condition (Figure 3.1 b). Prior to mechanical stimulation, cell-seeded constructs were incubated in the intracellular calcium ion dye Fluo-4 AM (2.5 µM; Life Technologies) in complete media containing 0.25 mM sulfinpyrazone (to reduce the leakage of the de-esterified indicator) at 25°C for 90 minutes. Constructs were washed for 10 minutes in complete media containing 0.25 mM sulfinpyrazone. Using this procedure, in excess of 90% of the cells were positively stained with Fluo-4. After washing, the labeled cell-seeded gels were mechanically stimulated, as described above (in complete media without sulfinpyrazone). Immediately after stimulation, the constructs were imaged at a depth of 200 µm (maximum penetration of the laser) at the sample centre-line to avoid barreling edge effects, by confocal microscopy (IX81, Olympus Canada, Richmond Hill, ON, Canada) with a 10X objective (excitation: 488 nm; emission 516 nm). Images (640x640 pixels) were captured every 5 s over a 5 min imaging period (12 frames per minute) with the shutter closed between acquisitions to reduce photo-bleaching.

Captured images were then analyzed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA) by selecting circular regions of interest around individual cells that were in plane and visibly experienced Ca^{2+} transients. Fluorescent intensity traces of these labelled cells (as a function of time) were then plotted and the number of peaks (i.e. transients) were counted. Only large changes in fluorescent intensity (at least two times the amplitude above the trace baseline noise) were counted as transients and only the first three transients were counted. For each construct, the following data were recorded: (i) the number of cells experiencing at least one transient, and (ii) the number of cells experiencing multiple transients (i.e. 2 or more).

3.2.4 Effect of Ca^{2+} transient inhibition

The effect of the SERCA inhibitor, thapsigargin, was determined on a subset of stimulation conditions to confirm the association between matrix synthesis and Ca^{2+} signaling. Constructs were stimulated under previously identified responsive (5% strain amplitude, 20 minutes) and unresponsive (5% strain amplitude; 60 minutes) conditions in the presence or absence of thapsigargin (1 μM) added to the complete culture media immediately prior to stimulation. Matrix synthesis and Ca^{2+} signaling were then determined as previously described. Control constructs for Ca^{2+} signaling were incubated with the inhibitor during the 10 minute wash period (after Fluo-4 staining).

3.2.5 Determination of an optimal rest period for additional stimulation

To determine the optimal refractory period (Figure 3.1 c), cell-seeded constructs were first mechanically stimulated under identified synthetic conditions determined in the previous experiment (5% compressive amplitude for 20 minutes) with Ca^{2+} signaling assessed

periodically over the next 24 hours (1, 2, 3, 4, 6, and 24 hours). To confirm the recovery of mechanical sensitivity after the refractory period, separate constructs were subjected to first application of mechanical stimulation, followed by a defined refractory period (1, 2, 3, 4, 6, or 24 hours), and then subjected to a second application of mechanical stimulation. All mechanical stimulation was conducted using the previously identified synthetic conditions (5% compressive amplitude for 20 minutes). Immediately after stimulation, matrix synthesis was determined as described previously. Matrix synthesis was normalized to time-matched controls (samples treated in the same fashion without the second application of mechanical stimulation) to account for any matrix synthesis occurring from the first application of mechanical stimulation.

3.2.6 Statistical Analyses

All experiments were performed at least twice using cell obtained from different donors. Collected data from multiple experiments was pooled, resulting in sample sizes of $n \geq 6$. All results were expressed as the mean ± standard error of the mean (SEM). Collected data was analyzed statistically using a one-way or two way ANOVA (depending on the experiment) and the Tukey's post hoc test (SPSS version 16, SPSS Inc., Chicago, IL, USA) to determine the effect of stimulation duration. Pearson's product-moment correlation coefficient was computed to assess the relationship between levels of Ca^{2+} signaling and synthesis of collagen and proteoglycans. All data were checked prior to performing statistical tests for both normality and equal-variance. Significance was associated with p-values less than 0.05 and trends were associated with p-values between 0.05 and 0.1.

3.3 Results

3.3.1 Sensitization/desensitization to dynamic compressive loading

Matrix synthesis in response to dynamic compression under different amplitudes (2.5, 5, and 10%) and increasing duration (0 – 90 min or 0 – 5400 cycles) was assessed by radioisotope incorporation (Figure 3.2). Under a 2.5% compressive amplitude, synthesis of both collagen and proteoglycans was supressed by 35-45% after 10 and 20 minutes of stimulation (600 and 1200 cycles) ($p<0.05$) and was unchanged relative to control after longer durations of stimulation. Under a 5% compressive amplitude, synthesis of both molecules was unchanged after 10 minutes (600 cycles), increased by 25-30% after 20 minutes (1200 cycles, $p<0.05$), and then returned to unchanged levels upon further stimulation. A compressive amplitude of 10% resulted in two observable increases in matrix synthesis: a small increase in proteoglycan synthesis (12%) after 20 minutes (1200 cycles, $p<0.05$) and a 15-20% increase in collagen and proteoglycan synthesis after 60 minutes (3600 cycles, $p<0.05$). All other stimulation durations elicited no observable changes in matrix synthesis.

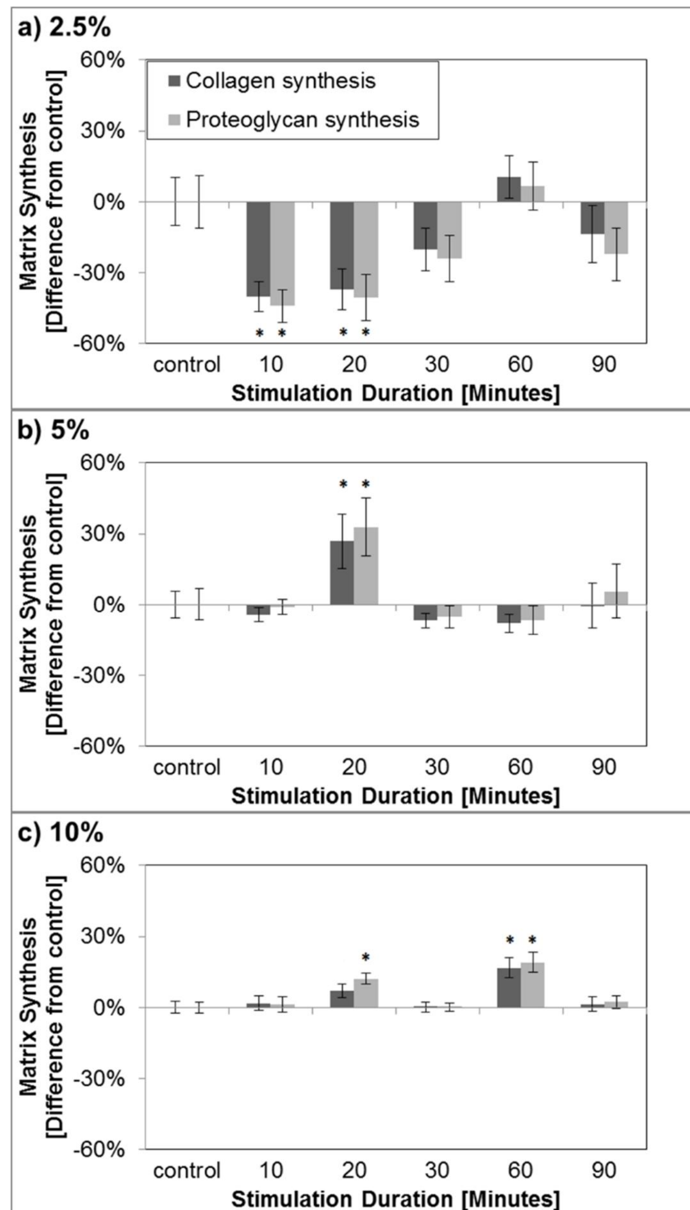

Figure 3.2: Collagen and proteoglycan synthesis after a 2.5% (n=6) (a), 5% (n=6) (b), or 10% (n=12) (c) dynamic compressive strain amplitude for 0 to 90 minutes. * Different from control ($p<0.05$)

Evaluation of DNA content (as measured by the PicoGreen assay) showed no significant changes in cellularity as a result of dynamic mechanical stimulation under either a 2.5% or 5% compressive amplitude (Table 3.1). However, a reduction in DNA content of 10% was observed

after 60 minutes of stimulation (3600 cycles) under a 10% compressive amplitude ($p<0.05$) (Table 3.1).

Table 3.1: Changes in DNA content after a 2.5%, 5%, or 10% dynamic compressive strain amplitude for 0 to 90 minute duration (mean ± SEM).

	Dynamic Compressive Amplitude		
Duration [min]	**2.5%** (n=6)	**5%** (n=6)	**10%** (n=12)
control	0 ± 6%	0 ± 6%	0 ± 3%
10	-39 ± 22%	0 ± 5%	-7 ± 2%
20	-37 ± 22%	4 ± 4%	-8 ± 2%
30	17 ± 6%	8 ± 3%	-6 ± 2%
60	4 ± 6%	6 ± 2%	-10 ± 3%*
90	18 ± 5%	8 ± 6%	1 ± 2%

*Different from control ($p<0.05$)

Cells loaded with the calcium-sensitive dye Fluo-4 were used to evaluate changes in Ca^{2+} signaling under the same mechanical stimulation conditions. Measurement of fluorescent intensity showed a range of Ca^{2+} signaling responses within the same construct, with cells experiencing either no Ca^{2+} transients, a single Ca^{2+} transient, or multiple Ca^{2+} transients (Figure 3.3). On average, each construct contained about 170 labelled particles that were visible in each image. Of these, by selecting for size and circularity, approximately 20% were considered to be cells in plane. Of the cells in plane, on average, about 40% were active and experiencing one or more transient. While the duration of each transient appeared similar for all cells (experiencing transients), the amplitude of the transients appeared to reduce with successive transients experienced by the same cell. In addition, the time elapsed between successive transients (transient refractory period) appeared to be random in nature.

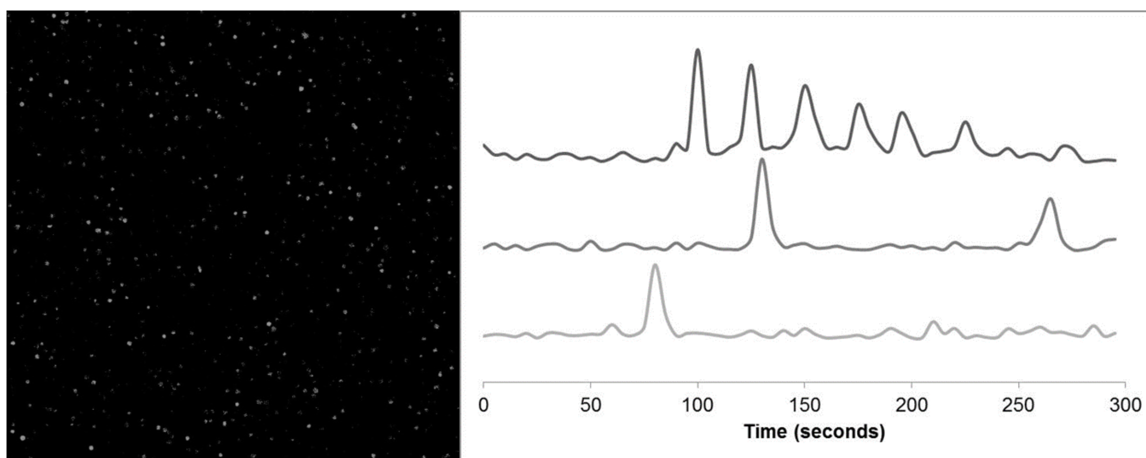

Figure 3.3: (a) Representative fluorescence image of Fluo-4 labelled cells (original magnification 10X). Just under 300 particles were labeled with the dye, however only 50 of these labeled particles were considered as cells in plane. (b) Traces of overall cell fluorescent intensity (arbitrary units) for 3 different cells imaged with Fluo-4 and confocal microscopy within the same construct: two cells experiencing multiple transient (top and middle traces) and one cell experiencing a single transient (bottom trace)

For each sample, the number of cells experiencing at least one transient or the number of cells experiencing multiple transients (two or more) were determined and plotted as a function of stimulation duration (Figure 3.4). The variability in overall signaling activity as determined by the number of cells experiencing at least one transient was large and showed no differences with respect to stimulation duration. However, an increasing trend was observed between the number of cells experiencing multiple transients with stimulation duration (p=0.07). Thus, to account for the distribution in cellular responses within a particular construct, the ratio of the number of cells experiencing multiple transients to the total number of cells experiencing at least one transient was determined.

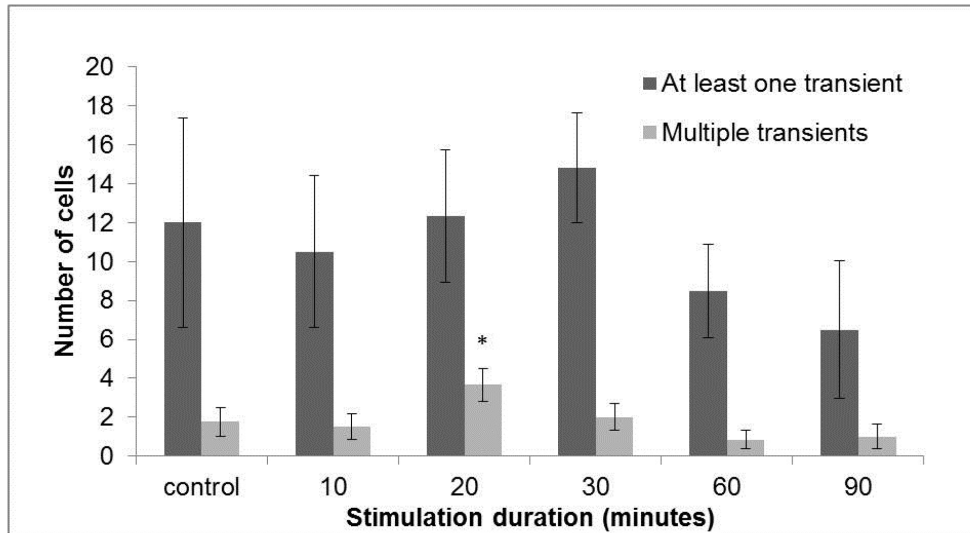

Figure 3.4: Cells exhibiting at least one transient and cells exhibiting more than one transient over a 5 minute imaging period in constructs subjected to 5% dynamic compressive strain amplitude for 0 to 90 minute duration (n=6). 177±39 particles were visible in each image analyzed; 25%±4% were considered as cells in plane and 44%±12% of these in plane cells were active. * Increasing trend from control (p=0.07)

Using this ratio, similar responses with respect to stimulation duration were observed (Figure 3.5). No changes in Ca^{2+} signaling were observed under a 2.5% compression amplitude. Under a 5% compressive amplitude, signaling was increased after 20 minutes of stimulation (1200 cycles, $p<0.05$). A compressive amplitude of 10% resulted in two observable peaks in signaling: one after 20 minutes (1200 cycles, p=0.09) and another after 60 minutes (3600 cycles, $p<0.05$).

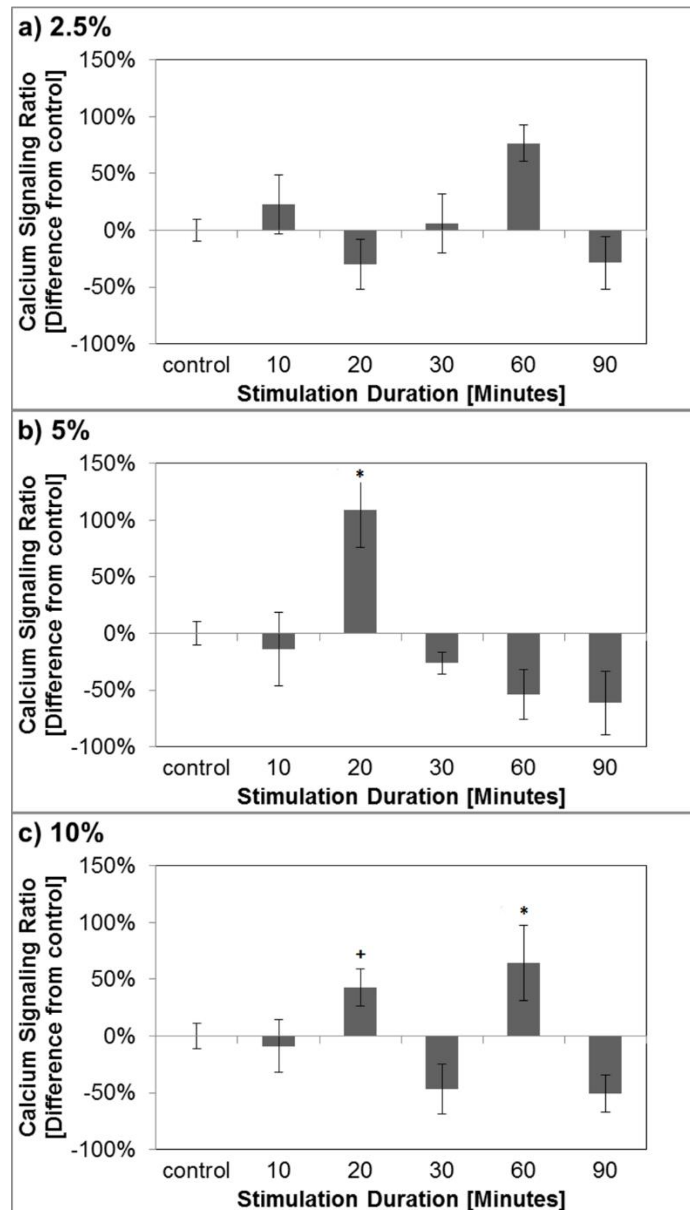

Figure 3.5: Ca^{2+} signaling (one or more to multiple transient ratio) expressed relative to control after a 2.5% (a), 5% (b), or 10% (c) dynamic compressive strain amplitude for 0 to 90 minute duration (n=6). About 40% of in plane cells were active and used to calculate the signaling ratios. *Different from control ($p<0.05$); + Trend from control ($p<0.08$)

To further explore the potential correlation between Ca^{2+} signaling and matrix synthesis, Ca^{2+} signaling and matrix synthesis results were plotted against one another for each stimulation amplitude (Figure 3.6) with statistical relationships determined by Pearson's product-moment

correlation. Positive correlations between Ca^{2+} signaling were observed for both collagen and proteoglycan synthesis at the 5% strain amplitude ($r=0.920$ and 0.872; $p<0.05$) and 10% strain amplitude ($r=0.848$ and 0.874; $p<0.05$). However, under a 2.5% strain amplitude, no correlation between Ca^{2+} signaling and matrix synthesis was observed. While increases in Ca^{2+} were associated with increased matrix synthesis, reductions in Ca^{2+} signaling appeared to be associated with both inhibitory and null effects on matrix synthesis.

