The Role of Chondrocyte Age in Cellular Response to External Cues and their Implications in Tissue Engineering

by

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A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Department of Chemical and Biological Engineering

2012

This thesis entitled:
The Role of Chondrocyte Age in Cellular Response to External Cues and their Implications in Tissue Engineering
written by Nikki Lynn Farnsworth
has been approved for the Department of Chemical and Biological Engineering

Stephanie J. Bryant

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Date

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Farnsworth, Nikki Lynn (Ph.D., Chemical Engineering)

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Thesis directed by Prof. Stephanie J. Bryant

Osteoarthritis is a degenerative joint disease for which there is no cure, but therapies such as tissue engineering offer hope. One of the challenges is that clinical therapies utilizing autologous chondrocytes require tissue engineering strategies that are suitable for adult chondrocytes, however additional questions remain as to whether cells isolated from donors of different ages affects how cells sense and respond to their external cues and regenerate new tissue. The overall goal of this thesis is to improve current strategies for cartilage tissue engineering by gaining a fundamental understanding of how chondrocytes respond to their external environment and the role of cell age in this response. First, we aim to improve strategies for the encapsulation of chondrocytes in photopolymerizable hydrogels, thus enhancing their survival and ECM synthesis. Second we aim to investigate the fundamental mechanisms by which environmental cues, specifically physiological and injurious loading, impact anabolic and catabolic activities in chondrocytes and the role of age in this response. Third we aim to investigate the role of a charged environment in regulating tissue production through intracellular calcium signaling with dynamic loading. The results of this thesis have concluded that a physiological osmolarity and the presence of a PCM does increase viability and ECM synthesis for adult chondrocytes photoencapsulated in PEG gels and protects cells from oxidative damage incurred during photoencapsulation. Physiological and injurious loading conditions differentially regulate tissue production with respect to cell age, due to age-related changes in mechanotransduction pathways. Finally, the addition of negative charges into PEG hydrogels regulated tissue production with dynamic loading and may be mediated by intracellular calcium signaling. This thesis has shown that chondrocyte age is a major factor in cellular response to physiological cues and that tissue engineering strategies utilizing PEG hydrogels will require specific

cues based on the age of the patient. The presence of a charged environment has proved to be a potentially useful tool in guiding cartilage tissue production, however specific mechanisms of tissue regulation through calcium signaling remain to be elucidated. This thesis has provided a greater understanding age-associated changes in how chondrocytes sense their environment and has laid the ground work for future studies to investigate the differential regulation of calcium signaling and tissue production by ionic and osmotic effects, such that the specific cues responsible for regulation of tissue production can be isolated towards developing better tissue engineering strategies. Investigations into the mechanisms by which chondrocytes respond to physiological cues will provide a tool set for tissue engineering strategies to better engineer cartilage with specific mechanical properties and will also further current knowledge of chondrocyte biology, which may provide insights into development and treatments for joint disease, such as osteoarthritis.

Acknowledgements

I would like to first acknowledge my research advisor, Stephanie Bryant, for her guidance throughout this project, encouragement to succeed and her mentorship throughout my degree. This project has presented us with many challenges that we have overcome and I am grateful to her for all she has done. I would also like to thank my committee members: Kristi Anseth, Theodore Randolph, and John Kisiday for all of their guidance throughout this project. I would especially like to thank Amy Palmer for the use of her miscroscope, for teaching me to think like a biochemist and for all of her advice throughout my project.

I would also like to thank all of the Bryant group members past and present for their continued support and advice, with a special thanks to Idalis Villanueva for her help in the osmolarity studies as well her continued friendship and support. I would like to acknowledge my undergraduates; Mark Kissler, Claire Bensard, Krista Donahue and Ben Mead for their outstanding work and dedication to their projects. A special thanks goes to Lorena Antunez for all the work she has done on the injury, physiological loading and calcium studies, without which I could not have accomplished all that I have.

I would like to thank my family, the Bishops and the Farnsworths, for all their support throughout my career. Thank you for always believing in me and encouraging me to achieve my dreams. Lastly, I would like to thank my husband, John, for his patience, support, and love that have helped me to persevere through every challenge that I have faced. I am so grateful to share this achievement with you and I am confident that we can see each other through anything to achieve all of our dreams.

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Introduction

1.1 Motivation

Articular cartilage covers the surfaces of bones in joints and serves to distribute loads and facilitate smooth motion during joint articulation. Cartilage is a complex and dynamic tissue, which is subjected to compressive, shear and tension loading due to physical activities causing a number of changes in the tissue. Repair of acute damage to cartilage due to injury or disease is limited because cartilage has essentially no access to blood flow or to nerves [1]. Acute damage to cartilage often leads to cartilage degeneration resulting in severe joint pain and loss of mobility and if serious the need for joint replacement.

Current treatments for damaged cartilage resulting in focal defects involve surgical interventions that aim to replace the damaged areas by i) implanting healthy cartilage taken from non-load bearing regions, ii) fracturing the subchondral bone to stimulate cartilage repair, or iii) implanting autologous cells to encourage new cartilage growth [2]. Cartilage produced by these therapies tends to have inferior mechanical properties compared to the surrounding tissue [3]. These therapies have had some success however they are only recommended for younger, healthy patients and generally do not present a permanent solution to the underlying disease.

Overall the current clinical outcome for repairing damaged cartilage is suboptimal and is even worse for older patients. Therefore, there is a clear need for improved therapies that are minimally invasive and regenerate functional articular cartilage with sufficient mechanical properties. Tissue engineering has grown to fill this need, where cells, a scaffold and biochemical cues are used in

combination to produce cartilage tissue [4]. Functional tissue engineering, which has gained more following recently, uses biomechanical cues to improve tissue production and mechanical properties of the engineered tissue [5]. The goal of cartilage tissue engineering is to provide cells with appropriate cues that stimulate tissue production and ultimately lead to development of functional tissue with similar mechanical properties to that of the native tissue. Many studies using synthetic or natural scaffolds have had success in developing cartilage-like tissue with some success for improving the functional properties; however many of these studies have used juvenile cells from animal models. Questions then arise as to the effect of age, where patients who will require this therapy are predominantly older patients [6, 7].

Aging produces a number of changes in cartilage structure and cellular function, the most notable change being a decrease in tissue production rates. As most patients who will require tissue replacement therapies are older and as most therapies utilize cells from the patient, the developing tissue engineering strategies may not be effective. Few studies have investigated the impact of age on the development of engineering tissue and it is likely that cells may require different cues to produce functional tissue with respect to age. To develop better tissue engineering strategies, it will be important to understand how age affects cellular response to biochemical and biomechanical cues. Therefore, the overall objective of this thesis is to improve tissue engineering strategies by providing a better understanding of how the role of age influences the cells response to physiological cues.

1.2 Articular Cartilage

1.2.1 Extracellular Matrix

Cartilage is composed of specialized molecules that allow the tissue to function under the dynamic conditions within a joint [8]. The composition of cartilage can described by three components: 1) a charged solid component termed the extracellular matrix 2) a fluid component composed mostly of water and 3) an ion component composed of dissolved electrolytes, such as Na^{+}, Ca^{2+} and

Cl^-, as shown in Figure 1.1 [9, 8]. Together, these components provide tensile strength, compressive resistance and a medium for mechanoelectrical signaling events [10]. The solid component of cartilage is composed of a tightly woven matrix of collagens, mainly type II collagen, which provides tensile strength and is the limiting factor for water absorption into the matrix [11]. The second most prevalent component of the solid matrix is proteoglycans, of which aggrecan is the most common [12]. Proteoglycans are composed of a core protein with negatively charged glycosaminoglycans (GAGs) attached to a specific globular domain, where in aggrecan the main GAGs are chondroitin and keratin sulfate [12, 11]. Proteoglycans are typically found in aggregates, non-covalently bound together by hyaluronan, dispersed throughout the collagen matrix. The negative charges associated with the proteoglycans gives the tissue its load-bearing properties and attracts water and ions allowing the tissue to swell [13]. These GAGs give cartilage a fixed charge density, which serves to regulate the local concentrations of Na^+, K^+, and Cl^- ions in the interstitial fluid according to Donnan osmotic equilibrium [10, 8, 14]. The local concentration of ions in the interstitial fluid influences the local osmolarity, giving cartilage an unusually high osmolarity when compared to most other tissues.

Cartilage cells, or chondrocytes, are sparsely dispersed within the cartilage matrix and are responsible for maintaining a delicate balance between tissue production and tissue remodeling to maintain mechanical integrity of the tissue [15, 16]. Chondrocytes regulate the turnover of cartilage through production of matrix metalloproteinases (MMPs) and aggrecanases, which are responsible for degradation of aggrecan and collagen, while suppression of this turnover is achieved by production of tissue inhibitors of matrix metalloproteinases (TIMPs) [17]. Cartilage turnover by these molecules is thought to be regulated, in part, by radical oxygen species (ROS) produced by chondrocytes [18]. ROS, along with many other signaling molecules, play an integral role as an intermediate signaling molecule in cartilage homeostasis and are balanced by the antioxidant barrier in chondrocytes [19].

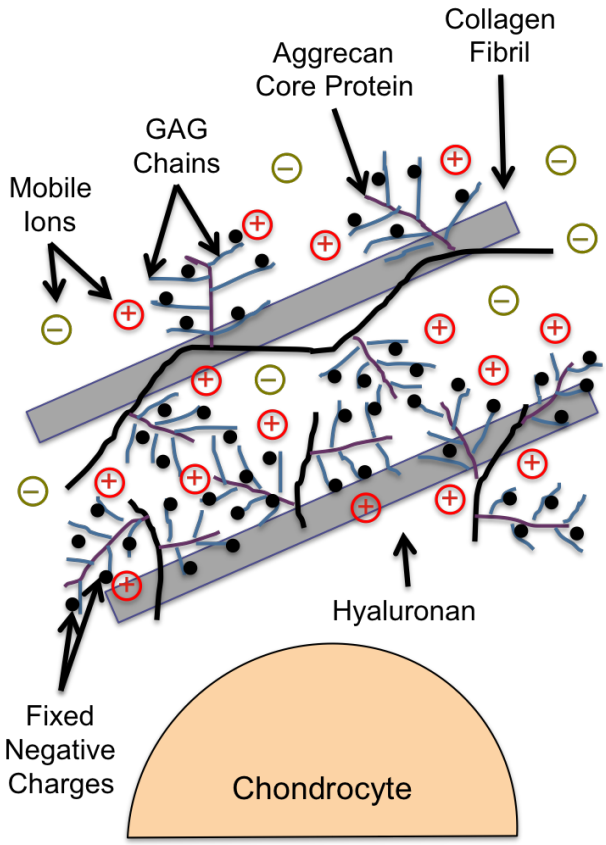

Figure 1.1: Schematic representation of the composition of articular cartilage.

1.2.2 Pericellular Matrix

The pericellular matrix (PCM) is the narrow region of matrix immediately surrounding chondrocytes [20]. The PCM is differentiated from the ECM by its composition, which is mostly collagen type VI and proteoglycans such as aggrecan [21, 22]. The pericellular matrix also has unique mechanical properties, most notably that the PCM is significantly less stiff than the ECM, but stiffer than a chondrocyte [23, 24]. It is thought that the PCM is responsible for filtering and transferring external stimuli to chondrocytes and has been found to be chondroprotective under injurious mechanical and biochemical stimuli [25, 22]. The PCM can also act an osmotic buffer zone, with a higher turnover rate of proteoglycans in the PCM, the local concentrations of proteoglycans is highly controlled, thereby regulating the local ion concentration independently of the ECM [22].

1.3 Effects of Aging on Articular Cartilage

1.3.1 Age-related Changes in the Cartilage Matrix

Age-related changes in the extracellular matrix of cartilage are characterized by several distinct changes in the tissue. For example, there is increased crosslinking between collagen fibers and sugar residues leading to a stiffer, less elastic cartilage matrix with lower water content [26, 27]. Biochemical changes in the size and chemistry of the aggrecan matrix molecules occur with age [28, 29, 30]. Aging leads to decreased proteoglycan and aggregate sizes and a shift in GAG content from predominantly chondroitin sulfate to keratin sulfate [31]. Decreases in the thickness of the cartilage and cell density have also been observed with increasing age [11]. These age-related changes in the tissue structure and composition can be directly correlated to changes in the mechanical properties of aging cartilage [29, 32, 33]. Increased stiffness and decreased water content of the tissue are well documented changes in tissue properties with age, along with many other age related changes, may make adult cartilage more susceptible to disease, however the link between age and osteoarthritis is not clear [32, 34].

1.3.2 Age-related Changes in Chondrocytes

Aging in chondrocytes is often characterized by reduced tissue synthesis and increased tissue degradation. MMP-13 is often elevated in aging cartilage and is also a biomarker of osteoarthritis [35]. Reduced capacity for tissue production in chondrocytes may be linked to decreases cell metabolism as a result of age [36]. This observation is partially due to a slowed cell cycle or senescence, which also results in shortening of the ends of chromosomes, or telomeres, that significantly reduces the cells metabolic activity [37, 38]. Increased production of radical oxygen species (ROS) has also been reported with aging, however it is still unclear whether this observation is due to an increase in ROS production or a decrease in antioxidant defenses [39, 40]. Taken together, these changes likely contribute to the cells reduced ability to maintain cartilage homeostasis and its greater susceptibility for inducing tissue degrading events, a hallmark of osteoarthritis [34].

1.4 Osteoarthritis

Osteoarthritis (OA) is a disease that effects joints and is characterized by degradation of cartilage, subchondral bone remodeling and inflammation [41]. Tissue degradation has been linked to the cell death observed in OA tissue, where the main mechanism of death is apoptosis, a ROS mediated event that is described as cellular suicide [42]. Increases in ROS production have also been observed in OA, where prolonged exposure to high levels of ROS can lead to further tissue degradation by upregulation of MMPs and inflammation through upregulation of inflammatory cytokines [43, 40]. Degradation of cartilage tissue in disease is distinctly recognizable from healthy turnover of matrix, due to upregulation of specific enzymes that are biomarkers of OA. Collagen degradation in OA is mainly due to increases in MMP-9, which can cleave the triple helical structure of collagen, and produce a large collagen fragment that has been detected in abundance in OA tissue [44]. Degradation of aggrecan in disease is characterized by increases in the aggrecanases ADAMTS4 and ADAMTS5, which cleave aggrecan into fragments that can diffuse out of the matrix and be detected in synovial fluid of the joint [45, 46]. The degradation of collagen and aggrecan with disease lead to changes in the mechanical properties of the tissue, such as increased water content and decreased tensile and compressive strength, leaving the tissue weakened and more susceptible to damage by mechanical loading [27].

1.5 Mechanical Loading of Cartilage

1.5.1 Mechanotransduction

The process of transducing a mechanical stimulus into a biochemical signal is termed mechanotransduction [47]. There are many types of mechanical stimuli that occur with compression of cartilage, such as tissue deformation, fluid flow, and ion flow. Tissue deformation causes deformation of the cell and compression of the actin cytoskeleton, which is attached to the extracellular matrix by focal adhesions, transferring the mechanical stimuli to the cell and activating biochemical signaling pathways [48]. Fluid flow can induce shear stress on the cell membrane, causing transfer of

mechanical energy to the cell, which can also activate biochemical signaling pathways [24]. Fluid flow and tissue deformation in concert, can also lead to flow of ions out of the matrix, causing a change in the local ionic and osmotic environment, which can directly activate intracellular signaling pathways [49]. These mechanical stimuli are translated into biochemical signaling pathways by a variety of mechanisms. One such mechanism is activation of ion channels on the cell surface due to changes in ionic and osmotic environment, causing a flux of ions across the cell membrane and activating ion channels that convert the mechanical stimulus into an intracellular biochemical signal [50]. These ion channels include slow conductance calcium-potassium channels, L-type calcium channels, and stretch activated ion channels, all of which are known to influence intracellular calcium signaling [51]. Cell deformation, compression of the actin cytoskeleton and shear stress induced by fluid flow all activate transmembrane integrins, which link the cell cytoskeleton to its surrounding matrix, and directly convert these stimuli into biochemical signals [52]. Integrins, such as $\alpha 5 \beta 1$, have been found to regulate hyperpolarization of the cell membrane in response to mechanical cues, which activates many ion channels, which has been linked to tissue production [53]. Soluble mediators, such as interleukin-4, that are released from chondrocytes in response to mechanical loading, can also activate intracellular signaling pathways that regulate anabolic and catabolic activity, however the mechanisms that lead to release of these factors are not well understood [54]. All of these mechanisms provide potential starting points for the transfer of mechanical cues into chemical signals, however many of the downstream mechanisms that lead to regulation of GAG and collagen production, as well as tissue remodeling enzymes like MMPs and aggrecanases, are unknown [55, 56, 48].

1.5.2 Physiological Loading

Mechanical stimulation of cartilage and chondrocytes is required to maintain homeostasis in the tissue, as dynamic compression has been found to regulate chondrocyte biosynthesis as well as tissue remodeling [57, 49, 48]. Remodeling is essential to replace damaged tissue and cells such that tissue integrity is maintained [50]. As such, many tissue engineering strategies have aimed to form

functional tissues that use physiological mechanical cues in an attempt to bolster tissue production and produce an engineered tissue with similar properties to that of native cartilage [53, 58, 59].

Mechanical cues are not all transduced the same, with different loading regimes resulting in changes to different cell functions, and therefore differences in tissue production [60, 49, 61]. Physiological loading regimes can have varying effects on chondrocytes based on load frequency and strain, however connections between loading regime and tissue production are not well defined. Furthermore, *in vitro* study of mechanotransduction with cartilage explants has been done without regard for the age of the tissue or chondrocytes, where age may affect chondrocyte response to loading regime. Loading regimes of 0 to 15% strain and 0.3 to 1Hz, which fall within the physiological range, have been employed in cyclic compression in tissue engineering strategies and these conditions have shown improved tissue synthesis, however these results depend on the structure and composition of the scaffold employed, as well as the age of the chondrocytes [62, 23, 63, 54, 64]. Taken together, this highlights the need for optimization of loading regimes with respect to chondrocyte age and tissue engineering scaffold, as well as a better understanding of mechanotransduction pathways with respect to age.

1.5.3 Injurious Loading

Evidence in the literature strongly supports the importance of age, when linked to injury, in the development of osteoarthritis. For example, *Roos et al.* reported that injury to the anterior cruciate ligament, resulting in compressive impact of the cartilage, in patients over 30 years of age developed measureable signs of osteoarthritis ∼10 years earlier than patients between 17-30 years of age [65]. Impact injury, such as this, which occurs over short periods and with high impact strains and forces, can cause an immediate loss of integrity of the tissue, given a great enough force of impact, and upregulates catabolic events in chondrocytes [66, 67, 68]. Immediately post-impact, cell death due to necrosis can be detected and matrix damage such as cracks or fissures are visible and are typically accompanied by release of matrix molecules, mostly collagen due to tensile failure [69, 70, 68]. Weakening of the tissue due to injury can lead to altered responses to physiological

loading, suggesting a potential mechanism by which injury develops into disease [71]. Soon after injury, ROS production is increased in chondrocytes, as a result of direct oxidative damage to mitochondrial electron transport complexes or to the DNA [72]. Within hours of injurious impact cartilage begins to swell, due to the loss of the collagen matrix no longer restraining the swelling pressure of GAGs in the tissue [73, 74]. Chondrocytes also upregulate GAG synthesis initially in response to injury, as evidenced by release of newly synthesized GAGs radiolabeled with $^{35}SO_4$ to the media, however following these immediate changes, GAG and collagen synthesis are suppressed [75]. The acute response to impact injury involves upregulation of matrix degrading enzymes, such as MMPs and aggrecanases, similar to what is seen in OA [71, 76, 77]. Cell death by apoptosis following injury has been observed within the first 24 hours of injury, with the greatest amount of apoptosis occurring at 24 hours post-injury [78]. Loss of cellularity by necrosis and apoptosis are thought to mediate the observed reduction in tissue synthesis [79].

Injury in cartilage can have differential effects depending on the age of the patient. In cartilage explant studies, adult cartilage showed a much smaller response to injury than juvenile cartilage, with smaller decreases in tissue production, smaller increases in catabolic activity and less apoptosis [71]. This is contradictory to studies that have found a faster onset of OA in older patients with joint injuries than in juvenile. The age-related changes in mechanical properties may play a role in the differential response to injury, however age effects on chondrocyte function may also be important. Adult chondrocytes already have higher levels of ROS, increased catabolic activity and decreased anabolic activity when compared to juvenile chondrocytes, therefore loading may produce similar levels of these activities with age, while the relative change from healthy to injured tissue will be very different. Some studies suggest that the increased presence of ROS in adult chondrocytes may condition chondrocytes to a more catabolic environment, thus decreasing the effects of injurious impact compared to juvenile cells, however further study is need to confirm this hypothesis [80]. A system that could isolate the response of chondrocyte to injury with respect to age, rather and a combination of age and tissue properties could provide valuable insight into the aging process and how mechanotransduction of injurious mechanical cues is changed with respect

to age.

1.6 Osmolarity

One of the unique biochemical characteristics of cartilage is its high fixed charged density. The negative charges allow the tissue to imbibe large amounts of water and provide cartilage with its unique ability to withstand large loads [8, 81]. These charges also affect the local concentrations of ions, attracting Na^+ and K^+ in the interstitial fluid [8, 14, 10]. Osmolarity is defined as the concentration of solute in a solution. Given the high concentration of mobile ions due to the negatively charged GAGs in cartilage matrix, this leads to a high osmolarity in cartilage tissue ($\sim$350-450mOsm), compared to other tissues [82, 83]. With dynamic loading, water is expelled from the matrix, along with ions, dynamically changing the osmotic environment [24, 84, 85]. Changes in the ionic and osmotic environment have been linked to changes in cartilage tissue production in isotonic culture, however the link between these is not well understood [86, 81, 87]. It is thought that these dynamic changes in the microenvironment of cartilage that arise as a result of the fixed negative charges may be an important cue for chondrocytes, however the role of the charges within the cartilage matrix with respect to cartilage homeostasis has not been studied in depth.

It has been hypothesized that these observed changes in tissue production, occur as a result of cell regulatory volume mechanisms, specifically regulatory volume decrease associated with an increase in osmolarity [88, 82, 89, 90, 91]. When cells sense an osmotic pressure gradient, water is rapidly transferred across the membrane to equilibrate this pressure, which is followed by a change in cell volume [88, 91]. This change in cell volume is initiated by activation of K^+ and anion channels on the cell surface, which leads to changes in proton and intracellular calcium concentrations when membrane co-transporters are activated, and ultimately leads to changes in cell volume [90, 92, 93, 94]. Changes in intracellular calcium signaling have been observed with changes in extracellular osmolarity however the link has not been well characterized or understood and the connection to tissue production has yet to be studied [90, 82]. In cartilage tissue, the concentrations of ions and their activities leads to osmotic pressure generated from the charged matrix as well as

changes in ionic strength [82, 88]. Many studies have looked at the combined effects of these by the ionic environment through changes in salt ion concentration, however decoupling these effects may prove valuable in determining the signaling mechanisms responsible for effecting the changes in tissue production and intracellular calcium seen with changes in osmotic environment.

1.7 Intracellular Calcium Signaling

Calcium is a versatile signaling molecule that regulates many different cellular functions [95]. Calcium signaling is determined by the balance between biochemical reactions that introduce Ca^{2+} into the cytoplasm and mechanisms to remove Ca^{2+}, such as cell surface pumps and exchangers [96]. Intracellular calcium transients have been observed and characterized in chondrocytes in steady state culture and have a characteristic shape, where a fast increase in calcium is observed with a slightly slower decrease to basal levels, occurring on a time scale of seconds to minutes [97]. Calcium signaling that occurs on this time scale has been associated with regulation of cell metabolism and transcription, with one transient being sufficient to effect a change in cellular response in some cases [95, 96]. Intracellular calcium signaling initiated by dynamic changes in osmolarity has been reported in other studies [50], however there are many different theories as to how calcium signaling effects changes in tissue production. Regulatory volume decrease (RVD), initiated by an influx of Ca^{2+} ions, is one such mechanism that has received much attention as a potential pathway for regulation of tissue production [98, 90, 99]. RVD has been shown to activate many surface ion channels, such as transient receptor potential vanilloid 4 (TRPV4), which is a stretch activated ion channel permeable to calcium and has been characterized as an osmotically sensitive channel [100, 101, 94]. This suggests a role for intracellular calcium signaling in regulating the response of chondrocytes to osmotic environment. For example, TRPV4 deficient mice were shown to develop osteoarthritis, suggesting a role for this channel in regulating tissue production [94]. Studies utilizing specific inhibitors of TRPV4 in a dynamic osmotic environment could provide further evidence of the role of intracellular calcium signaling in regulating tissue production. One other potential pathway that is activated by RVD and could potentially regulate calcium signaling

in response to osmotic changes is the Na^{+}-Ca^{2+} exchanger (NCE) of the cell surface. *Sanchez et al.* found that NCE is partially responsible for the increase in intracellular calcium seen with hypotonic challenge, however the connection between NCE and tissue production is not clear [100]. Although the connection between osmotic regulation of tissue production and intracellular calcium signaling is complex and will require significant study to fully understand, inhibitors of intracellular calcium in a controlled dynamic osmotic environment could provide valuable insight into this link and would lay the ground work for future studies to determine the specific mechanisms involved.

1.8 Hydrogels for use in Tissue Engineering Strategies

1.8.1 Hydrogels in Tissue Engineering and as Models to Study Mechanobiology

Hydrogels have been widely used as platforms for three-dimensional culture of cells because of their high water contents that provide a tissue-like environment and promote facile nutrient diffusion [102]. Hydrogels are commonly used in tissue engineering strategies as a scaffold to culture cells in a 3D environment, where stimuli can be tightly controlled to direct tissue production [103]. This also makes hydrogels ideal models to study mechanobiology, as the effects of physiological stimuli, such as cell deformation, can be studied without complications from the extracellular matrix [104]. Hydrogels can be formed from many natural or synthetic compounds. Natural hydrogels include agarose, alginate, hyaluronan, and collagen, which can all be non-covalently crosslinked under physiological temperature and pH [103]. Synthetic hydrogels, composed of polymers such as poly(vinyl alcohol) and poly(ethylene oxide), are also appealing choices for scaffolds as their chemistries can be tightly controlled, resulting in more reproducible hydrogels [105].

1.8.2 Photopolymerizable Hydrogels

Synthetic hydrogels formed through photopolymerization are of particular interest in tissue engineering applications because this process affords spatial control over the polymerization, occurs rapidly on clinically relevant timescales, and can be performed at physiological tempera-

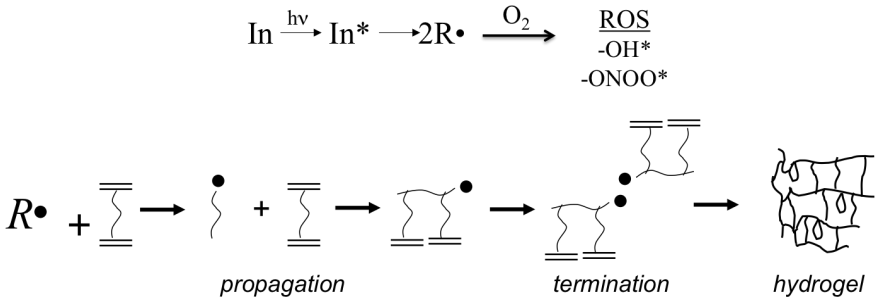

Figure 1.2: Schematic of photopolymerization reaction with PEGDM and the mechanisms for radical production.

ture with minimal heats of reaction [106, 107]. Moreover, photopolymerizable hydrogels can be formed from a wide range of natural and synthetic polymer precursors to produce gels tailored to mimic native cartilage that can be polymerized in situ [108, 109]. While photopolymerization has many desirable attributes for cell encapsulation and *in vivo* delivery of cells, reduced cell viability post-encapsulation has been reported in poly(ethylene glycol) hydrogels [110]. Because photopolymerizations are radical initiated chain polymerizations, the presence of radicals can generate a harsh environment.

During photopolymerization, photoinitiator molecules absorb photons of light energy and dissociate into radicals, as shown in Figure 1.2 with PEG macromers. Initiator radicals, activated by UV light, react with functional groups on macromolecular monomers forming macroradicals that lead to chain propagation [108]. Initiating radicals and macroradicals may attack cells causing direct or indirect damage and ultimately leading to cell death. In addition, initiating radicals have a high propensity to react with oxygen that is present during photoencapsulation of cells, having reaction rate constants five orders of magnitude greater for oxygen over typical monomers [111]. When radicals react with oxygen, ROS are formed, which are known to trigger oxidative stress in cells [112]. In chondrocytes, oxidative stress has been linked to cell death, inhibition of tissue synthesis, and upregulation of tissue degrading enzymes, e.g. matrix metalloproteinase-13 (MMP-13) [113, 114]. Therefore, photoencapsulation may also adversely affect cells through polymerization-induced ROS, leading to oxidative stress in cells and ultimately reducing tissue

regeneration capabilities in photopolymerized hydrogels. This suggests that photoencapsulation conditions may need to be optimized for cell survival and functional tissue production.

1.8.3 Structure and Mechanical Properties of PEG

Photopolyermized hydrogels are ideal candidates for tissue engineering scaffolds and models to study mechanobiology, due to the ability to control the mechanical properties of the gel and option to incorporate matrix molecules in a controlled manner. The mechanical properties of these gels can be tailored by controlling the crosslinking density, through variations in macromer molecular weight or the weight percent of macromer in solution [115]. Increasing crosslinking density leads to a decrease in water content and increase in the compressive strength of the hydrogel, which can alter transport properties within the gel, as well as cellular function [116]. Transport properties can also be controlled through the pore size of the gels, which increases with molecular weight of the PEG macromer [117].

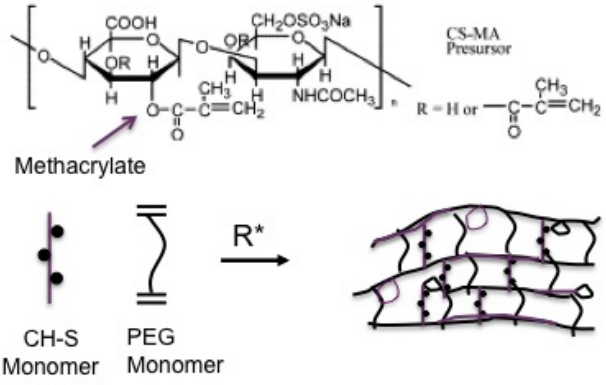

Figure 1.3: Structure of chondroitin sulfate methacrylate with a schematic representation of co-polymerization with PEGDM.