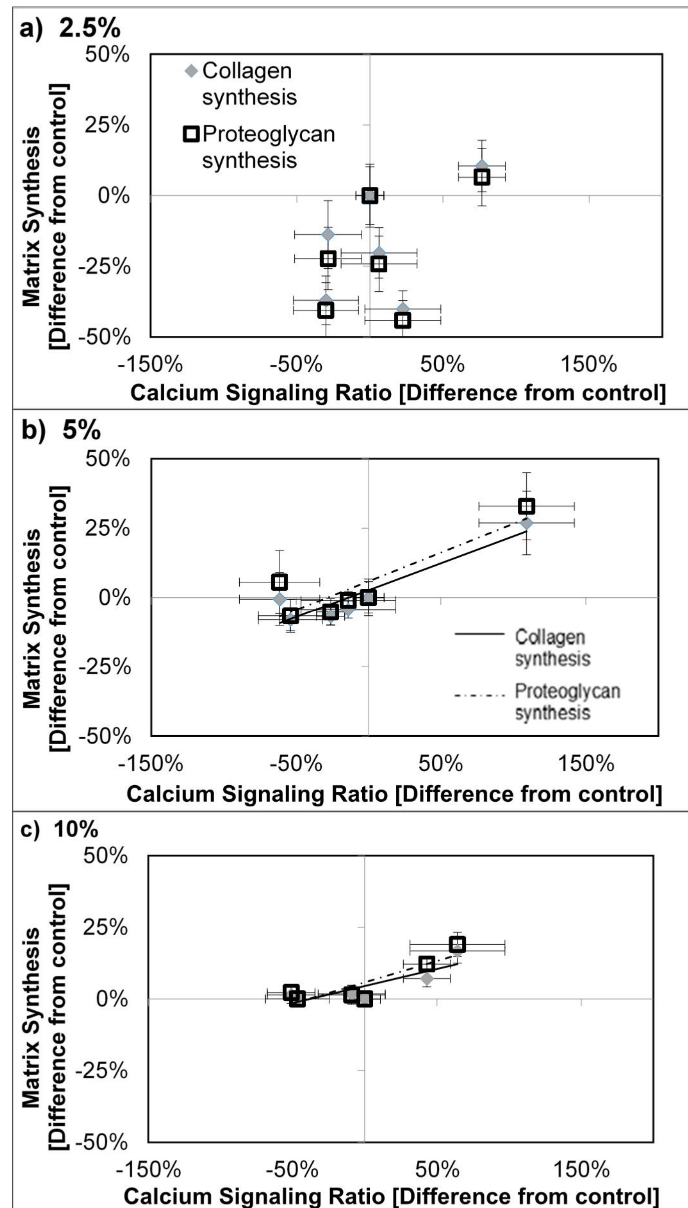

Figure 3.6: Correlation between Ca^{2+} signaling and matrix synthesis after a 2.5% (a), 5% (b), or 10% (c) dynamic compressive strain amplitude. Positive correlations were only observed between Ca^{2+} signaling and collagen (solid line) and proteoglycan (dashed line) synthesis under the 5% strain amplitude (r=0.920 and 0.872, n=6, $p<0.05$) and 10% strain amplitude (r=0.848 and 0.874, n=6, $p<0.05$) with no correlations found under 2.5% strain amplitude (r=0.524 and 0.537, n=6, p=0.286 and 0.272)

3.3.2 Effect of Ca^{2+} signaling inhibition

To confirm the correlation between Ca^{2+} signaling and matrix synthesis, additional experiments were conducted in the presence of the SERCA inhibitor, thapsigargin. In the presence of the

inhibitor, matrix synthesis was almost completely inhibited to a similar degree in both the stimulated and unstimulated samples ($p<0.05$) (Table 3.2). Similarly, in the presence of thapsigargin, no multiple Ca^{2+} transients were observed in any of the samples, and single transients were only observed in 17 – 67% of the samples (Table 3.2). Alternatively, the DNA content of the constructs appeared to slightly increase ($p<0.05$) as a result of treatment with the inhibitor (Table 3.2).

Table 3.2: Effect of thapsigargin on matrix synthesis (collagen and proteoglycan), DNA content and Ca^{2+} signaling for 5% dynamic compressive strain for 0 to 60 minute durations (mean ± SEM).

	Collagen Synthesis [Difference from control]	**Proteoglycan Synthesis** [Difference from control]	**DNA Content** [Difference from control]	**Ca^{2+} Signaling Ratio**
control (n=6)	0 ± 2%	0 ± 6%	0 ± 1%	0.15 ± 0.03
control + Tg (n=6)	-72 ± 1%*	-72 ± 1%*	16 ± 4%*	0^{2} ± N/A
20 min (n=6)	9 ± 4%*	9 ± 4%*	-5 ± 4%	0.23 ± 0.04*
20 min +Tg (n=6)	-70 ± 2%*	-69 ± 2%*	8 ± 4%*	0^{4} ± N/A
60 min (n=6)	-1 ± 4%	-4 ± 4%	1 ± 4%	0.06 ± 0.04
60 min + Tg (n=6)	-75 ± 1%*	-77 ± 1%*	10 ± 3%*	0^{1} ± N/A

+ Tg indicates the presence of thapsigargin (1 µM). Superscript indicates number of samples that were used to calculate the Ca^{2+} ratio.
*Different from control ($p<0.05$)

3.3.3 Using Ca^{2+} signaling to determine optimal rest periods for additional stimulation

In the next set of experiments, we explored the possibility of using Ca^{2+} signaling as a means to determine the optimal rest period between successive applications of the mechanical stimuli. Based on the previous results, constructs were stimulated under synthetic conditions (5% compressive amplitude for 20 minutes) and Ca^{2+} signaling tracked periodically over the subsequent 24 hours. Ca^{2+} signaling increased immediately after stimulation and continued to be

elevated for 1 hour after stimulation ($p<0.05$). Activity then dropped, reaching levels below control at 4 hours ($p=0.08$) and returning to pre-stimulated (control) levels at 6 hours (Figure 3.7a).

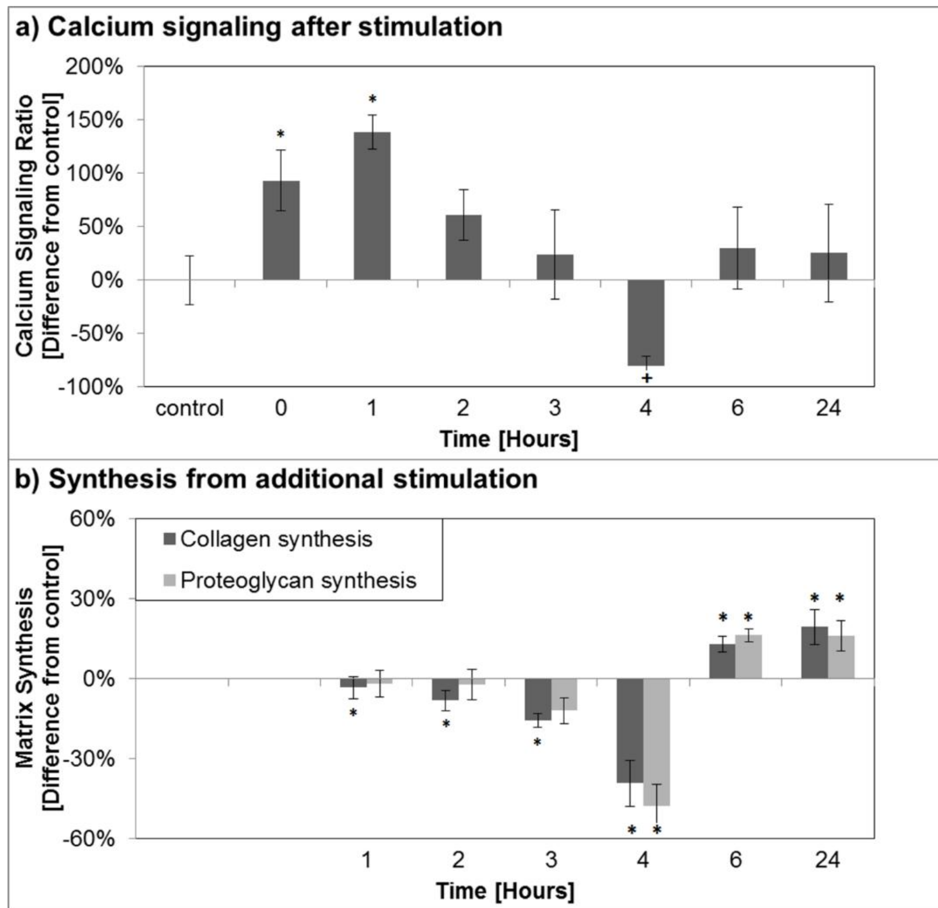

Figure 3.7: (a)Time course of calcium signaling of constructs subjected to 5% compressive strain amplitude for 20 minutes (n=6). (b) Matrix synthesis arising from a second application of mechanical stimuli as a function of rest period (n=6) * Different from control ($p<0.05$); + Trend from control ($p<0.10$)

Cell seeded constructs then underwent a second application of mechanical stimulation under previously identified synthetic conditions (5% compressive amplitude for 20 minutes) with matrix synthesis determined periodically over the next 24 hours. Matrix synthesis was

normalized to time matched controls to account for the synthesis occurring from the first application of stimulation (Figure 3.7b). When the second application of stimulation occurred after 1 to 4 hours of rest, collagen synthesis was supressed (3-39%, $p<0.05$) whereas only a significant suppression of proteoglycan synthesis after 4 hours of rest (48%, $p<0.05$). After 6 hours or greater of rest, the second application of mechanical stimulation resulted in a synthetic response with increased collagen and proteoglycan synthesis (13-19%, $p<0.05$).

3.4 Discussion

Dynamic mechanical loading influences development and maintenance of healthy articular cartilage in vivo[111]. For this reason, several studies have used dynamic mechanical stimuli to enhance both tissue formation and the mechanical properties of tissue engineered cartilage constructs[8–10,65,95]. Despite the numerous studies conducted in this area, there is little consensus on the optimal stimulation conditions which are typically determined empirically[13,14,79]. In the present study, we investigated the short-term temporal response (sensitization and desensitization) on matrix biosynthesis by chondrocytes subjected to dynamic mechanical stimuli and whether calcium signaling could be used as a predictor of the biosynthetic response.

Previous studies have shown changes in the Ca^{2+} signaling response to various different types of mechanical stimuli. Osmotic pressure[51,52], micropipette distortion[48,55], fluid shear[49], hydrostatic pressure[23,53], and dynamic compression[43] of cartilage explants and/or cell-seeded chondrocyte constructs have all been shown to induce increases in Ca^{2+} signaling as observed by fluorescent Ca^{2+} cytoplasmic dyes (e.g. Fluo-4). While Ca^{2+} signaling is an essential part of the

mechanotransduction cascade in chondrocytes, there has not been an established link between Ca^{2+} signaling and matrix anabolism. By correlating matrix synthesis and Ca^{2+} signaling in chondrocytes subjected to different loading protocols (amplitudes and durations), it was observed that the relative number of cells experiencing multiple Ca^{2+} transients (compared to the population of cells that experienced at least one Ca^{2+} transient) could be used as a predictor of an anabolic response. Increases in this Ca^{2+} signaling ratio correlated with increases in matrix biosynthesis whereas a decline correlated with unresponsive states with no changes in matrix synthesis observed. The correlation between Ca^{2+} signaling and matrix synthesis was further established by conducting similar experiments in the presence of the SERCA inhibitor, thapsigargin. By blocking Ca^{2+} sequestration, almost complete inhibition was observed in both matrix synthesis and the number of cells experiencing Ca^{2+} transients. This effect was observed in both the control and mechanically stimulated constructs, suggesting that Ca^{2+} transients are essential for chondrocytes to synthesize ECM macromolecules. Pingguan et al. (2005) showed that as few as one compression cycle elicited an increase in the number of cells experiencing transients. While they did not explicitly quantify matrix synthesis, they concluded that increases in the number of cells experiencing Ca^{2+} transients were also generally associated with increased matrix biosynthesis. In the present study, the number of cells experiencing at least one Ca^{2+} transient in the unstimulated control cultures varied from batch-to-batch. Randomly induced Ca^{2+} transients can be observed in labeled cells which may be a result of the imaging process[121], thereby leading spurious relationships between Ca^{2+} signaling and matrix synthesis. Alternatively, in the present study, a positive correlation between Ca^{2+} signaling and matrix synthesis was only achieved when this population of cells was effectively “thresholded” out. It should be noted, however, that the magnitude of biosynthetic response could not be predicted

with this method – only whether or not an anabolic response had occurred. Specific increases in Ca^{2+} signaling were different under different compressive amplitudes suggesting that the applied loading conditions may influence the extent of Ca^{2+} signaling in response to mechanical perturbation. Similarly, while the duration of the observed Ca^{2+} transients in the present study were similar to previous studies of chondrocytes embedded in agarose[50,54] and cartilage in situ[47,56], cells grown in monolayer[49,53] generally experience longer Ca^{2+} transient lengths – also indicating that the Ca^{2+} signaling response to mechanical stimuli may be dependent on the specific loading mode.

When subjected to an increasing number of dynamic mechanical loading cycles, it was observed that not only a minimum threshold of mechanical loading was required to stimulate matrix synthesis, but also that the cells rapidly desensitized to the imposed stimulus. This threshold effect appeared to be amplitude dependent. Small compressive amplitudes (2.5% strain amplitude) did not appear to elicit any observable changes in matrix synthesis and larger compressive amplitudes (5 and 10% strain amplitudes) generally requiring more loading cycles to elicit changes in biosynthesis. In addition, under higher compressive amplitudes (10% strain amplitude), peak increases in proteoglycan synthesis were observed after both 20 minutes (1200 cycles) and 60 minutes (3600 cycles) of loading. While the reason for this observed duration dependency is at present unclear; this effect appears to be independent of the amount of mechanical energy applied to the cell-seeded constructs suggesting a complex mechanism in how chondrocytes become sensitized to dynamic mechanical loading. Interestingly, the observed desensitization to mechanical loading was typically observed immediately after the peak increases in matrix synthesis were achieved. Previous studies have also observed chondrocyte

desensitization to dynamic mechanical loading under various different loading modes (e.g. compression, shear, high frequency vibrations, tension and reciprocal friction)[9,17,18,65,95,122]. In all cases, similar maximal increases in matrix synthesis were observed after 15-30 minutes (900-1800 cycles) of loading followed by an apparent complete inhibition beyond 60 minutes (> 3600 cycles) of continual loading[9,17,18,65,95]. While changes in the relative number of cells experiencing multiple Ca^{2+} transients does not provide an explanation to the observed desensitization response to prolonged dynamic mechanical loading, the amplitude of successive Ca^{2+} transients appeared to decrease. This effect has been observed in other studies[43,56] suggesting that Ca^{2+} sequestration may be a rate limiting step and may possibly be one of the factors responsible for this desensitization[123,124]. Alternatively, the release of Ca^{2+} from the sarcoplasmic reticulum mediated through ryanodine receptors has been shown in muscle to fatigue over time indicating another possible avenue for desensitization[125]. However, intracellular Ca^{2+} regulation is complex and involves several different pathways. Changes in cytosolic calcium concentrations are affected by the operation of both intracellular calcium stores (e.g. sarco/endoplasmic reticulum, mitochondrial) and trans-membrane calcium channels (e.g. voltage-gated, mechanically-gated, store-operated). Currently, it is unclear which of these pathways are responsible for the Ca^{2+} signaling associated as a result of mechanical stimuli.

While earlier studies have investigated the effect of continuous loading on chondrocytes[12,79], more recent studies have investigated intermittent mechanical stimulation in attempts to elicit changes over long-term culture[9,80,86,126]. In these studies, rest periods between successive applications of mechanical stimuli have in general not been optimized thereby potentially not fully exploiting the full beneficial effects of mechanical stimulation. In the present study, optimal

refractory periods between successive applications of mechanical stimuli was determined to be when Ca^{2+} signaling was allowed to return to baseline levels. The application of mechanical stimuli elicited an a sustained increase in Ca^{2+} signaling immediately after stimulation which then dropped below control levels before returning and remaining at control levels after 6 hours post stimulation. The application of a second stimulation within this dynamic Ca^{2+} signaling range (1 – 4 hours post stimulation) had an inhibitory effect on the synthetic response of cells, whereas the application of additional stimulation at baseline levels (≥ 6 hours post stimulation) elicited a stimulatory response. Previous studies on bone have shown that the osteoblasts rapidly desensitize to continuous dynamic mechanical stimulation[101,127–129] and that 8 hours of recovery was sufficient to restore full mechanosensitivity[101]. While the cellular mechanisms involved in the short-term recovery of bone to mechanical loading were not determined in this study, they suggested that this process may involve Ca^{2+} ion sequestration into intracellular stores as one potential mechanism[101]. It should be noted that the optimal rest periods determined in this study were likely a function of the applied stimulus and may only be applicable to the specific stimulation conditions investigated. However, this study illustrates the successful use of Ca^{2+} signaling to determine optimal recovery periods which could be used for other stimulation conditions and/or experimental models.

Although the results of this study demonstrate that Ca^{2+} signaling can be used a predictor of the biosynthetic response of chondrocytes to dynamic mechanical loading, there are a few limitations that should be mentioned. Despite the fact that cell-seeded agarose gels do not necessarily reflect the in situ behaviour of chondrocytes and may not be the optimal approach to develop tissue engineered cartilage constructs, this model was chosen for its simplicity, ability to

allow the cells to remain in their 3-dimensional phenotype[130], and ubiquitous use in mechanotransduction studies. The agarose model reduces confounding factors of native tissue by creating a homogenous, distributed cell population. In addition, as the pericellular matrix has not been fully re-established[131], imposed chondrocyte strains within embedded agarose gels are of similar magnitude, globally and at the intercellular level[54,130]. However there are some drawbacks of this model including the inability of the cells to attach to and interact with the gel as they would normally within their native ECM. Another limitation of the study was that Ca^{2+} signaling and induced strains could not be measured during the application of dynamic mechanical loading as the stimulation device used could not be mounted on a confocal microscope stage. A short period of time (order of minutes) elapsed between mechanical loading and imaging by confocal microscopy which may have reduced the ability to determine peak changes in Ca^{2+} signaling. While there have been a few reports of devices that are capable of measuring Ca^{2+} signaling events during the application of mechanical loading[56], there are limitations to size of the constructs that can be used as well as focal plane within the sample that can be measured.