PEG hydrogels were chosen for this thesis because chondrocytes encapsulated in these hydrogels retain their phenotype and deposit cartilaginous tissue [108]. Another benefit of using PEG hydrogels is that their chemistry allows for incorporation tissue specific molecules into the matrix, such as the cell adhesive peptide RGD, collagen derived enzyme-sensitive peptides, and growth factors like VEGF [105]. Previous studies have incorporated cartilage specific molecules into PEG

gels to study cellular interactions with ECM molecules, while others have used matrix molecules to provide a more physiological environment that will bolster tissue production [118, 119]. For this thesis, a negatively charged environment, designed to mimic the charged matrix in cartilage, was created by incorporation of chondroitin sulfate into PEG hydrogels. Chondroitin sulfate was functionalized with methacrylate groups, as described in Figure 1.3, and co-polymerized with PEG functionalized PEG monomers to form an integrated network of chondroitin sulfate [120]. In mimicking the fixed charge density in native cartilage, we aim to investigate the effects of charged matrix on tissue production in chondrocytes with physiological loading. In using PEG as a model to study mechanobiology and the effects of the incorporated charges, we can control signaling events, such as intracellular calcium signaling, to determine their role in regulating tissue production.

1.9 Summary

Osteoarthritis is a prevalent disease for which there is no cure, however therapies such as tissue engineering offer hope. Currently, no therapy has yet yielded engineered cartilage with native biochemical and biomechanical characteristics that can withstand physiological loads long-term, thus restoring function. This shortcoming is in part due to the fact that we still do not fully understand the pathways that regulate tissue production in chondrocyte and as a result the cues chondrocytes need to synthesize a functional engineered tissue have not been discovered. Additional questions remain as to whether cells isolated from donors of different ages affects how cells sense and respond to their external cues and regenerate new tissue. Understanding the mechanisms by which chondrocytes respond to their external environment and how these mechanisms are affected by age are critical to designing successful cartilage tissue engineering strategies. The overall goal of this thesis is to improve strategies for cartilage tissue engineering by gaining a fundamental understanding of how chondrocytes respond to their external environment and whether age effects this response. The results of this thesis will help to develop more robust tissue engineering therapies and will provide insight into basic chondrocyte biology.

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Chapter 2

Objectives

Osteoarthritis is a prevalent disease that is characterized by degradation of cartilage. To date, there is no cure for osteoarthritis, but therapies such as tissue engineering offer hope. However no therapy has yet yielded engineered cartilage with native biochemical and biomechanical characteristics that can withstand physiological loads long-term, thus restoring function. This shortcoming is in part due to the fact that we still do not fully understand the pathways that regulate tissue production in chondrocyte, and as a result, the cues chondrocytes need to synthesize a functional engineered tissue have not been discovered. This limitation is further complicated by the fact that clinical therapies utilize autologous chondrocytes and thus require tissue engineering strategies that are suitable for older patients. Additional questions remain as to whether cells isolated from donors of different ages affects how cells sense and respond to their external cues and regenerate new tissue. Understanding the mechanisms by which chondrocytes respond to their external environment and how these mechanisms are affected by age are critical to designing successful cartilage tissue engineering strategies.

The overall goal of this thesis is to improve strategies for cartilage tissue engineering by gaining a fundamental understanding of how chondrocytes respond to their external environment and whether age effects this response. Towards this overall goal, this thesis focuses on three key aspects. First, we aim to improve strategies for the encapsulation of chondrocytes in photopolymerizable hydrogels, thus enhancing their survival and ECM synthesis. Second we aim to better understand the mechanisms by which environmental cues, specifically physiological and injurious

loading, impact anabolic and catabolic activities in chondrocytes. Third, we aim to investigate the role of a charged environment, similar to that in native cartilage, in regulation of cellular responses and the mechanisms involved in this regulation. To better understand how chondrocytes respond to their environment, three distinct but related objectives were developed:

2.1 Objective 1

The first objective was to improve strategies for encapsulation of chondrocytes in PEG hydrogels. Previous studies have reported cell death during photoencapsulation with up to ∼20% decrease in viability [1] with up to a 50% decrease in viability one day after encapsulation, and further decreases with increasing culture time. A loss in cell number will reduce tissue production capacity in these hydrogels, thus requiring a greater number of cells to overcome this loss. It is hypothesized that, radicals produced during photoencapsulation creates a harsh environment for cells, as they are highly reactive and have the potential to damage cells and cause cell death, thus leading to the reduction in cell viability.

The first objective of this thesis was to better understand the effects of photoencapsulation on chondrocytes and develop ways to improve the survival of chondrocytes photoencapsulated in PEG hydrogels. In Chapter 3, we tested the hypothesis that a more physiological environment during encapsulation, such as presence of a nacent pericellular matrix (PCM) and a more physiological osmotic environment increase survivability of chondrocytes during photoencapsulation. To test this hypothesis, adult chondrocytes were encapsulated in chondrocyte media at a physiological osmolarity and with a visible PCM developed, while chondrocyte viability was monitored. Some cell types have been successfully encapsulated in PEG hydrogels with negligible decreases in viability at similar cell densities, suggesting that chondrocytes are more sensitive to photoencapsulation conditions. Photoencapsulation produces many radical species that have the potential to damage cells; however, few studies have characterized the effects of photoencapsulation on cells beyond monitoring viability. Therefore, in Chapter 4, we investigated how the radicals produced during photoencapsulation adversely affected adult chondrocyte survival and tissue production in PEG

hydrogels, as well as the potential for a more physiological environment, specifically presence of a PCM, to enhance survival and better support extracellular matrix expression and synthesis.

2.2 Objective 2

With improved photoencapsulation conditions in PEG hydrogels determined in objective 1, the second objective was to characterize anabolic and catabolic events under physiological and injurious loading and to determine if chondrocyte age plays a role in the response to dynamic compressive loading. Mechanical compression of cartilage is required to maintain the structure and function of cartilage tissue *in vivo*. Many studies have shown the ability of dynamic loading to bolster tissue production in chondrocytes encapsulated in 3D scaffolds; however, the optimal loading regimes will depend on numerous factors of the scaffold including its composition and mechanical properties. Chondrocyte age is another factor that may impact how chondrocytes respond to physiological loads of varying stresses and strains. The first aim of this objective was to characterize anabolic and catabolic activities, with respect to strain and frequency of load, in skeletally immature and mature chondrocytes encapsulated in PEG hydrogels (Chapter 5). Anabolic and catabolic activities were analyzed to define optimal loading regimes, which enhance tissue production, with respect to chondrocyte age. We hypothesize that different loading regimes will be required to bolster ECM deposition in chondrocytes of different ages. These studies will lay the groundwork for future investigation into the mechanotransduction pathways that are altered with age.

Injurious loading, in conjunction with age, is one of the leading risk factors for developing osteoarthritis. Injury in the joint leads to degradation of cartilage tissue and desensitization of chondrocytes to physiological loads. The risk of developing OA after an injury increases with increasing age, suggesting that age-related changes in chondrocytes and/or matrix properties play a role in development of OA. The second aim of this objective was to characterize chondrocyte anabolic activity, catabolic activity and cell death under injurious loading with respect to chondrocyte age (Chapter 6). To investigate the role of chondrocyte age in cartilage response to injurious loading, PEG hydrogels were employed such that similar mechanical cues could be imparted in

the cells, while removing the effects of matrix interactions, and the response of the cell to injury could be isolated. Previous studies in cartilage explants have suggested that juvenile chondrocytes have a greater response to injury with decreases in anabolic activity and decreases in catabolic activity compared to adult chondrocytes. We hypothesize that similar results will be found in PEG hydrogels and that the response of chondrocytes to injury with age will be mostly dictated by the age-related changes in the chondrocyte, rather than changes in the tissue.

2.3 Objective 3

Chondrocytes are exposed to a dynamic environment *in vivo*, with dynamic loading leading to changes in cell deformation, fluid flow, ion and nutrient transfer and extracellular osmolarity. Objective 2 has investigated the effect of physiological loading on chondrocytes encapsulated in PEG hydrogels; therefore, the final objective was to investigate the role of a dynamic loading in charged hydrogels, in an attempt to mimic the ionic and osmotic environment in cartilage and isolate the response of chondrocytes to these stimuli. The aim of this objective was to investigate role of a charged matrix in regulating tissue production with dynamic loading and to begin to investigate the mechanisms through which this response is mediated (Chapter 7). A negative charged matrix, similar to that observed in cartilage, was achieved by incorporation of the negatively charged GAG, specifically chondroitin sulfate, into PEG hydrogels to achieve a physiological charge density that produced changes in the ionic and osmotic environment with the application of loading. We hypothesize that the addition of negative charges into the hydrogel will increase tissue production and that intracellular calcium signaling may be involved in regulating tissue production in response to dynamic loading. To test this hypothesis, aggrecan and collagen production was monitored with the application of dynamic loading in charged hydrogels and was compared to uncharged hydrogels where loading was applied. Regulation of GAG and collagen synthesis was analyzed in the presence and absence of intracellular calcium signaling to determine if there is a significant link between addition of charges into the hydrogel, calcium signaling and tissue production.

A sub-aim of this objective was to investigate further the effects of a negatively charged

matrix, by differentiating between osmotic and ionic effects, on calcium signaling and tissue production. We hypothesize that ionic and osmotic effects will differentially regulate calcium signaling and tissue production. Through increasing medium osmotic pressure independently from ionic strength in isotonic culture, we can begin to understand if chondrocytes respond to these aspects of their environment differentially and use these insights to hypothesize potential mechanisms involved in regulating calcium signaling and tissue production through ionic and osmotic environment to be tested in future studies.

2.4 Summary

In summary, the first objective of this thesis will improve photoencapsulation strategies for chondrocytes and will help to understand the effects that photoencapsulation has on cells. This approach will provide an improved tissue engineering system, with increased viability and tissue production from previous methods used in PEG hydrogels and will serve as an improved model to study cellular response to physiological cues. Using what has been learned from the first objective, we can begin to understand how chondrocytes respond to physiological and injurious loading regimes and to determine if chondrocyte age plays a role in that response. Characterization of physiological loading regimes will provide a valuable tool to help guide future tissue engineering strategies, particularly with respect to cell age and the response of cells to injurious loading will provide a better understanding of how cell age contributes to development of degenerative joint disease and will aid in development of preventative therapies. Finally, investigating the response of cells in charged gels with physiological loading regimes will provide further insight into the response of chondrocytes to the unique physiological environment of cartilage, by helping to understand the role of charge in regulating tissue production. Investigations into the mechanisms by which chondrocytes respond to these physiological cues will provide a tool set for tissue engineering strategies to better engineer cartilage with specific mechanical properties and will also further current knowledge of chondrocyte biology, which may provide insights into development and treatments for joint disease, such as osteoarthritis.

2.5 References

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Chapter 3

Medium Osmolarity and Pericellular Matrix Development Improves Chondrocyte Survival When Photoencapsulated in Poly(Ethylene Glycol) Hydrogels at Low Densities

(As appears in Tissue Engineering: Part A 15(10):3037-3048 (2009) in collaboration with Idalis Villanueva)

The ability to encapsulate cells over a range of cell densities is important towards mimicking cell densities of native tissues and rationally designing strategies where cell source and/or cell numbers are clinically limited. Our preliminary findings demonstrate that survival of freshly isolated adult bovine chondrocytes dramatically decreases when photoencapsulated in poly(ethylene glycol) hydrogels at low densities (4 million cells/ml). During enzymatic digestion of cartilage, chondrocytes undergo a harsh change in their microenvironment. We hypothesize that the absence of exogenous antioxidants, the hyposmotic environment, and the loss of a protective pericellular matrix increase chondrocytes susceptibility to free-radical damage during photoencapsulation. Incorporation of antioxidants and serum into the encapsulation medium improved cell survival 2-fold compared to phosphate buffered saline. Increasing medium osmolarity from 330 to 400 mOsm (physiological), improved cell survival by 40% and resulted in ∼2-fold increase in ATP production 24 hours post-encapsulation. However, cell survival was only temporary. Allowing cells to reproduce some PCM prior to photoencapsulation in 400 mOsm medium resulted in superior cell survival during and post-encapsulation for up to 15 days. In summary, the combination of antioxidants, physiological osmolarity and the development of some PCM result in an improved robustness

against free radical damage during photoencapsulation.

3.1 Introduction

Tissue engineering holds great promise for replacing damaged and/or diseased tissues with regenerated healthy living tissues [1]. One attractive approach to engineering living tissues involves the encapsulation of cells in 3D hydrogels [2]. Hydrogels are characterized by their high water contents and tissue-like elastic properties making them ideal environments for cell and tissue growth. In addition, the gelation process is often mild permitting in vivo delivery of cells. Hydrogels formed via photopolymerization are particularly attractive because the process occurs on clinically relevant time scales, allows for spatial and temporal control over the polymerization reaction, and can be tuned to obtain a range of macroscopic properties and degradation profiles [3]. Furthermore, synthetic and natural polymers have been modified with polymerizable functionalities (e.g., (meth)acrylate) to create 3D environments suited for a range of cell encapsulation and tissue engineering applications including encapsulation of osteoblasts [4], islets of Langerhans [5], chondrocytes [6, 7], and mesenchymal stem cells[8].

Photopolymerization of hydrogels occurs through a photoinitiated free radical chain polymerization involving initiation, propagation, and termination. The process is described by Figure 3.1. For cell encapsulation strategies, the precursors include multi-functional macromolecular monomers (i.e., macromers), photoinitiator molecules, and light. Upon exposure to light, photoinitiator molecules absorb photons of light energy and dissociate into radicals that initiate the polymerization reaction to form growing kinetic chains. During propagation, the rate of polymerization increases dramatically with conversion as a result of diffusion controlled termination kinetics leading to autoacceleration [9]. During autoacceleration, there is a large increase in the concentration of propagating chains, i.e. macroradicals. Termination occurs through bimolecular termination or chain transfer between two propagating chains. Chain transfer may also occur with other solutes or molecules present in the polymerization medium, including proteins and/or molecules associated with cells.

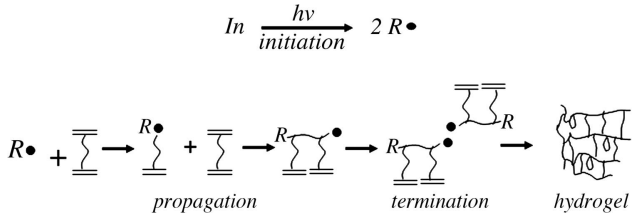

Figure 3.1: A schematic of the process for fabricating hydrogels by photopolymerization, which are used in cell encapsulation strategies. The photopolymerization process occurs via a photoinitiated free radical chain polymerization involving initiation, propagation, and termination. Photoinitiator molecules absorb photons of light energy and dissociate into radicals (initiation). The initiator radicals react with unreacted double bonds (C=C) on macromolecular monomers (e.g., poly(ethylene glycol) dimethacrylate) to form growing kinetic chains (propagation). Termination occurs through either bimolecular termination or chain transfer between two propagating chains.

For cell encapsulation strategies, the photopolymerization conditions must be carefully chosen to minimize cellular damage from free radicals associated with the initiating radicals and propagating macroradicals. It is well known that free radicals can damage cell membranes, nucleic acids and proteins that can ultimately lead to cell death [10, 11, 12]. Several studies have examined the cytocompatibility of different photoinitiators and their resulting radicals from exposure to light [13, 14, 15, 16]. Earlier work by Bryant et al. [15] demonstrated that initiator chemistry, initiator concentration, and their resulting radicals dramatically affected cell viability of NIH/3T3 fibroblasts. The initiating system, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone) or Irgacure 2959® under low intensity UV light (365nm), was determined to be cytocompatible. This initiating system has since been used to encapsulate a number of different cell types in photopolymerized hydrogels without adversely affecting cell viability or cellular functions [8, 15, 16].

To develop a clinically relevant tissue engineering strategy for cartilage regeneration, cell source and availability are important considerations. In addition, one of the limitations involving the use of primary chondrocytes is the fact that chondrocytes are known to de-differentiate rapidly when expanded in 2D cultures [17, 18, 19]. Therefore, developing successful strategies that employ low cell densities are attractive from a clinical perspective.

Our lab focuses on encapsulating chondrocytes in photopolymerized poly(ethylene glycol) (PEG) hydrogels fabricated from PEG di(meth)acrylate macromers and the Irgacure 2959® initi-

ating system towards engineering functional cartilage tissues. PEG hydrogels provide a 3D environment that maintains the chondrocyte phenotype, promotes cartilage-specific matrix synthesis and when designed to biodegrade leads to macroscopic tissue development [6, 20, 21, 22]. Traditionally, to enhance matrix deposition, we and others have employed high cell concentrations ($50x10^6$cells/ml) when encapsulating primary chondrocytes in hydrogels for cartilage regeneration [6, 7, 20, 21, 22]. Towards developing strategies that employ low cell densities, unpublished observations from our lab have found that when freshly isolated bovine chondrocytes are photoencapsulated in PEG hydrogels at cell densities similar to adult cartilage (i.e., $4x10^6$cells/ml), very few cells survived the encapsulation process. This finding was particularly surprising because other cell types have been successfully encapsulated in photopolymerized PEG hydrogels at low cell densities including bone marrow stromal cells [23], endothethial cells [24], and osteoblasts [4].

Primary chondrocytes are obtained from enzymatic digestion of cartilage tissue explants. The collagenase digestion process dissolves the extracellular matrix (ECM) and strips the cells of their pericellular matrix (PCM) resulting in a suspension of isolated single cells [25, 26, 27]. In addition, the isolation process for chondrocytes is typically performed in standard culture medium with osmolarities that are lower than native cartilage resulting in a hyposmotic environment [28]. We hypothesize that this harsh change in the microenvironment surrounding the chondrocyte enhances their susceptibility to free radical damage associated with radicals of the polymerization. These negative effects may be more pronounced when low cell densities are employed due to the higher concentrations of free radicals per cell.

Because free radicals are normally present in cells and tissues, cells inherently have protective capabilities against free radical damage. For example, the unsaturated bonds in lipids that are present on the cell membrane are key targets for free radical reactions and oxidative damage, which can lead to adverse cellular functions and even cell death [10, 29]. To prevent oxidative damage to the cell membrane, antioxidants released by the cell and/or present exogenously serve to quench and scavenge free radicals [29]. The ECM may also have potential to act as radical scavengers where previous studies have reported the ability of collagen type I to scavenge radicals in vitro [30].

Furthermore, the change in the osmotic environment during the isolation can result in rapid cell swelling that can deplete the cell of important osmolytes, such as potassium, which has been shown to inhibit free radical formation in other cell types [31]. Therefore, the loss of pericellular matrix, the hyposmotic environment, and the absence of exogenous antioxidants may act to increase the chondrocytes susceptibility to free radical damage.

Therefore, this study explores the role of the encapsulation medium and the importance of a pericellular matrix on cell survival and function during photoencapsulation of primary bovine chondrocytes in PEG hydrogels. Specifically, we examine several different media including i) a basic phosphate buffered saline (PBS) solution, which does not provide any protective components against radical damage, ii) PBS supplemented with medium nutrients, which include exogenous antioxidants, such as ascorbic acid, and serum, which has antioxidant capabilities [32], iii) standard culture medium, which contains nutrients and serum, and iv) media with varying osmolarities. To assess the importance of a PCM, isolated chondrocytes were allowed to reform some of the PCM prior to encapsulation. To assess cell survival, we assessed cell viability semi-quantitatively with fluorescence microscopy and metabolic activity quantitatively by ATP production. Chondrocyte function was assessed through proteoglycan production. Our findings indicate that a combination of physiological osmolarity and restoration of some of the PCM is necessary to maintain chondrocyte survival during photoencapsulation when low cell densities are employed and this environment promotes cartilage-specific matrix deposition in long-term culture.

3.2 Materials and Methods

3.2.1 Hydrogel Preparation

Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized by reacting linear PEG (3000MW, Fluka) with methacrylic anhydride (Sigma) at a molar ratio of 1:10 using microwave irradiation [33]. The final product was purified by dissolution in methylene chloride followed by precipitation in cold ethyl ether. The degree of methacrylate substitution for PEGDM was 90% as

Table 3.1: Cell Encapsulation Densities for Chondrocytes Photoencapsulated in Poly(Ethylene Glycol) Hydrogels

Description	Cell encapsulation density (cells/mL macromer solution)
Low	4×10^6
Intermediate	10×10^6
High	50×10^6

determined through ^{1}H NMR (Varian VYR-500MHz) by comparing the area under the integral for the vinyl resonances ($\delta = 5.7$ppm and $\delta = 6.1$ppm) to that of the methylene protons ($\delta = 4.3$ppm) in the PEG backbone.

3.2.2 Chondrocyte Isolation

Bovine articular cartilage (1-2 years; Arapahoe Foods Inc.) was removed within several hours of slaughter under sterile conditions from metacarpalphalangeal joints of 8 steers in two separate isolations. The cartilage was washed in PBS supplemented with 1% penicillin streptomycin (PBS-P/S, Invitrogen), diced finely, and digested in 0.2% collagenase type II (Worthington Biochemical Corp) in Dulbeccos Minimal Essential Medium (DMEM, Invitrogen) and 10% fetal bovine serum (FBS, Invitrogen) for 16 h at 37°C. Isolated chondrocytes were washed in PBS/PS+0.02% ethylene-diaminetetraacetic acid to deactivate the collagenase followed by centrifugation at 1200rpm for 10 minutes. Cell viability was determined by the tyrpan-blue exclusion to be ∼97%.

3.2.3 Chondrocyte Encapsulation

Immediately after isolation, chondrocytes were maintained in PBS-P/S for 30 minutes at 37°C. After which time, chondrocytes were recovered by centrifugation and gently mixed with macromer solution at concentrations of 4, 10, or 50 million cells/ml. The macromer solution consisted of 10% w/v PEGDM, 0.05% w/w photoinitiator (1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one, Irgacure 2959®, Ciba Specialty Chemical) in an encapsulation medium. Encapsulation media included (Table 3.2): i) PBS, ii) PBS + medium nutrients (10mM HEPES,

Table 3.2: Recipes For Encapsulation and Culture Media

Encapsulation Medium	Medium Recipe[a]
PBS	PBS, pH 7.4, 330mOsm
PBS + medium nutrients	PBS supplemented with HEPES, nonessential amino acids, l-proline, L-ascorbic acid, penicillin, streptomycin, and amphoterecin B
Basal medium	DMEM + medium nutrients
Basal medium + FBS	DMEM + medium nutrients + 0-5% FBS
Standard chondrocyte medium (330mOsm)	Standard chondrocyte medium supplemented with potassium chloride (2.6mg/mL) and sodium chloride (5.2mg/mL)
Chondrocyte medium (450mOsm)	Standard chondrocyte medium supplemented with potassium chloride (2.9mg/mL) and sodium chloride (5.9mg/mL)
Chondrocyte medium (500mOsm)	Standard chondrocyte medium supplemented with potassium chloride (3.3mg/mL) and sodium chloride (6.6mg/mL)

0.1M non-Essential Amino Acids, 0.4 mM L-proline, 50mg/L L-ascorbic acid, 1% P/S, 0.5μg/mL of amphoterecin B (all from Invitrogen), iii) basal medium (high glucose DMEM+medium nutrients) supplemented with varying concentrations of FBS (0-10%), iv) standard chondrocyte medium (basal medium + 10% fetal bovine serum, or v) standard chondrocyte medium supplemented with salts to adjust the medium osmolarity. Based on the assumption that standard chondrocyte medium has an osmolarity of 330mOsm (per manufacturer), the addition of salts (potassium chloride (2.6, 2.9, 3.3 mg/ml) and sodium chloride (5.2, 5.9, 6.6 mg/ml)) will increase the medium osmolarity to 400, 450 or 500mOsm, respectively [28]. The isolated cells were mixed with PBS-P/S for 30 minutes at 370°C prior to combining with their respective macromer solution. The cell-macromer solution was exposed to 365 nm light ($\sim$4mW/cm^2) for 10 minutes to form cylindrical hydrogel constructs (5mm in diameter and height). Post-encapsulation, the cell-laden constructs were cultured in standard chondrocyte medium with the appropriate osmolarity.

3.2.4 Cell Viability

At specified time points, PEG constructs (n = 3) were sliced in half lengthwise and chondrocyte viability assessed using a LIVE/DEAD® membrane integrity assay (Invitrogen), in which live cells fluoresce green and dead cells fluoresce red. Three images within 200μm from the cut side

of the gel were obtained for each construct using an inverted confocal laser scanning microscope (CLSM, Zeiss LSM 510) equipped with a 10x water immersion objective. Percent cell viability was semi-quantified by counting manually live and dead cells in each image.

3.2.5 Metabolic Activity Assay based on ATP Production

At specified time points, PEG constructs (n = 5-8) were removed and immediately frozen in liquid nitrogen. PEG constructs were crushed using a tissue homogenizer in 200μl of lysis buffer (20mM Tris, 2mM EDTA, 150mM NaCl and 0.5% Triton-X-100 in DI water). The solution was transferred into individual wells of a 96 well plate, combined with an equal volume of the CytoTox-Glo^{TM} cytotoxicity substrate solution (Promega), and incubated at 37°C for 10 minutes. ATP production was measured following the manufacturers protocol by luminescence (Fluostar Optima) and normalized to gel wet weight.

3.2.6 Caspase-3/7 Apoptosis Assay

PEG constructs photopolymerized in PBS (n = 3) were frozen in liquid nitrogen immediately post-encapsulation and crushed using a tissue homogenizer in 200μl of lysis buffer described above. Apoptosis was detected by measuring for caspase-3/7 activity using the Apo-ONE® (Promega) assay following manufacturers protocol. Cell pellets (n = 3) comprised of the same number of cells as in the PEG constructs (320,000 cells) was used as the control. The cell pellets were formed by centrifugation and cultured for 24 hours.

3.2.7 Pericellular Matrix (PCM) Development and Visualization

Isolated chondrocytes (10 million/dish) were placed in 100mm tissue culture dishes supplemented with 10ml of standard chondrocyte medium (330 or 400mOsm) for 24 hours to allow the cells to deposit some of their own PCM prior to encapsulation. Chondrocytes that did not attach to the surface of the tissue culture dish were removed and viability was assessed using tyrpan blue dye. The cells were then encapsulated at a concentration of 4x10^{6}cells/ml as described above in

standard chondrocyte medium (330 or 400mOsm). The hydrogel constructs were cultured in their respective culture media. Immediately post-encapsulation, immunohistochemistry was performed on control gels to visualize whether the freshly digested cells were devoid of a pericellular matrix. Additional gels were allowed to culture for an 24 hours post-encapsulation in each medium osmolarity and PCM development was assessed through immunohistochemistry. Hydrogel constructs were cut in half and placed in PBS supplemented with 0.5 units/ml Chondroitinase ABC (Sigma) and 1% BSA solution for 30 minutes followed by treatment with mouse anti-chondroitin-6-sulfate (clone MK302, Chemicon) (1:50) in DMEM + 20% FBS for 1 hour. Each construct was rinsed with Earles Balanced Salt Solution without phenol red (Invitrogen) and placed in DMEM+20% FBS supplemented with goat anti-mouse Alexa Fluor 546 (1:20) (Invitrogen) for 1 hour. The cytoplasm of live cells was counterstained using 2μL of Calcein AM (Invitrogen) for 30 minutes. Constructs were imaged by CLSM with a 40x oil immersion objective. The cytoplasm of live cells stains green and chondroitin-6-sulfate stains red providing an indication of PCM development.

3.2.8 Matrix Synthesis and Deposition

To assess matrix synthesis, hydrogel constructs were cultured in medium supplemented with 10μCi/ml $^{35}SO_4^{2-}$ (Perkin Elmer) for 24 hours. The constructs were removed, crushed using a tissue homogenizer, and digested by papain [125mg/ml papain (Worthington Biochemical), 10mM of L-cysteine-HCl (Sigma), 100mM of phosphate (Sigma) and 10mM EDTA (Biorad) at a pH of 6.3] for 16-17 h at 60°C. Incorporation of [$^{35}SO_4^{2-}$] into newly synthesized proteoglycans (cpm/g gel wet weight) was determined using alcian blue precipitation [34]. A sample size of 3 was used. To assess long-term matrix deposition, constructs cultured for 1 day, 1 week, or 2 weeks were digested in papain. Sulfated glycosaminoglycan (GAG) content was assayed by dimethylmethylene blue dye [35] in the papain digests. Total DNA content was determined by Hoeschst 33258 (Polysciences) in the digest [36]. GAG production was normalized to its corresponding DNA content at each timepoint. A sample size of 6 was used.

3.2.9 Statistical Analysis

Data are reported as mean ± standard deviation. Single factor ANOVA was used and a confidence level of 0.95 was considered significant.

3.3 Results

Bovine articular chondrocytes were seeded at low, intermediate, and high cell encapsulation densities (Table 3.1) in PEG hydrogels in one of three encapsulation media: (i) PBS, (ii) PBS + medium nutrients and (iii) standard chondrocyte medium containing 10% FBS (Table 3.2).Cellular viability was assessed by a membrane integrity assay while metabolic activity was assessed by ATP production. Prior to encapsulation, cell viability was ∼97%. Upon encapsulation in photopolymerized hydrogels with PBS, cell viability was dramatically reduced to 37% with low cell encapsulation densities (Figure 3.2A). However, incorporation of medium nutrients to the PBS or employing standard chondrocyte medium enhanced cell survival resulting in cell viabilities that were 2-fold higher compared to encapsulation in PBS. ATP production was similar for PBS and PBS supplemented with media nutrients in PBS, but was 2-fold higher in standard chondrocyte medium (Figure 3.2B).

For the intermediate cell encapsulation densities, cell viability was markedly improved in PBS compared to lower cell encapsulation densities. Cell viability (Figure 3.2C) was significantly higher when medium nutrients were incorporated into the PBS and in standard chondrocyte medium. ATP production (Figure 3.2D) was 2-fold higher in the PBS+medium nutrients 3-fold higher in standard chondrocyte media.

In the high cell encapsulation density, cell viability was not affected by the encapsulation medium (Figure 3.2E). However, ATP production (Figure 3.2F) was ∼30% lower in the PBS+medium nutrients compared to PBS while encapsulation in standard chondrocyte media resulted in the highest ATP production.