3.5 Conclusion

In conclusion, this study has demonstrated that chondrocytes require a minimum amount of mechanical stimulation to elicit an anabolic response and that they can quickly become insensitive to the imposed stimulus. The synthetic response appeared to be amplitude dependent which could be predicted through measuring the resultant changes in Ca^{2+} signaling. In addition, these changes in Ca^{2+} signaling were also used to determine the optimal recovery period between successive applications of intermittent mechanical loading in which full mechanosensitivity was

achieved when Ca^{2+} signaling was allowed to return to baseline (control) levels. The use of Ca^{2+} signaling to predict the effectiveness mechanical stimuli as well as to determine optimal refractory periods appears to be advantageous over empirical approaches. This method is both non-destructive and allows the same samples to be used at multiple time points, thereby minimizing potential inter-sample variances. Future work will investigate the process of intracellular Ca^{2+} regulation to elucidate potential desensitization mechanisms to dynamic mechanical loading.

Chapter 4 - Study 2: Short-term effects of Stochastic Resonance on Mechanosensitivity

The work from this chapter has been accepted for publication in the Journal of Orthopaedic Research with co-author Stephen D Waldman[132].

4.0 Introduction

A substantial effort has been placed on the development of tissue engineered articular cartilage for repair and reconstruction in damaged joints resulting from trauma or disease. While generally a promising approach, engineered tissue constructs are typically unable to accumulate the same amount of extracellular matrix and consequently possess inferior mechanical properties compared to native tissue[8,133,134]. One widely used strategy to enhance the growth and properties of engineered cartilage is through the application of mechanical stimuli[7–10]; based on the premise that the mechanical environment helps regulate the development and maintenance of cartilage[111]. Several studies have demonstrated that extracellular matrix metabolism and subsequent tissue organization can be controlled through the application of mechanical loading[112–116]. While dynamic mechanical stimulation has generally been shown to be beneficial for the development of tissue engineered cartilage constructs[7–10,117,135], the applied stimulation parameters (i.e. strains, durations, frequency of application) tend to vary significantly between studies. This is partly due to empirical optimization needed given the differences in response to mechanical loading as a result of species, age, and culture model[12–14,79,136]. Thus, within every study, it is important to specifically determine the stimulation parameters which elicit a synthetic (anabolic) response. Another confounding issue with mechanical stimulation, is that chondrocytes can rapidly desensitize to the imposed stimulus under prolonged dynamic loading. This desensitizing effect manifests as either an inhibition or decrease in matrix biosynthesis compared to shorter loading durations[9,16–18,65,95]. Therefore, in order for mechanical stimulation to be an effective treatment

in the development of tissue engineered cartilage, it is important that the cells within the construct remain sensitive to the applied stimulus.

One potential approach to mitigate the effects of load-induced cellular desensitization is by modifying the applied loading protocols. Specifically, cellular sensitivity to mechanical stimuli can be altered by superimposing random mechanical vibrations (or noise) on the loading waveforms. This approach, termed "stochastic resonance", is observed in non-linear systems and characterized by an increase in sensitivity to a weak input signal due to the presence of noise[102]. This phenomenon has been observed in a multitude of biological systems from the molecular level systems of DNA transcription in gene expression[103] up to the whole body level control systems involved with maintaining homeostasis of blood pressure[104], blood oxygenation[105,106], and balance[107] where stochastic resonance devices have been developed for regular use[105]. In addition, previous studies on bone (both whole bone *in vivo*[108] and isolated osteoblasts *in vitro*[109]) have also attempted to harness this effect by using loading protocols superimposed with broad frequency (0 - 50 Hz) mechanical vibrations[108,109]. Combined vibration-compressive loading applied both *in vivo*[108] and *in vitro*[109] resulted in increased extracellular matrix synthesis compared to compressive loading alone. Although encouraging results have been reported, the underlying mechanisms responsible are currently unknown. However, it is likely that the effects of stochastic resonance are transduced through calcium signaling pathways. Mechanical stimulation typically results in intracellular calcium signaling in mesenchymal cells[39]; either directly by calcium influx through mechanically sensitive ion channels or indirectly as a secondary messenger following receptor binding[14,38]. Similarly, increased intracellular calcium

signaling has been correlated with increased extracellular matrix synthesis in chondrocytes subjected to either dynamic compression or stimulation by ultrasound[88,89,110,137].

4.1 Specific Aim

Thus, stochastic resonance could present an opportunity in cartilage tissue engineering to increase cellular sensitivity to mechanical stimuli, thereby leading to the development of more effective stimulation protocols. Therefore, the specific objectives of this study were to: (i) determine whether superimposed mechanical vibrations results in a stochastic resonance response in chondrocytes and (ii) to determine the underlying mechanisms responsible.

4.2 Methods

4.2.1 Preparation of Chondrocyte-Agarose Constructs

Primary bovine articular chondrocytes were isolated through sequential enzymatic digestion. Briefly, fresh cartilage slices were obtained from the metacarpal-phalangeal joints of skeletally mature cattle (12-18 months old) from local slaughterhouses. The cartilage slices from several joints (up to 4 for each isolation) were pooled and incubated in a 0.5% protease (w/v) (Sigma Aldrich Ltd., Oakville, ON, Canada) for 1.5 hrs at 37°C followed by 0.15% collagenase A (w/v) (Sigma Aldrich) for 18 hrs at 37°C. Chondrocytes were then separated by passing the digest through a 200-mesh filter (Sigma Aldrich). Viable chondrocytes, determined by Trypan blue dye exclusion[138], were then seeded in type VII low melting point agarose (Sigma Aldrich) prepared with phosphate buffered saline (PBS, pH 7.4) forming a 2% (w/v) gel with a seeding density of 10×10^6 cells/mL. Cylindrical constructs, 3 mm in diameter by 3 mm in height, were cut using a biopsy punch, allowing for multiple constructs to be created from the each isolation. Constructs

were cultured under static (no load) conditions for 24 hrs at 37°C in an environment supplemented with 5% CO_2 in complete media: Ham's F12 media containing 20% v/v fetal bovine serum (FBS, Sigma Aldrich), 20 mM HEPES, 100 µg/mL ascorbic acid (Sigma Aldrich) and 2x antibiotics/antimycotics (Sigma Aldrich) before mechanical stimulation. For each experiment, at least two separate cell isolations from different animals were used to account for potential donor-to-donor variability.

4.2.2 Application of Mechanical Stimuli

Mechanical compression and vibrational stimuli (Figure 4.1) were applied using a Mach-1 micromechanical testing machine (Biomomentum, Laval, QC, Canada) equipped a vibration actuator (LFA-10, Equipment Solutions Inc., Sunnyvale, CA, USA)[8] mounted on the baseplate of the Mach-1 system. Sinusoidal compression was applied using the Mach-1 actuator operated in displacement control (position resolution: 0.5 µm, load resolution: 1 mN) and a custom-designed loading jig to stimulate 6 constructs simultaneously[95]. Superimposed vibrations (average maximal vibration actuator displacement < 70 µm) were applied underneath the cultures through the vibration actuator controlled through a separate controller (SCA754 Servo Amplifier, Equipment Solutions) and a LabView interface (National Instruments, Austin, TX, USA).

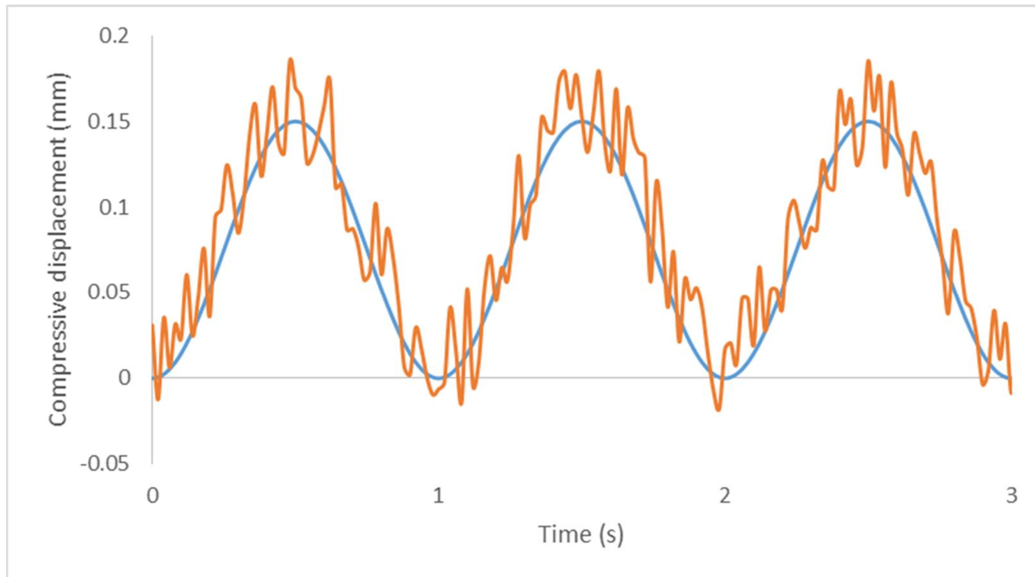

Figure 4. 1: Representative loading waveform of dynamic compression with superimposed vibrations

The effect of three vibrational amplitudes (0.5, 1, and 2 g) and four vibrational bandwidths (20-50, 30-60, 40-70 Hz) on three different compressive amplitudes (2.5, 5, and 10%) at a frequency 1 Hz were examined to determine the best superimposed vibration conditions for a 20 min stimulation duration. These conditions were then used for examining the effect on 4 loading durations (20, 30, 60, and 90 mins). Prior to mechanical stimulation, constructs were individually placed in the wells of a 24-well plate fitted with rapid prototyped retaining rings to prevent shifting during loading. Constructs were fed with 400 µL of fresh complete media, enough to cover the surface of the sample.

4.2.3 Quantification of Biosynthesis

Radioisotope incorporation of [^{3}H]-proline and [^{35}S]-sulfate (Perkin Elmer, Waltham, MA, USA) were used to determine collagen and proteoglycan synthesis, respectively. 5 µCi of each isotope was added to the culture media of each construct immediately following mechanical stimulation. Cultures were then incubated for 24 hrs in the presence of the isotopes at 37°C in an environment

supplemented with 5% CO_2. At harvest, constructs were washed 3 times with PBS to remove unincorporated isotope and then enzymatically digested by papain (40 µg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM DTT) for 72 hrs at 65°C. The accumulation of newly synthesized proteoglycans and collagen in the matrix were then estimated by quantifying radioisotope incorporation from aliquots of the papain digest using a β-liquid scintillation counter (Beckman Coulter LS6500, Mississauga, ON, Canada). The amounts of synthesized molecules were calculated relative to the DNA content of the tissue, determined from aliquots of the papain digest using the PicoGreen DNA assay (Life Technologies, Burlington, ON, Canada)[120]. Results of both DNA content and biosynthesis were normalized to unloaded control values.

4.2.4 Intracellular Ca^{2+} Imaging

Constructs were first incubated in the presence of the calcium ion dye Fluo-4 AM (4 µM; Life Technologies) for 90 mins then washed for 20 mins in complete media for de-esterification prior to mechanical stimulation. Following stimulation, completely intact constructs were imaged at a depth of 200 µm (maximum laser penetration) at 5x magnification on a confocal microscope (DMIRE, Leica Microsystems, Wetzlar, Germany) (ex/em: 495/506 nm) at 5 s intervals for 5 mins. Images were post processed in ImageJ (US National Institutes of Health, Bethesda, MD, USA) to identify and locate cells within the focal plane experiencing Ca^{2+} transients. The extent of Ca^{2+} signaling was calculated as the ratio of the number of cells experiencing multiple transients (2 or more) to the number of cells experiencing at least one transient[110]. To examine the regional distribution of Ca^{2+} signaling, two concentric regions of equal area were defined and analyzed separately for each construct (termed core and periphery). The temporal aspect of Ca^{2+} signaling was also analyzed by reimaging the same construct for an additional 5 mins at hourly

intervals for up to 6 hours post-stimulation. To avoid potential photo-bleaching, samples were protected from light and unnecessary laser exposure by closing the shutter between successive acquisitions.

4.2.5 Statistical Analyses

All results were expressed as mean ± standard error of the mean (SEM). Collected data were analyzed statistically using SPSS (version 17, SPSS Inc., Chicago, IL, USA) with one-way or two-way ANOVA (depending on the experimental design) and Dunnett's post hoc testing for subsequent pair-wise comparisons. To avoid unnecessary comparisons between all of the study groups (e.g. comparison between combined loading at one frequency bandwidth with the vibration-only loading of a different frequency bandwidth), two Bonferroni-corrected Dunnett's comparisons were used to compare the data to the two study controls: (i) unstimulated control group, and (ii) compression-only stimulated group. Prior to conducting the parametric statistical tests, all data were deemed normally distributed with equal-variance as determined by the Shapiro-Wilk and Levene's tests, respectively. P-values less than 0.05 were defined as significant, while p-values between 0.05 and 0.1 were defined as trends.

4.3 Results

4.3.1 Effects of Superimposed Vibrations on Matrix Synthesis

Matrix synthesis in response to dynamic compression under different amplitudes (2.5 – 10% at 1 Hz for 20 min) with, or without, superimposed vibrations (0.5 – 2 g at discrete 30 Hz bandwidths between 20 – 70 Hz) was assessed by radioisotope incorporation (Table 4.1). Dynamic compressive loading in the absence of vibrational loading elicited no observable changes in matrix synthesis under any of the strain amplitudes investigated. Vibrational loading, also on its

own, largely had no effect on matrix synthesis; with the exception of vibrations of 20 - 50 Hz at 1 g which resulted in an 18% increase in proteoglycan synthesis ($p<0.05$). However, when dynamic compression and vibrational loading were combined, significant changes in matrix synthesis were observed which appeared to be dependent on the compressive strain amplitude as well as the frequency bandwidth and amplitude of vibrational loading. Under a 2.5% compressive strain amplitude, collagen synthesis was increased under all 1 g superimposed vibration bandwidths with higher bandwidths (30 – 60 Hz and 40 – 70 Hz) showing a greater (21 – 36%) effect ($p<0.05$). Proteoglycan synthesis followed somewhat similar trends and was increased (by 17 – 30%) under the low and high frequency bandwidths (20 – 50 Hz and 40 – 70 Hz) ($p<0.05$). At 5% strain amplitudes, only vibrations of 20 – 50 Hz at 1g increased the synthesis of collagen by 38% ($p<0.05$) and proteoglycans by 41% ($p<0.05$). However, under 10% compressive strain amplitudes, none of the superimposed vibration frequency bandwidths tested affected matrix synthesis. Vibrations at either 0.5 or 2 g also did not appear to elicit any effect on matrix synthesis when superimposed on any of the compressive strain amplitudes investigated (data not shown).

Table 4.1: Effect of mechanical vibrations during dynamic compressive mechanical stimulation (at 1 Hz for 20 min) on matrix synthesis (collagen and proteoglycans) compared to both vibration-only and unstimulated controls. Data presented as mean ± standard error (SEM), n ≥ 4/group.

			Superimposed Vibrations			
			No Vibration	**1 g 20-50 Hz**	**1 g 30-60 Hz**	**1 g 40-70 Hz**
Collagen Synthesis	**Strain Amplitude**	0%	100 ± 1%	107 ± 3%	112 ± 8%	104 ± 8%
		2.5%	90 ± 3%	110 ± 3% [b]	121 ± 8% [B]	136 ± 5% [AB]
		5%	105 ± 4%	138 ± 10% [AB]	111 ± 20%	109 ± 20%
		10%	97 ± 3%	109 ± 5%	99 ± 12%	104 ± 5%
Proteoglycan Synthesis	**Strain Amplitude**	0%	100 ± 1%	109 ± 3%	118 ± 8% [A]	108 ± 8%
		2.5%	89 ± 4%	117 ± 3% [B]	109 ± 6%	130 ± 10% [AB]
		5%	103 ± 4%	141 ± 12% [AB]	123 ± 18%	108 ± 16%
		10%	94 ± 4%	109 ± 4%	85 ± 11%	91 ± 5%

A – Statistical difference from control ($p<0.05$)
B – Statistical difference from compression-only ($p<0.05$)
b – Trend from compression-only ($0.05<p<0.10$)

Evaluation of DNA content (as measured by the PicoGreen assay) also showed significant changes in cellularity as a result of superimposed vibrations during dynamic mechanical stimulation (Table 4.2). Similar to the effect on matrix synthesis, dynamic compressive stimulation or vibrational stimulation, when applied separately, generally elicited no observable effect on DNA content of the cell-seeded constructs under any of the conditions investigated. However, under combined vibration-compressive mechanical stimulation, significant effects on DNA content were observed. Under either a 2.5% or 5% compressive strain amplitude, a decline in DNA content was observed with superimposed vibrations of 1 g at either the 30 – 60 Hz or 40 – 70 Hz bandwidths (15 – 24% decrease, $p<0.05$) with the lower 20 – 50 Hz bandwidth apparently having no effect. Under a 10% compressive strain amplitudes, superimposed

vibrations of 1 g at all investigated frequency bandwidths had no apparent effect on DNA content. While vibrations at either 0.5 or 2 g largely had no effect on DNA content, there was a slight proliferative effect of 2 g vibrations at 20 – 50 Hz were superimposed on a 10% compressive strain amplitude (18% increase, $p<0.05$) (data not shown).

Table 4.2: Effect of mechanical vibrations during dynamic compressive mechanical stimulation (at 1 Hz for 20 min) on DNA content compared to both vibration-only and unstimulated controls. Data presented as mean ± standard error (SEM), n ≥ 4/group.

		Superimposed Vibrations			
		No Vibration	**1 g 20-50 Hz**	**1 g 30-60 Hz**	**1 g 40-70 Hz**
Strain Amplitude	0%	100 ± 1%	91 ± 1% ^{A}	94 ± 3%	95 ± 3%
	2.5%	97 ± 4%	102 ± 4%	76 ± 3% ^{AB}	85 ± 4% ^{A}
	5%	95 ± 2%	90 ± 4% ^{A}	85 ± 6% ^{A}	91 ± 5%
	10%	103 ± 3%	91 ± 5%	105 ± 12%	90 ± 10%

A – Statistical difference from control ($p<0.05$)
B – Statistical difference from compression-only ($p<0.05$)

Since the greatest effect of stochastic resonance was observed under a 5% compressive strain amplitude with superimposed vibrations at 1 g between 20 – 50 Hz, this condition alone was selected for further study. Additional experiments were then conducted to determine the influence of stimulation duration and effect on subsequent intracellular signaling.

4.3.2 Effect of Superimposed Vibrations on Different Stimulation Durations

In this series of experiments, the effects of superimposed vibrations of 1 g between 20 – 50 Hz on matrix synthesis during dynamic 5% compressive strain amplitude for durations of 20 – 90 minutes were investigated (Figure 4.2). In this study, dynamic mechanical stimulation, in the absence of superimposed vibrations, applied for 20 or 30 minutes generally resulted in increased

matrix synthesis (~ 40% increase in collagen and proteoglycan synthesis; $p<0.05$). Matrix synthesis was apparently unaffected as a result of longer durations (60 or 90 min). In contrast, vibrational stimulation alone did not elicit any changes in matrix synthesis for any of the durations investigated. Irrespective of the duration of stimulation, combined vibration-compressive loading appeared to elicit additional increases in matrix synthesis to an overall similar magnitude (40 – 65% increase in collagen and proteoglycan synthesis, $p<0.05$). This effect was observed under compressive-only stimulation conditions which were both sensitive (20 and 30 min durations) and insensitive (60 and 90 min durations) to mechanical stimuli. Construct cellularity was largely unaffected by dynamic mechanical stimulation both in the presence and absence of superimposed vibrations. However, under combined loading, the stimulation duration of 30 min experienced a moderate decrease (23%, $p<0.05$) in DNA content (Table 4.3).

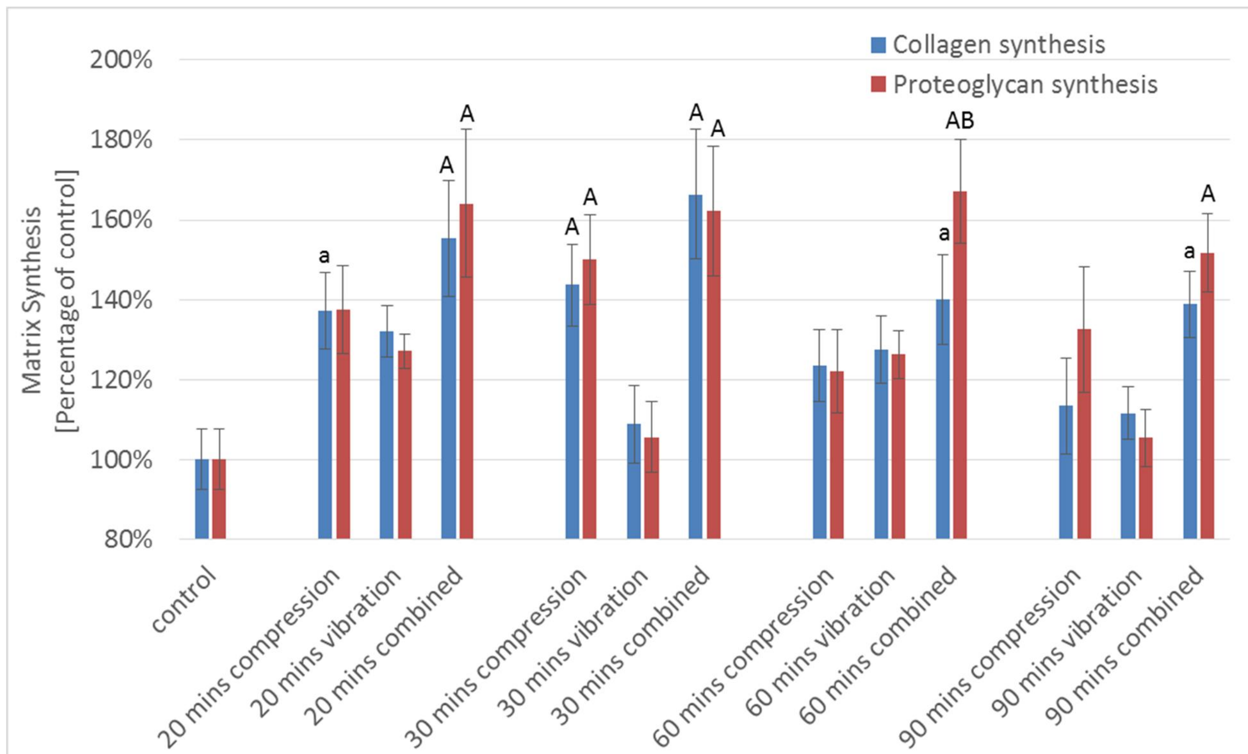

Figure 4.2: Effect of mechanical vibrations (1 g at 20 – 50 Hz) during dynamic compressive mechanical stimulation (5% strain amplitude at 1 Hz) of different durations (20 – 90 min) on matrix synthesis (collagen and proteoglycans). Data presented as mean ± standard error (SEM); n ≥ 6/group. A – Significant difference from unstimulated control ($p<0.05$); a – trend from unstimulated control ($0.05<p<0.10$); B – significant difference from dynamic compression alone ($p<0.05$).

Table 4.3: Effect of mechanical vibrations (1 g at 20 – 50 Hz) during dynamic compressive mechanical stimulation (5% strain amplitude at 1 Hz) of different durations (20 – 90 min) on DNA content. Data normalized to control (0 min stimulation duration) and presented as mean ± standard error (SEM); n ≥ 6/group.

Stimulation Duration [min]	Stimulation Condition: **Compression** (*5% strain amplitude*)	**Vibration** (*1 g 20-50 Hz*)	**Combined Compression-Vibration** (*5% strain amplitude and 1 g 20-50 Hz*)
0	100 ± 3%	100 ± 3%	100 ± 3%
20	95 ± 3%	102 ± 3%	84 ± 6%
30	94 ± 3%	103 ± 5%	77 ± 7% [A]
60	98 ± 3%	104 ± 4%	93 ± 4%
90	84 ± 8%	95 ± 3%	98 ± 4%

A – Significant difference from unstimulated control ($p<0.05$)

4.3.3 Effect of Superimposed Vibrations on Calcium Signaling

In the last series of experiments, the effect of superimposed vibrations of 1 g between 20 – 50 Hz during dynamic 5% compressive strain amplitude stimulation on intracellular calcium signaling was investigated. Following mechanical stimulation, Fluo-4 labelled constructs were imaged by confocal microscopy to locate cells experiencing Ca^{2+} transients. For each cell experiencing Ca^{2+} transients, both the number of transients experienced (over the 5 min imaging period) and its spatial location (radial distance from the construct center) within the construct were recorded (Figure 4.3). The extent of Ca^{2+} signaling (ratio of the number of cells experiencing multiple transients to the number of cells experiencing at least one transient) was then determined for the entire construct as well as the construct core and peripheral regions (concentric regions of equal area) (Figure 4.4). When determined over the entire construct, Ca^{2+} signaling was increased by 52% over control in response to dynamic compressive loading ($p<0.05$) whereas vibrational loading alone had no apparent effect (Figure 4.4). Combined loading appeared to further increase overall Ca^{2+} signaling by 73% over control ($p<0.05$) (Figure 4.4). This effect appeared to be

exclusively localized within the peripheral region of the construct where signaling was increased by 96 and 141% ($p<0.05$) for compressive and combined loading, respectively. In contrast, no detectable changes in Ca^{2+} signaling of cells within the core of the construct under either compressive or combined loading (Figure 4.5).

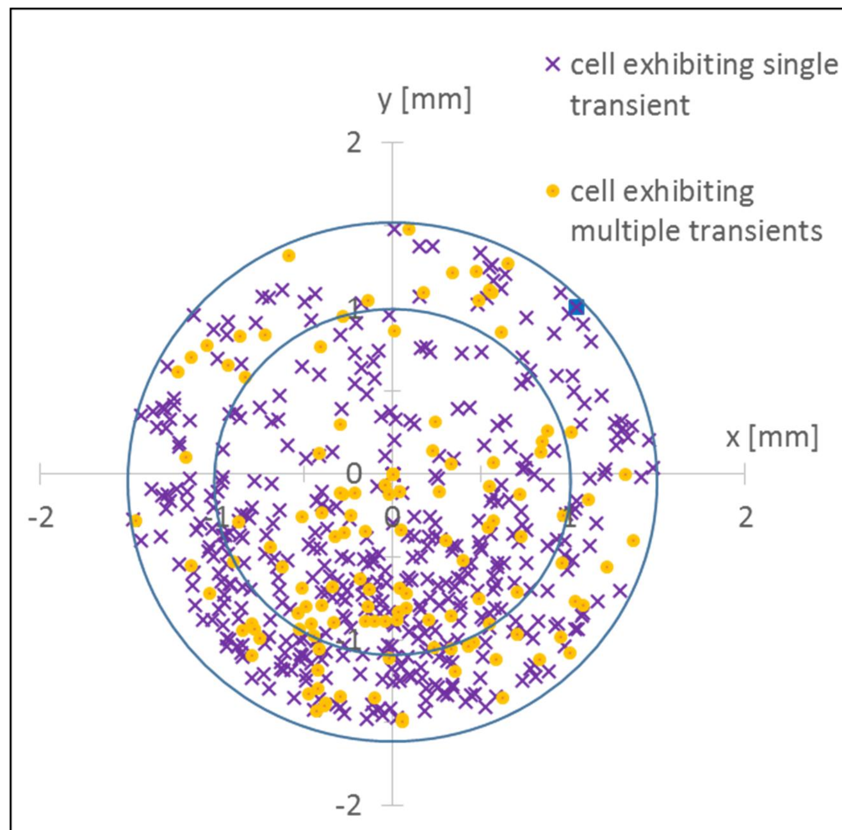

Figure 4.3: Graphical illustration cells experiencing Ca2+ transients in a mechanically stimulated construct. Cells experiencing only a single transient are denoted by “x” whereas cells experiencing multiple transients (more than one) are denoted by “•”. Solid lines denote the inner and outer radii (at 1.05 and 1.50 mm, respectively) used to separate the construct into two concentric regions of equal area (core and periphery, respectively).

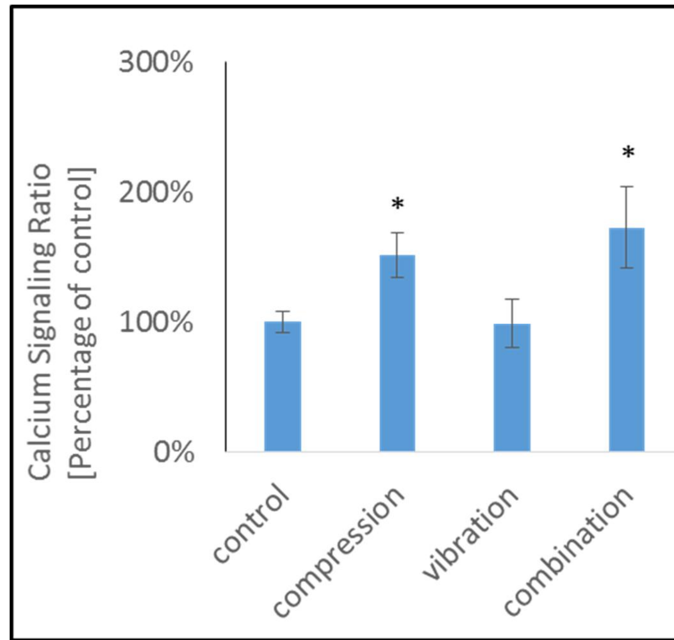

Figure 4.4: Effect of mechanical vibrations (1 g at 20 – 50 Hz) during dynamic compressive mechanical stimulation (5% strain amplitude at 1 Hz for 20 min) on intracellular calcium signaling. Calcium signaling ratio was defined as the number of cells experiencing multiple transients (2 or more) to the number of cells experiencing at least one transient and presented relative to the unstimulated controls. Data presented as mean ± standard error (SEM); n ≥ 6/group. * - Statistical difference from unstimulated control ($p<0.05$).

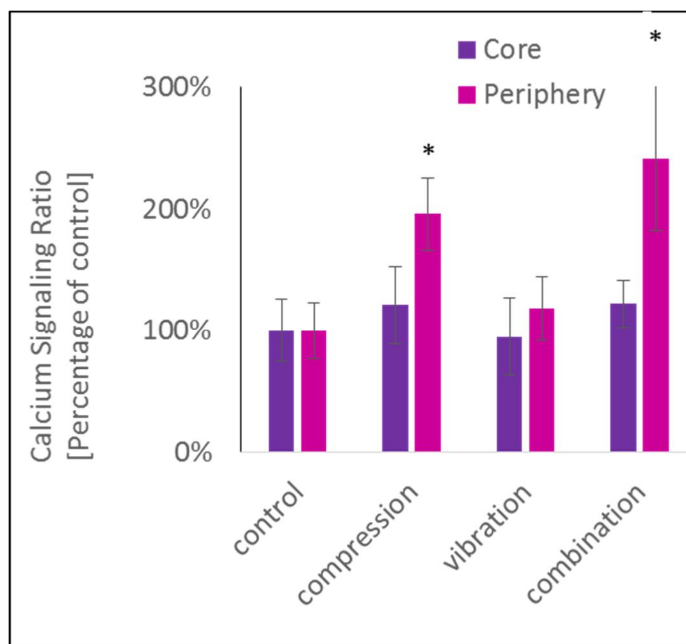

Figure 4.5: Effect of mechanical vibrations (1 g at 20 – 50 Hz) during dynamic compressive mechanical stimulation (5% strain amplitude at 1 Hz for 20 min) on intracellular calcium signaling within the core or periphery of the construct. Calcium signaling ratio was defined as the number of cells experiencing multiple transients (2 or more) to the number of cells experiencing at least one transient and presented relative to the unstimulated controls. Data presented as mean ± standard error (SEM); n ≥ 6/group. * - Statistical difference from unstimulated control ($p<0.05$).

Additional experiments were conducted to determine whether combined vibration-compressive loading would affect the Ca^{2+} signaling recovery (refractory) period. Ca^{2+} signaling was tracked periodically over a 6 hour period after mechanical stimulation (dynamic compression, vibration, or combined loading) (Figure 4.6). Based on the previous results, vibration-only stimulated constructs were omitted from this study as vibrational loading alone (at 1 g 20 – 50 Hz) did not elicit any observable changes in Ca^{2+} signaling. For both dynamic compression and combined vibration-compressive loading, Ca^{2+} signaling increased immediately after stimulation and continued to be elevated for up to 3 hours after stimulation. At 4 hours post stimulation, Ca^{2+}

signaling levels dropped below control for constructs subjected to dynamic compression alone ($p<0.05$) (Figure 4.6). However, for constructs subjected to combined vibration-compressive loading, at 4 hours post stimulation, Ca^{2+} signaling levels only dropped to levels no different than control ($p=0.99$). By 5 hours post stimulation, Ca^{2+} signaling returned to, and appeared to stay at, baseline (control) levels for all stimulation protocols.

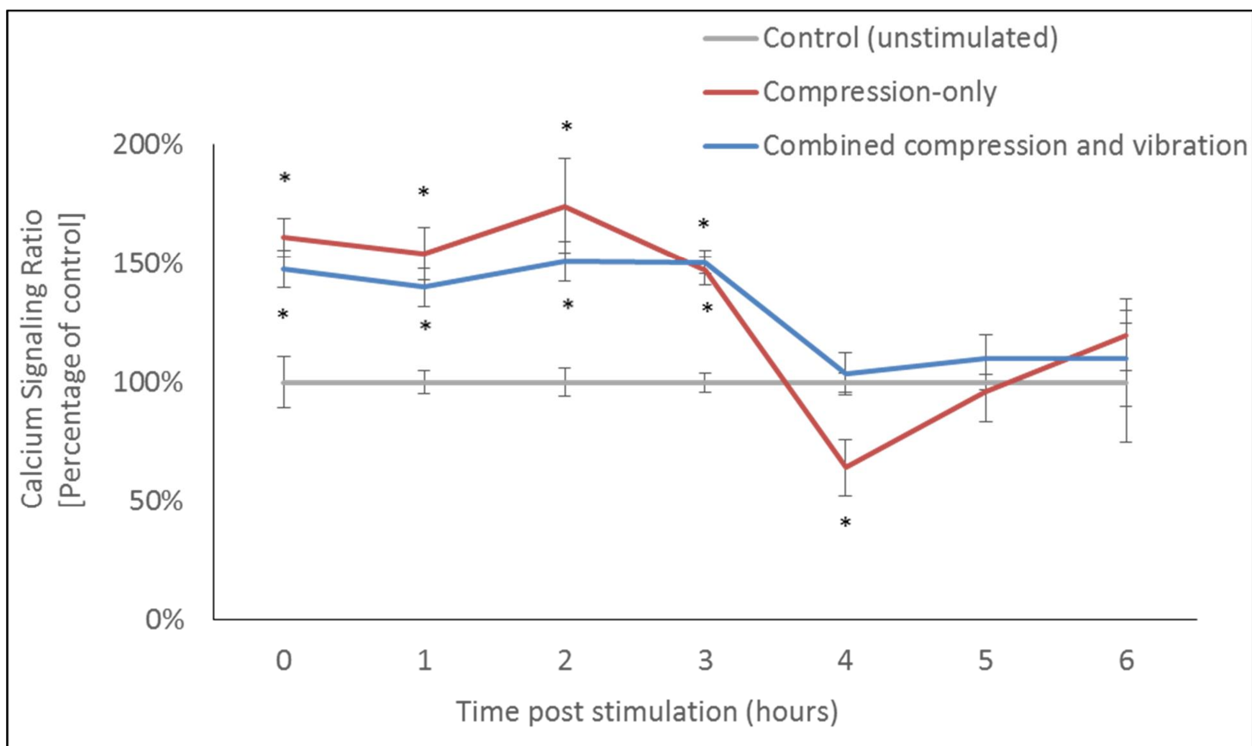

Figure 4.6: Time course of calcium signaling after mechanical stimulation of constructs subjected to 5% compressive strain amplitude (at 1 Hz for 20 min) with, or without, superimposed vibrations (1 g at 20 – 50 Hz). Data presented as mean ± standard error (SEM); n = 6/group. * - Statistical difference from unstimulated control ($p<0.05$).

4.4 Discussion

Mechanical stimulation is a widely used method to enhance both tissue formation and the mechanical properties of tissue engineered cartilage constructs[7–10]. Although generally quite

successful, there are several confounding issues with this approach. Due to the differences in response to mechanical loading as a result of species, age, and/or culture model, applied stimulation parameters (i.e. strains, durations, frequency of application) generally need to be empirically optimized for each individual study[12–14,79]. Additionally, as chondrocytes can rapidly desensitize to the imposed stimulus under prolonged loading[9,16–18,65,95], it is critical that the cells resident in the construct remain sensitive to the applied stimulus. Therefore, the development of stimulation protocols that be universally applied to different experimental systems that also maintain cellular sensitivity are warranted. With this in mind, we investigated the effect of stochastic resonance as a method to improve the biosynthetic response of chondrocytes to dynamic mechanical stimuli. Stochastic resonance is the phenomenon where the sensitivity to a weak input signal can be increased due to the presence of noise[102]. This method has been successfully applied to a wide array of biological systems[103–107], including the response of bone to mechanical loading[108,109].