To assess the mechanism of cell death for chondrocytes photoencapsulated at low densities and in PBS, caspase-3/7 activity was assessed (Figure 3.3). As a control, chondrocytes were cultured

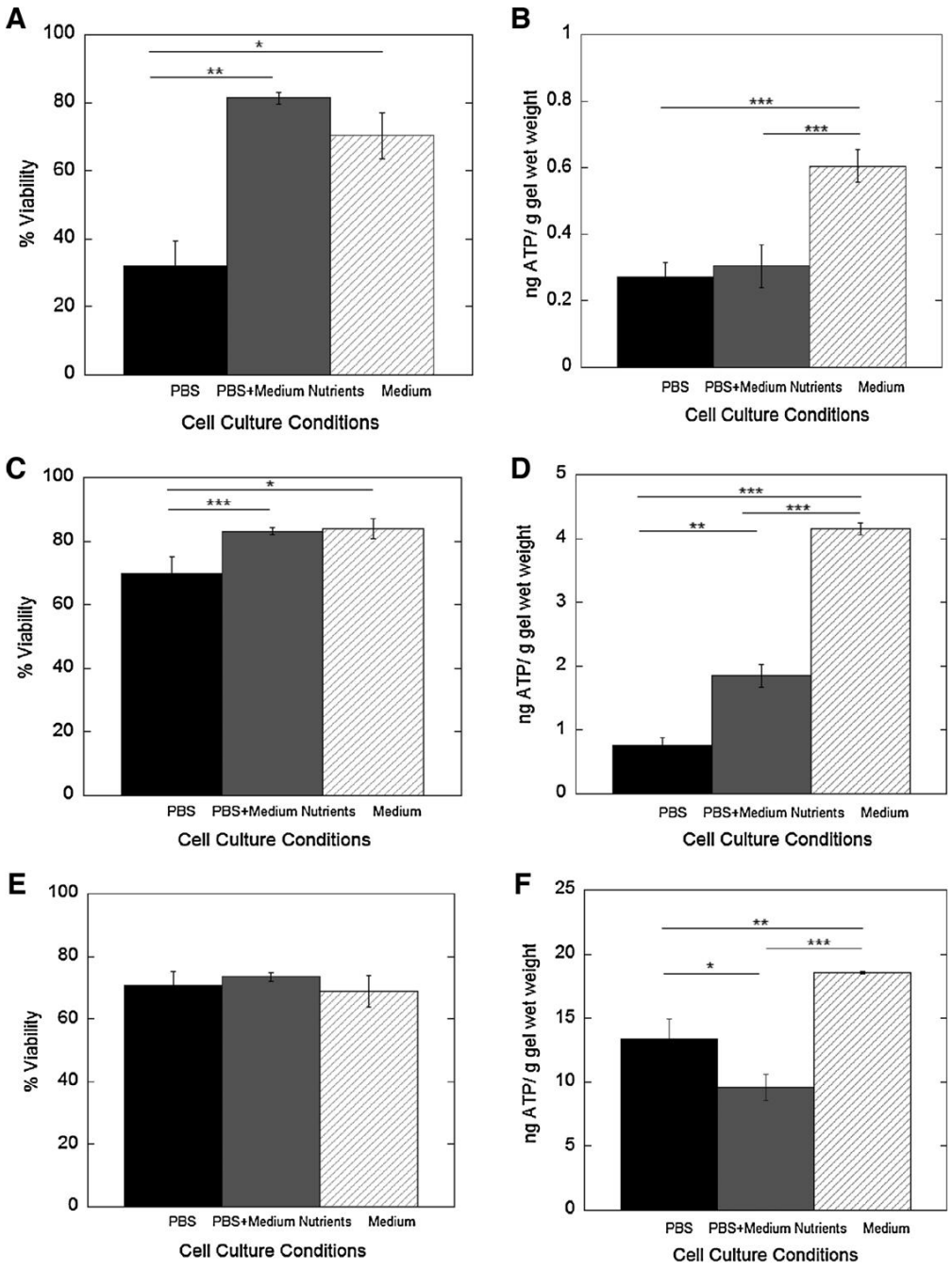

Figure 3.2: The effects of cell density and encapsulation medium on cell viability (A,C,E) and ATP production (B,D,F) immediately after encapsulation in photopolymerized PEG hydrogels. Three cell encapsulation densities were studied: 4×10^6cells/ml (low; A,B), 10×10^6cells/ml (intermediate; C,D) or 50×10^6cells/ml (high; E,F). Three different encapsulation media were studied: phosphate buffered saline (PBS, pH 7.4), PBS+medium nutrients, or standard culture medium. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Percent cell viability (n = 3) and ATP production (n = 5-8) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

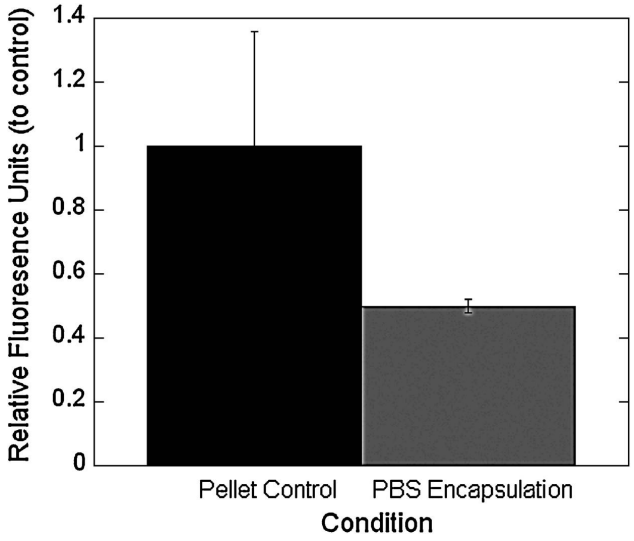

Figure 3.3: Caspase-3/7 activity for chondrocytes photoencapsulated in PEG hydrogels using PBS as the encapsulation medium and for the low cell encapsulation density. The relative fluorescence is directly associated with caspase-3/7 activity where activity in chondrocytes encapsulated in PEG hydrogel constructs was normalized to the activity of chondrocytes cultured in a 3D cell pellet (control).The total number of cells was the same for both the control and the samples. Data is given by mean ± standard deviation (n = 3).

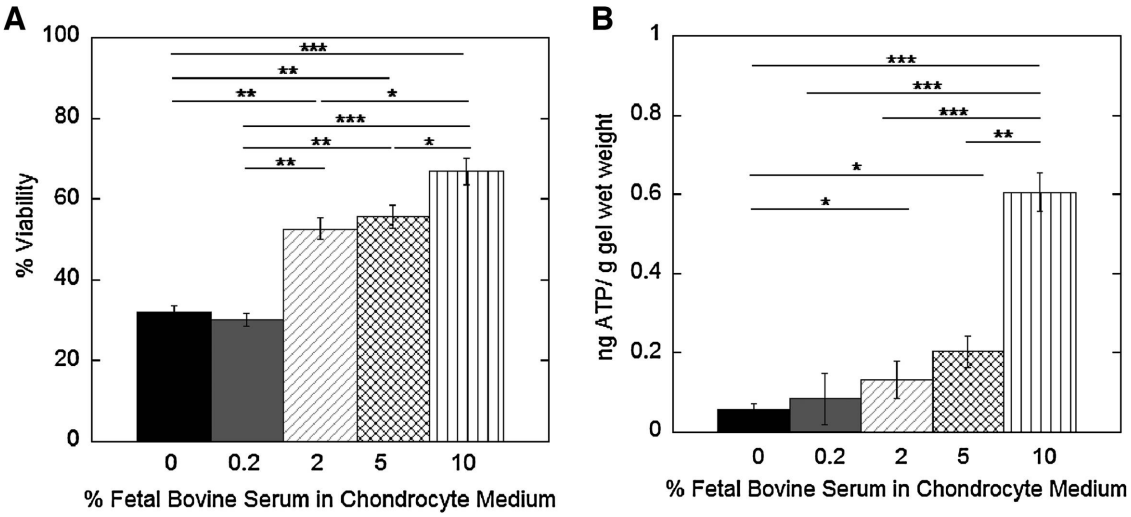

Figure 3.4: The effect of serum concentration on cell viability (A) and ATP production (B) immediately after photoencapsulation in basal medium containing 0, 0.2, 2, 5, or 10% fetal bovine serum for the low cell encapsulation density. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Percent cell viability (n = 3) and ATP production (n = 5-7) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

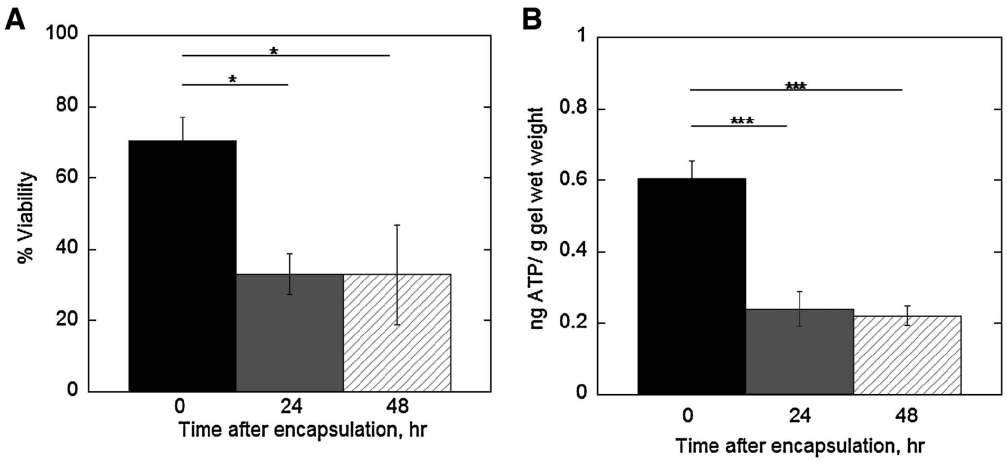

Figure 3.5: The effect of culture time on cell viability (A) and ATP production (B) for chondrocytes photoencapsulated in PEG hydrogels at the low cell encapsulation density using standard chondrocyte medium (330mOsm). The constructs were cultured for 0, 24 or 48 hours in similar medium. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Percent cell viability (n = 3) and ATP production (n = 5-8) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

in a cell pellet, which served as a 3D control to compare the 3D culture environment within PEG hydrogels. The mean relative fluorescence decreased by ∼50% for chondrocytes encapsulated within PEG hydrogels when compared to the cell pellet control, but was not statistically significant.

The effect of serum concentration on chondrocyte survival was assessed under low cell encapsulation densities. Chondrocytes were photopolymerized in basal medium supplemented with 0, 0.2, 2, 5 or 10% FBS (Figure 3.4A). An increase in FBS concentration resulted in higher cell viabilities immediately post-encapsulation ($p<0.001$). Similarly, an increase in serum concentration led to higher ATP levels ($p<0.001$, Figure 3.4B).

To assess cell survival post cell encapsulation, chondrocytes encapsulated at low densities in standard chondrocyte medium (i.e., containing 10% serum) were cultured for 0, 24, or 48 hours (Figure 3.5). After 24 hours of culture, cell viability decreased by 30% and remained low after 48 hours (Figure 3.5A). Similarly, ATP production was highest immediately after encapsulation, but decreased 3-fold 24 hours post-encapsulation and remained low after 48 hours (Figure 3.5B).

The effect of medium osmolarity on cell viability (Figure 3.6A) and ATP production (Fig-

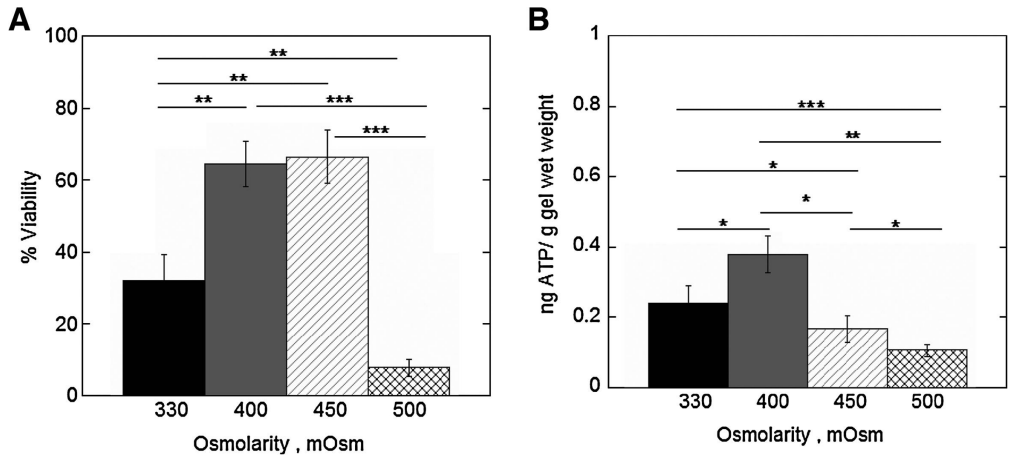

Figure 3.6: The effect of medium osmolarity on cell viability (A) and ATP production (B) for chondrocytes isolated, encapsulated at the low cell encapsualtion density, and cultured for 24 hours in chondrocyte medium at 330 (i.e. standard chondrocyte medium), 400, 450, and 500mOsm. Medium osmolarity was adjusted by the addition of potassium chloride and sodium chloride. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Percent cell viability (n = 3) and ATP production (n = 5-8) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

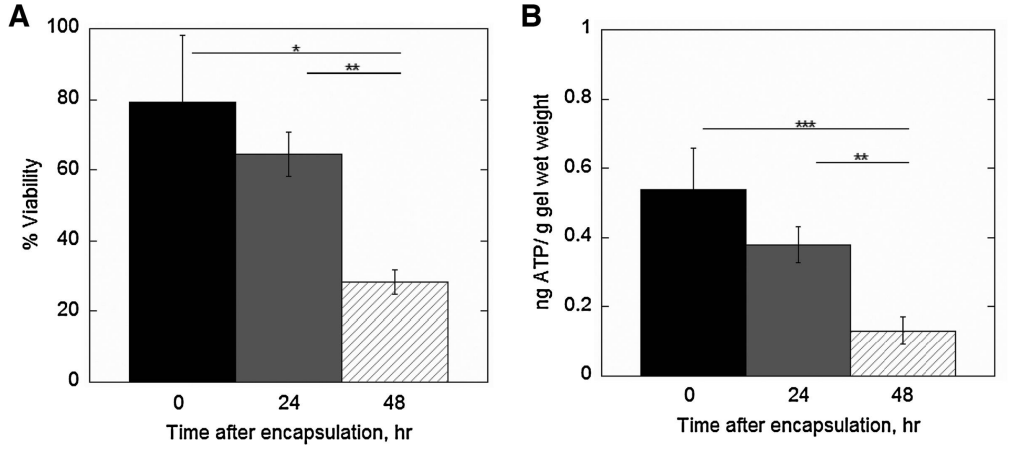

Figure 3.7: The effect of culture time on cell viability (A) and ATP production (B) for chondrocytes photoencapsulated in PEG hydrogels at the low cell encapsulation density using chondrocyte medium at 400mOsm, representative of the physiological osmolarity of native cartilage. The constructs were cultured for 0, 24 or 48 hours in similar culture medium. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Percent cell viability (n = 3) and ATP production (n = 5-8) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

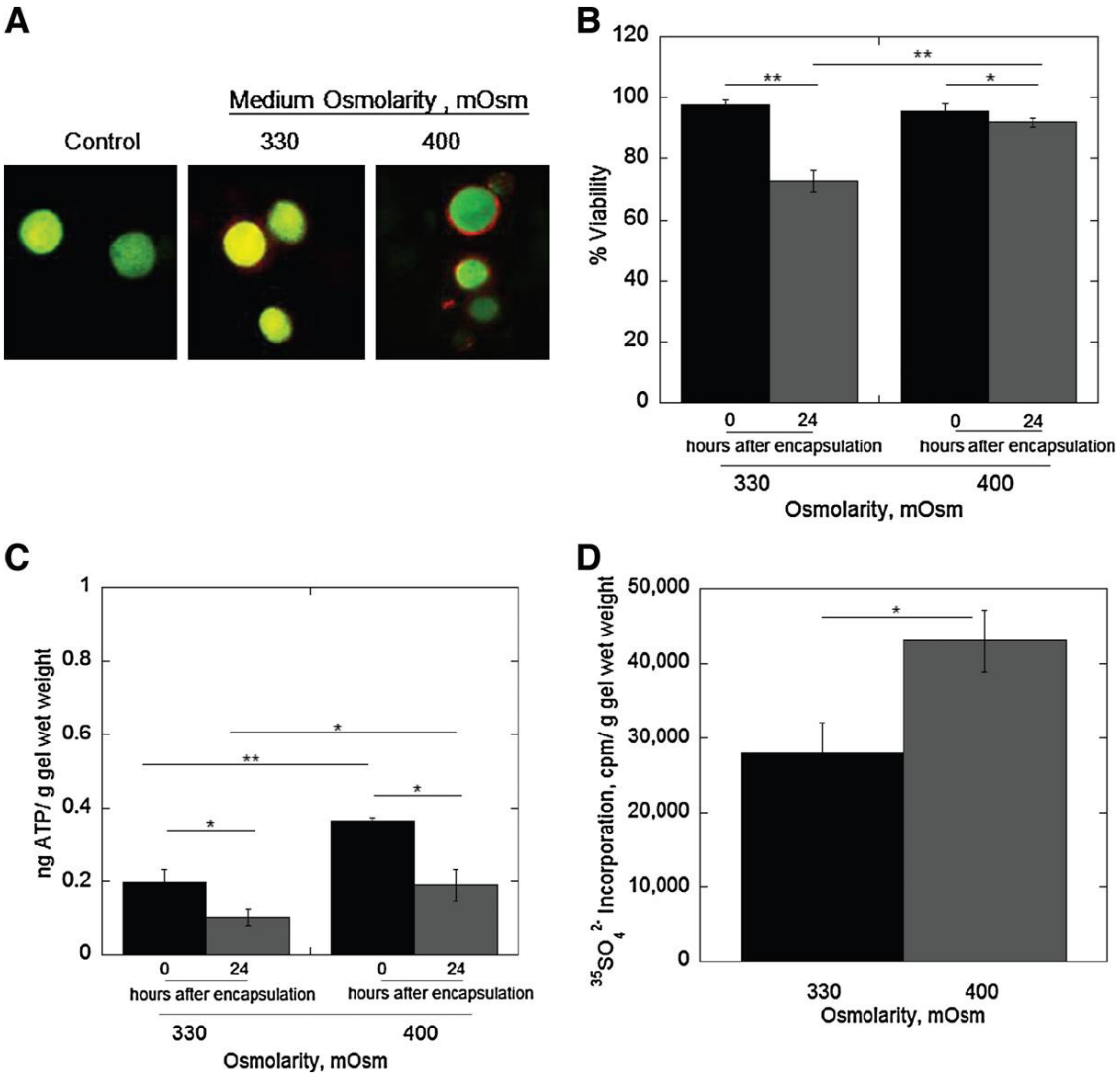

Figure 3.8: (A) Representative confocal microscopy images of chondrocytes (green) and their surrounding pericellular matrix (red) immediately after encapsulation. The chondrocytes were cultured in monolayer for 24 hours in either 330 or 400mOsm culture medium to allow them to re-form some of their own pericellular matrix prior to photoencapsulation at the low cell encapsulation density. The red staining indicates that the cells have deposited chondroitin sulfate, a major glycosaminoglycan found in aggrecan. The green stain indicates the cytosol of a live cell. Original magnification is 40x oil. For chondrocytes, which were pre-plated prior to encapsulation, their cell viability (B) and ATP production (C) were assessed immediately after encapsulation (0hr) and 24 hours post-encapsulation for the 330 or 400mOsm medium. ATP production (ng) for each gel was normalized to its respective gel wet weight (g). Proteoglycan synthesis (D) was assessed by $^{35}SO - 4^{2-}$ incorporation normalized to total DNA content during the first 24 hour of culture post-encapsulation in the 330 or 400mOsm medium. Percent cell viability (n = 3), ATP production (n = 5-8), and proteoglycan synthesis (n = 3) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

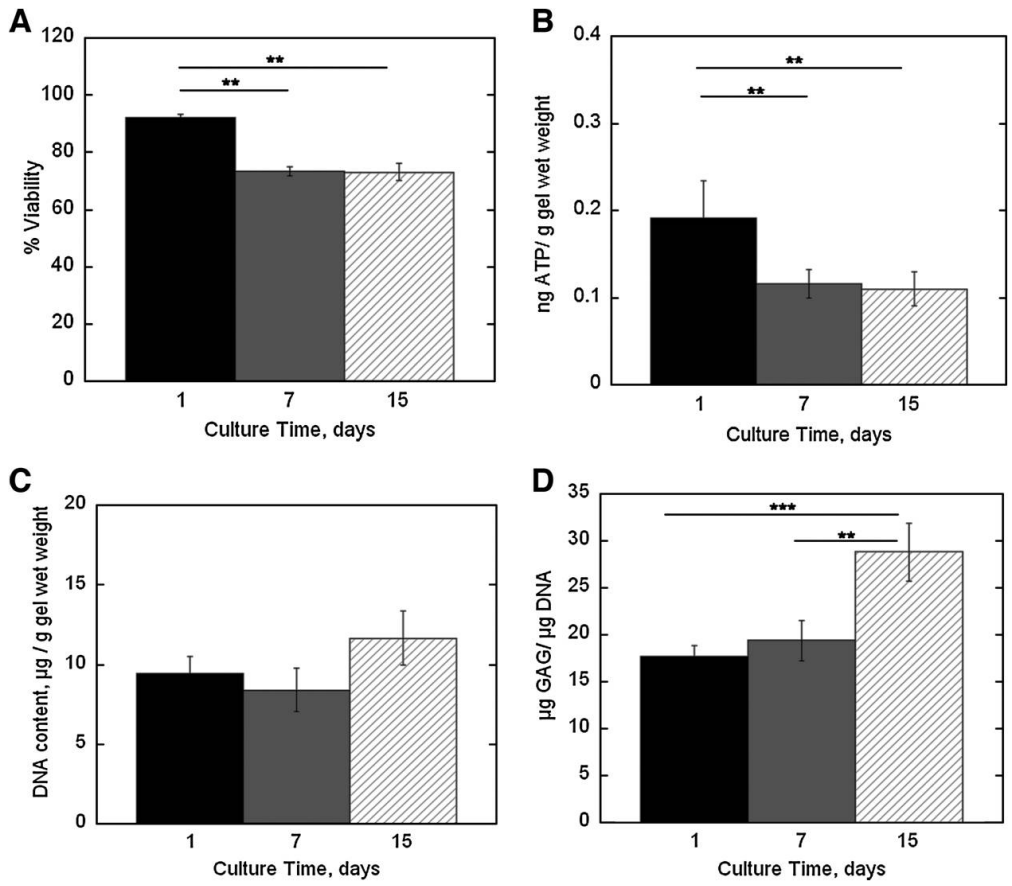

Figure 3.9: The effects of long-term culture on chondrocyte cell viability (A), ATP production (B) and glycosaminoglycan (GAG) production (C) for chondrocytes that were pre-plated for 24 hours then photoencapsulated and cultured in 400mOsm medium for 1, 7, and 15 days. ATP production (ng) for each gel was normalized to its respective gel wet weight. GAG production was normalized to total DNA content. Percent cell viability (n = 3), ATP production (n = 3), and GAG production (n = 5-6) are given by mean ± standard deviation; $*p<0.05$, $**p<0.01$, $***p<0.001$.

ure 3.6B) was assessed for chondrocytes encapsulated in low densities and in standard chondrocyte medium with osmolarities ranging from 330 to 500mOsm. After 24 hours of culture post-encapsulation, an increase in medium osmolarity from 330 to 400 or 450mOsm resulted in 40% higher cell viabilities with only ∼8% of chondrocytes surviving under 500mOsm medium. The 400mOsm medium resulted in the highest ATP levels while 500mOsm chondrocyte medium resulted in the lowest ATP levels.

For the 400mOsm chondrocyte medium, cell viability and ATP production were assessed as a function of culture time (Figure 3.7A and 3.7B, respectively). Immediately after encapsulation,

cell viability was ∼80% and did not show a significant change in viability after a 24 hour culture period. After 48 hours of culture, cell viability decreased by ∼63%. Similarly, ATP production was highest immediately post-encapsulation and did not significantly change after 24 hours of culture. However, after 48 hours of culture ATP production decreased by 90%.

To assess the role of the PCM in mediating the negative effects due to the photoencapsulation process, chondrocytes were allowed to reform some of their own PCM prior to encapsulation under 330 or 400mOsm medium. Immediately post-encapsulation, the presence of a PCM was confirmed by positive staining for chondroitin sulfate surrounding the chondrocytes (Figure 3.8A). Cell viability was high at ∼98% for both culture media immediately post-encapsulation. After 24 hours of culture, cell viability declined by ∼25% and ∼3% in the 330 and 400mOsm medium, respectively. At 0 and 24 hours post-encapsulation, ATP production was higher in the 400mOsm medium compared to the 300mOsm. However, for both culture media, ATP production decreased significantly with culture time. We additionally assessed the chondrocytes ability to function and produce cartilage-specific matrix, specifically through proteoglycan synthesis, when encapsulated with a PCM. Proteoglycan synthesis was evident under both culture media with the 400mOsm medium resulting in 35% more matrix deposition (Figure 3.8D).

Long-term chondrocyte survival and function were assessed under the best encapsulation condition where chondrocytes were pre-plated for 24 hours and encapsulated in 400mOsm chondrocyte medium (Figure 3.9). The cell-laden constructs were cultured in the 400mOsm medium for 1, 7 or 15 days. Cell viability (Figure 3.9A) decreased by 21% after 7 days of culture from day 1, but was not affected by longer culture times of 15 days. Similarly, ATP production (Figure 3.9B) decreased by 26% after 7 days of culture but no further changes were found after 15 days. Chondrocyte function was assessed by matrix deposition for sulfated glycosaminoglycan (Figure 3.9C). GAG content increased by ∼35% after 15 days of culture.

3.4 Discussion

In this study, we have shown that when freshly isolated bovine chondrocytes are photoencapsulated in PEG hydrogels via free radical photoinitiated polymerization, their viability and metabolic activity are dramatically influenced by the cell density employed during encapsulation, encapsulation medium, and presence of a pericellular matrix.

When high cell encapsulation densities (10-50 million cells/ml) were employed, the percentage of cells that remained viable immediately after encapsulation was greater than 70% in the different encapsulation mediums. High cell encapsulation densities of 50 million cells/ml have been used successfully to grow cartilaginous tissue within similar photopolymerized PEG and biodegradable PEG hydrogels [6, 7, 37]. However, only ∼35% of chondrocytes survived when a low cell encapsulation density and PBS were used. For cell encapsulation strategies involving photopolymerization, the polymerization must occur in the presence of oxygen to maintain cell survival. However, oxygen is a well-known inhibitor of free radical polymerizations where radicals react with molecular oxygen to produce a less reactive peroxy radical [10, 29]. Oxygen radicals, however, can be highly toxic to cells most notably through damage to cellular membranes by lipid peroxidation, which can lead to numerous adverse effects including altered membrane permeability and if severe, cell death via necrosis. The rapid cell death that occurred during the 10 min encapsulation process, as measured by a membrane integrity assay, indicates that cell death is likely through necrosis. The 50% mean decrease observed in Caspase-3/7 activity also suggests necrosis.

For each encapsulation condition, the concentrations of photoinitiator and polymerizable double bonds were similar suggesting that the number of initiator radicals and macroradicals generated during the polymerization process was similar. Based on the percentage of viable cells and the cell density at encapsulation, the number of cells that did not survive the encapsulation process in PBS was similar for the 4 and 10 million cells/ml conditions (∼220,000 cells per gel). Interestingly, the total number of cells that did not survive the encapsulation process for the 50 million cells/ml density was approximately 5 times higher. During necrosis, dying cells release a plethora of enzymes,

which can trigger a chain reaction of cell death. It is possible that this phenomenon may have occurred due to the high cell concentration and close proximity of neighboring cells to a dying cell. Alternatively, the higher cell concentration may increase the viscosity of the solution enhancing the autoacceleration effect and leading to an overall higher concentration of macroradicals during polymerization [38]. Taken together, our findings indicate that high cell encapsulation densities lead to significantly more cell death when assessing total numbers of dead cells suggesting either high cell density and/or alterations in the polymerization reaction enhance cellular death via free radical damage. Regardless, the percentage of live cells in the gels containing high cell densities is higher than the gels with lower cell densities, suggesting that although more cells die via radical damage at the high densities there are still a vast number of live cells available for macroscopic tissue development.

At the low cell concentration, the presence of medium nutrients, which include ascorbic acid and HEPES, and serum in a concentration dependent manner, significantly improved cell viability immediately post-encapsulation. All of these components have known antioxidant capabilities. For example, ascorbic acid reacts directly with superoxides, radicals and singlet oxygen [29]. The presence of HEPES was found to reduce DNA damage during photoencapsulation of plasmid DNA due to its radical scavenging abilities [39]. Serum has been shown to protect cells against damage due to oxidative stress [40]. In general, metabolic activity as measured by ATP production mirrored cell viability with the exception of when chondrocytes were encapsulated in the presence of glucose and serum, precursors of cellular respiration and ATP production. As expected, ATP production was significantly higher in chondrocytes encapsulated in standard culture medium compared to PBS supplemented with medium nutrients even though cell viability was similar (i.e., Figure 3.2). Nonetheless, cell viability and metabolic activity were not maintained with culture time resulting in a 50% and 60% decline in cell viability and ATP production, respectively, after 1 day of culture. This finding suggests that the presence of antioxidants in medium nutrients and serum minimize acute cellular damage during encapsulation, but are insufficient at preventing radical damage and cell death.

As noted earlier, the typical isolation process for chondrocyte occurs in a hyposmotic environment and leaves the chondrocyte deprived of any protective pericellular matrix. The hyposmotic environment initially causes cell swelling, but through regulatory volume decrease mechanisms, chondrocytes are able to respond quickly by counteracting cell swelling through the removal of intracellular osmolytes, typically KCl and organic solutes [28]. Prolonged exposure to non-physiological osmotic environments and long-term loss of osmolytes, however, can be detrimental to cells [28]. This phenomenon is often characteristic of many pathological conditions, including osteoarthritis [41]. We hypothesized that this decrease in osmolytes associated with the hyposmotic environment may increase the cells susceptibility to oxidative degradation. After 24 hours post encapsulation, cell viability and metabolic activity was highest under physiological medium osmolarity of 400 mOsm, while chondrocyte survival was significantly compromised under hyposmotic and hyperosmotic media. However, by 48 hours viability and ATP production decreased markedly suggesting that free radical damage during encapsulation still occurred.

In native cartilage, chondrocytes interact directly with their pericellular microenvironment to receive biomechanical and biochemical signals [42]. Freshly isolated chondrocytes, although deprived of their PCM, are known to begin reforming a PCM within 24 hours after their isolation [43]. Here, we demonstrate that after 24 hours in monolayer culture, freshly isolated chondrocyte have deposited pericellular chondroitin sulfate, one of the major glycosaminoglycans in aggrecan. Previous studies have reported collagen type VI and keratan sulfate deposition in the pericellular regions of chondrocytes 24 hours post-isolation [43]. The presence of some of the PCM components improved cell viability when encapsulated and cultured in a hyposmotic environment, which is likely due to the antioxidant capabilities of many extracellular matrix molecules [31, 44]. The combination of PCM and a physiological osmotic environment, however, resulted in superior cell survival after 24 hours post-encapsulation. Chondrocyte function was also enhanced by the isotonic culture environment as evident by increased proteoglycan synthesis during the first 24 hours of encapsulation compared to the hyposmotic environment. Previous studies have reported that a physiological osmotic environment for chondrocytes results in the highest glycosaminoglycan pro-

duction [45]. Over long-term cell cultures (of 15 days), cell viability, metabolic activity, and matrix deposition were maintained under these encapsulation and culture conditions.