To highlight the difficulty in determining mechanical loading protocols to which the cells are sensitive to stimulus, dynamic compressive loading alone applied to chondrocyte-seeded agarose gels did not appear to elicit any observable effects on matrix molecule synthesis for any of the three strain amplitudes (2.5, 5 or 10%) investigated. However, in presence of superimposed random mechanical vibrations, the biosynthetic response of chondrocytes to dynamic mechanical loading was significantly increased. This effect appeared to be dependent on the amplitude of the applied dynamic compressive strains, with increased collagen and proteoglycan synthesis occurring in the presence of superimposed vibrations under low-to moderate (2.5 and 5%) strain amplitudes (30 – 40% increase over control), but not higher (10%) strain amplitudes. These

effects were not attributable to the applied vibrations alone, as vibrational stimulation in the absence of dynamic compression elicited almost no observable changes in matrix synthesis. Interestingly, while there were some differences with the respect to the types of superimposed random vibrations applied, superimposed vibrations with a bandwidth of 20 – 50 Hz at an amplitude of 1 g were generally the most successful at eliciting an increased biosynthetic response. These results were similar to previous studies on bone where superimposed mechanical vibrations between 0 – 50 Hz increased the extracellular matrix synthesis when applied to either isolated osteoblasts[109] or whole bone *in vivo*[108]. It is important to note that the observed increases in matrix synthesis were apparently not a result of changes in construct cellularity. While combined compression-vibrational loading induced a decline in DNA content (up to -23%), these changes were lower than the elicited increases in matrix synthesis. This result was not unexpected as decreases in DNA content in response to dynamic compression have been previously reported[110,117].

Determining the optimal duration to apply mechanical stimuli is complex as cells require a long enough treatment to become sensitized to the stimulus, but short enough not to induce desensitization[110]. Previous studies have shown that dynamic loading of 20 –30 min induces maximal changes in matrix synthesis, whereas durations exceeding 60 min elicits no effect[9,17,18,65,95]. While the present study reproduced these findings, stochastic resonance appeared to be successful at limiting the desensitization effect observed under prolonged loading. Combined vibration-compressive loading elicited additional increases in matrix synthesis to an overall similar magnitude (40 – 60%), irrespective of the duration of stimulation even under previously observed desensitized conditions. This effect may potentially be beneficial for long-

term stimulation studies where it is important to achieve greater sensitivity to the mechanical stimulus. Although promising, it should be noted that as only stimulation durations of up to 90 min were investigated, it is not presently clear whether the phenomenon of load-induced chondrocyte desensitization can be completely avoided using this approach.

In addition to limiting load-induced desensitization, the recovery of sensitivity after stimulation is another important consideration for long-term, intermittent stimulation studies. Specifically, it is of interest to determine the optimal refractory period between successive applications of mechanical loading to ensure the return of mechanosensitivity as chondrocytes have been observed to be inhibited by mechanical stimuli when in an apparent refractory state[110]. Through the use of monitoring intracellular calcium signaling (a ubiquitous second messenger involved in chondrocyte mechanotransduction[88,110,137]) after the application of mechanical stimuli[110], we investigated whether this refractory period would be affected by stochastic resonance. Similar to previous results[110], the application of dynamic compressive loading caused an immediate increase in calcium signaling, followed by an inflection below control (at 4 hours post stimulation), before returning to baseline levels (by 6 hours post stimulation). In the presence of superimposed random vibrations, a similar time course in calcium signaling was observed with the exception that calcium signaling levels did not appear to drop below control — suggesting that mechanosensitivity was re-established as early as 4 hours after stimulation. As calcium ion (re)sequestration has been identified as potentially the rate limiting step in matrix metabolism[123,124], stochastic resonance could allow for the earlier re-application of mechanical stimuli, thereby[123,124] leading to improved extracellular matrix deposition as a result of long-term mechanical stimulation.

In attempts to determine the underlying mechanisms responsible for improved biosynthetic response of chondrocytes subjected to combined vibration-compressive loading, we investigated the spatial pattern of calcium signaling over the cross-sectional area of the construct. When determined over the entire construct, the number of cells experiencing calcium transients was increased in response to both dynamic compression and combined vibration-compressive loading, whereas vibrational stimulation alone had no effect. These increases appeared to exclusively occur within the periphery of the construct. This result directly correlates with the previous finding that newly synthesized matrix macromolecules as a result of dynamic compression also occurred primarily within the periphery of chondrocyte-seeded agarose gels — the region of maximal fluid flow induced due to the unconfined sample geometry[79]. As combined loading appeared to only elicit relatively small additional increases in intracellular calcium signaling compared to dynamic compression, potentially other mechanisms may be responsible. The lack of changes in intracellular calcium signaling due to vibrational stimulation may be due to the frequency bandwidths examined. While ultrasound stimulation has been shown to elicit matrix synthesis mediated through calcium signaling[88,137], the frequency bandwidths used in this study were orders of magnitude lower, suggesting that the vibrations served only to amplify the compressive stimulation response and were not capable of eliciting an effect on their own.

Although the results of this study demonstrate that the application of superimposed vibrations can enhance the biosynthetic response of chondrocytes to dynamic mechanical loading, there are a few limitations of the study that should be mentioned. While cell-seeded agarose model used

in this study is not likely the optimal scaffold to develop tissue engineered cartilage constructs, this model was chosen due to its common use in mechanotransduction studies where its simplicity, homogeneity, and maintenance of 3-dimensional phenotype[54,130,131] reduce confounding factors. However, one of the drawbacks of this model is that cellular interactions with agarose are not necessarily the same as in their native ECM. Another limitation of this study was that Ca^{2+} signaling could not be measured simultaneously during the application of mechanical stimulation which has been accomplished previously[56]. A short period of time (< 5 min) elapsed between mechanical loading and confocal imaging which may have resulted in a reduced ability to detect peak changes in Ca^{2+} signaling. In addition, the depth at which the constructs could be imaged was limited by the focal plane of the microscope. Finally, another potential limitation was the use of serum in the culture media which may have potentially influenced the observed results. Thus, for future translation of this approach for the development of engineered human cartilage constructs, confirmatory studies should be undertaken with the defined, serum-free media used to support growth and function of human chondrocytes.

4.5 Conclusion

In conclusion, this study has demonstrated that it is possible to achieve a stochastic resonance response in chondrocytes by superimposing random vibrations during compressive mechanical loading. Superimposed vibrations not only served to increase cellular sensitivity to mechanical loading, but also appeared to be successful at limiting the effect of load-induced desensitization. Thus, the application of stochastic resonance appears to be a valuable tool during the mechanical stimulation of cartilage constructs, even when suboptimal stimulation conditions are used. This is an important effect to consider as the cellular response to mechanical loading can vary due to

differences in species, age, and/or culture model causing previously determined synthetic stimulations to be ineffective. Future work will focus on the effectiveness of stochastic resonance on extracellular matrix accumulation and construct properties when applied over the long-term (i.e. order of weeks).

Chapter 5 – Study 3: Age and Stochastic Resonance

5.0 Introduction

Cellular mechanosensitivity is an important issue to consider when using mechanical stimulation as a means to improve the results of tissue engineered cartilage constructs. Mechanical desensitization commonly occurs in chondrocytes that are subjected to extended stimulation protocols[9,16–18], however; innate mechanical insensitivity has also been observed[132]. This observation of donor to donor variability may be due to several different causes including species, disease state, age, etc.

Age-related changes have been extensively studied in chondrocytes and many important differences in the functionality of these older cells have been observed which impact their suitability for tissue engineering. In native cartilage, cellularity[139,140], tissue hydration[140], and proteoglycan content[140,141] decrease with age and the size and structure of the chondron and pericellular matrix also experiences changes[142]. Isolated cells from older tissue display decreased chondrogenic capacity which is characterized by an impaired ability to synthesize the chondrocyte-specific matrix molecules (e.g. collagen and sulfated proteoglycans) as well as reduced proliferation[139,143,144]. Older chondrocytes also respond differently to growth factor stimulation. Reduced sensitivities to TGF-β[145] and IGF-1[146] have been observed; however, an increased response to PDGF-AA has been noted[145]. Aged chondrocytes not only display reduced anabolism, but also an increased tendency towards increased catabolism, such as an increased sensitivity to exogenous IL-1 and increase production of both IL-1 and MMP-13[147]. In addition, when subjected to mechanical stimulation, aged chondrocytes show a reduced chondrogenic response[144,148]. Thus, without special treatments, these factors combine to result in tissue

engineered cartilage constructs from aged chondrocytes that are biochemically and mechanically inferior compared to constructs developed from younger counterparts[143].

It is especially important to consider the response of aged cells in cartilage tissue engineering as these cells would most likely be used clinically. Maintaining cellular sensitivity to a particular loading protocol is essential and is particularly important in the case of older chondrocytes which inherently have a poor anabolic response. Previous work has shown that stochastic resonance can increase cellular sensitivity to mechanical stimuli and mitigate mechanically induced desensitization of young cells making it important to determine if these effects can be translated to overcoming the inherent insensitivity of older cells[132].

5.1 Specific Aim

Thus the specific aim of this study was to determine whether the beneficial effects of stochastic resonance could be observed when applied to cells from old animals which have been previously seen to be inherently insensitive to mechanical stimulation.

5.2 Methods

5.2.1 Preparation of constructs

Full thickness cartilage slices were obtained from of both young (12-18 months old) and adult (>30 months old) bovine metacarpal-phalangeal joints. Cells were released by sequential enzymatic digestion: 1 hour in 0.5% w/v protease (Sigma Aldrich) followed by 18 hours in 0.15% w/v collagenase A (Sigma Aldrich), both prepared in Ham's F-12 (Thermo Fisher)

supplemented with 25 mM HEPES (Bioshop Canada). The cell suspension was filtered through a 200-mesh filter (Sigma Aldrich) then washed and resuspended at 20x10^6 cells/mL with Ham's F-12 with 25 mM HEPES. A 4% w/v solution of autoclaved low melt type VII agarose (Sigma Aldrich) was prepared with sterile phosphate-buffered saline (pH 7.4). The clean cell suspension was mixed 1:1 with the molten agarose and transferred to a 100 mm petri dish to create a 4 mm thick disc. After gelation (~15 minutes at room temperature), a 4 mm biopsy punch was used to cut cylindrical constructs. Constructs were transferred to a new petri dish and cultured undisturbed for 24 hours at 37°C with 5% CO_2 with complete media (Ham's F-12 supplemented with 20% v/v FBS (Sigma Aldrich), 100 µg/mL ascorbic acid (Sigma Aldrich), 20 mM HEPES, and antibiotics/antimycotics (Sigma Aldrich).

5.2.2 Quantification of Biosynthesis

The incorporation of the radioisotopes [^{3}H]-proline and [^{35}S]-sulfate (Perkin Elmer) were used to determine collagen and proteoglycan synthesis respectively. Immediately following mechanical stimulation, 5 µCi of each isotope was added to the culture media for each construct. After a 24 hr incubation period in the presence of the isotopes (37°C with 5% CO_2), the constructs were washed with PBS to remove any unincorporated isotope. The constructs were papain digested (40 µg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM DTT) for 72 hrs at 65°C and aliquots of the digest were used for β-liquid scintillation counting (Beckman Coulter) and DNA quantification through the PicoGreen DNA assay (Life Technologies)[120]. Radioactivity content was then normalized to the DNA content of each construct.

5.2.3 Application of Mechanical Stimuli

Compressive and vibrational stimuli were applied using a Mach-1 system[95] (Biomomentum) with a vibration actuator[65,132] mounted to the baseplate. A specialized loading jig with adjustable loading platens in custom fitted lid for a 24 well plate was used to keep the constructs sterile. The constructs were stimulated under several combinations of conditions with a 5% compressive strain amplitudes, durations ranging from 0-90 minutes, and in the presence or absence of stochastic vibrations which have previously shown an anabolic response (an amplitude of 1 g with a bandwidth of 20-50 Hz, average maximal strain of $< 1.75\%$)[132]. Before stimulation, constructs were transferred to 24-well plate fitted with rapid prototyped plastic retaining rings and fed with 400 μL of fresh complete media.

5.2.4 Statistics

Experiments were performed at least twice using cells obtained from different donors. Data from the multiple experiments were pooled resulting in sample sizes of $n \geq 6$. All results were expressed as mean ± standard error of the mean (SEM). Data were statistically analyzed using SPSS (version 17) with two-way ANOVAs and Dunnett's post hoc testing for subsequent pairwise comparisons with Bonferroni-correction for multiple Dunnett's tests to the two study control groups: unstimulated and compression-only groups. Before statistical testing, all data were checked for normal distribution and equal variance through Shapiro-Wilk and Levene's tests. P-values 0.05 or less were defined as significant and p-valued between 0.05 and 0.1 were defined as trends.

5.3 Results

Matrix synthesis in response to 5% strain amplitude dynamic compression, with and without superimposed vibrations (1 g amplitude with frequencies between 20 – 50 Hz) and increasing duration (0-90 minutes) was assessed by radioisotope incorporation on two different populations of cells: young and old. A direct comparison of stimulation effects between groups was achieved by normalizing radioactivity by DNA content (Figure 5.1). As expected, a significant effect of age was observed with constructs created of different aged cells responded differently to the same stimulus. Baseline (unstimulated) collagen and proteoglycan synthesis of the older cells was approximately 50% of the younger cells ($p<0.05$). Conversely, the DNA content (Table 5.1) of the older cell constructs was approximately 25% greater than the younger cell constructs ($p<0.05$). In the older cell constructs, dynamic compressive stimulation alone had no apparent effect on matrix synthesis, except after 90 minutes of stimulation where the effect was detrimental (-30%, $p<0.05$). Younger chondrocytes however responded positively to the compression-only stimulation at 30 minutes ($p<0.05$), but were otherwise insensitive for any of the other durations tested. Vibration-only loading was apparently ineffectual on the younger chondrocytes; however, older chondrocytes showed sensitivity at two durations (20 and 60 minutes) increasing synthesis by ~15-30% ($p<0.05$). Both older and younger chondrocytes responded positively to combined compression-vibration loading, with increases in synthesis of both matrix molecules for the majority of the conditions tested, an increase of about 30% for proteoglycans ($p<0.05$) and a 10-50% increase for collagen ($p<0.05$). While combined loading was effective in increasing matrix synthesis of the older cells, the amounts synthesized were approximately 60% of the unstimulated younger cell constructs and around 50% or less of those that were mechanical stimulated ($p<0.05$).

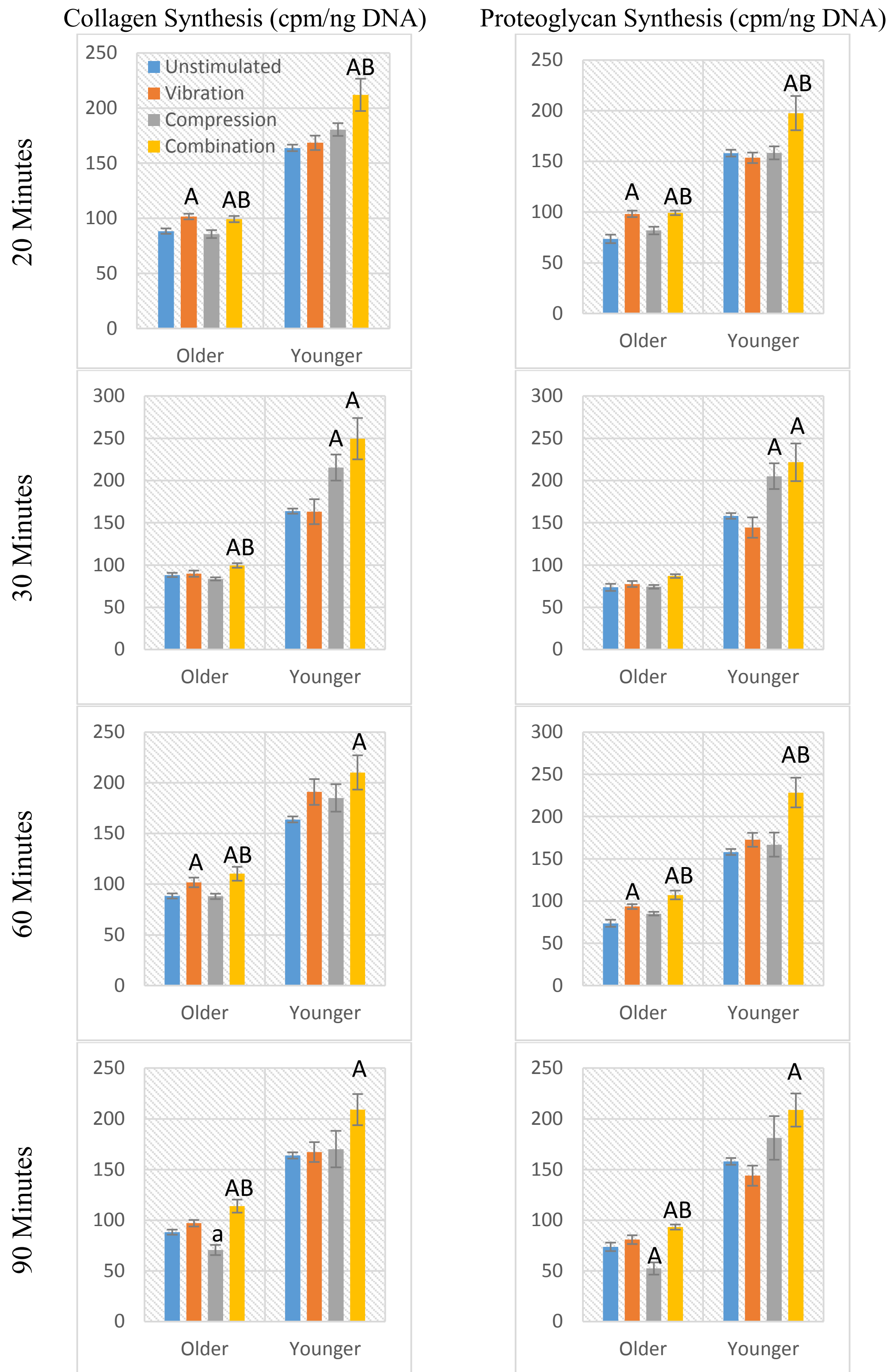

Figure 5.1: Effect of mechanical stimuli on matrix synthesis (collagen and proteoglycan). Data presented as radioactivity normalized to DNA content (cpm/ng DNA), mean ± standard error (SEM); n≥6/group. A – Difference from unstimulated control ($p<0.05$); a – trend from unstimulated control ($0.05<p<0.10$); B – Difference from corresponding compression-only stimulation ($p<0.05$)

Table 5.1: Effect of mechanical stimuli on DNA content (ng). Data presented as mean ± SEM; n≥6/group.