In summary, for chondrocytes that were photoencapsulated in low cell encapsulation densities in PEG hydrogels, the combination of antioxidants, physiological osmolarity, and the development of some PCM, resulted in an improved robustness against free-radical damage during photoencapsulation. Our findings indicate that primary isolated cells, particularly chondrocytes, are more susceptible to free radical damage. These findings may be important in other tissue engineering applications where freshly isolated cells are employed with photoencapsulation strategies. Nonetheless, we report suitable encapsulation and culture conditions that maintain chondrocyte survival and function at least for several weeks post-encapsulation towards developing strategies that employ low encapsulation densities and photopolymerization.

3.5 Acknowledgements

This work was supported by a research grant from the NIH (K22 DE016608), NIH Pharmaceutical Biotechnology Training Fellowship to NF, and a NASA Harriett Jenkins Predoctoral Fellowship and a Department of Educations Graduate Assistantship in Areas of National Need Fellowship to IV. Confocal microscopy was performed at the Nanomaterials Characterization Facility at the University of Colorado. A very special thanks to Sara K.Gladem for her technical assistance in this study.

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Chapter 4

The Role of the PCM in Reducing Oxidative Stress Induced by Radical Initiated Photoencapsulation of Chondrocytes in Poly(ethylene glycol) Hydrogels

(Accepted to Osteoarthritis and Cartilage, In Press 2012)

Objective: The objectives for this study were to determine whether radical initiated photopolymerizations typically employed for cell encapsulations lead to oxidative stress incurred by chondrocytes and whether the development of a pericellular matrix (PCM) decreases this oxidative stress and has longer-term benefits on chondrocyte function. *Methods*: Freshly isolated bovine chondrocytes were encapsulated in poly(ethylene glycol) (PEG) hydrogels devoid of a PCM or with a PCM, confirmed by immunocytochemistry, and cultured for up to 2 weeks. ROS production and damage to cell membrane by lipid peroxidation was accomplished using carboxy-H_2DFFDA and by MDA content, respectively. Gene expression and proteoglycan synthesis were analyzed using RT-qPCR and $^{35}SO_4$ incorporation, respectively. *Results*: The photopolymerization reaction, which alone generates radicals and extracellular ROS, led to oxidative stress in chondrocytes evidenced by increased intracellular ROS and lipid peroxidation. The presence of a PCM decreased intracellular ROS and abrogated membrane lipid peroxidation, improved aggrecan, collagen II and collagen VI expression, and enhanced proteoglycan synthesis. *Conclusions*: The development of the PCM prior to photoencapsulation in PEG hydrogels reduces oxidative stress and improves chondrocyte anabolic activity. Our data suggest this reduction occurs by decreased ROS diffusion into the cell and decreased membrane damage. Our findings suggest that minimizing oxidative stress, such

as through the presence of a PCM, may have long-term beneficial effects on tissue elaboration when employing photopolymerizations to encapsulate chondrocytes for cartilage tissue engineering applications.

4.1 Introduction

Hydrogels have been widely used as platforms for three-dimensional culture of chondrocytes because they promote a rounded cellular morphology that maintains the chondrocyte phenotype, have high water contents for facile nutrient diffusion, and encourage cartilage-like tissue deposition [1, 2]. Hydrogels formed through photopolymerization are of particular interest because this process affords spatial control over the polymerization, occurs rapidly on clinically relevant timescales, and can be performed at physiological temperature with minimal heats of reaction [2, 3, 4]. Moreover, photopolymerizable hydrogels can be formed from a wide range of natural and synthetic polymer precursors [5, 6] to produce gels tailored to mimic native cartilage [7].

While photopolymerization has many desirable attributes for cell encapsulation and *in vivo* delivery of cells, reduced cell viability post-encapsulation has been reported [8, 9, 10]. Because photopolymerizations are radical initiated chain polymerizations, the presence of radicals can generate a harsh environment. During photopolymerization, photoinitiator molecules absorb photons of light energy and dissociate into radicals. Initiator radicals react with functional groups on macromolecular monomers forming macroradicals that leads to chain propagation [11, 5]. Initiating radicals and macroradicals may attack cells causing direct or indirect damage and ultimately leading to cell death. In addition, initiating radicals have a high propensity to react with oxygen that is present during photoencapsulation of cells, having reaction rate constants five orders of magnitude greater for oxygen over typical monomers [12]. When radicals react with oxygen, common byproducts include peroxy radicals [13, 14], a type of reactive oxygen species (ROS) known to trigger oxidative stress in cells [15, 16, 17]. In chondrocytes, oxidative stress has been linked to cell death [17], inhibition of tissue synthesis, and upregulation of tissue degrading enzymes, e.g. matrix metalloproteinase-13 (MMP-13) [18, 19]. Therefore, photoencapsulation may also adversely

affect cells through polymerization-induced ROS, leading to oxidative stress in cells and ultimately reducing tissue regeneration capabilities in photopolymerized hydrogels.

Previous studies from our group have shown that chondrocyte death resulting from photoencapsulation can, in part, be mitigated by allowing cells to reform some of their own pericellular matrix (PCM) prior to encapsulation [20]. These findings suggest that the PCM is able to protect chondrocytes possibly by reducing damage from radicals and ROS. To better understand the photopolymerization process and the protective role of the PCM, this study aimed to address three research questions. 1) *Does the photopolymerization process, under conditions employed for cell encapsulations, lead to oxidative stress incurred by chondrocytes?* 2) *Does the presence of a PCM alleviate oxidative stress incurred by chondrocytes during photoencapsulation?* and 3) *Does oxidative stress impact chondrocytes over short and longer term cultures?* Specifically, markers for oxidative stress were measured by chondrocyte generated intracellular ROS and damage to the cell membrane. Our model was bovine articular chondrocytes encapsulated in poly(ethylene glycol) (PEG) hydrogels formed from PEG dimethacrylate precursors. Longer term, chondrocyte anabolic activity was evaluated by aggrecan, collagen II and collagen VI gene expression and proteoglycan synthesis and chondrocyte catabolic activity was assessed by MMP-13 gene expression.

4.2 Materials and Methods

4.2.1 Chondrocyte Isolation

Articular cartilage from metacarpalphalangeal joints of 1-2 year old steers (Arapahoe Foods, Lafayette, CO) was harvested several hours after slaughter under sterile conditions. Cartilage was processed in one of two ways: 1) cartilage from three different animals was harvested separately and cells from a single donor were used to give three biological replicates (referred to as single donor chondrocytes) or 2) cartilage was harvested from four to six animals in two separate isolations and for each isolation the cells were pooled to give two biological replicates (referred to as pooled donor chondrocytes). All solutions were adjusted to a physiological osmolarity of 415±14mOsm by supple-

mentation with 3.64g/L NaCl and 0.45g/L KCl determined by freezing point osmometry (Precision Systems Inc, Natick, MA). Cartilage slices were washed in PBS with 1% penicillin/streptomycin (PBS+P/S, Invitrogen, Carlsbad, CA), diced finely, and enzymatically digested in 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ) in Dulbeccos Minimal Essential Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 16 hours at 37°C on a shaker. Isolated chondrocytes were washed twice in PBS+0.02% EDTA and resuspended in PBS+P/S. Cell viability was 90-96% determined by trypan blue exclusion.

4.2.2 Chondrocyte Studies in Suspension and Encapsulated

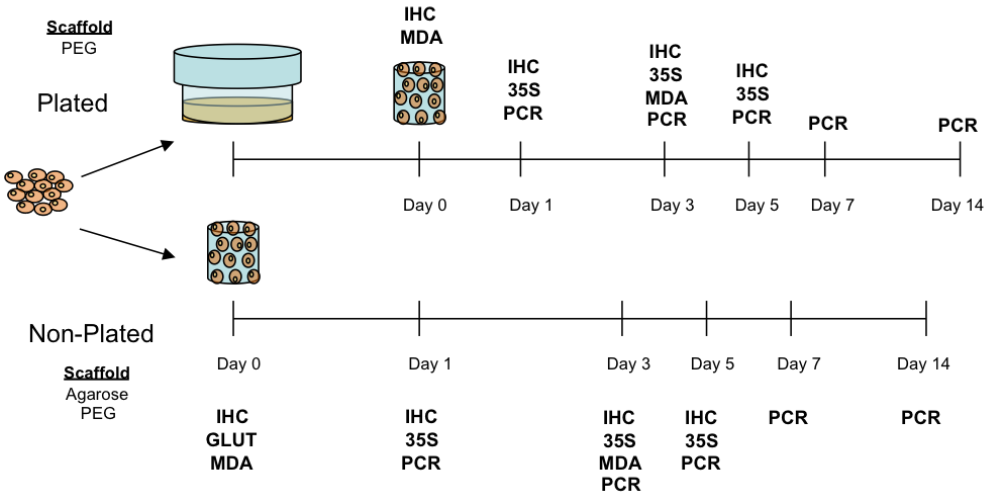

Figure 4.1: The experimental design. Freshly isolated primary bovine chondrocytes were cultured under one of two conditions, referred to as non-plated or plated. Under plated conditions, chondrocytes were cultured in 2D on non-tissue culture treated dishes for 24 hours and subsequently used in 2D studies to assess intracellular ROS generation or encapsulated in PEG hydrogels. Under non-plated conditions, chondrocytes were immediately used after isolation in 2D studies to assess intracellular ROS generation or encapsulated in PEG hydrogels. In 3D, cell-laden hydrogels were analyzed by immunocytochemistry (IC), malondialdehyde quantification (MDA) for lipid peroxidation, ^{35}Sulfate incorporation ($^{35}SO_4^{2-}$) into new synthesized proteoglycans, and gene expression by quantitative PCR (qPCR) at the time points indicated.

Chondrocytes were pre-cultured under non-plated or plated conditions (Figure 4.1). Non-plated condition describes cells that were used immediately after enzymatic digestion. Plated condition describes cells that after enzymatic digestion were plated in two-dimensional culture at

10 million cells/10 mL standard chondrocyte medium (defined below) per dish (100 mm diameter non-tissue culture treated) for 24 hours at 37°C, enabling chondrocytes to re-form some of their native PCM. Cell viability after plating was 83-96% by trypan blue exclusion. Chondrocytes were investigated in suspension cultures (details provided below) or encapsulated in PEG hydrogels.

For cell encapsulations, PEG dimethacrylate (PEGDM) was synthesized by reacting linear PEG (3000 MW, Fluka, Milwaukee, WI) with methacryloyl chloride (97% pure, Sigma-Aldrich) in the presence of triethylamine (Sigma-Aldrich) at a molar ratio of 1:4.4:4 at 60°C for 24 hours. PEGDM was purified by filtration over alumina powder and precipitated in cold ethyl ether. ^{1}H NMR analysis confirmed 75% of end hydroxyls were methacrylated by comparing the area under the vinyl group peaks (∼5.6 and 6.1ppm) to that of the methylene group peaks (∼3.6-4ppm). Chondrocytes (4x10^{6}/mL) from non-plated and plated conditions were combined with 10% w/v PEGDM and 2 mM photoinitiator (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY) in standard chondrocyte medium (DMEM with 4g/L $CaCO_3$, 5.24g/L NaCl, 0.26g/L KCl, 10mM HEPES, 0.1M non-essential amino acids, 0.4mM L-proline, 50mg/L L-ascorbic acid, 1% P/S, 10% FBS, 0.02mg/mL gentamicin and 0.5μg/mL fungizone (HEPES, nonessential amino acids, gentamicin: Invitrogen, others from Sigma-Aldrich)). This solution was exposed to 365nm light (∼4mW/cm^{2}) for 10 minutes to form stable hydrogels. Chondrocytes (4x10^{6} cells/mL) from non-plated conditions were combined with 3% (w/v) agarose type IX-A (Sigma Aldrich) at 37°C, which was previously dissolved in Earls Balanced Salt Solution (Invitrogen) at 60°C. Gelation occurred by reducing the temperature to 4°C for 20 minutes. Cylindrical hydrogels (4.5mm height, 4.5mm diameter) were formed. Hydrogels were cultured in standard chondrocyte medium for up to 2 weeks at 37°C and 5% CO_2. Cell viability, determined by LIVE/DEAD® (Invitrogen) analysis post-encapsulation was similar between non-plated and plated conditions throughout the 2 weeks of culture (data not shown).

4.2.3 PCM Development and Visualization

PCM was assessed by immunocytochemistry for collagen II, collagen VI, and chondroitin sulfate, the main constituents of the PCM in cartilage [21, 22]. Pooled donor chondrocyte-seeded PEG constructs were pre-treated with 2080U/mL hyaluronidase (Sigma-Aldrich) and 500mU/mL chondroitinase-ABC (Sigma-Aldrich) in PBS with 1% bovine serum albumin (Sigma-Aldrich) for 30 minutes at 37°C. Constructs were treated with anti-collagen II (US Biologicals, C7510-20F, Swampscott, MA), anti-collagen VI (Abcam, ab6588, Cambridge, MA) or anti-chondroitin sulfate (Chemicon International, MK302, Temecula, CA) in DMEM+20% FBS (1:50) for one hour at 37°C. Constructs were treated with secondary antibodies at 1:100 (AlexaFlour546 goat anti-mouse IgG, Invitrogen) or 1:2500 (Dylight549 goat anti-rabbit IgG, Rockland Immunochemicals, Gilbert, PA) in DMEM+20% FBS for one hour at 37°C. Cytosol was counterstained using 8μM calcein AM (Invitrogen) in PBS for 30 minutes at 37°C. Cells and PCM were imaged using confocal laser scanning microscopy (Zeiss LSM 510, Thornwood, NY). One biological replicate was performed with two technical replicates (n=1).

4.2.4 Quantification and Visualization of ROS Production using carboxy-H_2DFFDA

The cell-permeant dye, carboxy-2,7-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA, Invitrogen) was used to quantify and visualize radicals and ROS extracellularly and intracellularly [23, 24]. Once the diacetate is hydrolyzed, carboxy-H_2DFF can react with reactive oxygen species (e.g., hydroxyl radicals, peroxyl radicals, superoxide anions, and hydrogen peroxide) to produce a stable fluorescent product [23, 24]. In the absence of cells, the diacetate group was hydrolyzed with 200 μM KOH for one hour [25]. The hydrolyzed dye was added at a final concentration of 20 μM to chondrocyte medium containing 0, 1, 2, 4.5 or 11mM photoinitiator. Wells were either left untreated or exposed to 365nm light ($\sim$4mW/cm^2) for 10 minutes and fluorescence immediately assayed on a FLUOstar Optima plate reader (BMG Labtech, Cary, NC) with 488nm excitation and 525nm emission. Six independent measurements were taken for each PI concentration (n=6). To measure

chondrocyte-generated ROS by the photoinitiation process, single donor chondrocytes (non-plated or plated) in suspension culture were incubated with 20 μM carboxy-H_2DFFDA for 20 minutes to allow for its transport into cells and subsequent cleavage of the diacetate followed by several rinses in PBS via centrifugation. Cells ($4x10^6$ cells/mL) were resuspended in chondrocyte medium with 0, 1, 2, 4.5 or 11mM photoinitiator and transferred to 96 well plates (80μl/well). Wells were either left untreated or exposed to 365nm light and fluorescence immediately assayed on the plate reader as described above. Three biological replicates were performed with two technical replicates per experiment (n=3). In a separate experiment, intracellular generation of photoinitiator radicals and/or ROS resulting from photoinitiator molecules being taken up by cells followed by UV exposure was investigated in non-plated cells (termed photodynamic effect). Pooled donor chondrocytes were incubated with 0-11mM photoinitiator for 20 minutes to allow cells to take up the photoinitiator molecules. Cells were rinsed in PBS by centrifugation, treated with carboxy-H_2DFFDA and UV light, and assessed by fluorescence as previously described. Two biological replicates were performed with two technical replicates per experiment (n=2). In 3D culture, single donor chondrocytes (non-plated or plated) were incubated with carboxy-H_2DFFDA for 20 minutes, rinsed in PBS by centrifugation, and encapsulated in PEG hydrogels as described above directly in 96 well plates. Immediately post-encapsulation, fluorescence was assessed on the plate reader or imaged on a CLSM. Three biological replicates were performed with three technical replicates per experiment (n=3).

4.2.5 Quantification of Malondialdehyde (MDA)

Malondialdehyde, MDA, is a stable byproduct of the reaction between high-energy radicals and lipids in the cell membrane. Single donor chondrocytes from plated and non-plated conditions were encapsulated in PEG hydrogels as described above and cultured up to three days. At prescribed times, gels were snap frozen and homogenized in lysis buffer (20mM Tris, 2mM EDTA, 150mM NaCl and 0.5% Triton X-100 in deionized water, Sigma-Aldrich). MDA content was determined using a thiobarbituric reactive species assay kit (Cayman Chemical, Ann Arbor, MI) and

FLUOstar Optima plate reader per manufacturer. Three biological replicates were performed with three technical replicates per experiment (n=3).

4.2.6 Gene Expression

Single donor chondrocytes from plated and non-plated conditions were encapsulated in PEG hydrogels as described above and cultured for two weeks. At prescribed times, gels were snap frozen and homogenized in TRK lysis buffer (Omega Bio-Tek, Norcross, GA) with 2% β-mercaptoethanol (Sigma-Aldrich). Samples were eluted through a homogenizer column (Omega Bio-Tek) at 13,000 rpm for 5 minutes to separate PEG from lysate. RNA was extracted by centrifugation using micro-elute RNA binding columns (Omega Bio-Tek) per manufacturer. RNA was converted to cDNA using high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA). All primers (Table 4.1) were designed to span an entire intron (Integrated DNA Technologies, San Diego, CA).

Table 4.1: Primer sequences designed for use in real time PCR gene expression analysis

Gene of Interest	Forward Primer	Reverse Primer
Aggrecan	5-GCGGGTGCGGGTCAA-3	5-ATCACCTCGCAGCGTAGAATC-3
Collagen II	5-AGTCCCTCAACAACCAGATCG-3	5-CGATCCAGTAGTCTCCGCTCTT-3
Collagen VI	5-GGCCGGACTCCACTGAGA-3	5-TCTCCATAGGCTTCATGTTTCATG-3
MMP-13	5-TCCTGGCTGGCTTCCTCTT-3	5-GTAGCTCTCTGCAAACTGGAAGTCT-3

Quantitative PCR was performed (7500 Fast real-time PCR system, Applied Biosystems). Normalized gene expression is defined as [26]

$$\text{Normalized Expression} = \frac{(E_T)^{\Delta C_{TT}(Calibrator-Test)}}{(E_R)^{\Delta C_{TR}(Calibrator-Test)}}$$

where E_T and E_R are the efficiencies of the target and housekeeping genes, respectively, C_{TT} and C_{TR} are the difference in C_T values between calibrator and test sample for the target and housekeeping genes, respectively. The ribosomal protein L30 was used as the stable housekeeping gene. Calibrator was day 0. Three biological replicates were performed with two technical replicates per experiment (n=3).

4.2.7 Proteoglycan Synthesis

Proteoglycan synthesis was assessed in PEG hydrogels encapsulated with pooled donor chondrocytes from non-plated and plated conditions and cultured in chondrocyte medium supplemented with 10μCi/mL $^{35}SO_4$ (Perkin Elmer, Shelton, CT) for 24 hours prior to analysis. At prescribed times, medium was removed for analysis and hydrogels were homogenized and enzymatically digested (125μg/mL Papain (Worthington), 10mM L-cysteine, 100mM phosphate and 10mM EDTA (Sigma-Aldrich) in DI water (pH 6.3)) overnight at 60°C. Newly synthesized proteoglycans were assayed by Alcian blue precipitation [27]. Briefly, Alcian blue (0.2% w/v) was added to media and construct digest samples, which binds to and precipitates proteoglycans. The precipitate was captured by filtration (Multiscreen HTS Filter Plate, Fisher Scientific, Pittsburgh, PA), and released by incubation with 1.34g/mL guanidine-HCl (Sigma) in deionized water/isopropanol solution (2:1 v/v) (Sigma). Total proteoglycans were quantified in cpm on a Beckman LSC 6500 (MDA 80.3pCi) and normalized to gel wet weight. Two biological replicates were performed with three technical replicates per experiment (n=2).

4.2.8 Statistical Analysis

Data are reported as the mean of the biological replicates or as individual data points in a dot plot. Error bars represent 95% confidence intervals of the mean. One-way analysis of variance was used with Tukeys post-hoc analysis and $p<0.05$ considered significant. All data followed a Gaussian distribution and exhibited homogeneous variance. The analysis unit for each experiment was cells in solution or encapsulated, either from one animal or from several animals pooled together, which were subjected to a treatment (e.g., photoinitiator, UV exposure) applied at random.

4.3 Results

Freshly isolated chondrocytes were largely devoid of a pericellular matrix evidenced by a lack of staining for chondroitin sulfate and collagens II and VI, as shown in Figure 2. A 24-hour plating

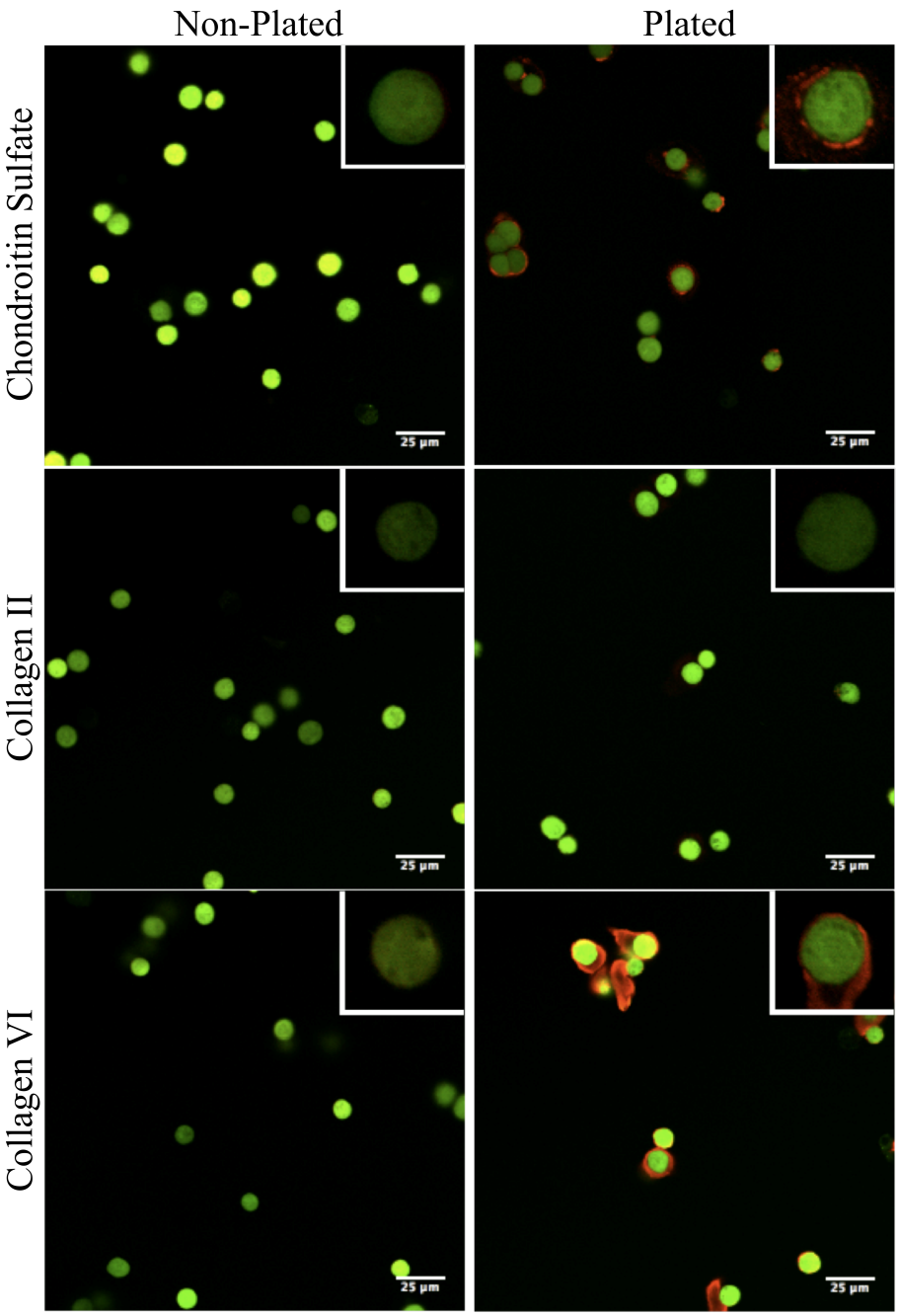

Figure 4.2: The presence of pericellular matrix (PCM) in live chondrocytes from non-plated and plated conditions encapsulated in PEG hydrogels. The PCM molecules examined were chondroitin sulfate, collagen II and collagen VI (red) by immunocytochemistry, while the cytosol of the live cells was counterstained with calcein AM (green)(n=1 biological replicate).

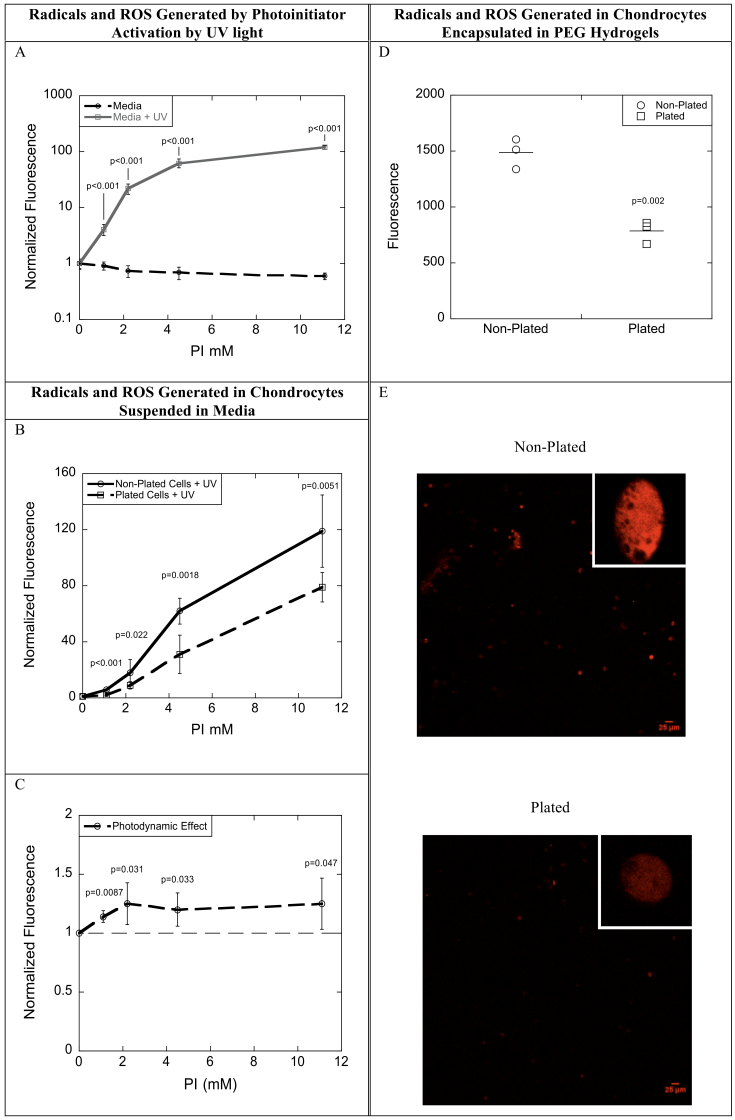

Figure 4.3: (A) ROS generation measured using carboxy-H_2DFFDA in media with and without UV exposure, normalized to samples with no photoinitiator (n=6). (B) ROS generated intracellularly in chondrocytes from non-plated and plated conditions, exposed to UV light, and normalized to samples with no photoinitiator but exposed to UV (n=3 biological replicates). (C) ROS generated in chondrocytes resulting from cells taking up photoinitiator molecules and subsequent exposure to UV light (photodynamic effect), normalized to samples with no photoinitiator and compared (dashed line) to samples with no PI but with UV exposure (n=2 biological replicates). (D) ROS generation intracellularly immediately after photoencapsulation in PEG hydrogels. (E) Confocal microscopy images of intracellular ROS (red) immediately after photoencapsulation of chondrocytes in PEG hydrogels (n=3 biological replicates). In A-C, data are presented as the mean and error bars represent the 95% confidence interval of the mean. P values represent significance between experimental conditions for a given PI concentration. In D, data points from non-plated and plated conditions are presented. P values represent significant differences from the non-plated condition.

period, however, was sufficient for chondrocytes to re-form some of their own PCM comprised of chondroitin sulfate and collagen VI, although collagen II was not yet present (Figure 4.2).

When photoinitiator molecules dissolved in chondrocyte medium in the absence of cells were exposed to a single dose of UV light in the presence of oxygen and hydrolyzed carboxy-H_2DFFDA, fluorescence increased significantly with increasing photoinitiator concentration (Figure 4.3A). Fluorescence was significantly higher for each corresponding photoinitiator concentration without UV exposure. For example, fluorescence was 268-fold higher ($p<0.001$) for the 11mM photoinitiator sample with UV exposure than without UV exposure.

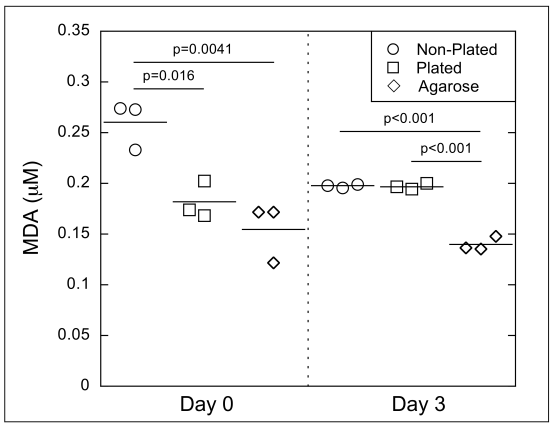

Figure 4.4: Detection of lipid peroxidation by malondialdehyde (MDA) content in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions after photoencapsulation in PEG hydrogels and in non-plated chondrocytes (without PCM) encapsulated in agarose hydrogels. Data points for each condition (non-plated, plated, and agarose) and time point are presented from three independent experiments(n=3 biological replicates). P values represent significant differences between conditions for a given time point.

When cells were present in solution, photoinitiator radicals led to elevated intracellular ROS, which was a function of photoinitiator concentration and dependent on cell plating condition (Figure 4.3B). Non-plated conditions had 1.5 to 3-fold higher ($p<0.001$) intracellular ROS levels for photoinitiator concentrations of 1.1mM and greater, respectively over plated conditions. For non-plated cells, transport of photoinitiator molecules into cells and subsequent intracellular ROS generation by UV light (photodynamic effect) was assessed (Figure 4.3C). The photodynamic effect

resulted in significantly higher intracellular ROS when compared to untreated cells exposed to UV light for all photoinitiator concentrations. Specifically, fluorescence increased by 1.25-fold at 2mM photoinitiator and remained constant with higher photoinitiator concentrations.

When cells were photoencapsulated in PEG hydrogels with 2mM photoinitiator, non-plated conditions showed 1.89-fold higher ($p=0.002$) levels of intracellular ROS when compared to plated conditions (Figure 4.3D). Qualitatively, confocal microscopy images also showed higher levels of intracellular ROS immediately post-encapsulation in non-plated over plated conditions (Figure 4.3E).