	Older	Younger
unstimulated	2143 ± 67	1539 ± 20
20 mins vibration	2138 ± 89	1459 ± 31
20 mins compression	2105 ± 101	1437 ± 43 [a]
20 mins combination	1981 ± 51	1383 ± 55 [A]
30 mins vibration	2140 ± 117	1609 ± 78
30 mins compression	2217 ± 89	1465 ± 51
30 mins combination	1858 ± 110	1207 ± 114 [AB]
60 mins vibration	1854 ± 112	1619 ± 58
60 mins compression	2296 ± 136	1459 ± 69
60 mins combination	1698 ± 99 [B]	1534 ± 50
90 mins vibration	1567 ± 89	1484 ± 44
90 mins compression	1533 ± 140	1317 ± 119 [A]
90 mins combination	1416 ± 163 [A]	1493 ± 59

A – Difference from unstimulated control ($p<0.05$); a – trend from unstimulated control ($0.05<p<0.10$); B – Difference from corresponding compression-only stimulation ($p<0.05$)

Further comparisons were achieved by normalizing all data to their age-matched unstimulated controls (Figure 5.2). Beneficial mechanical stimuli conditions (those conditions that elicited an increase in matrix synthesis) caused a similar percentage increases in the synthesis of proteoglycans in constructs for both age groups (~25-35%, p=0.875). While in the younger aged group, this increase was generally accompanied by a similar magnitude increase in collagen synthesis; however, in the older age group, increased collagen synthesis under beneficial stimulation conditions was generally only about 50% of increased proteoglycan synthesis (i.e. 10–25%, $p<0.05$). In younger cell constructs, cellularity as measured by DNA content (Table 5.2) was decreased by about 10% when subjected to compressive or combined loading that resulted in an anabolic response. In the older cell constructs, DNA content apparently decreased

(~30%) with any change in matrix synthesis (positive or negative) resulting from compressive or combined loading.

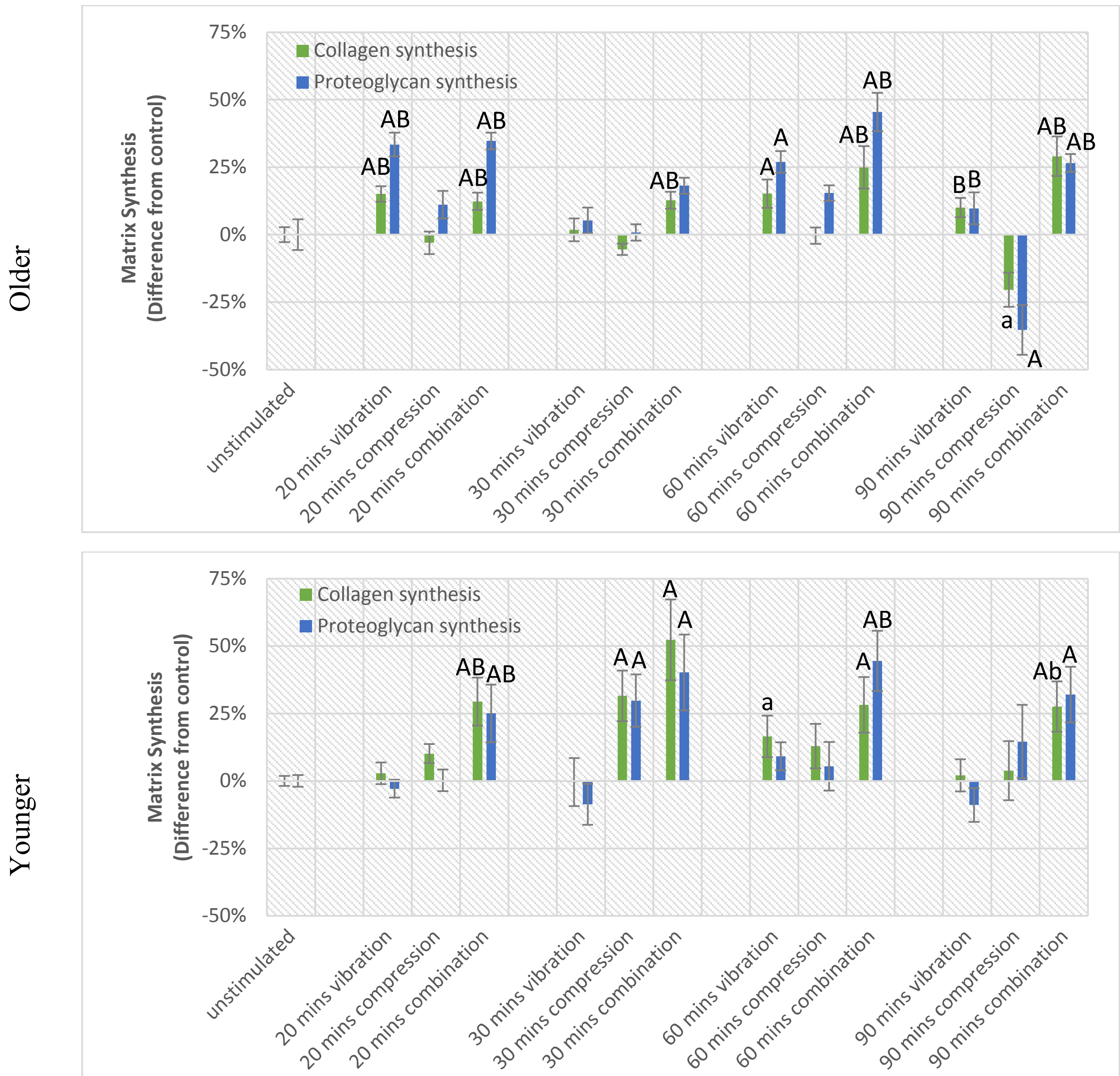

Figure 5.2: Effect of mechanical stimuli on matrix synthesis (collagen and proteoglycan). Data presented as percent difference from unstimulated controls (%), mean ± standard error (SEM); n≥6/group. A – Difference from unstimulated control ($p<0.05$); a – trend from unstimulated control ($0.05<p<0.10$); B – Difference from corresponding compression-only stimulation ($p<0.05$); b – trend from compression-only condition ($0.05<p<0.10$).

Table 5.2: Effect of mechanical stimuli on DNA content (Percent difference from unstimulated control). Data presented as mean ± SEM; n≥6/group.

	Older	Younger
unstimulated	0.0% ± 3.1%	0.0% ± 1.3%
20 mins vibration	-0.3% ± 4.2%	-5.2% ± 2.0% [a]
20 mins compression	-1.8% ± 4.7%	-6.6% ± 2.8% [a]
20 mins combination	-7.6% ± 2.4%	-10.2% ± 3.6% [A]
30 mins vibration	-0.2% ± 5.5%	4.5% ± 5.0%
30 mins compression	3.4% ± 4.2%	-4.8% ± 3.3%
30 mins combination	-13.3% ± 5.1%	-21.6% ± 7.4% [AB]
60 mins vibration	-13.5% ± 5.2%	5.2% ± 3.7%
60 mins compression	7.1% ± 6.3%	-5.2% ± 4.5%
60 mins combination	-20.8% ± 4.6% [AB]	-0.3% ± 3.2%
90 mins vibration	-26.9% ± 4.2% [A]	-3.5% ± 2.9%
90 mins compression	-28.5% ± 6.5% [A]	-14.4% ± 7.8% [A]
90 mins combination	-33.9% ± 7.6% [A]	-3.0% ± 3.8% [b]

A – Difference from unstimulated control ($p<0.05$); a – trend from unstimulated control ($0.05<p<0.10$); B – Difference from corresponding compression-only stimulation ($p<0.05$); b – trend from compression-only condition ($0.05<p<0.10$).

5.4 Discussion

Mechanical stimulation is commonly applied to tissue engineered cartilage constructs to enhance tissue formation and mechanical property development through the accumulation of extracellular matrix molecules [7–10]. While much success has been observed, inherent differences in sensitivity to mechanical loading as a result of age, species, and/or culture model as well as induced desensitization due to prolonged stimulation restricts the reliability of this approach [9,16–18,65,95]. For mechanical stimulation treatments to be successful, it is critical that the cells within the constructs are sensitive and remain sensitive to the applied stimuli. Previous work has shown that mechanosensitivity of chondrocytes isolated from younger animal can be increased and prolonged through use of stochastic resonance (superimposed random vibrations during dynamic compression)[132]. Given the promising results of this work, we investigated the effects of

stochastic resonance on unresponsive chondrocytes isolated from older aged animals. In the present study, cell-seeded agarose constructs were grouped by animal age and subjected to different types of mechanical stimuli: dynamic compression-only, vibration-only, and combined compression-vibration loading. The short term effects of these stimuli on matrix synthesis was assessed using radioisotope incorporation and construct cellularity was measured through DNA content.

As expected, age had a significant effect on the manner in which the chondrocytes responded to the mechanical stimuli. Most distinct was the magnitude of matrix synthesis between the two age groups. Differences in matrix synthesis responses were also apparently greater in magnitude for the younger cells compared to the older cells. Younger cells displayed some responsiveness to compression-only stimulation (at durations of 30 minutes) while older cells were typically insensitive to all durations tested. Conversely, vibration stimulation alone was able to elicit increase matrix synthesis in older cells at 20 and 60 minutes while the younger cells remained essentially unaffected by vibration-only stimulation. With combined compression-vibration loading, generally synthesis of both matrix molecules was increased at all loading durations for both older and younger cells. Differences in the anabolic response of chondrocytes with age may explain some of the differences observed in the response of older chondrocytes to mechanical stimuli. Older chondrocytes have been shown to display a reduced ability to synthesize both collagen and proteoglycans[139,143], reduced sensitivity to some chondroprotective growth factors (e.g. TGF-β and IGF-1) and reduced endogenous production of these same factors[145,148–150], increased sensitivity to other growth factors[145], and both decreased sensitivity and response to mechanical stimuli[144,148]. Decreases in anabolic response with age are most likely not the only

age-related changes that account for the observed differences in the response to mechanical stimuli of the older cell constructs. With age, increased catabolic activity in chondrocytes has also been observed. Specifically, MMP-13 production is increased in aging chondrocytes[147]. MMP-13 is an important factor in matrix remodeling is regularly produced in conjunction with anabolic factors[73,111,151]. While the present study did not measure catabolic markers, elevated gene expression and production of MMP-13 and other catabolic factors produced in older cells may help to explain the reduced responsiveness in terms of matrix molecule synthesis in the older chondrocytes.

While much of the age-related research concentrates on the decreased ability of old chondrocytes to produce a proteoglycan-rich extracellular matrix[139,141], collagen production is also an extremely important matrix constituent, especially its contribution to the mechanical functionality of the generated tissues[143]. In aged chondrocytes, both vibration-only and combined compression-vibration loading were able to increase proteoglycan synthesis (by 20-40%); however, under most conditions, collagen production was much lower (by 10-20%). In contrast, younger chondrocytes displayed similar magnitude changes in both proteoglycan and collagen synthesis when subjected to beneficial stimulation conditions. This result was not entirely unexpected as the synthesis and accumulation of collagen, including over long-term cultures, is notoriously difficult to stimulate[9,17].

The effect of mechanical stimulation on construct cellularity also differed depending on the age of the cells. In the older cell constructs, decreases in DNA content were associated with

compressive and combined stimuli which resulted in a change in matrix synthesis, regardless of whether the change was positive or negative. For younger cells constructs, it was generally the shorter stimulation durations which experienced small decreases in cellularity, which has been previously observed[110,117]. Interestingly, although initial seeding density (construct cellularity) was the same, the younger cell constructs generally contained less DNA than the older cell constructs, irrespective of whether subjected to mechanical stimuli. This is contrary to the decrease in DNA content that is usually observed with aging, where increased apoptosis leads to a decline in tissue DNA content[152].

The phenomenon of stochastic resonance describes when the sensitivity to a weak input signal is enhanced when noise is introduced to the system[102]. In this study, the addition of superimposed random vibrations onto a dynamic compressive stimulus increased the sensitivity of the cells to this stimulus. This effect was generally observed for both the younger as well as older chondrocytes. The ability of stochastic resonance to rescue an anabolic response in aged chondrocytes suggests that superimposed vibrations may be effective with diseased cells (e.g. osteoarthritic) that also display an impaired response to mechanical stimuli[153]. Some research has been conducted into how to mitigate age-related difference in chondrocyte cultures. Pre-treatment with growth factor cocktails has been used to increase proliferation during the expansion phase of older chondrocytes[139]. This pre-treatment was also successful at increasing proteoglycan production during pellet culture; however, this ability was still age-dependent[139]. The use of bioreactors have also been examined with both old and young chondrocytes cultured under the same conditions able to create tissue engineered constructs that were histologically comparable[154]. While the results of the current study demonstrate that matrix synthesis can be

increased for both older and younger chondrocytes through stochastic resonance methods, older cells were only able to achieve about half of the matrix synthesis compared to younger cells. That being said, mechanical stimulation methods, such as stochastic resonance, are generally more specific treatment options and therefore may be preferred over the use growth factors which can elicit numerous different responses[155].

While this study demonstrated the differences in response of aged cells to mechanical stimuli and the ability of superimposed vibrations to enhance the biosynthetic response of these aged cells, there are some limitations to the study that should be discussed. The cell-agarose model is not a perfect model of the organization and intercellular interactions of native tissue, nor is it an optimal scaffold for tissue engineering. It was however used for its ability to reduce confounding factors by creating homogenous constructs, maintaining the 3-dimensional chondrocyte phenotype, and not interfering with measurement techniques (i.e. assays)[130,131]. In addition, a full spectrum of ages was not investigated in this study.

5.5 Conclusion

In conclusion, cellular insensitivity is a major barrier for the successful use of mechanical stimulation to enhance tissue growth and properties of tissue engineered cartilage. The use of stochastic resonance to improve the response of cells to dynamic compressive loading is a simple and effective method to guarantee the success of most mechanical loading protocols. This study demonstrated that the addition of superimposed vibrations on mechanical loading waveforms generally improved the biosynthetic response of both younger and older chondrocytes while

having only a small detrimental effect on construct cellularity. It is important to consider this older population of cells as their behaviour is likely more representative of the adult cells that would be seen clinically. The ability of stochastic resonance to elicit an increased anabolic response in these older cells suggests that superimposed vibrations may be effective with other cells that also display an impaired response to mechanical stimuli (i.e. diseased or osteoarthritic cells). Future research should be conducted to determine the effects of stochastic resonance on diseased cells as well as the long-term efficacy of superimposed vibrations on both young and aged chondrocytes.

Chapter 6 – Study 4: Long-Term Effects of Stochastic Resonance with Mechanical Stimulation

6.0 Introduction

The functionality and integration of tissue engineered cartilage constructs after implantation relies on the constructs having biochemical and mechanical properties similar to healthy articular cartilage. One approach is to use mechanical stimulation to enhance the growth of the engineered construct. Through the use of mechanical stimuli, it has been possible to accelerate the synthesis and accumulation of cartilage-specific matrix molecules and obtain near functional mechanical properties of the developed tissues[7–10,79,86,110,117].

While mechanical stimulation is a simple and relatively straightforward treatment method in tissue engineering, it does have a few limitations. Due to differences in species, age, and culture model, stimulation parameters often need to be optimized for each set of conditions[12–14,79,136,143] and some conditions, especially in the case of age, display an inherent insensitivity to mechanical stimuli[132]. In addition, desensitization to prolonged stimuli can occur thereby reducing the efficacy of the protocols[9,17,18,65,95,110]. This variability in mechanical sensitivity is especially crucial to consider when dealing with cells that are derived from a less homogeneous subject group, a case which is more representative of what might be encountered clinically.

Stochastic resonance in the form of randomly generated vibrations superimposed on the loading waveform has been used to increase cellular mechanosensitivity of both sensitive and insensitive cells to dynamic compressive stimuli over short term, single-stimulus treatments[132]. In a previous study, the addition of stochastic resonance was able to mitigate both inherent

mechanical insensitivity as well as desensitization caused by prolonged loading, effects which would both be beneficial for dealing with sub-optimal stimulation conditions. Thus, given the beneficial effects observed with stochastic resonance during short-term culture, it is important to determine if these effects can be translated over the long-term by helping to maintain mechanosensitivity throughout each stimulation period.

6.1 Specific Aim

The previous two chapters demonstrated that stochastic resonance has the ability to increase cellular sensitivity to mechanical stimuli after a single application of loading. For stochastic resonance to be a useful tool, it is important to determine its long-term effects. Therefore, the specific objective of this study was to determine whether the beneficial short-term effects of stochastic resonance are maintained over repeated applications of stimulation.

6.2 Methods

6.2.1 Chondrocyte agarose constructs

Fresh articular cartilage slices from bovine metacarpal-phalangeal joints (18-30 months of age) were incubated in protease (0.5% w/v in Ham's F-12 with 25 mM HEPES) for 1 hour followed by 18 hours in collagenase A (0.15% w/v in F-12 with 25 mM HEPES) to release the cells. The tissue digest was filtered through a 200-mesh filter to remove undigested particulates. The cells suspension was washed 3 times by centrifugation and resuspension with fresh F-12 with 25 mM HEPES. The resulting cell suspension was mixed with type vii molten (50°C) agarose (resulting in a cell concentration of $10x10^6$ cells/mL in a 2% gel) and a 4 mm thick layer was pipetted into a 100 mm petri dish to cool. After gelation (15-20 minutes at room temperature), cylindrical

constructs were extracted using a 4mm diameter biopsy punch. Constructs were individually transferred to 24-well plated fitted with plastic retaining rings then fed with 400 µL complete media (Ham's F-12 with 20% FBS, 20 mM HEPES, 100 µg/mL ascorbic acid, and antibiotics/antimycotics). The constructs were allowed to culture undisturbed for 24 hours (at 37°C with 5% CO_2) before beginning stimulation treatments.

6.2.2 Application of Mechanical Stimuli

Dynamic compression was applied to the top of the constructs with a Mach-1 Micromechanical testing system with a specially fitted lid for a 24-well plate and 6 individual loading platens. Vibrational loading was applied from the bottom of the cultures with a vibration stimulation system consisting of a voice coil, well plate support stage, and separately controlled software interface. A compressive strain amplitude of 5% was used at a frequency of 1 Hz for a 20 minute stimulation durations with, or without, superimposed random vibrational loading between 20-50 Hz at an amplitude of 1 g (average maximal strain of < 1.75%). Cultures were stimulated three times a week and harvested after 2 and 4 weeks (6 and 12 stimulation cycles, respectively). Immediately prior to each stimulation, conditioned media was removed and stored at -20°C, then each construct was supplied with 400 µL of fresh complete media.

6.2.3 Determination of DNA, Collagen, and Proteoglycan Content

Constructs were removed from culture at 2 and 4 weeks, weighed, frozen (-20°C), then lyophilized. The dried constructs were re-weighed before papain digestion (40 µg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM DTT) for 72 hrs at 65°C. DNA content was quantified through the fluorometric PicoGreen DNA assay[120]. Proteoglycan content was

measured through the use of the dimethylmethylene blue (DMMB) sulfated glycosaminoglycan binding assay and standardized to bovine cartilage chondroitin sulfate A (Sigma Aldrich)[156]. Hydrolyzed aliquots of the papain digested solution were used to quantify hydroxyproline residues through the colorimetric chloramine-T/Ehrlich's reagent assay standardized to L-hydroxyproline (Sigma Aldrich)[157]. Collagen content was estimated with the assumption that 10% of collagen mass is made up of hydroxyproline[158].

6.2.4 Media analysis of Collagen and Proteoglycan Content

Conditioned media samples were thawed then split into two aliquots and pooled by week. Proteoglycan extraction was accomplished through cold ethanol precipitation[159]. Proteoglycan precipitates were pelleted through centrifugation, washed with cold ethanol, dried, and stored at -20°C. Proteoglycan pellets from collected media were resuspended in 4 M guanidium hydrochloride and quantified using the DMMB assay [156].

Collagen was extracted through ammonium sulfate precipitation[160,161]. Collagen precipitates were pelleted, washed, dried, and stored at -20°C. Collagen pellets from media were hydrolyzed and assayed through chloramine-T/Ehrlich's reagent[157]. Collagen content was estimated with the assumption that 10% of collagen mass is made up of hydroxyproline[158].

6.2.5 Histology

Representative samples were fixed in 4% paraformaldehyde (24 hrs) then paraffin embedded and cut into 5 μm sections. Sections were stained with either safranin-O and fast green counterstain or picrosirius red with Weigert's haematoxylin counterstain for visualization of proteoglycan and

collagen distribution, respectively. Sections were analyzed by light microscopy with images acquired using a Nikon Eclipse TS100 microscope image capture system.

6.2.6 Mechanical Testing

The equilibrium modulus of each construct was determined through unconfined compressive stress relaxation tests using a Mach-1 mechanical tester. After sample harvest, sample height and several representative sample diameters were measured using a digital caliper. A pre-load of 5 mN was applied to ensure contact with the compression platen surface. Sequential step strains of 2.5% were applied to the samples up to a maximum of 20% strain. For each step, the resulting forces were recorded until equilibrium was reached (<2 mN/min change)[17,57,162]. The equilibrium stress was plotted against the equilibrium strain and fitted with a first degree polynomial. The equilibrium modulus was then determined by the slope of the fitted line.

6.2.7 Statistics

All experiments were performed at least twice using cells obtained from different donors. All results were expressed as mean ± standard error of the mean (SEM). Data were analyzed with a one-way ANOVA treating each stimulation combination and time point as its own group. Post hoc Dunnet's pairwise comparisons were done twice with Bonferroni's correcting treating the unstimulated group and the compression-only group as two controls. P-values 0.05 or less were defined as significant and p-values between 0.05 and 0.1 were defined as trends.

6.3 Results

6.3.1 Biochemical properties of mechanically stimulated constructs

The effect of long term dynamic compressive stimulation (5% strain amplitude), with or without superimposed random vibrations (1 g amplitude with frequencies between 20-50 Hz) on the in vitro formation and properties of tissue in cell-agarose constructs was assessed for up to 4 weeks in culture.

Water content of the tissue constructs remained at a consistent ~95% across all conditions regardless of culture time or stimulation condition (Table 6.1). Culture time, alternatively, had a significant effect on construct cellularity (Table 6.1). Samples cultured over a 4 week period consistently had greater DNA content (~60% more) than their 2 week counterparts; however, little variation was observed with mechanical stimulation (compression, vibration or combined loading).

Table 6.1: Extracellular matrix accumulation. Data presented as mean ± standard error (SEM); n ≥ 6/group.

	unstimulated		vibration		compression		combined	
	2 week	4 week	2 week	4 week	2 week	4 week	2 week	4 week
Hydration (%)	95 ± 0	95 ± 0	95 ± 0	95 ± 0	95 ± 0	95 ± 0	95 ± 0	95 ± 0
DNA/dry weight (µg/mg)	0.67 ± 0.08	1.03 ± 0.08^{A}	0.75 ± 0.12	1.09 ± 0.06^{A}	0.58 ± 0.04	0.93 ± 0.06^{A}	0.54 ± 0.03	1.05 ± 0.03^{A}
Proteoglycan (µg)	88.1 ± 3.4	73.6 ± 6.7^{a}	106.0 ± 10.9	75.9 ± 7.9^{A}	78.3 ± 5.7	67.1 ± 13.3	88.5 ± 6.6	88.9 ± 13.9
Proteoglycan/dry Weight (µg/mg)	37.0 ± 2.7	33.4 ± 3.0	43.7 ± 4.9	32.1 ± 3.4	34.8 ± 2.5	32.8 ± 6.2	35.8 ± 3.8	42.7 ± 6.1
Proteoglycan/DNA (µg/µg)	60.1 ± 1.0	27.6 ± 1.3^{A}	65.0 ± 6.0	26.0 ± 2.5^{A}	62.1 ± 7.4	31.3 ± 4.5^{A}	71.8 ± 5.5^{b}	34.2 ± 2.5^{Ab}
Collagen (µg)	24.6 ± 0.9	21.6 ± 0.9^{a}	33.4 ± 5.2^{C}	31.9 ± 4.3^{c}	17.7 ± 2.8	21.6 ± 1.6	21.2 ± 2.8	37.1 ± 4.9^{ABC}
Collagen/dry Weight (µg/mg)	10.3 ± 0.5	9.0 ± 0.5	13.9 ± 2.2	12.5 ± 1.4	8.0 ± 1.3	17.0 ± 0.8^{AB}	8.6 ± 1.2	20.8 ± 1.8^{AB}
Collagen/DNA (µg/µg)	16.8 ± 0.4	8.0 ± 0.3^{a}	21.4 ± 3.3	11.6 ± 1.3^{A}	14.2 ± 1.8	10.9 ± 1.3	17.3 ± 1.6	14.8 ± 1.8^{b}
Collagen to proteoglycan Ratio	0.28 ± 0.01	0.26 ± 0.01	0.32 ± 0.04	0.44 ± 0.07	0.23 ± 0.04	0.42 ± 0.09^{a}	0.24 ± 0.02	0.45 ± 0.07^{A}

A - Significant difference from 2 week time point ($p<0.05$); a – Trend from 2 week time point ($0.05<p<0.10$); B – Significant difference from unstimulated controls ($p<0.05$); b – Trend from unstimulated controls ($0.05<p<0.10$); C – Significant difference from compressive loading ($p<0.05$); c – Trend from compressive loading ($0.05<p<0.10$).

Irrespective of stimulation condition, the absolute proteoglycan content of the constructs remained mostly unchanged between the two time points. Proteoglycan accumulation normalized by construct cellularity was decreased approximately 50% between the 2 week and the 4 week time point for all conditions ($p<0.05$); however, a small positive difference was observed between the combined loaded conditions and the unstimulated controls ($p=0.070$).

Compared to proteoglycan accumulation, mechanical stimulation had more of an effect on collagen content and accumulation (Table 6.1). Under combined loading after 4 weeks, the collagen content of the constructs was increased relative to the 2 week time point and the unstimulated controls ($p<0.05$). While a slight decrease in collagen accumulation normalized to construct cellularity was observed after 4 weeks between the vibration-only loaded samples and the unstimulated controls, combined compression-vibration loading was apparently effective in maintaining steady collagen accumulation ($p<0.05$). Due to the gains in collagen content due to the combined loading and compressive loading protocols, an increase in the collagen-to-proteoglycan ratio of almost 50% was observed for these conditions ($p<0.05$ and $p=0.099$, respectively) (Table 6.1). Alternatively, this ratio remained relatively unchanged for the unstimulated controls as a function of culture time.

6.3.2 Mechanical properties of mechanically stimulated constructs

Mechanical properties as measured by equilibrium modulus were also affected by culture time (Table 6.2), with both compressive and combined stimuli at 4 weeks showing a significant decrease (~25%) compared to 2 weeks of culture ($p<0.05$). Combined compression-vibration loading resulted in a slight increase in modulus after 2 weeks of culture ($p<0.05$); however, this effect was lost after 4 weeks of culture ($p=0.498$). Construct height and diameter were measured before mechanical testing, but no difference were observed compared to pre-cultured conditions (data not shown).

Table 6.2: Effect of stimulation condition and culture time on the properties of cell-agarose constructs. Data presented as mean ± standard error (SEM); n ≥ 6/group.

		Equilibrium Modulus (kPa)
2 Weeks	unstimulated	6.9 ± 0.5
	vibration	9.1 ± 1.0
	compression	9.4 ± 0.7
	combined	11.0 ± 1.5^{B}
4 Weeks	unstimulated	8.1 ± 0.7
	vibration	8.4 ± 0.3
	compression	7.6 ± 0.6^{A}
	combined	7.5 ± 0.2^{A}

A - Significant difference from 2 week time point (p<0.05); B – Significant difference from unstimulated controls (p<0.05).

6.3.3 Analysis of released collagen and proteoglycans

A sizeable amount of synthesized matrix components were also lost into the media. Conditioned media throughout the culture period was collected and analyzed for the presence of collagen and proteoglycans (Figure 6.1). Increased culture time generally resulted in more collagen present in the media (p<0.05). As a result of mechanical stimulation, the amount of collagen released into the media tended to increase after 2 weeks of culture for both the compression (p=0.074) and combined (p=0.086) loading protocols; however, this effect was undetectable after 4 weeks of stimulation (p=0.196). Increased culture time also had a similar effect of increasing proteoglycan content in the media (p<0.05). At 4 weeks of culture, compressive and combined stimulated samples tended to have increased proteoglycan content compared to the unstimulated controls (p=0.068 and p=0.064, respectively).

Total collagen and proteoglycan synthesis was calculated by combining the amount of ECM found in the media with the amount of ECM retained within the constructs. Overall, total synthesis of both molecules was elevated after 4 weeks of culture compared to 2 weeks of culture ($p<0.05$). Mechanical stimulation mode (vibration, compression or combined loading) had no apparent effect at either time point on the total matrix synthesis of either collagen ($p=0.405$ and $p=0.106$, respectively), or proteoglycan ($p=0.421$ and $p=0.079$, respectively).

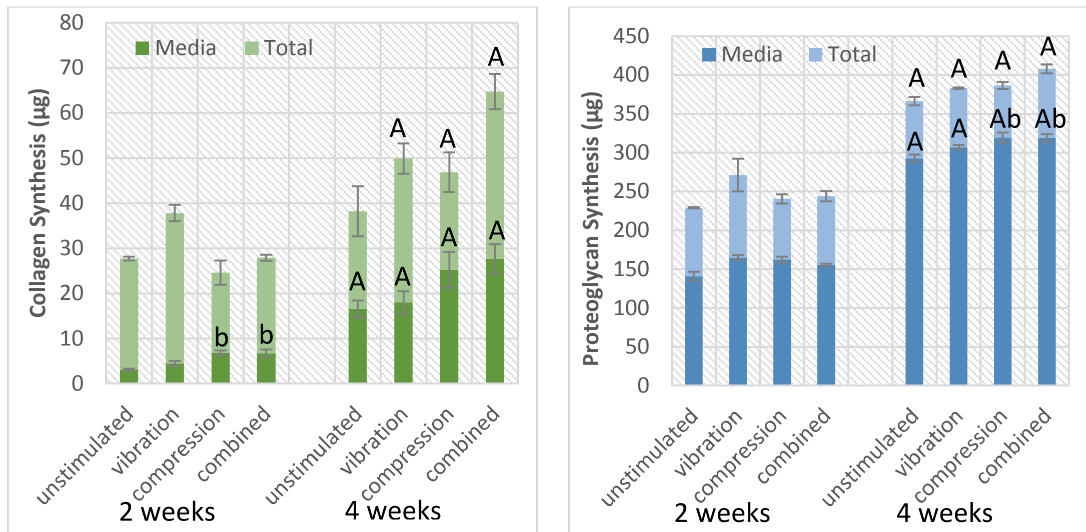

Figure 6.1: Effect of stimulation condition and culture time on total matrix production. Total synthesis shown as full bar height, component found in the media superimposed in the darker bar; presented as mean ± SEM; $n \geq 4$/group. A – Significant difference from the 2 week time point ($p<0.05$); B – Significant difference from the unstimulated controls ($p<0.05$).

To further examine the differences between matrix synthesis and accumulation, the percent retention of collagen and proteoglycan were calculated (Table 6.3). Generally, more of the synthesized collagen was retained in the construct compared to proteoglycans, in which the majority of the synthesized molecules released into the media. For both collagen and proteoglycan retention, time in culture was a significant factor. Retention of both constituents

significantly decreased with culture time ($p<0.05$). With the exception of proteoglycan retention at 4 weeks, there was no apparent effect of mechanical stimulation on matrix retention. After 4 weeks, compressive stimulation displayed reduced proteoglycan retention ($p<0.05$) compared to unstimulated controls which appeared to be most rescued by combined compression-vibration loading.

Table 6.3: Effect of stimulation condition and culture time on retention of matrix molecules produced. Data presented as mean ± standard error (SEM); n ≥ 4/group.

		Proteoglycan retained (%)				Collagen retained (%)			
2 Weeks	unstimulated	37.2	±	1.4		87.6	±	0.56	
	vibration	38.1	±	3.0		83.7	±	1.45	
	compression	29.7	±	1.0		74.1	±	4.77	
	combined	39.1	±	1.3		79.6	±	2.26	
4 Weeks	unstimulated	20.4	±	0.6	A	55.7	±	1.48	A
	vibration	18.6	±	0.9	AC	64.0	±	2.80	A
	compression	11.7	±	1.3	AB	49.9	±	3.52	A
	combined	17.1	±	0.6	AC	49.1	±	4.03	A

A - Significant difference from the 2 week time point ($p<0.05$); B - Significant difference from the unstimulated controls ($p<0.05$); C – Significant difference from compressive stimulation.

6.3.4 Histological appearance of mechanically stimulated constructs

Histological staining (Figure 6.2) of the constructs show that the majority of collagens and proteoglycans retained in the constructs appeared to be located in the pericellular and territorial spaces immediately surrounding the cells with little staining in the inter-territorial area observed. The staining was also most prominently observed at the edges of the constructs (data not shown). After 2 weeks in culture, little differences were observed as a result of mechanical stimulation;

however, by 4 weeks in culture, all stimulated samples showed noticeable increases in the territorial area for both collagen and proteoglycan distribution compared to the unstimulated controls.

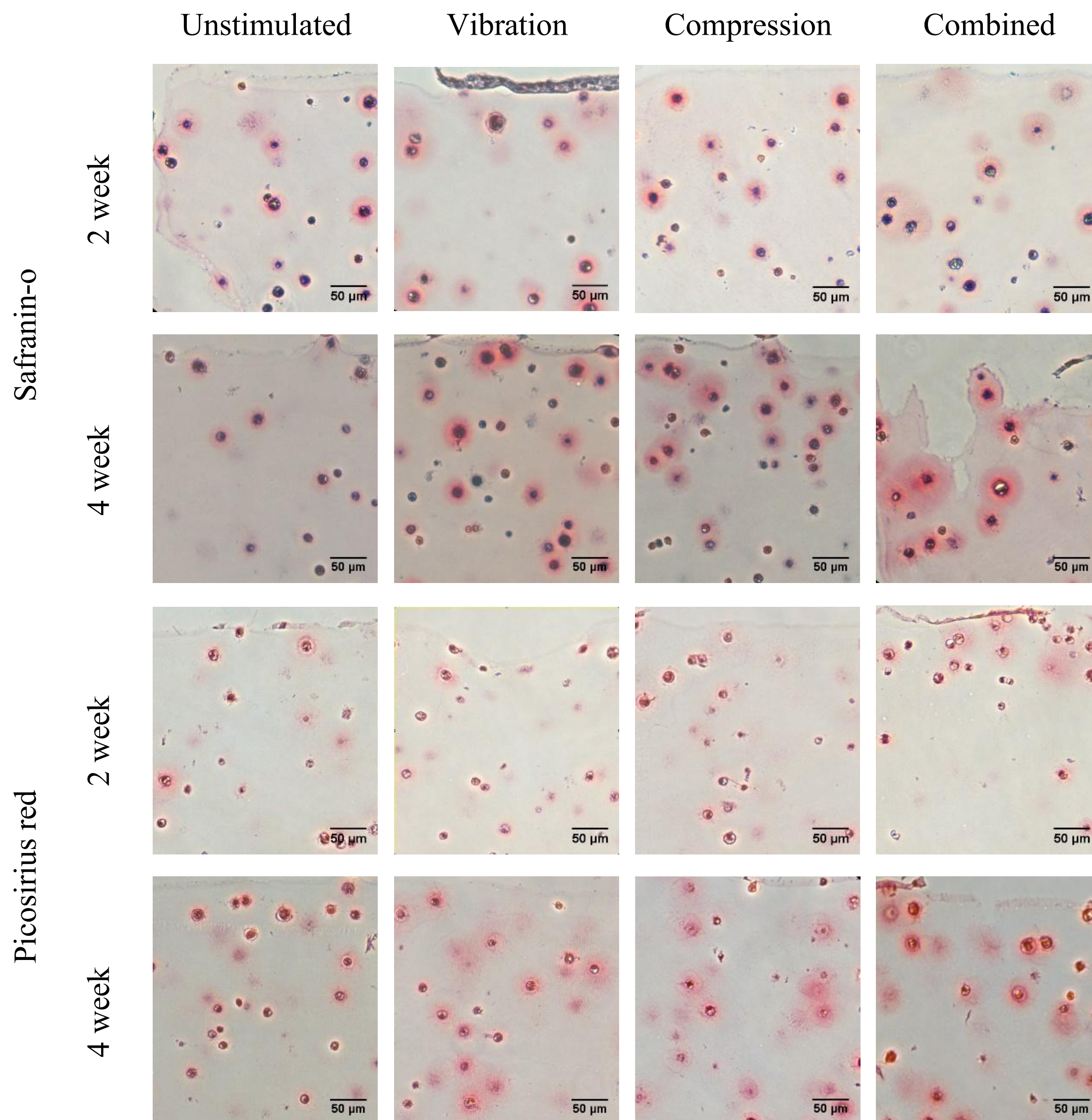

Figure 6.2: Histological staining. Top rows - safranin-o (red) staining for proteoglycans. Bottom row - picrosirius red staining for general collagens. Staining was mostly concentrated in the pericellular regions. Scale bar: 50 μm.

6.4 Discussion

Dynamic compressive mechanical stimulation is widely used to accelerate chondrogenesis of tissue engineered cartilage constructs[7–10]. Innate insensitivity and induced desensitization to mechanical stimuli can limit the effectiveness of these protocols. Stochastic resonance has previously been used in short-term culture to enhance the effect of dynamic compressive stimuli in a chondrocyte-agarose model by mitigating desensitization and shortening apparent required recovery periods[132]. Stochastic resonance has also been applied to the culture of osteoblasts both in vitro and in vivo resulting in increased bone synthesis compared to compressive loading alone over long term cultures[108,109,163]. This method has also been found to have beneficial effects in whole body systems to such a degree that clinically used treatment devices have incorporated broadband noise in the devices[105–107]. These studies demonstrate the ability of stochastic resonance to improve system responses in a wide variety of applications. With such a prevalent positive response in such a diverse field of applications, the use of stochastic resonance appears to be a valuable tool for mechanical stimulation of cartilage constructs, especially when less than optimal conditions are used. Given the encouraging results of this work, we investigated the effects of stochastic resonance over long-term culture with repeated applications of mechanical stimuli. In the present study, cell-seeded agarose constructs were subjected to dynamic mechanical compression, with or without, superimposed random vibrations applied repeatedly for up to 4 weeks in culture and assessed for changes in tissue growth and properties.