Damage to the cell membrane as a result of photoencapsulation was assessed by MDA content (Figure 4.4). Immediately post-encapsulation, non-plated conditions in PEG had 1.45-fold higher ($p=0.016$) MDA content when compared to plated conditions in PEG and 1.69-fold higher in non-plated conditions in agarose ($p=0.0041$), both of which had similar MDA levels. By day 3 MDA levels were similar among conditions in PEG.

Relative expression of anabolic and catabolic genes in PEG hydrogels encapsulated with chondrocytes from non-plated or plated conditions is shown in Figure 4.5. All anabolic genes examined (aggrecan, collagen II and collagen VI) decreased ($p<0.001$) during the first week of culture for both non-plated and plated conditions. During week two, all anabolic genes increased over time. Plated condition, however, led to generally higher gene expression levels by 0.83 to 920-fold for all anabolic genes when compared to non-plated conditions over the two weeks. Relative expression for the catabolic gene MMP-13 did not significantly change over the two-week period for the plated condition, but was 54.7% lower ($p<0.001$) on average for the non-plated condition compared to the plated condition.

Proteoglycan synthesis was measured by $^{35}SO_4$ incorporation into newly synthesized GAGs (Figure 4.6). Proteoglycan synthesis was 25-fold higher ($p<0.001$) for the plated condition one day post-encapsulation over the non-plated condition. By days 3 and 5, synthesis rates were comparable.

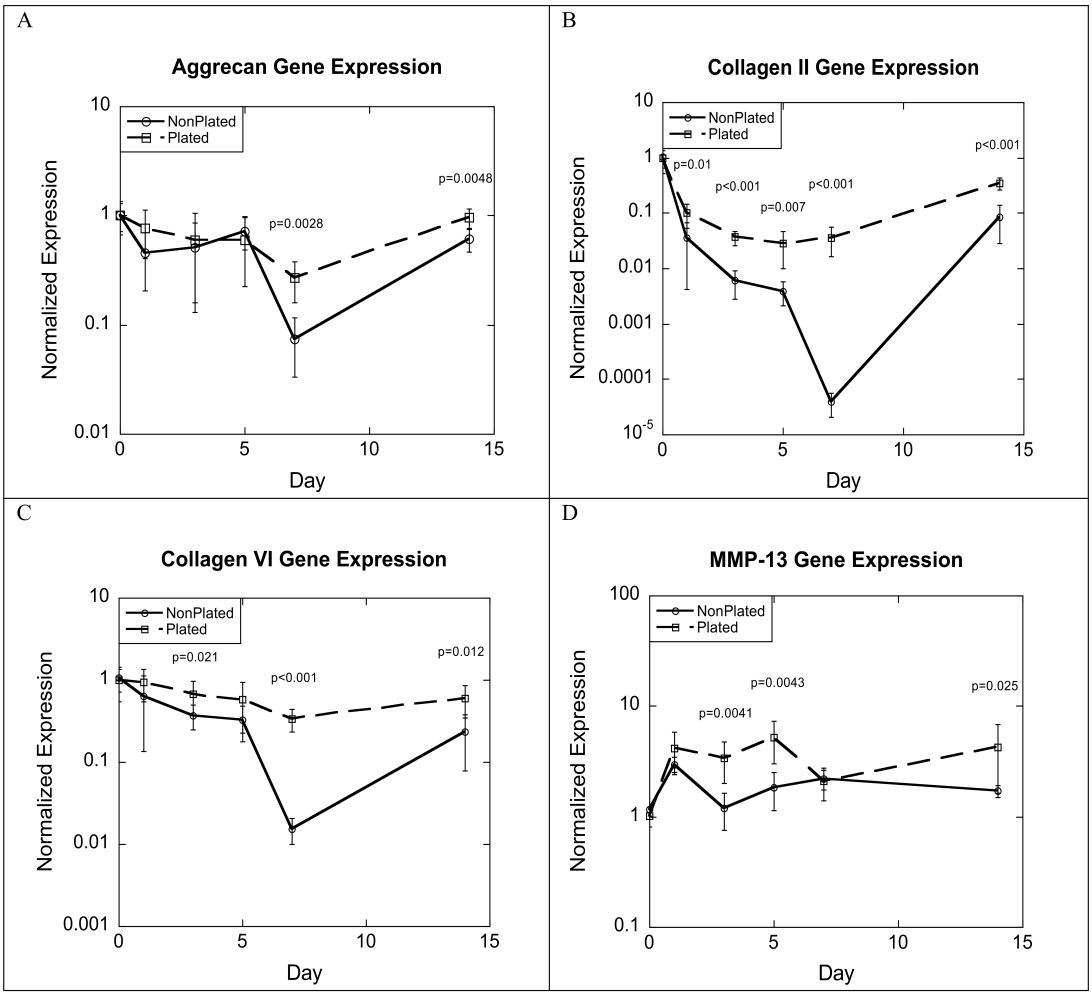

Figure 4.5: Normalized gene expression for (A) aggrecan, (B) collagen type II, (C) collagen type VI, and (D) matrix metalloproteinase-13 (MMP-13) in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions when photoencapsulated and cultured in PEG gels. Normalized expression represents relative expression for each gene normalized to the average expression at day 0 for the respective condition. Data are presented as the mean and error bars represent the 95% confidence interval of the mean from three independent experiments (n=3 biological replicates). P values represent significant differences between experimental conditions for a given time point.

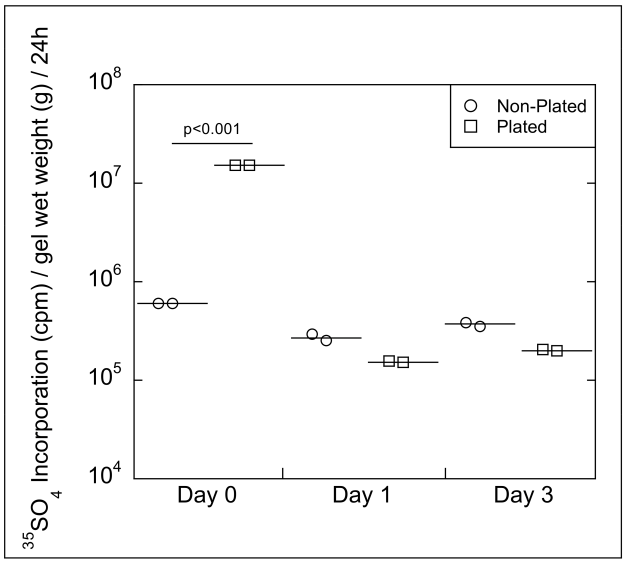

Figure 4.6: Proteoglycan synthesis measured by $^{35}SO_4$ incorporation over a 24 hour period in chondrocytes from non-plated (without PCM) and plated (with PCM) conditions when encapsulated and cultured in PEG gels as measured in counts per minute (cpm) and normalized to the wet weight of the construct in grams. Data points for each condition (non-plated and plated) and time point from two independent experiments are presented (n=2 biological replicates). P values represent significant differences from the non-plated condition for a given time point.

4.4 Discussion

This study confirms that chondrocytes incur oxidative stress during photoencapsulation, evidenced by increased intracellular ROS and lipid peroxidation. The presence of PCM partly mitigates polymerization-induced oxidative stress leading to higher anabolic gene expression and improved tissue synthesis in bovine chondrocytes when photoencapsulated in PEG hydrogels. Our findings suggest that PCM serves as a natural defense mechanism against radicals and polymerization-induced ROS, thus protecting chondrocytes during photoencapsulation.

During photoencapsulation, chondrocytes are exposed to radicals generated by the photopolymerization reaction and by extracellular ROS generated from photoinitiator radicals reacting with oxygen. Both radicals and ROS have damaging effects on chondrocytes [15, 28, 29, 30] and our findings provide evidence of increased intracellular ROS in chondrocytes when exposed to photoinitiator molecules and light, i.e. the photoinitiation step of photopolymerization. We postulated

that intracellular ROS generation may result from several possible modes of action: 1) photoinitiator molecules are taken up by cells and upon activation by UV light react with intracellular oxygen produce intracellular ROS (photodynamic effect), 2) extracellular ROS generated during photoinitiation diffuses into cells and is detected as intracellular ROS, and 3) ROS produced by the polymerization and/or radicals formed during polymerization react with cells, e.g. the cell membrane, leading to downstream events that generate intracellular ROS.

To address the first mode, our findings investigating the photodynamic effect indicated that only a small fraction of photoinitiator molecules is taken up by chondrocytes causing intracellular ROS generation upon UV exposure, accounting for ∼1-9% (depending on photoinitiator concentration) of the total intracellular ROS. As carboxy-H_2DFFDA may be oxidized by both photoinitiator radicals and ROS, we cannot distinguish between photoinitiator radicals and ROS generated within the cell. Nonetheless, these findings suggest that the photodynamic effect is minimal.

For the second mode to occur, stability of radicals and ROS must be considered. Radicals have limited distances over which they can diffuse due to their short half-life, while more stable non-radical based ROS can diffuse over considerable distances and readily enter cells. This observation suggests that only a small fraction of ROS or photoinitiator radicals are likely to diffuse into cells, but that any stable ROS, e.g. hydrogen peroxide, which may form through subsequent reactions, could enter cells. Differences can be inferred between levels of radicals and ROS generated by the photoinitiation reaction extracellularly and levels of intracellular ROS. With the volume fraction of cells being ∼1%, similar levels of fluorescence detected under both scenarios indicate that intracellular ROS concentrations are likely substantially higher than radical/ROS concentration outside the cell. This observation points toward the last proposed mode of action, suggesting radicals and/or ROS generated by photoinitiation damages cells initiating downstream events that trigger intracellular ROS generation.

Indeed, photopolymerization induced lipid peroxidation was confirmed by a greater presence of MDA [31, 32] in cells that were photoencapsulated. Others have shown that lipid peroxidation leads to intracellular ROS generation in other cell types [33, 34]. Lipid peroxidation

requires highly reactive species and only certain radicals/ROS are capable of reacting with membrane lipids [35]. Possible candidates formed during photopolymerization include photoinitiator radicals, macroradicals formed during propagation, and highly reactive ROS, such as peroxy radicals, which are formed as a result of the photopolymerization reaction occurring in the presence of oxygen. Photopolymerization-induced lipid peroxidation was not sustained and by day 3 MDA levels were similar to agarose, suggesting a return to basal levels of lipid peroxidation, which has been observed in 2D chondrocyte cultures [36]. Taken together, our findings point towards the hypotheses that intracellular ROS generated in chondrocytes during photoencapsulation results from direct damage to the cell, triggering intracellular ROS generation, and to a lesser extent by extracellular photoinitiator radicals and ROS diffusing into chondrocytes.

When chondrocytes formed some of their own PCM prior to photoencapsulation, intracellular ROS generation was significantly reduced by ∼40% and the level of lipid peroxidation induced by photoencapsulation was partially abrogated. This observation suggests that radicals and/or extracellular ROS generated during photoencapsulation may diffuse into cells and that the presence of the PCM can reduce, but not abrogate this mode of action. It is well known that proteins and other matrix molecules can react with and terminate radicals [10, 36]. Nascent PCM was comprised of chondroitin sulfate, one of the main building blocks of aggrecan, and collagen VI, the main collagen in the chondrocyte PCM [37]. Of the molecules found in cartilage PCM, hyaluronan a building block of aggrecan is most well-known for its antioxidant properties, acting as radical scavengers [38]. While hyaluronan was not explicitly detected in the nascent PCM, the presence of chondroitin sulfate suggests that aggrecan and hyaluronan are present [39]. This property, along with the ability of the PCM to act as a physical barrier between the extracellular environment and the cell membrane, appears to have reduced radical damage to membrane lipids during photoencapsulation and reduced intracellular ROS.

While photoinitiation alone leads to oxidative stress, it is important to recognize that during photoencapsulation, the concentration of radicals rises exponentially. This effect, referred to as auto-acceleration, occurs because mobility of large macroradicals becomes increasingly more lim-

ited, while macromers can readily diffuse and continue propagation [11, 40]. For photoinitiator concentrations typically employed for cell encapsulation (i.e., 2mM), our data show that intracellular ROS generated during photoencapsulation is significantly higher than for cells simply exposed to photoinitiator radicals (i.e., comparing Fig 3B at 2mM with 3E). This finding suggests that in addition to photoinitiator radicals, macroradicals generated during polymerization also have damaging effects on cells leading to oxidative stress.

Oxidative stress in chondrocytes is known to have downstream effects, negatively impacting their metabolism and inducing tissue destruction [16, 41, 42]. In this study, photoencapsulation of chondrocytes lacking a PCM exhibited reduced anabolic gene expression and reduced matrix synthesis when compared to chondrocytes with a PCM. This finding suggests that oxidative stress incurred by chondrocytes during photoencapsulation may have longer-term negative effects on cell metabolism, which is supported by other studies. For example, induction of ROS in cartilage explants led to decreased collagen II and aggrecan gene expression and collagen II production [43]. *Ex vivo*, hydrogen peroxide treatment of cartilage explants induced lipid peroxidation and enhanced proteolytic activity [33]. ROS is closely linked to osteoarthritis and is thought to upregulate catabolic enzymes and induce tissue degradation in osteoarthritic cartilage [36]. In this study, catabolic activity, specifically gene expression of MMP-13, which is prevalent in osteoarthritic cartilage [34], decreased over time in chondrocytes lacking PCM during photoencapsulation. While oxidative stress has been shown to upregulate MMP-13 activity in chondrocytes [16, 44], exposure to radicals may be too short (i.e., on the order of minutes) to induce a catabolic response longer-term. In contrast osteoarthritic chondrocytes are exposed to ROS for prolonged periods [36]. It is possible that radicals and ROS may affect post-translational MMP-13 activity, which was not evaluated in this study. Interestingly, MMP-13 gene expression in chondrocytes encapsulated with a PCM was greater than without a PCM. In non-degrading PEG hydrogels the relatively tightly crosslinked network limits matrix elaboration to the pericellular space [44]. With increased matrix deposition in the plated condition, there may be a greater need for matrix turnover [45] where MMP-13 may be involved [46]. Future studies are needed to confirm these hypotheses. Nonethe-

less, our findings confirm the presence of PCM prior to photoencapsulation improves chondrocyte anabolism longer-term.

In conclusion, the photoencapsulation process is known to lead to the generation of photoinitiator radicals and macroradicals on propagating chains and to extracellular ROS when oxygen is present, which induce oxidative stress in chondrocytes. This oxidative stress appears to have longer-term negative effects on chondrocyte anabolism. The presence of a PCM, however, reduces the level of oxidative stress and improves chondrocytes anabolic activity. Our findings suggest that minimizing oxidative stress, such as through the presence of PCM, may have long-term beneficial effects on tissue elaboration when employing photopolymerizations to encapsulate chondrocytes for cartilage tissue engineering applications.

4.5 Acknowledgements

NF: Design of the study, acquisition and interpretation of data, drafting, revising and final approval of the article. CB: Design of the study, acquisition and interpretation of data, drafting and final approval of the article. SJB: Design of the study, interpretation of data, drafting, revising and final approval of the article.

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Chapter 5

Physiological Compressive Loading Regimes Differentially Regulate Chondrocyte Anabolic and Catabolic Activity with Age

Functional cartilage tissue engineering strategies employing dynamic compressive loading have emerged as an effective means to bolster tissue production towards producing cartilage with the required mechanical properties. As patients in need of new cartilage span a wide range of age groups, questions arise as to the role of cell age in response to physiological loading. The goal of this study was to characterize the anabolic and catabolic responses of chondrocytes to physiological loading, with respect to loading frequency and strain, and to determine if cell age plays a role in these responses. Bovine chondrocytes isolated from 3 week and 1-2 year old donors were encapsulated in poly(ethylene glycol) hydrogels and subjected to intermittent dynamic loading at 1 or 0.3Hz and 5 or 10% amplitude strain up to two weeks. Total sGAG production was highest with 0.3Hz and 5% loading in juvenile chondrocytes and with 1Hz and 5% loading in adult chondrocytes, while overall deposition of aggrecan, collagen II and collagen VI were highest with 1Hz and 5% loading in juvenile chondrocytes and 1Hz and 10% loading in adult chondrocytes. Both cell populations were anabolically and catabolically stimulated by the application of dynamic loading. The results from this study confirmed that chondrocytes in PEG hydrogels respond differentially to dynamic loading with respect to frequency and strain and demonstrated that chondrocyte age plays a role in how cells responded to loading. Overall, our findings suggest that tissue engineering strategies may need to be optimized for the age of the donor to enhance tissue production and minimize tissue destruction.

5.1 Introduction

Cartilage damage occurring from injury or degenerative disease, such as osteoarthritis, can cause significant pain and limit mobility. Because cartilage has a limited capacity for self-repair, cartilage tissue engineering has emerged as a promising therapeutic approach [1]. While significant progress has been made towards engineering cartilage, the mechanical properties of this tissue remain inferior to the native tissue [2, 3]. The application of physiological loading in the form of dynamic compressive loading or hydrostatic pressures [4] has been shown to bolster tissue production and improve mechanical properties of engineered cartilage [5, 6, 2]. Towards translating these technologies clinically, questions arise as to whether donor age will impact how cells respond to physiological loading. This question becomes particularly important as patients who require tissue replacement therapies are from a range of age groups. Few studies, however, have investigated the role of age in how chondrocytes respond to mechanical cues from their extracellular environment.

Aging causes distinct changes in the cartilage extracellular matrix and its properties as well as changes in chondrocyte metabolism. Articular cartilage, which is responsible for the dissipation of loads within joints, has a unique composition of collagen type II and aggrecan, which provides tensile and compressive strength [7, 8, 9, 10], respectively, and which changes with age [11, 12]. Cartilage is maintained and slowly remodeled by resident chondrocytes with a delicate balance between anabolism and catabolism [13]. However, aging leads to reduced capacity in tissue production concomitant with increases in the secretion of tissue degrading enzymes, such as matrix metalloproteinases (MMPs) and aggrecanases. These changes cause degradation of the extracellular matrix and contribute to age-related changes in the tissue and its mechanical properties [14, 15, 16, 17]. Therefore, chondrocyte response to mechanical cues from the extracellular environment will likely be age dependent and a function of age-related changes in ECM and the cell.

It is well known that mechanical stimulation of cartilage and chondrocytes regulates chondrocyte biosynthesis as well as tissue remodeling and is important for homeostasis [18, 19, 20]. A number of *in vitro* studies have shown that dynamic compressive loading impacts chondrocytes in

a frequency and strain dependent manner. For example, Lee et al. [21] have found that dynamic loading at 0.3Hz frequency had an inhibitory effect on sGAG synthesis while loading at 1Hz stimulated sGAG production in chondrocytes encapsulated in agarose gels. However, a direct comparison between different age groups has not yet been performed.

Photopolymerizable hydrogels based on poly(ethylene glycol) (PEG) hydrogels are promising candidates for cartilage tissue engineering. Their mechanical properties are tailorable. Degradable crosslinks are readily incorporated to facilitate macroscopic tissue growth [22]. Photopolymerization offers injectability on clinically relevant time scales [23]. However loading conditions have yet to be optimized for this system, particularly with respect to age [24, 25]. Therefore, the overall objective for this study was to better understand how loading regimes, based on dynamic loading frequency and strain influence anabolism and catabolism and how age affects this response. Specifically, bovine chondrocytes from different aged donors were subjected to intermittent dynamic loading at 1 or 0.3Hz and 5 or 10% strain, loading regimes which have successfully bolstered tissue production in other *in vitro* culture systems [26, 27]. We hypothesized that juvenile chondrocytes will produce more tissue than adult under free swelling and loaded conditions and that aggrecan and collagen production will stimulated by different loading regimes with respect to age. Anabolic activity, as defined by aggrecan/sGAG, collagen II and collagen VI deposition, and catabolic activity, as defined by aggrecan and collagen II degradation products, were analyzed. Taken together, findings from this study provide new insights into age-related responses to loading within PEG hydrogels and help to identify appropriate loading regimes for *in vitro* cartilage tissue engineering.

5.2 Methods

5.2.1 Chondrocyte Isolation

Chondrocytes referred to as adult (skeletally mature) were isolated from full depth articular cartilage harvested from the metacarpalphalangeal joints of 2-3 year old free range steers obtained from a local abattoir (Arapahoe Meat Co., Lafayette, CO) within 12 hours of slaughter. Two

separate isolations were performed with 3 animals per isolation and by pooling of all cells for each isolation. Chondrocytes referred to as juvenile (skeletally immature) were isolated from full depth articular cartilage harvested from the patellar-femoral groove of 1-3 week old calves (Research 87 Inc., Natick, MA) within 24 hours of slaughter. Two separate isolations were performed with one animal per isolation. Cartilage slices were rinsed in phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), finely diced, and enzymatically digested in 0.02% collagenase II (Worthington Biochemical Corp., Lakewood, NJ) in Dulbeccos Minimum Essential Medium (DMEM, Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C for 16 hours on a figure-8 shaker. Cells were washed twice in PBS with 0.02% EDTA and resuspended in PBS+P/S. Cell viability was 85-91% for all isolations determined by Trypan blue exclusion.

5.2.2 Hydrogel Formation and Chondrocyte Encapsulation

PEG dimethacrylate (PEGDM) was synthesized by reacting linear PEG (3000MW, Fluka) with methacrylic anhydride (94%, Sigma) at a molar ratio of 1:10 with trace amounts of hydroquinone (Sigma) under microwave radiation [28] followed by purification via precipitation. Percent methacrylation of end hydroxyls was 95% determined by ^{1}H NMR and comparing area under the vinyl peaks (δ=$\sim$5.6 and δ=$\sim$6.1ppm) and methylene peaks (δ=$\sim$3.6-4ppm). Chondrocytes at 20x10^6 cells/ml were mixed with a solution of 10% w/v PEGDM, 0.05% w/w Irgacure 2959 photoinitiator (Ciba Specialty Chemicals, Tarrytown, NY) in chondrocyte medium (DMEM, 10mM HEPES, 0.1M non-essential amino acids (Invitrogen), 0.4mM L-proline, 0.14mM L-ascorbic acid, 1% v/v P/S, 0.5 μg/ml amphoterecin B, 5.24 g/L NaCl, 0.26 g/L KCL, and 10% fetal bovine serum). Cell-macromer solution was exposed to 365 nm light ($\sim$4 mW/cm^2) for 10 minutes to form cylindrical gels (4.5 mm height, 4.5 mm diameter). Gels were cultured in chondrocyte medium for 24 hours before physiological loading to allow the gels to reach an equilibrium volume.

5.2.3 Physiological Loading

After 24 hours of free swelling culture, gels were placed in custom bioreactors [29, 25]. Hydrogels were dynamically loaded in unconfined compression 8 cycles per day of 30 minutes on and 90 minutes off up to two weeks. During rest periods, tare strains were ∼1% strain. Four loading regimes were investigated, 1 or 0.3Hz and 5 or 10% strain, based on previous results showing enhanced tissue production in PEG [29], agarose [30, 20] and peptide hydrogels [6]. A similar set of control gels were cultured under free swelling conditions. Gels and media were collected after 0, 3, 7, 10 and 14 days of culture for analysis, where day 0 corresponds to 24 hours after chondrocyte encapsulation. Cell viability was assessed over the two weeks using a LIVE/DEAD® stain of calcein AM and ethidium homodimer (Invitrogen). No significant differences in viability were observed between loading conditions over time or with age (data not shown).

5.2.4 Measurement of sGAG production

At prescribed times, gel and media samples were collected for analysis of sGAG content. Gels were homogenized and enzymatically digested (125 μg/ml Papain (Worthington), 10 mM L-cysteine, 100 mM phosphate and 10 mM EDTA in DI water (pH 6.3) overnight at 60°C). Media and gel samples were analyzed for sGAG content using the 1,9-dimethylmethylene blue dye method [31]. Total sGAG in gels and media was normalized to wet weight of each gel, as well as the day 0 time point. Two independent experiments were performed each with a sample size of three (n=3).

5.2.5 Immunohistochemistry

Gels were collected at 14 days and fixed in 4% (v/v) paraformaldehyde (Sigma) in PBS for 24 hours, transferred to 30% sucrose for 3 days, dehydrated and embedded in paraffin. Sections (10 μm) were pretreated with 2080 U/ml hyaluronidase (Sigma) and 100 mU/ml chondroitinase ABC (Sigma) for 30 minutes at room temperature followed by treatment with 0.05% Triton X-100. Sections were blocked with 1% BSA for 5 minutes. Primary antibody for mouse anti-human aggrecan IgG1 (1:2.5, US Biologicals, Swampscott, MA), rabbit anti-human collagen II IgG (1:25, US Biolog-

icals), rabbit anti-human collagen VI IgG (Abcam, Cambridge, MA), or rabbit anti-human C1, 2C IgG (1:100, IBEX Pharmaceuticals, Montreal, QC, CA) was applied at 4°C overnight. Secondary antibody, AlexaFluor 546 (1:200, Invitrogen) for aggrecan and AlexaFluor 488 (1:200, Invitrogen) for all others, was applied at room temperature for 2 hours. Nuclei were counter stained with DAPI. Sections were imaged on a CLSM (Zeiss 510 LSM). Two independent experiments were performed with a sample size of two per experiment. Semi-quantitative analysis of the images was performed, where the average fluorescence of aggrecan, collagen II, collagen VI or C1,2C was measured using NIH Image J and was normalized to the number of nuclei present in each image.

5.2.6 Western Blot Analysis

Medium was collected from day 11 to day 14 of loading for western blot analysis for the ARG fragment of aggrecanse-degraded aggrecan and the FFGV fragment MMP-3 degraded aggrecan [32, 33]. Protein was quantified using the BCA® assay (Thermo Scientific, Rockford, IL). Total protein (25 μg) was deglycosylated to reveal the antibody binding domain (0.1 U/ml keratinase II and 0.5 U/ml chondroitinase ABC (Sigma) at 37°C overnight), combined 1:1 with laemmli buffer (BioRad, Hercules, CA) containing 5% β-mercaptoethanol, then boiled for 10 minutes. Deglycosylated protein and a Kaleidescope Protein standard (BioRad) were loaded into a 4-12% polyacrylamide gel (BioRad) and run at 200 V for 45 minutes then transferred to a PVDF membrane (BioRad) and run at 100 V for 60 minutes. The membrane was blocked in 15% dry milk with 0.1% v/v Tween 20 (PBST, Sigma) for 4 hours at room temperature. Membranes were treated with primary antibody for the N-terminal neoepitope ARG-fragment or FFGV-fragment (1:100, MD Bioproducts, St. Paul, MN) at 4°C overnight and then treated with a secondary antibody (AlexaFluor 546) for 2 hours at room temperature. The membrane was imaged on a Versadoc imaging system (BioRad). Two independent experiments were performed with a sample size of n=2.

5.2.7 Statistical Analysis

Data are reported as the mean with a 95% confidence interval about the mean. To determine statistical significance an ANOVA with Tukeys post-hoc analysis was used with α=0.05. A three-way ANOVA with Tukeys post-hoc analysis was used to compare the effects of age, loading and time in culture with α=0.05. All data were from independent observations, followed a Gaussian distribution and exhibited a homogeneous variance.

5.3 Results

Total sGAG production by adult and juvenile chondrocytes in PEG hydrogels is shown in Figure 5.1A and B. For both cell populations, total sGAG production increased with culture time for free swelling and all dynamically loaded conditions. For adult chondrocytes, the dynamic loading regime of 1Hz and 5% strain led to the highest up-regulation in total sGAG production by 88% over free swelling controls after 14 days. All other loading regimes investigated led to a significant inhibition in total sGAG production or similar production when compared to free swelling controls after 14 days. For example, the loading regime of 0.3Hz and 10% strain was inhibitory throughout the 14 day culture period reaching maximum inhibition at day 14 by 45%. Contrarily, the loading regime 1Hz and 10% strain initially inhibited sGAG production by 33.6% up to day 10 but increased to levels similar to that of the free swelling controls by day 14. For juvenile chondrocytes, the dynamic loading regime of 0.3Hz and 5% strain led to the highest up-regulation in total sGAG production by 220% at day 7 and 280% at day 14 over free swelling controls. All other loading regimes had no effect on total sGAG production when compared to free swelling controls over 14 days with the exception of 1 Hz and 5% strain at 3 days, which was slightly but significantly higher than the controls. Three-way ANOVA analysis of the interactions between age, loading condition and time are presented in Table 5.1. All factors significantly affected sGAG production ($p<0.001$) and interactions between age and loading, age and time, and loading and time were all significant ($p<0.001$).

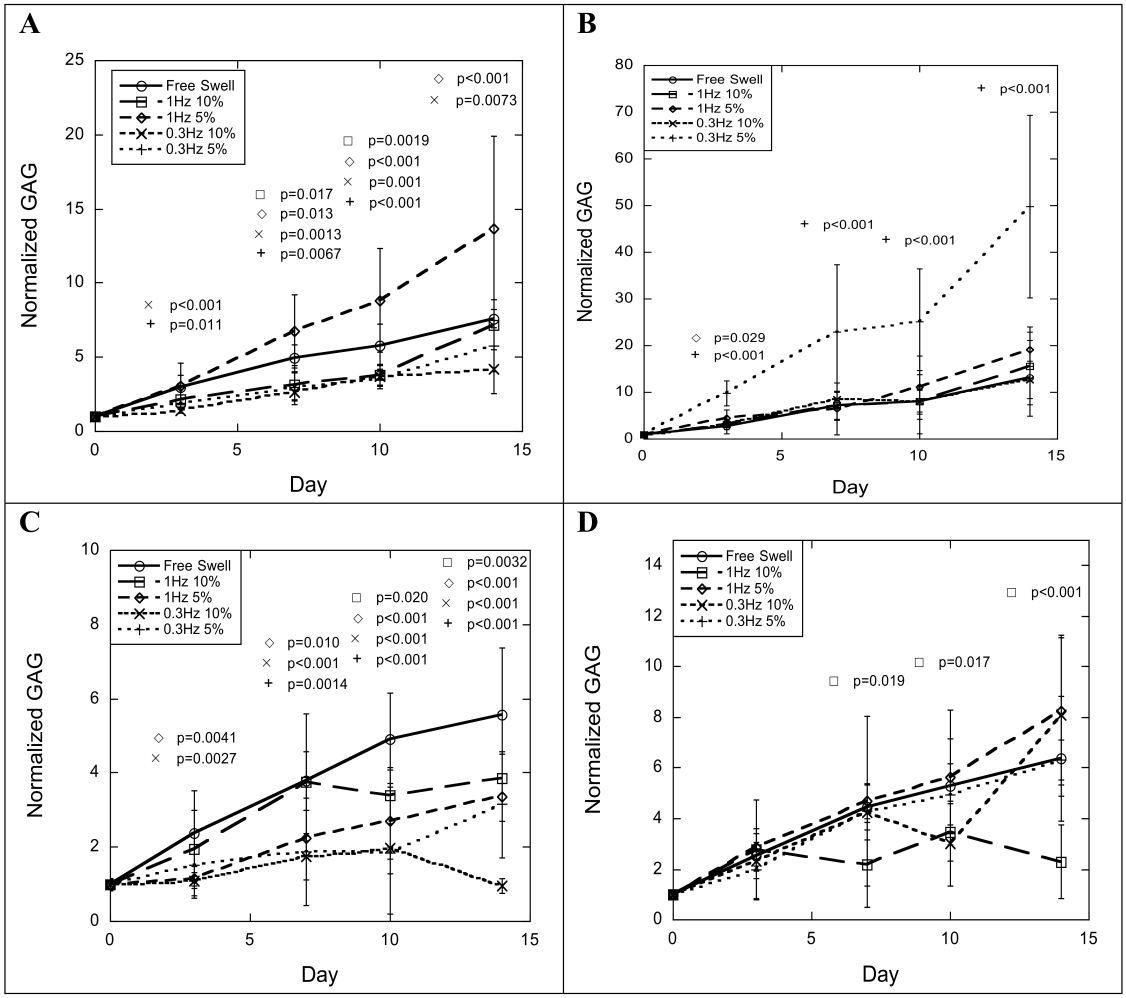

Figure 5.1: Total GAG in media and gels in adult (A) and juvenile (B) chondrocytes and Total GAG in gels only in adult (C) and juvenile (D) chondrocytes with 1 or 0.3Hz and 10 or 5% strain. Data represent the mean over a sample size of n=5 with a 95% confidence interval. Statistics presented are comparing loading conditions to the free swelling samples.

SGAG production in the gels only is presented in Figure 5.1C and D. SGAG production in the gels was markedly different than total sGAG production in adult and juvenile chondrocytes. For the adult chondrocytes, all loading conditions gave less sGAG in the gel than the free swelling condition, however 1Hz/10% loading gave the highest sGAG of all conditions, with an increase in 12.7-17.8% at day 14. In juvenile chondrocytes, loading at 1Hz/10% led to a 64.1% decrease in sGAG production in the gel, compared to free swelling at day 14. All other conditions were not significantly different than the free swelling condition.

Table 5.1: Three-Way Analysis of Variance Results

Source	DOF	SS	MS	F	P
Age	1	2767.7	2767.7	195.4	<0.001
Loading	4	2525.8	631.5	44.58	<0.001
Day	3	3489.1	1160.6	81.94	<0.001
Age/Loading	4	3219.1	804.8	56.8	<0.001
Loading/Time	12	903.7	75.3	5.3	<0.001
Age/Time	3	897.6	299.2	21.1	<0.001

Percent of total sGAGs produced but released from the gel into the media is shown in Figure 5.2 for adult and juvenile chondrocytes. For both cell populations, the majority of the sGAGs produced were released to the medium and ranged from 79% to 96% of total sGAG. For adult chondrocytes, the condition that led to the lowest percentage of sGAG release at day 7 was 1 Hz/10% strain. By day 14, mean percent sGAG release was higher for all loaded samples when compared to free swelling and was significantly higher by ∼10% for 1Hz/5% strain, 0.3Hz/10% strain and 0.3Hz/5% strain. For juvenile chondrocytes, the conditions at day 7, which led to the lowest percentage of sGAG release was free swelling and 1Hz/5% strain and which led to the highest percentage of sGAG release was 1 Hz/10% strain by 14.3% over free swelling controls. By day 14, all conditions had similar levels of sGAG loss to the media.

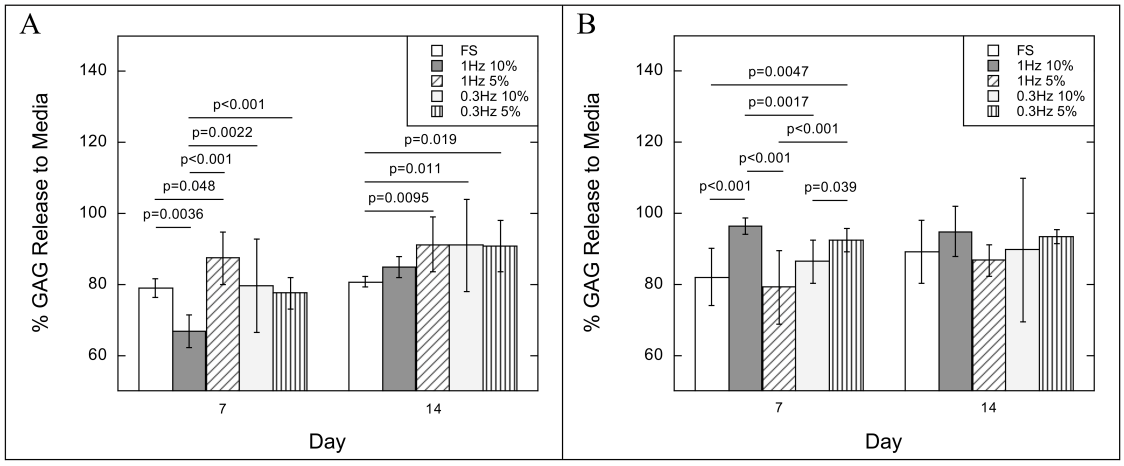

Figure 5.2: Percent GAG release to the media at day 7 and 14 of culture calculated from DMMB measurement of GAG in the gels and media from adult (A) and juvenile (B) chondrocytes encapsulated in PEG hydrogels. Data represent the mean over n=5 samples with a 95% confidence interval.

Spatial deposition is shown for aggrecan (Figure 5.3A), collagen II (Figure 5.4A) and collagen VI (Figure 5.5A) by adult and juvenile chondrocytes in the hydrogels. For both cell populations, aggrecan, collagen II and collagen VI deposition was detected in the pericellular region in free swelling and all loaded conditions. For adult chondrocytes, semi-quantitative aggrecan deposition (Figure 5.3B) was the lowest under 1Hz/5% strain and 0.3Hz/10% strain with a 80% and 59% decrease from the free swelling condition, respectively. For juvenile chondrocytes, semi-quantitative aggrecan deposition (Figure 5.3C) was 80% lower under 1Hz/10% strain, while all other loading conditions showed similar depositions to the free swelling condition. For adult chondrocytes, semi-quantitative collagen II deposition (Figure 5.4B) was the lowest under 1Hz/5% strain and 0.3Hz/5% strain at 70% and 38% below free swelling, respectively. In juvenile chondrocytes, semi-quantitative collagen II deposition (Figure 5.4C) was inhibited by 85% under 1Hz/10% strain compared to the free swelling condition. For adult chondrocytes, semi-quantitative collagen VI deposition (Figure 5.5B) was highest under 1Hz/10% strain and 0.3Hz/10% strain, with increases of 61% and 52% above swelling conditions, respectively. Semi-quantitative analysis of collagen VI deposition in juvenile chondrocytes (Figure 5.5C) was notably decreased under the 1Hz/10% strain, 0.3Hz/10% strain, and 0.3Hz/5% strain conditions, by 76%, 93% and 46%, respectively, when compared to the free swelling control.

Catabolic degradation of aggrecan was detected in the media between day 11 and day 14 by western blots for the FFGV-fragment (Figure 5.6A and B) and the ARG-fragment (Figure 5.7A and B). Increased staining for the FFGV-fragment was observed with application of loading in adult and juvenile chondrocytes, compared to the free swelling condition, while juvenile cells appeared to have a greater increase in staining compared to adult. No visible differences in FFGV staining were observed between loading conditions in either adult or juvenile samples. Increased staining for the ARG-fragment was also observed with application of loading in adult and juvenile chondrocytes, compared to the free swelling condition, where the juvenile cells appeared to have a larger increase than the adult. In adult chondrocytes, loading at 0.3Hz/5% strain appeared to increase staining for the ARG-fragment the most over the free swelling samples, while no stark differences were noticed

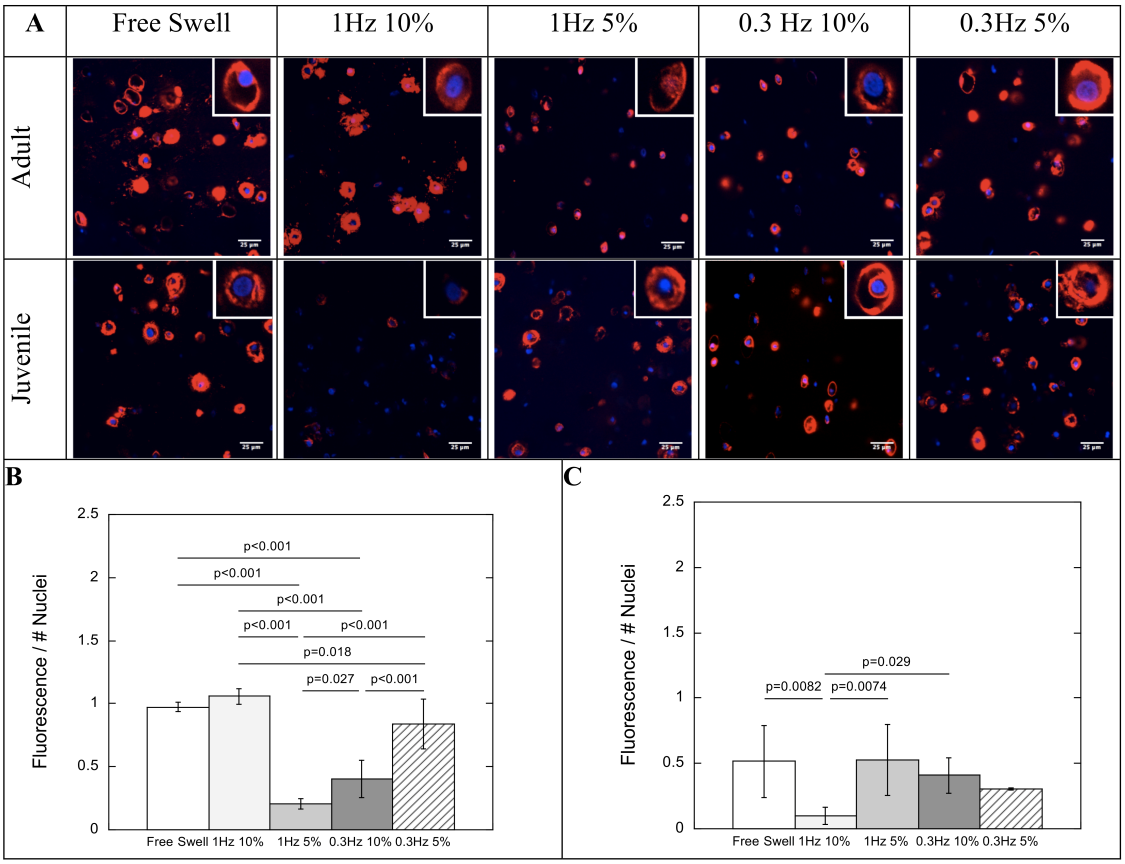

Figure 5.3: Immunohistochemistry images (A) and semi-quantitative image analysis for aggrecan in adult (B) and juvenile (C) chondrocytes encapsulated in PEG hydrogels after 14 days under dynamic physiological loading conditions. Images are representative of a sample size of n=2.

between loading conditions in the juvenile samples.

Catabolic degradation of collagen was detected by the presence of the C1,2C fragment through immunohistochemistry (Figure 5.8A) at day 14. For the adult chondrocytes (Figure 5.8B), the C1,2C fragment staining was decreased by 85% with application of 1Hz/5% strain, while loading at 0.3Hz/10% strain and 0.3Hz/5% strain increased staining by 45% and 66%, respectively, compared to the free swelling condition. Semi-quantitative analysis of images for the C1,2C fragment for juvenile chondrocytes (Figure 5.8C) showed no significant difference in staining between the free swelling and loaded conditions.

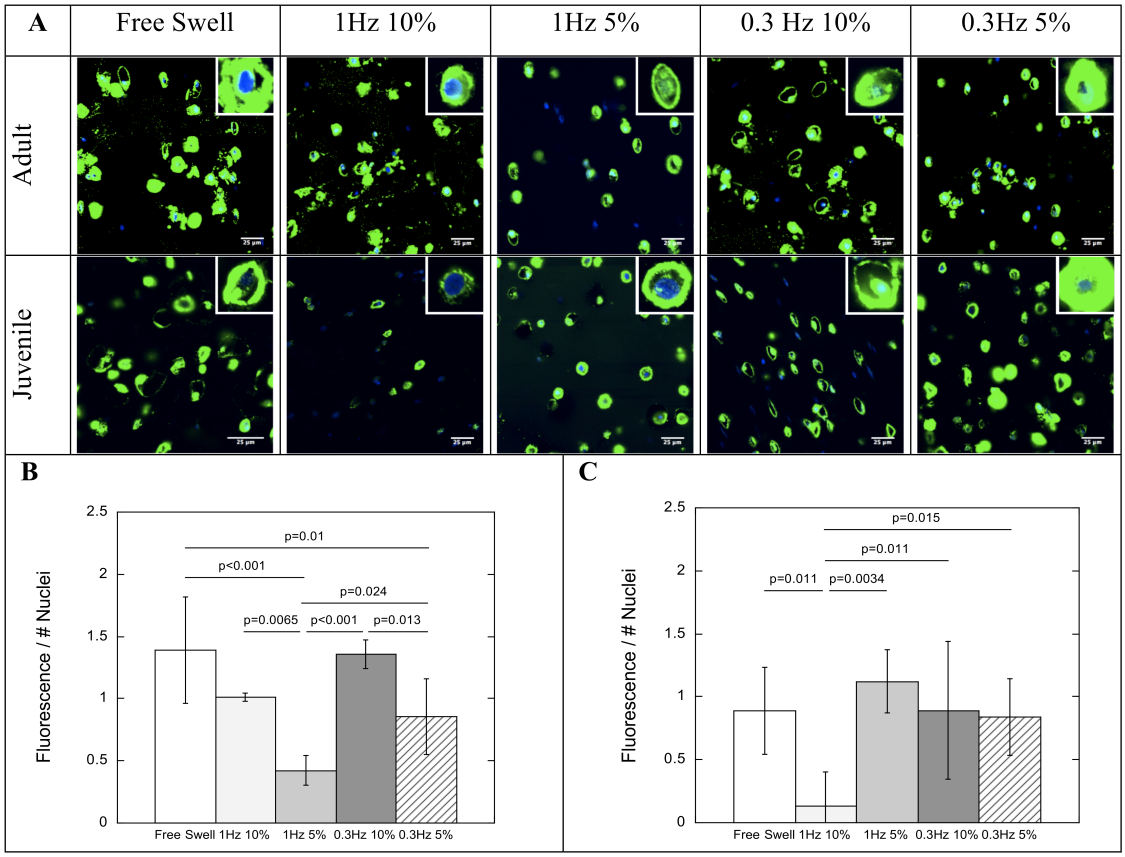

Figure 5.4: Immunohistochemistry images (A) and semi-quantitative image analysis for collagen II in adult (B) and juvenile (C) chondrocytes encapsulated in PEG hydrogels after 14 days under dynamic physiological loading conditions. Images are representative of a sample size of n=2.

5.4 Discussion

This study demonstrates that chondrocytes respond differentially to dynamic compressive loading with respect to frequency and strain when encapsulated in PEG hydrogels and that the response is dependent on age. Within the loading regimes investigated, juvenile chondrocytes responded favorably with increased sGAG production under 0.3Hz/5% loading regime. These results, however, were accompanied by catabolic activity. Adult chondrocytes, however, responded favorably with increased sGAG production and lower catabolic activity under 1Hz/5% and 1Hz/10% loading regimes depending on the type of ECM deposited. Taken together our findings reinforce the idea that the design of a cartilage tissue engineering strategy will need to be tailored to the age

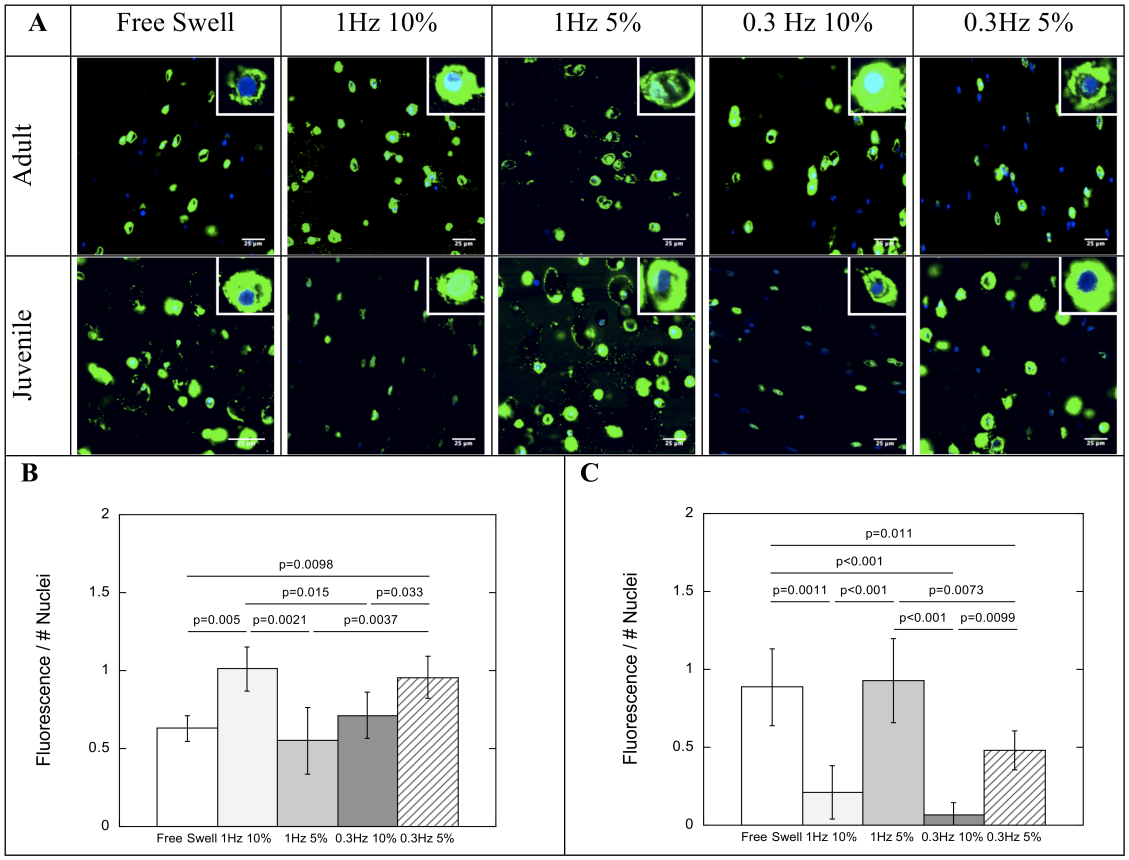

Figure 5.5: Immunohistochemistry images (A) and semi-quantitative image analysis for collagen VI in adult (B) and juvenile (C) chondrocytes encapsulated in PEG hydrogels after 14 days under dynamic physiological loading conditions. Images are representative of a sample size of n=2.

of the donor.

Sulfated glycosaminoglycans (sGAGs) are typically one of the first matrix molecules secreted in abundance by freshly isolated chondrocytes [34, 35]. While sGAGs are present in other matrix molecules such as decorin, and biglycan, they are predominantly associated with aggrecan through chondroitin and keratin sulfates. This study shows that chondrocyte age, loading regime and time in culture are all factors that influence sGAG production. Overall, adult chondrocytes produce less sGAG than juvenile chondrocytes, which is expected as tissue producing capabilities are known to decline with age [36]. Regardless of age, low strains led to marked improvements in total sGAG production, however adult chondrocytes required a higher frequency to respond favorably to loading.

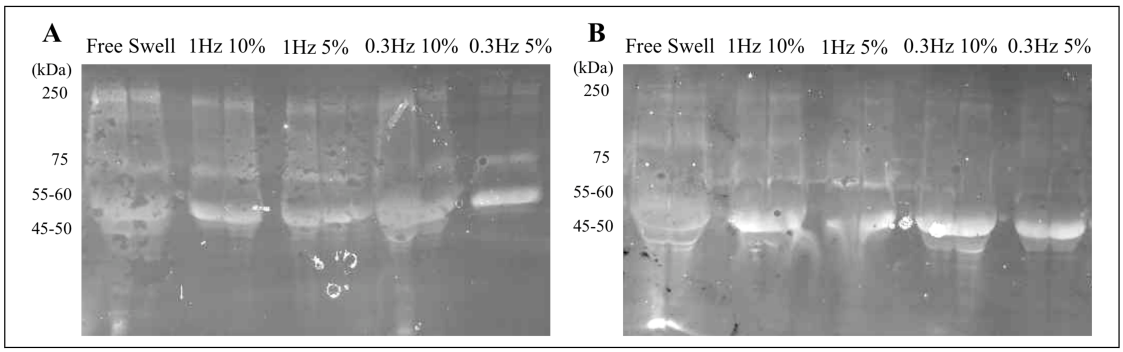

Figure 5.6: Western blot analysis of the FFGV-fragment of aggrecan detected in the media of adult (A) and juvenile (B) chondrocytes after 14 days of culture with a sample size of n=2.

Interestingly, optimal loading conditions that led to the highest sGAG production did not translate to the highest amount of aggrecan deposition within the hydrogel. In fact aggrecan deposition was either not affected or reduced in response to loading for adult and juvenile chondrocytes. For adult chondrocytes, the highest sGAG production resulted in the least amount of aggrecan deposition based on the presence of the G1 domain. Aggrecan is synthesized intracellularly from a core protein containing G1, G2 and G3 globular domains, which is then glycosylated in the golgi prior to its extracellular secretion [35]. Extracellularly, aggrecan molecules assemble with hyaluronan and link protein to form the large aggregan aggregates, which are too large to readily diffuse through the PEG hydrogels. Studies have shown that newly synthesized aggrecan can be rapidly processed extracellularly leading to the cleavage of a chondroitin sulfate-rich G3 domain [37]. This carboxyl-terminal processing is thought to be part of the normal extracellular processing of aggrecan and may be important to subsequent protease recognition of aggrecan [38]. Interestingly, the G3 degraded fragment has no known binding affinity to the ECM and is often released to the culture medium *in vitro* or synovial fluid *in vivo* [37]. Previously, our group has shown that in PEG hydrogels, the G1 domain of aggrecan is present only in the pericellular region while chondroitin sulfate can be detected throughout the hydrogel [39], agreeing with the idea that post-processing of aggrecan may lead to smaller sGAG-rich aggrecan fragments which can diffuse through the hydrogel. This idea is also supported by the high fraction of sGAG released measured in this study.

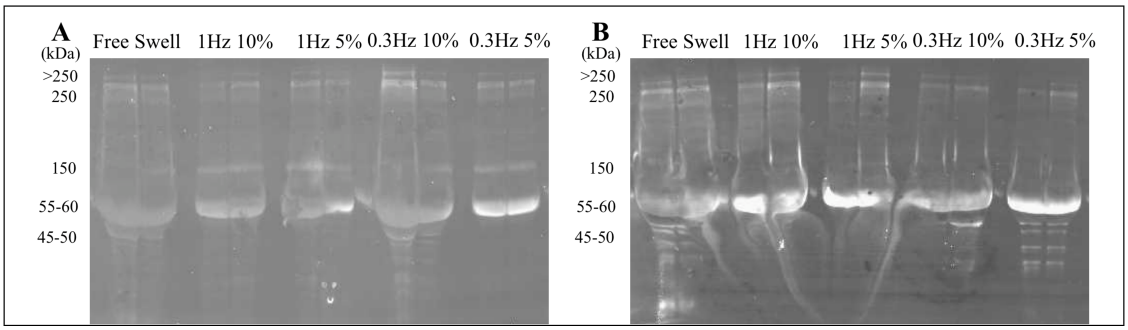

Figure 5.7: Western blot analysis of the ARG-fragment of aggrecan detected in the media of adult (A) and juvenile (B) chondrocytes after 14 days of culture with a sample size of n=2.

Therefore, the quantification of total sGAGs released and retained in the hydrogel may give a reasonably accurate indication of the amount of aggrecan synthesized by the chondrocytes.

The decreased presence of the G1 domain of aggrecan in the pericellular region, however, suggests that catabolic activity may have also been elevated in response to loading, which could also contribute to the high level of sGAG release to the media. MMP3 and aggrecanase cleaved aggrecan degraded products were detected in the culture medium for both adult and juvenile chondrocytes confirming catabolic activity. For both cell populations, it appears that aggrecan catabolism is elevated with loading; although it is not possible to make definitive conclusions based on the western blot analysis. Increases in aggrecan catabolism with dynamic loading [40, 41] or age are well documented for chondrocytes, where age associated increases in MMPs and aggrecanases can lead to greater matrix degradation [14, 40, 42, 41]. While the presence of ECM catabolism may be important to the development of functionally competent engineered cartilage [43], the presence of aggrecanase activity, which is closely associated with disease [44, 45], suggests that adverse catabolism may be occurring.

Collagen II is a large macromolecule that, similar to aggrecan, cannot diffuse through PEG hydrogels leading to its localization to the pericellular region. Collagen II deposition was either not affected or reduced under loading. In adult chondrocytes, collagen II was down regulated with 5% strain regardless of frequency. Contrarily in juvenile chondrocytes, collagen II was down regulated under 1Hz/10% loading. These findings indicate that collagen synthesis may also be

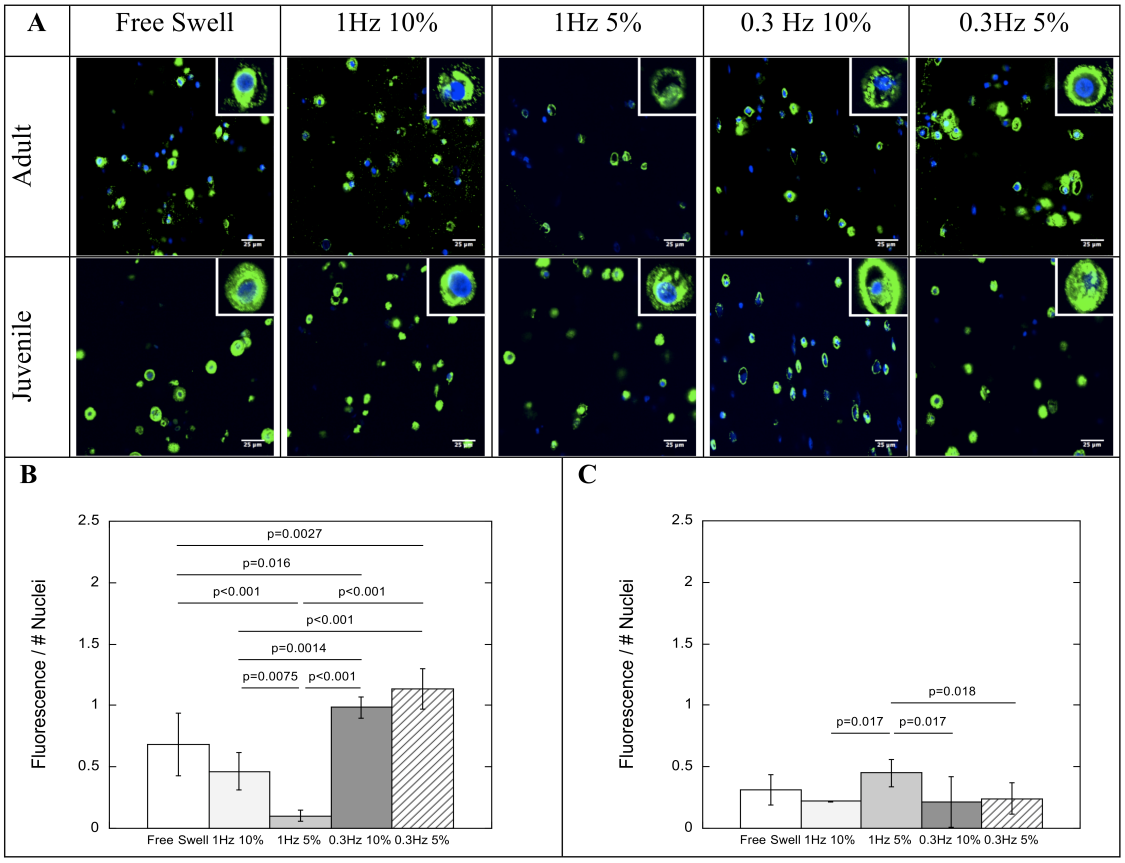

Figure 5.8: Immunohistochemistry images (A) and semi-quantitative image analysis for the C1,2C collagen degradation fragment in adult (B) and juvenile (C) chondrocytes encapsulated in PEG hydrogels after 14 days under dynamic physiological loading conditions. Images are representative of a sample size of n=2.

differentially regulated with age under loading. Collagen catabolism was also detected in both cell populations. For adult cells, 0.3Hz loading regime stimulated collagen catabolism regardless of frequency, while 1Hz/5% inhibited collagen catabolism. The latter was accompanied by lower collagen type II deposition, which may explain the lower collagen catabolism. However, the former was accompanied by collagen deposition that was similar or less to the free swelling conditions suggesting that low frequency for adult cells is detrimental to collagen elaboration. In juvenile chondrocytes, loading generally had no affect on collagen catabolism. This finding suggests that collagen catabolism is greater and more sensitive to loading in adult cells than in juvenile cells, which is supported by others [46].

Interestingly, the effects of loading and age on collagen VI deposition, the primary protein present in the PCM [47], was markedly different from that observed for collagen II. In juvenile chondrocytes, collagen VI was downregulated by all loading regimes except 1Hz/5%. Interestingly, collagen VI deposition in adult chondrocytes was significantly upregulated with loading under 1Hz/10% and 0.3Hz/5% and was not affected by loading for the other loading conditions examined. In cartilage explants, mechanical loading has been shown to more greatly influence matrix molecules in the pericellular matrix (PCM) than in the extracellular matrix [5, 48], suggesting that collagen VI regulation may occur on a shorter time scale than that of collagen II due to its periphery to the cell [48].

Overall our findings suggest that aggrecan and collagen production and their degradation are differentially regulated by both age and loading regime. *Ikenoue et al.* [49] reported similar findings with respect to loading, specifically for hydrostatic pressure, showing differential regulation of aggrecan and collagen gene expressions in chondrocytes. *Rotter et al.* [50] have demonstrated that while native septal cartilage shows differences in mechanical properties with age, septal chondrocytes cultured in free swelling conditions seeded on PLA coated PGA scaffolds, and then implanted subcutaneously, did not show any differences in mechanical properties of engineered tissue with aging, suggesting that physiological mechanical cues may lead to differences in tissue properties with age. Taken together, our findings together with other reports in the literature support the hypothesis that mechanical loading differentially effects the type of matrix synthesized and deposited by the cells and that this regulation is dependent on the age of the donor. Our findings suggest that there may need to be shift in the magnitude of the biomechanical cues during tissue development.