After two weeks of culture, differences in matrix content in the tissue constructs due to stimulation condition was difficult to detect. Collagen and proteoglycan levels were similar across all stimulation conditions and histological staining shows that the bulk of these molecules

were located in the pericellular matrix and a small territorial area surrounding the cells. Throughout all 2 week conditions, a similar distribution of these matrix molecules is observed. However, after four weeks, matrix content in the tissue constructs was significantly dependent on the mode of mechanical stimulation. Collagen content in the unstimulated controls remained unchanged, but increased by almost 2-fold when subjected to either the combined or compressive stimulation protocols. Collagen accumulation normalized to construct cellularity also generally decreased with increasing culture time, but combined loading was able to maintain this level between the two time points. The proteoglycan content of the tissue constructs was generally unaffected by both culture time and stimulation mode. When expressed relative to construct cellularity, a 50%-60% decrease in proteoglycans was generally observed with time; however, combined compression-vibration stimulation was able to mitigate the majority of this loss. This is in contrast to previous studies which have generally shown in increase in proteoglycan synthesis and accumulation with mechanical stimuli[9,58,79,80]. After four weeks of culture, some differences were observed histologically. Unstimulated constructs showed little difference in the distribution of matrix molecules, but all modes of mechanical stimulation showed an increase in the size of the territorial matrix stained. While a similar pattern of deposition was observed for both the collagen and proteoglycans, small collagen fibres can be identified surrounding the cells whereas the proteoglycan staining appeared to be more diffuse and homogeneous in this area. Similar to previous studies, histological assessment at both time points indicated that the majority of accumulated matrix molecules is associated with the cells in the periphery of the constructs[58].

Construct cellularity was only affected by culture time, with no observable differences between mechanical stimulation mode. A consistent increase in DNA content was observed regardless of stimulation, whereas previous studies have shown a small, but significant decrease in DNA content associated with compressive stimuli[110,132]. This may be due to the intermittent application of stimuli, allowing the constructs to rest (approximately 48 hours) between stimulation periods giving the cells a chance to proliferate without the interference of mechanical stimuli.

The mechanical properties, as measured by the equilibrium modulus, displayed a dependence on the mode of mechanical stimulation after two weeks of culture. A slight increase in equilibrium modulus was observed with combined compression-vibration stimulation. This effect was lost at four weeks and a decrease in modulus was also observed with compressive stimulation while the unstimulated and vibration-only stimulated samples experienced no apparent change. This decrease in mechanical properties was unexpected as increased culture time showed an increase in the collagen-to-proteoglycan ratio and the accumulation of matrix molecules has generally been associated with improved mechanical properties[164]. A possible explanation for the decreased mechanical properties could be fatigue of the agarose gels due to the cyclic strains applied under both compression and vibrational loading, a phenomenon which has been previously encountered[79]. Some irregularities in the construct surfaces were observed in the histological sections also suggesting that the integrity of the gel may have been compromised by the repeated loading protocols.

Total proteoglycan production was increased after 4 weeks of culture and much of the proteoglycans produced were released into the surrounding media. For compression and combined loaded constructs, an increased amount of proteoglycans could be detected in the conditioned media. Consequently, proteoglycan retention at 2 week of culture was approximately 35%, but at 4 weeks of culture dropped to 20% for both unstimulated and vibration stimulated constructs and to 10% for the compression stimulated constructs. The application of compressive stimulation could have induce fluid flow that facilitated the loss of proteoglycans into the media[165]. Interestingly, stochastic resonance was able to mitigate some of the load-induced proteoglycan loss resulting in retention values similar to that of the unstimulated constructs (decreased to about 20% retention).

A significant amount of collagen was also detected in the media. While a small load-associated increase in media collagen content was observed at 2 weeks, by 4 weeks of stimulation, no effect of mechanical stimulation was apparent. Compared to the proteoglycans, a higher percentage of collagen was retained within the constructs, about 80% at 2 weeks and approximately 55% at 4 weeks. Due to an overall increase in total collagen production, the increased amount of collagen found in the media was not due to loss from the tissue construct. It possible that the improved collagen retention (compared to the retention of proteoglycans) was due to the fibrous nature of collagen (and generally larger molecular weights) making it more difficult for these molecules to be released from the agarose constructs.

Contrary to many other long-term chondrocyte culture studies, a major increase in proteoglycan accumulation and associated increase in mechanical properties of the constructs was not observed in the present study. One possible explanation of this discrepancy could be due to these other studies using chondrocytes derived from skeletally immature animals. Many studies have documented the effects of aging on the chondrogenic potential of isolated cells and have found that with increasing age, beginning around normal adolescent growth and maturation[143], cell proliferation, matrix anabolism, and cellular sensitivity to important chondrogenic growth factors are diminished[139,143–146]. While the present study did not use elderly cells, the cells used were derived from skeletally mature animals. This may contribute to the limited matrix anabolism that was observed; however, the application of stochastic resonance was still able to elicit a significant positive effect in these cells. Another possible factor in the differences observed compared to previous studies may be the difference in culture model. In addition to the use of younger cells, many of these previous studies used different culture models (e.g. scaffold-free)[9,17,162]. The successful application of mechanical stimuli depends on the cells being able to sense the loading from the stimulus. In developing tissues in vivo, this is modulated by cell-matrix interactions[19,26,44]. As can be observed from the histological staining, little staining was observed in the spaces between the territorial matrix of the cells indicating a lack of matrix deposition between cells to facilitate these stimuli-matrix-cell or cell-matrix-cell interactions. Longer culture times may be needed to develop this inter-territorial space and the growth of the territorial matrix that was observed suggests that this may be possible. Another confounding factor may be the reformation of the pericellular matrix after construct formation. After enzymatic isolation, it can take up to 48 hours for the pericellular matrix to re-establish around the cells[131]. For short-term culture, this is beneficial since these cells without pericellular matrix

in an agarose scaffold can then experience cellular strains similar to the global strain applied to the construct[54,130]. In long-term culture, the pericellular matrix is re-established and may therefore serve to shield the cells from the strains induced in the construct during mechanical stimulation. In addition, increased culture time of cell-agarose constructs has been previously observed to decrease the effectiveness of vibrational induced effects, which may be attributed to accumulated matrix proteins attenuating the vibrations[65]. Therefore, the limited effect of stochastic resonance with mechanical compression during long-term culture of cell-agarose constructs observed in the present study could be due to the reformation of the pericellular matrix attenuating the vibrations necessary for eliciting stochastic resonance. To overcome some of these limitations, it may be necessary to change the vibration parameters (i.e. amplitude and/or frequency) as the tissue develops to ensure that the stochastic resonance vibrations are not completely dampened.

6.5 Conclusion

In conclusion, this study demonstrated that stochastic resonance applied over long-term culture was able to aid in the development of matrix constituents in tissue engineered cartilage constructs by augmenting the accumulation of collagen and mitigating strain induced proteoglycan loss. The ability of stochastic resonance to increase cellular sensitivity to mechanical stimuli makes it a worthwhile method to further investigate. Future work should focus on two points: first, the effectiveness of stochastic resonance and mechanical stimulation in long-term culture on unresponsive cell populations and second, the effect of tuning stochastic resonance vibrations (amplitude/frequency adjustment) throughout long-term culture to maintain the greatest effect possible.

Chapter 7: General Discussion and Conclusions

7.1 Summary

A common obstacle in the development of treatment methods for functional tissue engineered articular cartilage is the inability to guarantee the efficacy of a particular mechanical stimulation protocol. Variability in the mechanosensitivity of chondrocytes can be attributed to inherent differences between donors, which include factors such as: species, age, disease state, etc. It is therefore important to explore the progression and possible mechanisms of sensitization and desensitization as well as to develop new methods to increase mechanosensitivity. Therefore, the studies conducted in this thesis all revolve around examining and improving the sensitivity of chondrocytes to dynamic mechanical stimuli.

7.1.1 Sensitization and desensitization to dynamic mechanical loading

The complex phenomena of sensitization and desensitization to mechanical stimulation has not been fully described. In the first study, the short term temporal response of chondrocytes to dynamic compressive strain and the changes in intracellular calcium signaling were investigated using cells derived from a controlled source to limit as much variation as possible. Through radioisotope incorporation analyses and confocal imaging, it was observed that a minimum amount of mechanical stimulation was required to "sensitize" the cells (i.e. elicit a response), while prolonged stimulation led to cellular desensitization. This response could be predicted by measuring the resulting changes in calcium signaling. When changes in calcium signaling were expressed as a relative number of cells experiencing multiple transients, a positive correlation was observed between signaling and resultant matrix synthesis. This measure of calcium signaling was also effective in predicting the required recovery period for full

mechanosensitivity to be achieved thus illustrating a method to determine the minimum amount of time needed between successive applications of intermittent dynamic loading. The work from this study was published in Biomechanics and Modeling in Mechanobiology [110].

7.1.2 Stochastic resonance as a method to improve mechanosensitivity

Sustaining cellular mechanosensitivity is important for the successful use of mechanical stimulation to enhance the growth and maturation of tissue engineered cartilage. The complex nature of sensitization and desensitization to mechanical stimuli can limit the effectiveness of such treatments; however, one potential method to mitigate mechanical insensitivity is by superimposing noise on the loading waveforms — a phenomenon known as stochastic resonance. Therefore, the purpose of the second study was to investigate the effect of superimposed random vibrational loading on chondrocyte matrix metabolism to find optimal noise parameters to elicit a stochastic resonance response. A variety of vibrations of differing amplitudes and frequency bandwidths were examined through radioisotope incorporation analysis and confocal imaging. Stochastic resonance was able to improve cellular sensitivity to mechanical loading by further increasing matrix biosynthesis by 20-60%. This approach also appeared to be successful in mitigating the effects of load-induced desensitization by sustaining matrix synthesis after long loading durations. Although stochastic resonance had no apparent effect on the magnitude or spatial distribution of calcium signaling immediately after stimulation, a shorter refractory period in calcium signaling was achieved after stimulation suggesting that full mechanosensitivity could be achieved at a faster rate. The work from this study has been accepted for publication in the Journal of Orthopaedic Research [132].

7.1.3 The effect of stochastic resonance with age

Age is a key factor in how chondrocytes respond to various stimuli. With mechanical stimulation, sensitivity to dynamic compressive loading decreases with increasing age in terms of the biosynthesis of extracellular matrix macromolecules. The third study examined the effect of age on the anabolic response to mechanical stimulation with stochastic resonance through the use of two distinct age groups (younger and older) using radioisotope incorporation analyses. In all cases for both the younger and older cells, stochastic resonance improved cellular sensitivity and mitigated load-induced desensitization as evidenced by a consistent increase above control in matrix synthesis. Under certain conditions, stochastic resonance was also observed to rescue the response of desensitization in older cells. Vibrational loading alone also had a positive effect on the response from older cells, while the younger cells were generally insensitive to vibration-only stimuli suggesting that the older cells may be especially sensitive to the mechanism by which stochastic resonance acts. Although the absolute magnitude of biosynthetic response in the older cells was significantly lower than that of the younger cells, the change in synthesis relative to their respective controls was similar.

7.1.4 The effect of stochastic resonance during long term culture

The success of implanted tissue engineered cartilage constructs depends on the constructs obtaining biochemical and mechanical properties similar to the surrounding native tissue. Stochastic resonance has been shown to be effective in mitigating mechanical insensitivity in short-term studies, but the long-term efficacy of this intervention on any type of chondrocytes has not yet been studied. Therefore, the purpose of the fourth study was to study the effect of superimposed random vibrational loading on chondrocyte matrix metabolism throughout repeated stimulations over long-term culture through biochemical assays to assess matrix

constituents and DNA content, histology, and compressive mechanical testing. A positive effect on matrix accumulation by the end of the 4 week culture period was observed due to applying stochastic resonance methods. Both collagen accumulation was enhanced and strain-induced proteoglycan loss was mitigated resulting in increased matrix deposition in the developing constructs. While the overall effects of stochastic resonance observed in this study were relatively small in magnitude, there were no apparent detrimental effects of stochastic resonance, suggesting that this method can be applied over the long-term even if the cells are derived from a less responsive cell source.

7.2 General Discussion

Mechanical sensitivity is an important factor to consider when subjecting tissue engineered constructs to mechanical stimuli. The diversity in mechanical stimulation parameters throughout the literature is reflective of the complex nature of cellular sensitization and desensitization responses to mechanical stimuli [12–15,79]. Empirically optimizing stimulation protocols can be time consuming and involves much trial and error and careful control of the cell population to limit confounding factors between experiments. Then, even with a previously optimized stimulation protocol, there are factors such as age which change the sensitivity of the cells and can make such protocols ineffectual[144,148]. Therefore, the overall aim of this thesis was to investigate chondrocyte sensitivity to dynamic mechanical loading with a focus on determining an intervention that could be used to affect mechanical sensitivity.

The results of the first study in this thesis highlights the difficulty that exists in finding optimal stimulation conditions even for a controlled cell population: too little stimulation has no effect while too much stimulation induces desensitization, both of which could result in either no response or a detrimental response[9,16,17,110,117]. For mechanical stimulation to become a standard treatment in tissue engineering, there must be a way to guarantee that the method will work (or at least not be detrimental) for a wider range of cell sources as it would be impractical to determine the optimal stimulation conditions for each experiment. In addition, it is likely that cellular mechanical sensitivities change over long-term culture as the extracellular matrix develops[65], thus the effect of previously optimized conditions may be diminished.

In an effort to make mechanical parameter optimization easier, a calcium signaling approach was taken. Calcium signaling is a common downstream mechanotransduction pathway also responsible for initiating gene transcription[39,45,46]. In previous studies, calcium signaling is often observed during and/or immediately after mechanical stimulation[43,47–51,54–56]. In these studies, while a change in the number of cells responding and the number of transients experienced in response to mechanical stimuli have been observed, these changes were not correlated to, or compared with, resultant biosynthesis. By calculating the ratio of the number of cells experiencing multiple transients to the number of cells experiencing at least one transient, thereby effectively thresholding out randomly induced calcium transients, a positive correlation between calcium signaling and matrix synthesis was observed. While this method is unable to predict the magnitude of the induced matrix synthesis, it is possible to use it as a predictor of an anabolic response. Thus, this approach can provide another method through which dynamic compressive stimulation may be optimized.

It was found that calcium signaling could also be used to predict the return of mechanosensitivity indicating the earliest possible time for another application of stimuli. Determining the recovery (or refractory) period between stimulations is essential to maximize the beneficial effects of mechanical stimulation. If the reapplication of stimulation occurs before mechanosensitivity has fully returned, there can be a detrimental effect on matrix synthesis and presumably matrix accumulation. By tracking the extent of calcium signaling after stimulation, the required recovery time can be determined when calcium signaling levels return to the baseline, pre-stimulated values. Observing calcium signaling is a non-destructive method which allows the same sample to be tested over several time points, minimizing potential variance between

samples. Since calcium signaling can be used an effective predictor of the success of dynamic compressive loading protocols as well as to optimize refractory periods, this approach has several advantages over other empirically-based approaches (e.g. biochemical assays of stimulated constructs[156,157]).

Stochastic resonance is currently used in some clinical settings to enhance the responsiveness of biomedical systems (e.g. blood oxygenation, blood pressure, balance)[104–107]; however, it appears to be rarely using in tissue engineering applications with the exception of studies conducted on bone[108,109]. In this thesis, the stochastic resonance approach taken was in the form of superimposed random vibrations on dynamic compressive loading. Through the course of optimization studies, the most beneficial vibration parameters were with an average amplitude of 1 g and a bandwidth between 20-50 Hz. Cell-seeded constructs subjected to stochastic resonance under these conditions demonstrated an increase in mechanosensitivity manifesting in a greater biosynthetic response, and under specific conditions, could be used to rescue a detrimental response where biosynthesis was returned to control (unstimulated) levels. When unresponsive cells were used, such as the older cell group in study 3, a similar positive effect was observed suggesting that stochastic resonance may be effective with cells that generally display impaired mechanical sensitivity such as the case with diseased (e.g. osteoarthritic) cells[153].

Beyond the short term immediate effects of stochastic resonance, stochastic resonance may also prove to be useful when applied over the long-term. Stochastic resonance affects the change in calcium signaling levels that are observed after stimulation. The refractory period of combined

loaded cells appeared to be shorter than for compressive-only stimulated samples indicating that mechanosensitivity is recovered faster, suggesting that less time may be required between successive loading applications. This means that in the same time period, stochastic resonance stimulated constructs could be stimulated more times for a beneficial effect than normal compressive stimulated constructs, potentially leading to faster growth and maturation of the tissue engineered constructs. Stochastic resonance also proved to maintain a small but beneficial effect over regular long-term culture (i.e. not accelerated applications of stimulation). In these long-term studies, matrix accumulation within the constructs was enhanced under stochastic resonance stimulation. Proteoglycan loss associated with the compressive loading was mitigated and collagen production was boosted, a promising result given adequate collagen production and accumulation is difficult to achieve in engineered cartilage[8,133,166,167]. Thus, the use of stochastic resonance both in long term and short term culture periods appears to be a valuable tool to enhance the sensitivity of cells to mechanical stimulation, especially when optimized conditions cannot realistically be determined.

7.3 Directions of Future Work

It has been shown that stochastic resonance has the potential to increase the sensitivity of cells to mechanical stimulation. The model used in this thesis was a simple model geared towards easily measuring short term outcomes (i.e. radioisotope incorporation and confocal imaging for calcium signaling) while eliminating some confounding factors by using healthy cells, a homogeneous cell distribution, maintaining chondrocyte phenotype, and reducing cell-cell and cell-matrix interactions. Given the results of these studies and the limitations of the model, several directions for future work exist. These include:

1. Exploring the use of stochastic resonance with mechanical stimulation on a diseased population of cells (e.g. osteoarthritic) for both short-term and long-term studies.
2. Examining the effect of stochastic resonance with mechanical stimulation on other culture models such as cartilage tissue explants, scaffold-free constructs, and/or cell-seeded constructs intended for implantation in clinical trials.
3. Determining the mechanism through which stochastic resonance acts on mechanical sensitivity.
4. Looking at tuning stochastic resonance parameters (amplitude and/or frequency bandwidth) as a function of time for long-term studies
5. Exploring the use of other types of dynamic mechanical stimulation (e.g. shock loading) on the mechanical sensitivity of chondrocytes.

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