Our findings also indicate that age plays an important role in how chondrocytes sense and respond to loading. Most notably, our findings suggest that for sGAG deposition, adult chondrocytes require a higher frequency of loading when compared to juvenile chondrocytes. This observation may in part be due to the preconditioning of adult cells in a tissue that is generally stiffer and less elastic [51]. It has been shown that chondrocytes become less responsive to signaling cues, such as growth factors with increasing age [52]. Therefore, it is possible that adult cells may require

greater stimulation, such as higher frequency of loading, to give a measurable response to loading. This is further supported by the semi-quantitative analysis for aggrecan and collagens types II and VI which for 1 Hz show that high strains lead to better tissue deposition by adult cells while low strains are better for juvenile cells. Further study is warranted to identify the exact mechanisms responsible for the age-related differences in chondrocyte response to loading.

In conclusion, this study has demonstrated that chondrocytes respond differently to varying frequencies and strains with respect to age. For adult chondrocytes, GAG production was greatest with 1Hz/5% loading, however GAG production was greatest with 0.3Hz/5% loading in juvenile chondrocytes. While this study has investigated differences between skeletally immature and mature chondrocytes, further study is required to look at a broader range of age in skeletally mature chondrocytes. Further study is also required to understand if the cells are responding to cell deformation, fluid flow or changes in nutrient diffusion with mechanical compression and how these stimuli are perceived by chondrocytes with respect to age. This information will provide optimal conditions for developing functional engineered tissue in PEG hydrogels, but will also provide a basis for further study into changes in mechanotransduction with aging. The insights gained from this could potentially be used to develop therapies that prevent age related changes in tissue composition, which are thought to be a factor contributing to development of osteoarthritis, as regulated by mechanical cues [53].

5.5 Acknowledgements

This work was supported by a grant from the American Federation for Aging Research and an NIH Pharmaceutical Biotechnology Training Fellowship to NF. The authors declare no conflict of interest.

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Chapter 6

Influence of Chondrocyte Maturation on Acute Response to Impact Injury in PEG Hydrogels

(Submitted to The Journal of Biomechanics, Under Review 2012)

Age is a risk factor in developing osteoarthritis, but the link is not well understood. It is thought that age predisposes the tissue to osteoarthritis when other risk factors are involved, e.g. abnormal biomechanics. Therefore, this study aimed to test the hypotheses that chondrocyte response to injurious loading is dependent on donor age. Bovine chondrocytes were selected as model cells and isolated from skeletally immature (juvenile,1-3weeks) or mature (adult, 2-3 years) cartilage to represent different aged donors. Juvenile and adult chondrocytes were encapsulated in identical 3D poly(ethylene glycol) hydrogels and subjected to an initial compressive impact load of 25.6±7.5 kN/m^2 applied to 50% strain. Under free swelling culture, adult chondrocytes exhibited higher intracellular ROS levels and catabolism, specifically collagen degradation, when compared to juvenile chondrocytes. In response to injurious load, adult chondrocytes responded with higher cell death, while juvenile chondrocytes responded with greater apoptosis and greater increases in intracellular ROS. With respect to anabolism and catabolism in response to injurious load, adult chondrocytes exhibited decreased aggrecan and collagen deposition, while juvenile chondrocytes exhibited decreased proteoglycan synthesis and increased collagen degradation. Overall, chondrocytes responded to injury regardless of age, but exhibited age-dependent responses with respect to anabolism and catabolism. These findings confirm that age influences how chondrocytes respond to abnormal biomechanical cues warranting further study into the mechanisms of how cells, age,

and injury contribute to the onset of osteoarthritis.

6.1 Introduction

Osteoarthritis is a prevalent and debilitating disease [1]. While the underlying cause remains unknown, several risk factors have been identified including age, injury, genetics, and abnormal biomechanics, which together with inflammatory cues can initiate pathologic changes in cartilage and bone [2, 3]. In particular, age is thought to predispose cartilage to osteoarthritis when other risk factors are involved [4]. Clinical evidence suggests a role for biomechanics in the link between age and osteoarthritis, but its role is poorly understood.

Two distinct age-related changes in cartilage are the function of cartilage cells (chondrocytes) and the extracellular matrix (ECM). In chondrocytes, age leads to increased production of radical oxygen species (ROS) [5, 6], decreased tissue synthesis [7], and increased secretion of matrix degrading enzymes [8]. These cellular changes can alter cartilage homeostasis leading to imbalances in catabolism and anabolism [9]. Age-related changes in ECM include increased crosslinking between collagen fibers and glycosaminoglycans [10, 11], and changes in size and chemistry of aggrecan [12, 13, 14]. These ECM changes affect tissue mechanical properties leading to reduced tissue elasticity [15] and alter local biochemical and mechanical cues perceived by chondrocytes. Consequently, it is difficult to decouple age-related changes in the cell from that of the ECM.

There is strong evidence supporting the role of age, when linked to injury, in osteoarthritis. For example, Roos et al. [16] reported that patients over 30 years of age with an anterior cruciate ligament injury (results in impact to cartilage) developed measurable signs of osteoarthritis $\sim$10 years earlier than patients between 17-30 years of age. In an effort to better understand the acute response to injury, cartilage explants have been studied under impact loading. These studies have confirmed that injurious loading elevates secretion of matrix degrading enzymes [17, 18], and have indicated that skeletally immature chondrocytes respond more detrimentally to injurious loading evidenced by more cell death, higher glycosaminoglycan loss, and reduced tissue production [19]. Biomechanical cues due to age-related differences in ECM alone could not explain the results. These

findings highlight the need to study cellular contribution to injury.

Our overall goal is to isolate and decouple the effects of cellular age and age-related changes in ECM in the response of cartilage to injurious loading. Towards this goal, this study focused on age-related cellular responses to injurious impact loading by culturing chondrocytes isolated from different aged donors in a controlled 3D *in vitro* system based on bioinert poly(ethylene glycol) hydrogels [20, 21, 22]. This study tested the hypothesis that injurious loading of chondrocytes suspended in a PEG hydrogel in the absence of ECM leads to cell death, increased ROS production, decreased tissue production, and increased tissue catabolism, and that the response is dependent on donor age.

6.2 Materials and Methods

6.2.1 Chondrocyte Isolation

Adult chondrocytes were isolated from articular cartilage harvested from metacarpalphalangeal joints of 2-3 year old steers (3-4 animals/isolation, 2 isolations, 7 animals total) obtained from a local abattoir within 12 hours of slaughter. For each isolation, adult cells from all animals were pooled together. Juvenile chondrocytes were isolated from full depth articular cartilage harvested from the patellar-femoral groove of 1-3 week old calves (1 animal/isolation, 2 isolations, 2 animals total) (Research 87 Inc., Natick, MA) within 24 hours of slaughter. Chondrocytes were isolated as previously described [23]. Trypan blue exclusion confirmed >85% viable cells for all isolations. Experiments were performed using either one or two isolations referred to as one or two independent experiments, respectively, and performed in multiplicate.

6.2.2 Chondrocyte Characterization

Metabolic activity was measured by suspending freshly isolated chondrocytes ($1x10^5$ cells/ml) in 1% w/w MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) in PBS for four hours at 37°C. Cells were recovered by centrifugation, lysed in 0.04N hydrochloric acid

in isopropanol and absorbance (560nm) measured. One independent experiment was performed (n=6).

Telomerase activity was measured by suspending freshly isolated chondrocytes (5x10^6 cells/ml) in lysis buffer (Allied Biotech, Inc, Vallejo, CA). Telomerase activity was quantified by real-time PCR (7500 Fast, Applied Biosystems, Carlsbad, CA) with TSR standards per manufacturer (Allied Biotech, Inc). One independent experiment was performed (n=3).

6.2.3 Hydrogel Preparation

Linear poly(ethylene glycol) (PEG, 3000MW, Fluka) was reacted with methacrylic anhydride (Sigma) at 1:10 molar ratio with trace hydroquinone under microwave radiation [24] to produce PEG dimethacrylate (PEGDM) followed by purification. Methacrylation of end hydroxyls was 95%, confirmed by ^{1}H NMR. Chondrocytes (4x10^6 cells/ml) were suspended in 10% w/v PEGDM, 0.05% w/w Irgacure 2959 (Ciba Specialty Chemicals, Tarrytown, NY) in chondrocyte medium (DMEM, 10mM HEPES, 0.1M non-essential amino acids (Invitrogen), 0.4mM L-proline, 0.14mM L-ascorbic acid, 1% v/v P/S, 0.5μg/ml amphotericin B and 10% FBS). Cylindrical hydrogels (5.6mm height/5.6mm diameter) were formed by photo-polymerization (365nm light, $\sim$4mW/cm^2, 10 minutes).

6.2.4 Chondrocyte Strain

Twenty-four hours post-encapsulation cytosol of live cells was stained with 4nM calcein AM (Invitrogen) for 30 minutes. A single gel was placed into a custom straining device associated with a confocal laser-scanning microscope (CLSM, Zeiss LSM 510, Thornwood, NY). Cells were followed from 0 to 50% strain and imaged at full width half maximum height. Using NIH Image J, cell diameters parallel (x) and perpendicular (y) to the application of strain were measured. Cell deformation was assessed by diameter ratio (x-diameter/y-diameter) and cellular strain along the x- ($100x(D_0-D_{50})/D_0$, where D_0 and D_{50} are diameters at 0% and 50% strain, respectively) and y-axis ($100xD_{50}/D_0$) [25, 26]. Two independent experiments were performed (1-2 gels/experiment

and ∼10 cells/gel (n=30 total)).

6.2.5 Impact Loading

An impact load was simulated using the apparatus in Figure 6.1A. Twenty-four hours post-encapsulation, hydrogels were placed into individual porous Teflon® wells (40-70μm pores, Interstate Specialty Products, Sutton, MA) at a depth that produced 50% maximum strain. A weight (786g) was dropped from a height of 12in. The acceleration immediately before impact was 4.8±1m/s^2 determined by an accelerometer (U352B10, PCB Piezotronics, Depew, NY). Initial impact force was determined to be 25.6±7.5kN/m^2, similar to that reported for impact loaded cartilage explants [27]. A separate set of gels was not subjected to impact (control). Figure 6.1B depicts experimental design.

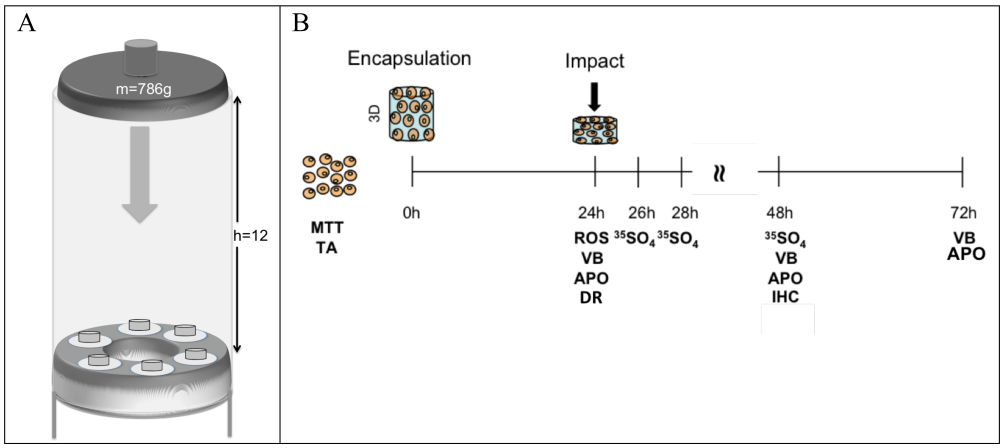

Figure 6.1: Drop tower design (A) consisting of a known mass dropped from 12 inches onto six hydrogels. Hydrogels are placed into wells in the base such that a single impact compresses the gels to 50% strain. Experimental design (B) of the study. Cells from different aged donors were analyzed by metabolic activity (MTT) and telomerase activity (TA) in cell suspensions immediately after isolation. Cells were encapsulated in PEG hydrogels at 0 hour and subjected to impact loading at 24 hour, followed by 48 hours of subsequent free swelling culture. Analysis of cells post-impact included imaging of ROS (ROS), live cell count (VB), apoptosis count (APO), GAG production ($^{35}SO_4$), immunohistochemistry (IHC) and cell diameter ratio measurements (DR).

6.2.6 Live Cell and Apoptosis Quantification

Standards were generated from known live cell concentrations (0.5, 4, 6 and 8 x10^5 cells). For apoptosis, a separate set of standards was generated by treating cells with 10mM dexamethasone for 3 hours. Hydrogels 0, 24 and 72 hours post-impact, controls and standards were snap frozen and homogenized in lysis buffer (0.05M Tris HCl (pH 7.5), 0.2M NaCl, 0.01M $CaCl_2$, 0.02% NaN_3, and 0.05% Triton-X in DI water) with a protease inhibitor cocktail (Roche, Indianapolis, IN). Supernatants were assayed with MultiTox-Glo assay (Promega, Madison, WI) for live cells or with the EnzChek® Caspase-3 Assay Kit 2 (Molecular Probes, Eugebe, OR) for apoptosis per manufacturer. One independent experiment was performed (n=6).

6.2.7 ROS Production

Chondrocytes encapsulated in PEG hydrogels were incubated with 20μM carboxy-2,7-difluoro-dihydrofluorescein diacetate (Invitrogen), which detects intracellular ROS, for 20 minutes prior to impact loading. Immediately post-impact, hydrogels were imaged by CLSM. Two independent experiments were performed (n=2/experiment, n=4 total).

6.2.8 Proteoglycan synthesis

Proteoglycan synthesis was measured by $^{35}SO_4$ incorporation. Impacted and control gels were placed in chondrocyte medium with 10μCi/ml $^{35}SO_4$ (Perkin Elmer, Shelton, CT) from 0-2, 2-4, or 4-24 hours post-impact. Hydrogels were homogenized and enzymatically digested (125μg/ml Papain (Worthington), 10mM L-cysteine, 100mM phosphate and 10mM EDTA in DI water (pH 6.3) overnight at 60°C). Proteoglycan synthesis in media and hydrogel was determined by Alcian blue precipitation [28] and a Beckman LSC 6500 (MDA 80.3pCi). Proteoglycan synthesis was normalized to total DNA content measured by PicoGreen® (Invitrogen) per manufacturer. Two independent experiments were performed (n=2-3/experiment, n=5 total).

6.2.9 Immunohistochemistry

Twenty-four hours post-impact, hydrogels were fixed in 4% v/v paraformaldehyde for 24 hours, dehydrated, and embedded in paraffin. Sections (10μm) were pretreated with hyaluronidase (2080U/ml Sigma) and chondroitinase ABC (100mU/ml, Sigma), Triton X-100, and blocked with 1% BSA. Sections were treated with primary antibody: mouse anti-human aggrecan IgG1 (1:2.5, US Biologicals, Swampscott, MA), rabbit anti-human collagen II IgG (1:25, US Biologicals), or rabbit anti-human C1,2C IgG (1:100, IBEX Pharmaceuticals, Montreal, QC, CA) at 4°C overnight. Secondary antibodies were AlexaFluor 546 (1:200, Invitrogen) for aggrecan and AlexaFluor 488 (1:200, Invitrogen) for all others. Sections were counter-stained with DAPI and imaged by CLSM. Two independent experiments were performed (n=2/experiment, n=4 total).

6.2.10 Western Blot

Twenty-four hours post-impact, medium and hydrogel were collected. Hydrogels were homogenized in lysis buffer with protease inhibitors and lysate collected. Proteins in lysate and media were quantified (BCA^{TM} assay, Thermo Scientific, Rockford, IL) and deglycosylated (0.1U/ml keratinase II, 0.5U/ml chondroitinase-ABC (Sigma), 37°C, overnight). Proteins from gel (10μg) and media (20μg) were combined 1:1 with laemmli buffer and run on a polyacrylamide gel with a Kaleidoscope protein standard (BioRad). Proteins were transferred to a PVDF membrane (BioRad), blocked in 15% dry milk with 0.1% v/v Tween 20, incubated with primary antibody (1:100) at 4°C overnight, and incubated with secondary antibody (AlexaFluor 546, 1:200) at room temperature for 2 hours. Images were acquired (Versadoc^{TM} imaging system, BioRad). Two independent experiments were performed (n=1/experiment, n=2 total).

6.2.11 Statistical Analysis

Data are reported as mean with 95% confidence intervals about the mean. One-way analysis of variance was used with Tukeys post-hoc analysis and $p<0.05$ considered significant.

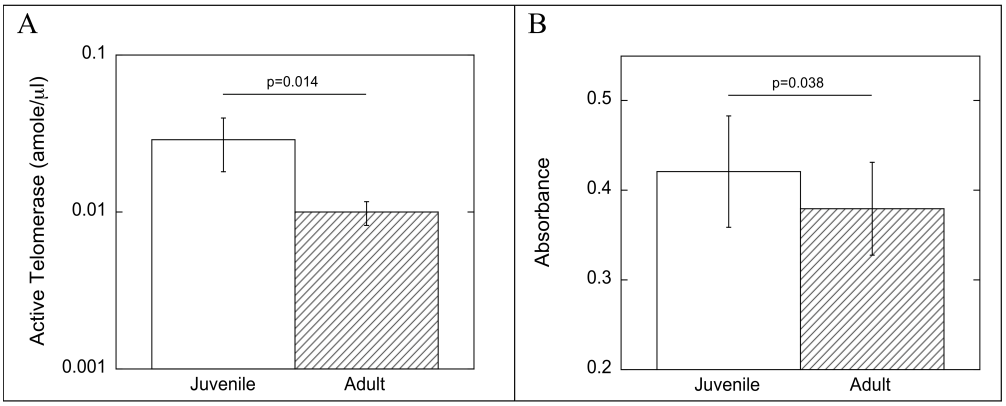

Figure 6.2: Telomerase activity (A) and metabolic activity as measured by the MTT assay (B) in freshly isolated juvenile and adult chondrocytes in suspension. Data represents the mean with 95% CI error bars (n=3 for telomerase activity and n=6 for metabolic activity).

6.3 Results

Chondrocyte maturation from different aged donors was assessed (Figure 6.2). Telomerase and metabolic activity were 65.6% and 9.7% lower in adult compared to juvenile chondrocytes, respectively.

Cell diameter ratio was similar for both cell populations (Figure 6.3) 24 hours post-encapsulation. The ratio decreased from unity to ∼0.6 with 50% strain applied to the gel. Cellular strain was similar for both cell populations at ∼24-26% and ∼124-126% in the direction parallel and perpendicular to the applied strain, respectively. Representative single cell images show a transition from round to ellipsoid with 50% strain.

Live cells (Figure 6.4A,B) and apoptotic cells (Figure 6.4C,D) were measured over 3 days post-impact. Initial number of live cells in controls was similar in adult ($9.76\text{x}10^5$) and juvenile ($9.34\text{x}10^5$) cells. Impact loading immediately lowered the number of live cells by 22.9% and 15.1% for adult and juvenile cells, respectively. Twenty-four hours post-impact, live cell number further dropped by 34% (adult) and 27% (juvenile) compared to controls. For both cell populations, impact loading led to significantly higher active caspase-3 levels at all time points. Caspase-3 activity was highest at 72 hours in adult cells ($\sim 25\text{x}10^3$ more apoptotic cells than controls) and at 24 hours in

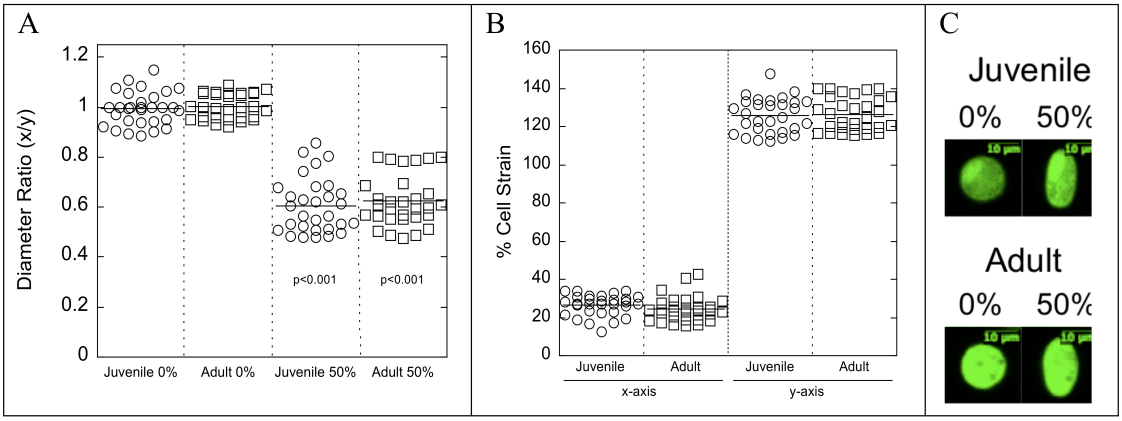

Figure 6.3: Diameter ratio (x/y) (A) at 0 and 50% gel strain. Percent cellular strain (B) along the x- and y-axis at 50% gel strain, and representative confocal microscopy images (C) at 0 and 50% gel strain. Juvenile and adult chondrocytes were encapsulated in PEG gels for 24 hours. X-axis represents the direction parallel to the applied gel strain. Y-axis represents the direction perpendicular to the applied gel strain. P-values represent significance between 0 and 50% strain in adult and juvenile chondrocytes (n=30 cells total for each cell population).

the juvenile cells ($\sim$33x10^3 more apoptotic cells than controls).

Representative images of intracellular ROS post-impact are shown (Figure 6.5). Qualitatively, impact loading elevated ROS levels in both cell populations. Adult chondrocytes in control hydrogels had more ROS initially than juvenile chondrocytes. After impact, juvenile chondrocytes had more ROS.

$^{35}SO_4$ incorporation (Figure 6.6) was not affected initially by impact in adult chondrocytes, but was elevated 4-24 hours post-impact by 2.8-fold. Proteoglycan synthesis in juvenile chondrocytes was 1.73-fold higher immediately post-impact, but was inhibited by 35% and 45% 2-4 and 4-24 hours post-impact, respectively. For adult chondrocytes, impact loading led to increased proteoglycan release 4-24 hours post-impact by 1.3-fold. For juvenile chondrocytes, impact loading did not affect proteoglycan release.

Representative images of collagen deposition, aggrecan deposition, and collagen degradation are shown (Figure 6.7). Impact loading decreased aggrecan deposition in both cell populations and decreased collagen II deposition in adult cells only. Impact loading led to increased collagen degradation (C1,2C fragment) in juvenile chondrocytes, while no apparent affect was observed in

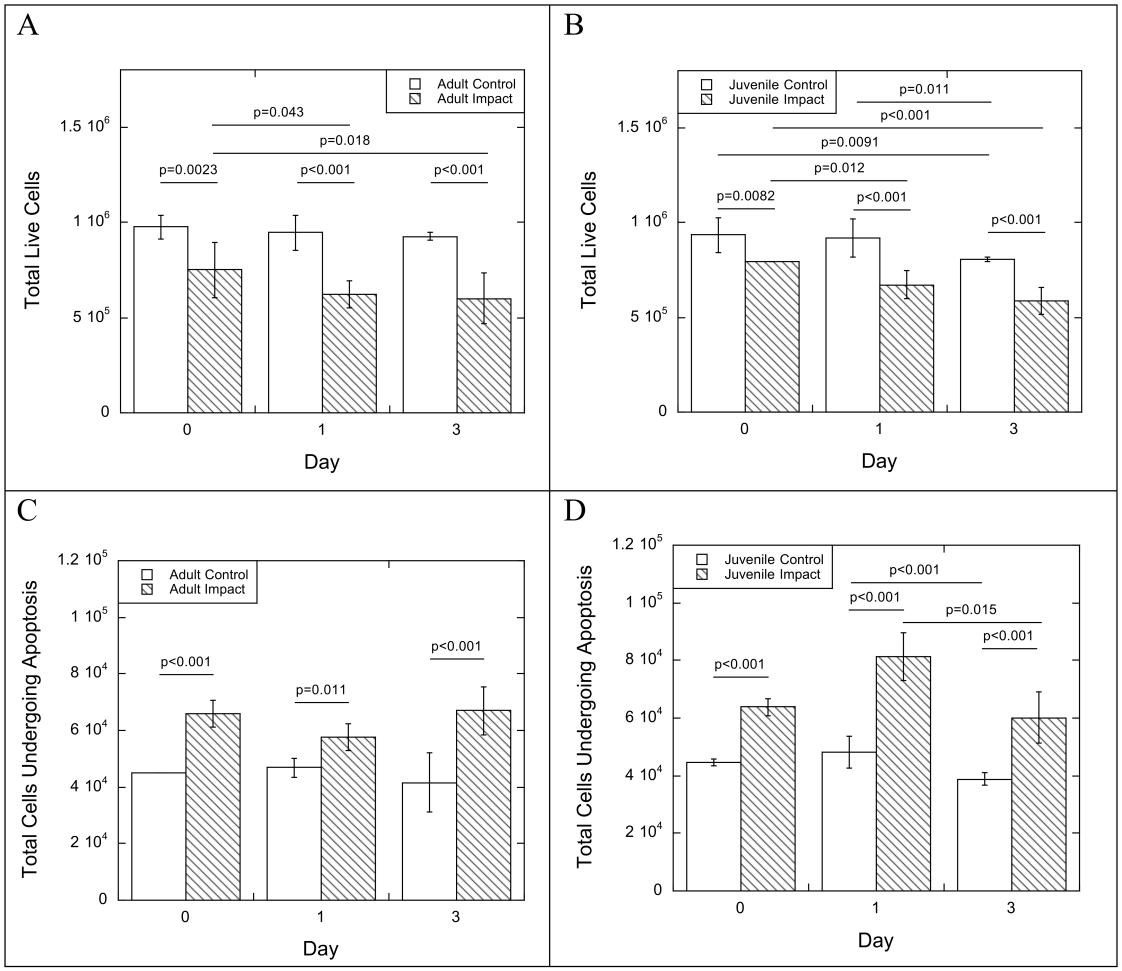

Figure 6.4: Number of live adult (A) and juvenile (B) chondrocytes and apoptotic adult (C) and apoptotic juvenile (D) chondrocytes encapsulated in PEG hydrogels and subjected to either no load (free swelling control) or an injurious impact load. Data are represented as mean with 95% CI error bars (n=5) for all conditions.

adult chondrocytes with impact loading.

Aggrecan catabolic activity was confirmed by the presence of ARG-fragment in the hydrogel and medium for both cell populations through western blot analysis (Figure 6.8). A range of ARG-fragments was detected with the most prevalent band being 60-65kDa after deglycosylation. Impact loads appeared to increase the ARG-fragment in both cell populations, where the increase was most noticeable in the media for adult chondrocytes.

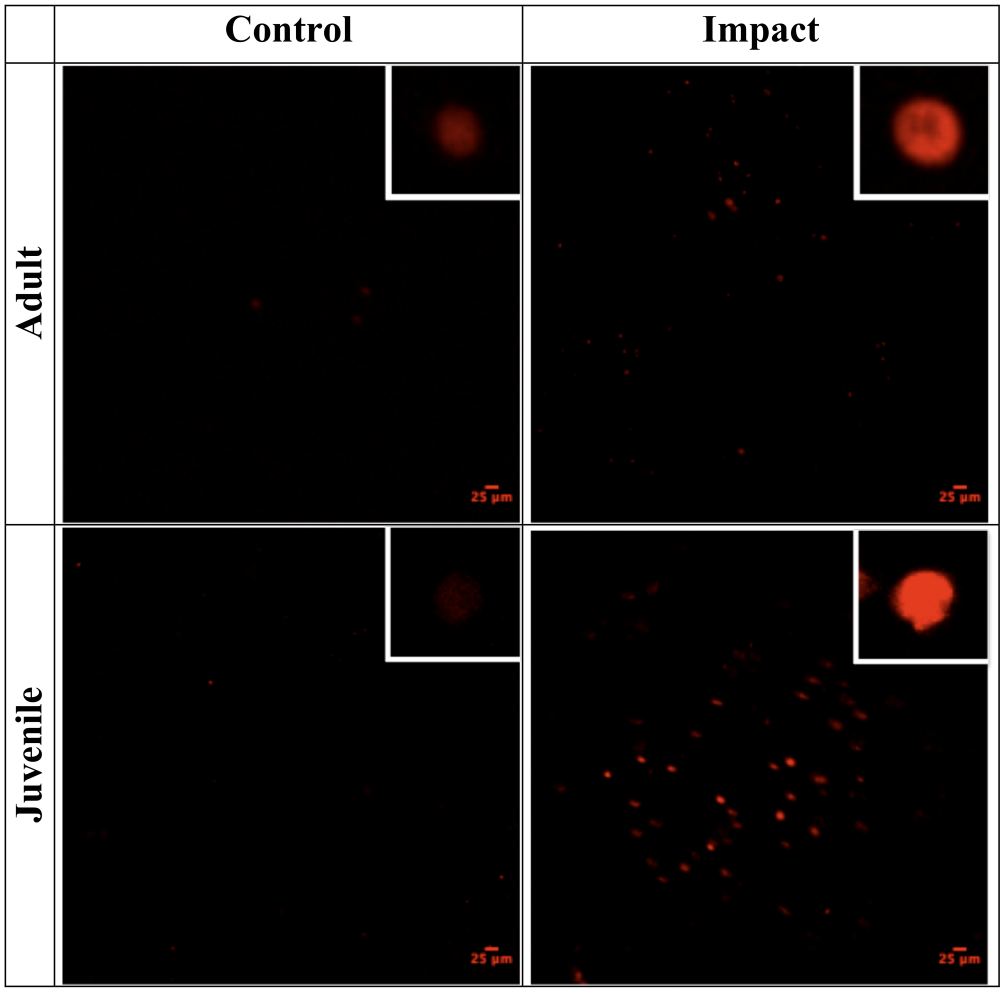

Figure 6.5: Representative confocal microscopy images of intracellular ROS generation in adult and juvenile chondrocytes, under free swelling controls and injurious loading conditions in PEG gels after 24 hours of free swelling culture and immediately after impact.

6.4 Discussion

An *in vitro* model system was developed with two different cellular populations, which exhibited distinctly different age-related characteristics, and a 3D culture environment that imparted similar local mechanical cues. Using this model, findings from this study confirm that chondrocytes, independent of age, respond to injurious mechanical loading with increased cell death, intracellular ROS, increased catabolism and a concomitant decrease in anabolic activity. The degree of the response, however, differed with age, with juvenile chondrocytes exhibiting an overall greater negative

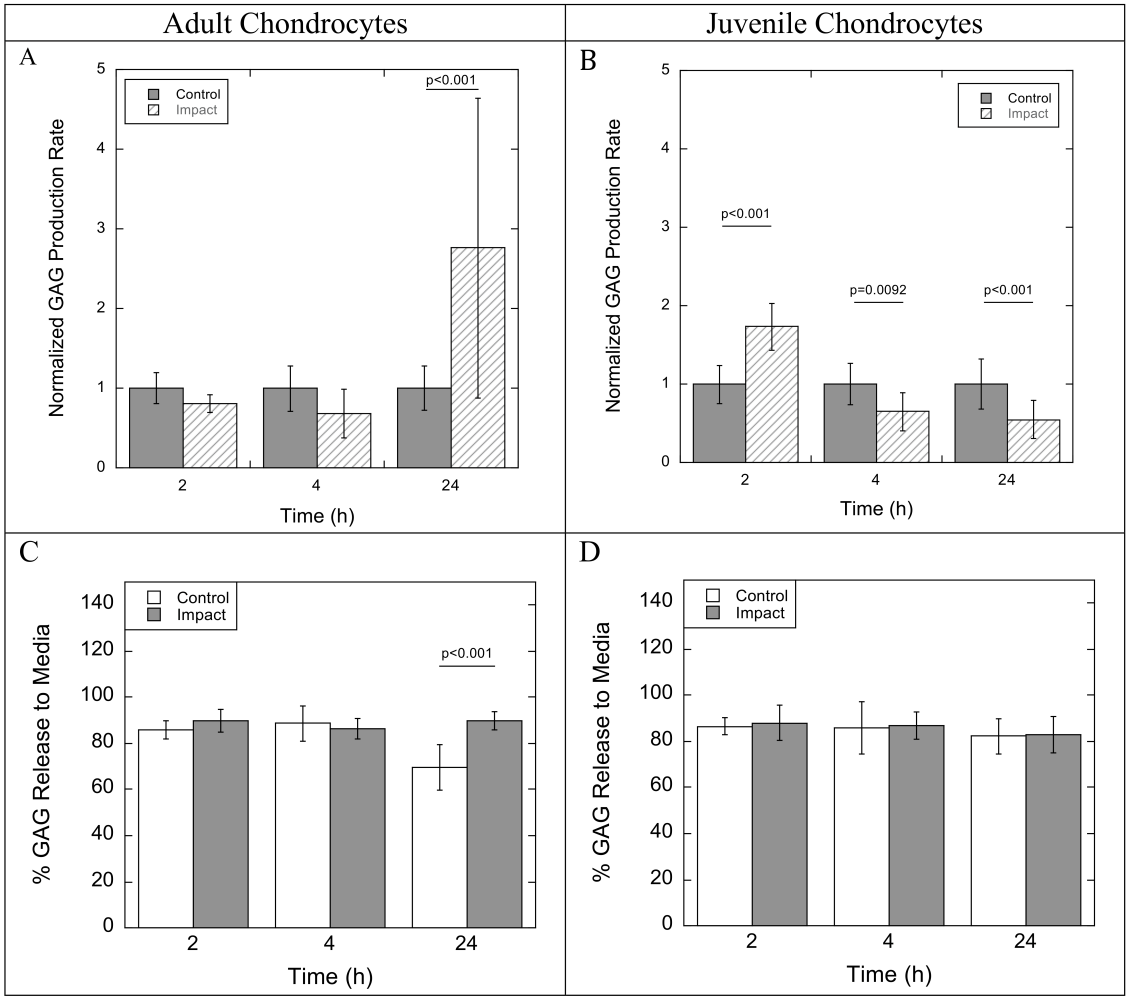

Figure 6.6: $^{35}SO_4$ incorporation into newly synthesized proteoglycans (cpm/μg DNA) in adult (A) and juvenile (B) chondrocytes in PEG gels normalized to the free swelling control at each time point. Percent proteoglycan release to the media in adult (C) and juvenile (B) chondrocytes. Data are represented as mean with 95% CI error bars (n=8).

response.

Cell death has been reported at the location of impact in cartilage explants [29, 27]. Our data show a similar response where both cell populations in PEG hydrogels undergo cell death as a result of injurious loads. Cell death appears to be largely by necrosis given the drop in live cells is far greater than the number of cells undergoing apoptosis for both cell populations. Adult chondrocytes appear to be more susceptible to impact loading evidenced by greater cell death. Contrarily, more juvenile chondrocytes underwent apoptosis, particularly one day after impact,

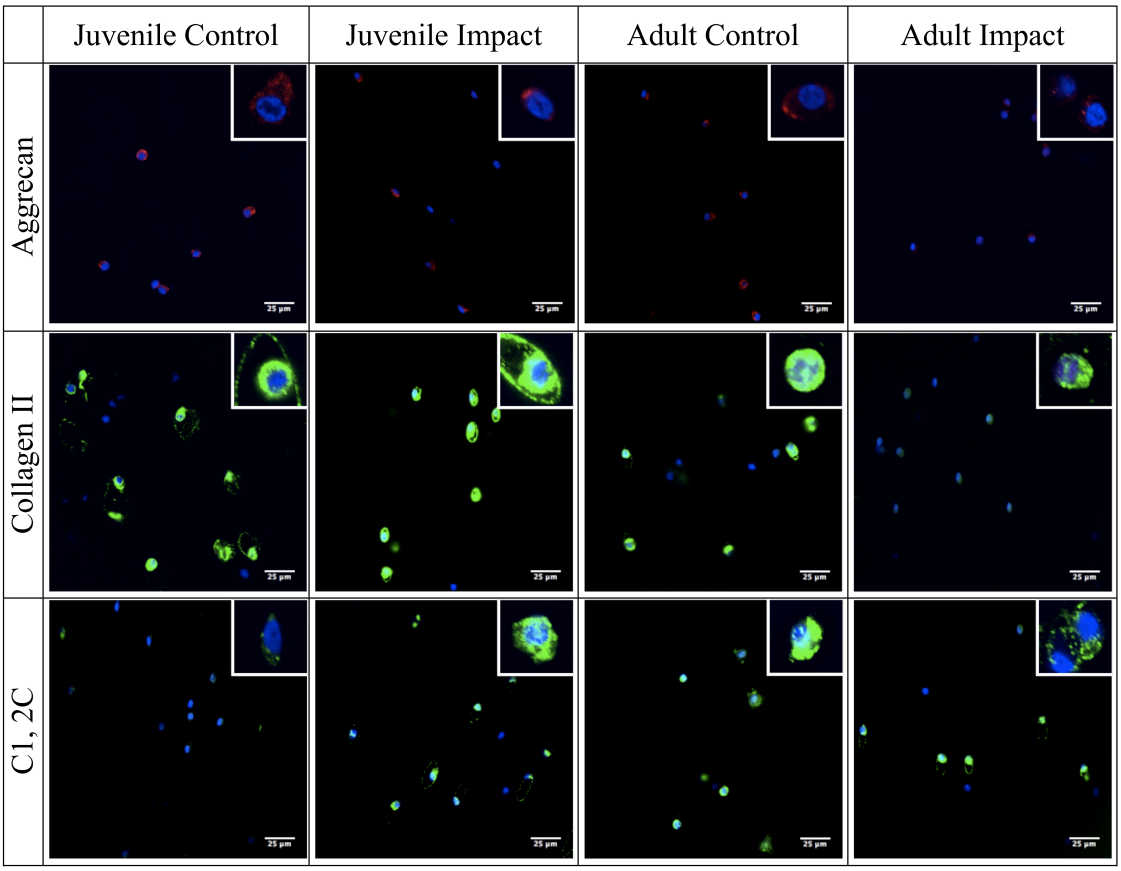

Figure 6.7: Representative immunohistochemistry images for aggrecan, collagen II, and the C1, 2C collagen fragment in juvenile and adult chondrocytes encapsulated in PEG gels and subjected to either no load (free swelling control) or an injurious impact load.

which agrees with findings for juvenile cartilage explants [17, 19].

Elevated intracellular ROS has been associated with age, trauma, and osteoarthritis and linked to apoptosis and catabolic activity in chondrocytes [30, 31, 32]. In this study, adult chondrocytes appeared to have higher intracellular ROS under control conditions, which follows the free radical theory of aging [33]. Post-impact, adult chondrocytes responded with a small but observable increase in intracellular ROS while juvenile chondrocytes responded with a large increase. Oxidant conditioning has been shown to protect chondrocytes from mechanically induced death, suggesting that chronic exposure to ROS may bolster the antioxidant response. Therefore, adult cells may more effectively scavenge intracellular ROS in response to injury [34]. As oxidative stress has downstream effects on anabolism and catabolism, age-related differences in ROS in response

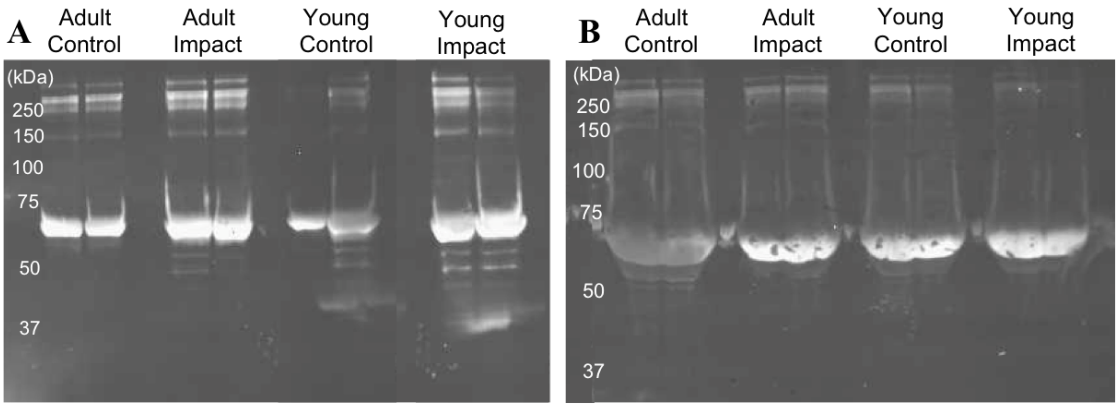

Figure 6.8: Western blot images for the ARG-fragment of degraded aggrecan in hydrogels (A), and in media (B) for juvenile and adult chondrocytes encapsulated in PEG hydrogels. Images are shown for a sample size of n=2.

to injury may lead to differences in tissue production and degradation [31].

Indeed, impact loading affected proteoglycan synthesis and was age-dependent. In juvenile chondrocytes, impact loading led to an initial up-regulation in proteoglycan synthesis followed by a down-regulation, which agrees with findings from joint injuries [35, 36] and early OA [37]. Surprisingly, this response was not observed in adult cells where a late up-regulation in proteoglycan synthesis was observed. Interestingly, the increased proteoglycan synthesis observed in adult chondrocytes was accompanied by a higher percentage of proteoglycan release, suggesting that the increased proteoglycan synthesis may be counterbalanced by enhanced catabolic activity.

Aggrecan degradation is one of the hallmarks of OA and injury and is detected in the synovium by the presence of the ARG-fragment, which is mediated by ADAMTS-4 or 5 [38, 39, 40]. The ARG-fragment was detected in both cell populations in control and impact conditions, suggesting that chondrocytes are anabolically and catabolically active when cultured in PEG hydrogels. While interpretation is limited, several differences are worth noting. Most notably, there appeared to be an age-dependent shift in the relative amount of ARG-fragment retained in the gel and that, which was released with impact loading. This observation suggests a difference in aggrecan glyco-

sylation, which has been reported to increase during adolescence [41], but also increase in cartilage explant injury models [42, 43].

Collagen II degradation, specifically the C1,2C collagen fragment produced by MMP-1 and MMP-13 [44] is a biomarker of osteoarthritis and is commonly found in the synovial fluid of patients with joint injury [45, 46]. Impact loading did not appear to affect collagen II deposition by juvenile chondrocytes, but did inhibit collagen II deposition by adult chondrocytes. Collagen degradation was evident in adult chondrocytes, but not in juvenile chondrocytes under control conditions suggesting that adult cells are inherently more catabolically active. Contrarily, impact loading led to up-regulations in collagen degradation in juvenile chondrocytes, but no additional up-regulation in adult chondrocytes. The former observation is supported by studies in juvenile cartilage explants where MMP-13 expression was significantly increased after injury [47].

Our findings indicate that, when subjected to injurious loads, juvenile and adult chondrocytes respond negatively to injury; however juvenile chondrocytes respond with larger decreases in anabolism and larger increases in catabolism. While it has been suggested that cartilage structure and/or biochemical composition may be responsible for the increased susceptibility of juvenile cartilage to injurious loads [17, 19], our findings point towards the chondrocyte. As reported here and in other tissues, immature cells experience greater oxidative stress in response to negative cues possibly due to reduced glutathione levels [48]. In addition, the inherent increased catabolism and ROS in adult cells may desensitize them to injury making adult cells less susceptible. Contrary to *in vivo* findings, our study shows that juvenile chondrocytes respond more detrimentally to impact injury, suggesting that other factors, such as inflammation, may play a role in accelerating the development of disease in older patients. However, it is important to acknowledge several limitations of this study. Although the local strains were similar for both cell populations, any nascent PCM formed prior to impact loading albeit mechanically inferior, may provide different biochemical signals to the cells altering their response to impact loading. Additionally, adult and juvenile chondrocytes were isolated from different joints and even though they are both load bearing and have characteristic hyaline cartilage, joint-specific responses may have contributed to the overall

findings. Nonetheless our findings agree well with cartilage explants and thus we hypothesize that the increased vulnerability of juvenile cartilage to injurious loading is in part a result of immature antioxidant pathways in juvenile cells.

With intensity of physical activity rising in young patients and a concomitant increase in joint injuries [49], therapies that target the cell may help to reduce post-traumatic osteoarthritis onset later in life. Further study is also needed to investigate the role of cell age in older, more clinically relevant, cell populations. This study has shown that understanding chondrocyte response to injury in the absence or minimal presence of ECM can provide insight into cellular contributions to injury.

6.5 Acknowledgements

This work was supported by the American Federation for Aging Research and an NIH Pharmaceutical Biotechnology Training Fellowship to NF.

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Chapter 7

The Role of Intracellular Calcium Signaling in Stimulation of Tissue Production with Dynamic Loading in Charged Hydrogels

In cartilage, mechanical forces induce changes in the local environment, which mediates tissue homeostasis. However, the exact mechanisms through which cells sense and respond to mechanical signals is still under investigation. One mechanical cue unique to cartilage results from its highly negatively charged extracellular matrix, which attracts mobile ions leading to an unusually high osmolarity, that changes under loading. While studies have shown that osmolarity influences chondrocytes and their tissue production, the role of extracellular osmolarity under dynamic loading in chondrocyte mechanotransduction remains largely unstudied. The goal of this study is gain a better understanding of the role of negative charges in cartilage in regulating tissue production, and whether intracellular calcium is involved in mediating this response. To achieve a 3D culture system where fixed charges could be incorporated in a controlled manner, chondroitin sulfate was functionalized and co-polymerized into PEG hydrogels as a concentration similar to that in native cartilage. Studies in static isotonic culture were also conducted to further investigate the link between ionic and osmotic environment to tissue production and calcium signaling. Our data suggests that changes in GAG and collagen production with the addition of negative charges may not be mediated by intracellular calcium signaling. Potential mechanisms of regulation of tissue production still remain to be determined, however we have shown that ionic and osmotic effects give differential responses with respect to intracellular calcium signaling as well as tissue production, suggesting that multiple pathways may be activated in response to loading in a charged

environment.

7.1 Introduction

Cartilage remodeling *in vivo* is mediated in part by mechanical signals, which help to maintain tissue homeostasis [1]. Mechanical forces produce many changes in the local environment, such as fluid flow, movement of mobile ions and cellular deformation. Cells sense these events and translate extracellular cues into intracellular signals that mediate the downstream effects through mechanotransduction. For example, cellular deformation has been found to play an important role in transmitting mechanical cues to cells, as cartilage cells have been shown to regulate their metabolism in a load-dependent manner [2]. However, many of the mechanotransduction pathways in chondrocytes are not well understood [3].

Cartilage cells or chondrocytes sense and respond to their environment through a wide range of extracellular cues arising from the extracellular matrix (ECM) and the mechanical environment. The ECM of cartilage is composed of a dense crosslinked collagen matrix, mainly type II collagen, and aggrecan, which is dispersed throughout the matrix. Aggrecan is the main proteoglycan in cartilage and is composed of highly negatively charged glycosaminoglycans (GAGs) [4, 5, 6]. During normal physical activity, cartilage is subjected to mechanical forces largely in the form of compressive forces, which are translated through the ECM into local biomechanical cues. These biochemical and biomechanical cues are important to the health and maintenance of cartilage as well as to developing strategies for cartilage tissue engineering. While a significant amount of research has focused on chondrocyte mechanotransduction, many of the mechanisms by which chondrocytes sense and respond to these cues is not well understood.

One of the unique biochemical characteristics of cartilage is its high fixed charged density. The negative charges allow the tissue to imbibe large amounts of water and provide cartilage with its unique ability to withstand large loads [7, 8]. These charges also affect the local concentrations of ions, attracting Na^+ and K^+ in the interstitial fluid [7, 9, 10]. This leads to a high osmolarity in cartilage ranging from $\sim$350 to 450mOsm, which is substantially higher than most tissues [11, 12].

During dynamic loading of cartilage, fluid is expelled from the tissue causing dynamic changes in the ionic and osmotic environment [13, 14, 15], which can activate intracellular signaling pathways [16]. It is thought that these dynamic changes in the microenvironment of cartilage that arise as a result of the fixed negative charges may be an important cue for chondrocytes, through activation of mechanotransduction signaling pathways.

One such pathway is activation of ion channels on the cell surface due to changes in ionic and osmotic environment, causing a flux of ions across the cell membrane and activating ion channels that convert the mechanical stimulus into an intracellular biochemical signal [17]. These ion channels include slow conductance calcium-potassium channels, L-type calcium channels, and stretch activated ion channels, all of which are known to influence intracellular calcium signaling [18]. Calcium is a dynamic signaling molecule that regulates many processes within the cell. In particular, intracellular calcium signals are known to be involved in numerous cellular functions, including gene expression, differentiation, and tissue synthesis [19, 20]. Extracellular osmolarity has also been linked to tissue production in chondrocytes [21, 8, 22]. Using isotonic cultures, medium osmolarity that most closely matches that of cartilage has been shown to increase tissue synthesis in chondrocytes, compared to medium with either higher or lower osmolarity than the physiological range [8, 23]. Taken together, this led us to hypothesize that dynamic change in osmolarity, which arise as a result of dynamic loading of cartilage, may influence chondrocytes and tissue synthesis, via calcium-mediated events.

The overall aim of this study was to better understand the role of fixed negative charges in cartilage, specifically related to tissue production, and whether intracellular calcium is involved in regulation of tissue production when cells are cultured in a charged environment and subjected to dynamic loading. We employed a 3D model where fixed negative charges could be incorporated in a controlled manner. Poly(ethylene glycol) hydrogels served as the base hydrogel platform, which are easily tailored and allow for incorporation of tissue specific molecules, such as chondroitin sulfate [24, 25, 26, 27]. This model system enabled the dynamic changes associated with movement of mobile ions to be recapitulated in a manner similar to that in cartilage under dynamic loading.

Using intracellular calcium chelators, calcium-mediated tissue production could be assessed as a function of loading in neutral and charged environments. Because the charged environment leads to changes in both osmolarity and ion concentration within the hydrogel, systematic studies were also performed using isotonic solutions containing sucrose and salts in an effort to decouple the role of osmolarity and ionic strength on intracellular calcium and tissue production. Overall, findings from this study suggest that changes in tissue production with the addition of negative charges may be mediated by intracellular calcium events. The mechanisms by which negative charges regulate tissue production remain to be determined, however our data suggests that the combination of ionic and osmotic effects contribute to this regulation.

7.2 Methods

7.2.1 Chondrocyte Isolation

Adult chondrocytes were isolated from full depth articular cartilage harvested from the metacarpalphalangeal joints of 2-3 year old steers, which were obtained from a local abattoir, within 12 hours of slaughter. Adult cells were isolated from 1-2 animals per isolation, where cells were pooled together from all animals, and all studies were completed with two separate isolations. Cartilage slices were rinsed in phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA), finely diced, and enzymatically digested in 0.02% collagenase II (Worthington Biochemical Corp., Lakewood, NJ) in Dulbeccos Minimum Essential Medium (DMEM, Invitrogen) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C for 16 hours on an orbital shaker. Cells were washed twice in PBS with 0.02% EDTA and resuspended in PBS+P/S. Cell viability was 92.3% and 90.6% for the two isolations, as determined by Trypan blue exclusion.

Table 7.1: Amounts of sodium chloride, potassium chloride or sucrose added to standard media at 330mOsm to adjust to the desired osmolarity. The average osmolarity over 2 independent measurements is shown with standard deviation.

Calculated Osmolarity (mOsm)	Added to 330mOsm NaCl (g/L)	Added to 330mOsm KCl (g/L)	Added to 330mOsm Sucrose (g/L)	Actual Osmolarity (mOsm)
330	-	-	-	327±1.0
400 (Salts)	2.62	0.13	-	404±4.2
430 (Salts)	2.34	1.17	-	425±0.7
460 (Salts)	3.02	1.51	-	464±1.4
400 (Sucrose)	-	-	17.62	402±1.4
430 (Sucrose)	-	-	27.61	434±2.1
460 (Sucrose)	-	-	37.08	462±2.1

7.2.2 Chondrocyte Media Formulation

Standard chondrocyte medium was composed of DMEM, 10mM HEPES, 0.1M non-essential amino acids, 0.4mM L-proline, 0.14mM L-ascorbic acid, 1% v/v P/S, 0.5 μg/ml amphoterecin B (Invitrogen) and 10% fetal bovine serum. The osmolarity of the standard chondrocyte medium was determined to be 330 mOsm. Medium was supplemented with either sodium chloride and potassium chloride or sucrose to adjust the final medium osmolarity to 400, 430, and 460 mOsm [8]. Table 1 lists the concentrations of salts or sucrose used for each media formulation and the resulting osmolarity, as measured by a freezing point osmometer (Model 5520 Vapro, Wescor Inc., Logan, UT).

7.2.3 PEG and Chondroitin Sulfate Methacrylation

Linear PEG (3000MW, Fluka) was reacted with methacrylic anhydride (94%, Sigma) at a molar ratio of 1:10 with trace amounts of hydroquinone (Sigma) under microwave radiation [28] to produce PEG dimethacrylate (PEGDM). The product was dissolved in dichloromethane (Sigma) and precipitated into cold ethyl ether (Sigma). The percent of methacrylation of end hydroxyls was determined to be 95% through ^{1}H NMR by comparing the area under the vinyl (∼5.6 and ∼6.1ppm) and methylene peaks (∼3.6-4ppm).

Chondroitin sulfate A (CHS, Sigma), containing 70% chondroitin-4-sulfate and 30% chondroit-

in-6-sulfate, was dissolved in deionized (DI) water at 25% w/v with methacrylic anhydride (Sigma) in a 1:8 dilution and was reacted for 24 hours at 4°C. During the first hour of reaction the pH was maintained at 8 by the addition of concentrated sodium hydroxide. The product was precipitated in cold methanol. The precipitate was dialyzed in DI water for 24 hours and was lyophilized to recover the product. The degree of methacrylation, as determined by ^{1}H NMR analysis of the area under the vinyl (∼5.6 and ∼6.1ppm) and acetyl (∼1.8ppm) peaks, was found to be 29.9%, indicating that on average 30 methacrylates have been attached to one molecule of CHS [27, 22, 25].

7.2.4 Hydrogel Preparation

For PEG-Only hydrogels, freshly isolated chondrocytes (4x10^6 cells/ml) were suspended in a solution of 10% w/v PEGDM, 0.05% w/w Irgacure 2959 photoinitiator (Ciba Specialty Chemicals, Tarrytown, NY) in chondrocyte medium at 330, 400, 430 or 460 mOsm adjusted with either salts or sucrose depending on the experiment. The cell-macromer solution was exposed to 365 nm light (∼4 mW/cm^2) for 10 minutes to form cylindrical gels 4.5 mm in height by 4.5 mm in diameter. Hydrogels were cultured in their respective medium. For PEG-CHS hydrogels, freshly isolated chondrocytes (4x10^6 cells/ml) were suspended in a solution of 10 to 15% w/v macromer with varying weight ratios of PEGDM:CHS (90:10, 80:20, 70:30, 60:40), 0.022% w/w Irgacure 2959 photoinitiator in standard chondrocyte medium. The gels were polymerized as previously described and cultured in standard chondrocyte medium.

7.2.5 Mechanical Testing

Tangent modulus under compression was determined for each hydrogel formulation. Compression tests were performed on hydrated samples at 0.02 mm/s to 15% strain under unconfined compression. The tangent modulus was calculated from the linear region of the stress-strain curve. A sample size of n=3 was used.

7.2.6 Cellular Strain Measurements

Cell strain was analyzed in PEG-Only and PEG-CHS hydrogels, with 15% w/v macromer and a PEG:CHS ratio of 80:20, 6 hours after encapsulation. Cells were stained with 4nM calcein AM (Invitrogen), which stains the cytosol of live cells, in PBS for 30 minutes at room temperature. A single hydrogel was placed into a custom straining device that sits on the stage of an inverted confocal laser scanning microscope (Zeiss 510). Images of cells were captured at their half height and maximum width under no gross strain and the same cells imaged under a 5% gross strain applied to the hydrogel. Cellular strain was determined by the difference in cell diameter in the direction of applied strain from 0 to 5% strain [29]. Two independent experiments were performed with a total of 10 cells per gel over 3 gels for a total sample size of n=30.

7.2.7 Measurement of Tissue Production

At prescribed times, gel and media samples were removed from culture for analysis of sulfated GAGs and total collagen content. All gels were halved and one half was homogenized and enzymatically digested in a papain solution (125 μg/ml Papain (Worthington), 10 mM L-cysteine, 100 mM phosphate and 10 mM EDTA in DI water (pH 6.3) overnight at 60°C). Media and papain digested gel samples were analyzed for GAG content using the 1,9-dimethylmethylene blue dye method, where a standard solution of GAG in chondrocyte media was used to determine GAG content [30]. The other gel half was homogenized in 1 mg/ml Pepsin A (Sigma) in 0.5M acetic acid for 24 hours at 4°C. Pepsin digested gel samples were analyzed for total collagen content using Sirius red [31]. DNA content in the gel was measured by PicoGreen® assay (Invitrogen) in the papain digest samples. Total GAG content, which represents GAGs in the gels and that which diffused into the medium, and total collagen content in gels were normalized to the DNA content of each gel. Two independent experiments were performed with a sample size of n=2 per experiment.

7.2.8 Intracellular Calcium Measurements

The intracellular calcium dye FURA-2 AM (Invitrogen) was used to visualize calcium transients in chondrocytes. Freshly isolated chondrocytes were plated in 2D on petri dishes with glass slides on the plating surface for 2 days in standard chondrocyte medium to allowed cells to adhere. Adhered cells were incubated with 4 μM FURA-2 AM in Hanks balanced salt solution (HBSS) with 1% (w/v) pluronic for 30 minutes, followed by several rinses in HBSS, and replaced with standard chondrocyte medium for 30 minutes. Approximately 15 chondrocytes per plate were imaged with excitation ratio imaging using 350/10 and 380/10 excitation filters, a 450-nm dichroic mirror, and a 535/45 emission filter on a Zeiss Axiovert 200M. Cells were imaged with either constant osmolarity at 330mOsm or during a change in osmolarity. Specifically, medium osmolarity was either increased from 330 to 400 mOsm, 330 to 430 mOsm or 330 to 460 mOsm or decreased from 460 to 330 mOsm, 430 to 330 mOsm, or 400 to 330 mOsm. For each condition, fluorescence images were captured for $\sim$1 minute before, during, and $\sim$2 minutes after osmotic perturbation every 10 seconds. After each experiment, cells were calibrated with a solution of 8 μM ionomycin salt (Sigma) in calcium free Hanks Balanced Salt Solution (Invitrogen) supplemented with 10 mM EGTA (Sigma) to chelate intracellular calcium followed by a rinse with HBSS and 20 mM calcium chloride to maximize intracellular calcium signals. Fluorescence ratio was analyzed for calcium transients, where a transient was considered to be a sharp increase in ratio ($\sim$10-30s) above 10% of the baseline, followed by a slower decrease in ratio ($\sim$30-60s). The percentage of cells exhibiting transients for each condition was averaged over 3 separate experiments where 12-19 cells were imaged per dish.

7.2.9 Intracellular pH (pH_i) Measurements

The intracellular dye 2, 7-bis-(-carboxyethyl)-5 -(and-6)-carboxyfluorescein acetoxymethyl (BCECF-AM) was used to measure intracellular pH (pH_i) as a function of extracellular osmolarity. For studies in 2D, freshly isolated chondrocytes were incubated in media at 330, 400, 430 or 460mOsm for 6 hours followed by incubation with 10 μM BCECF AM for 30 minutes. The dye

solution was rinsed and replaced with the respective media and the cells were transferred to a 96-well plate. A pH standard curve was prepared using cells cultured in solutions of varying pH (6.5-8) with 10 μM nigericin (Sigma) to equilibrate extra- and intracellular pH. Fluorescence was converted to a ratio of emissions at 535nm with excitations of 490nm and 440nm, read on a FLUOStar Optima plate reader (BMG Labtech). For studies in 3D, chondrocytes encapsulated in PEG hydrogels were cultured in media without phenol red at 330, 400, 430 or 460 mOsm, while PEG-CHS hydrogels (80:20) were cultured in 330mOsm media for 6 hours before incubation with 10 M BCECF for 30 minutes. The gels were rinsed and fluorescence was imaged using a Nikon A1R confocal and the background fluorescence, measured in gels with no cells and cultured in media, was subtracted from all samples.

7.2.10 Dynamic Loading of Cell-Seeded Constructs with Calcium Inhibition

The intracellular calcium chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) was used to chelate intracellular calcium during dynamic loading. PEG-Only hydrogels were prepared and cultured in 400mOsm media, while PEG-CHS (80:20) gels were prepared and cultured in 330mOsm media. After 5 hours of free swelling culture, 10μM BAPTA-AM was added to half of the PEG-Only and PEG-CHS (80:20). After one additional hour of free swelling culture gels designated for loading were placed in the loading bioreactors and fresh media containing either with or without BAPTA-AM was added to these gels [32, 33]. Gels designated for free swelling conditions were also replenished with fresh media with or without BAPTA-AM. Previous work in our lab has demonstrated that after 6 hours in culture in PEG-Only and PEG-CHS hydrogels, no visible amounts of tissue have been deposited [34], and as the native pericellular matrix can alter chondrocyte response to osmotic cues [35], the authors chose to begin loading at this time to ensure that chondrocytes would respond only to the osmotic cues presented and that newly synthesized GAG would be minimal. Hydrogels were loaded continuously at 15% strain and 1Hz for 6 hours. The loading regime applied to the gels was selected based on previous results showing stimulation of anabolic activity chondrocytes encapsulated in

PEG [34], agarose [16, 36] and peptide hydrogels [37]. After 6 hours of loading gels for analysis of GAG production were removed from loaded and free swelling culture, while gels for immunohistochemistry were left in free swelling culture without BAPTA-AM for 12 hours before being removed for analysis.

7.2.11 $^{35}SO_4$ Incorporation into Newly Synthesized GAGs

Proteoglycan synthesis was measured with $^{35}SO_4$ incorporation into newly synthesized GAG. Just before loading media supplemented with 10 μCi/ml $^{35}SO_4$ (Perkin Elmer, Shelton, CT) was added to all samples, free swelling and loaded, with or without BAPTA-AM. After 6 hours of dynamically loaded culture media with radioisotope was collected along with the respective gels. Hydrogels were crushed and enzymatically digested in a solution of 125 μg/ml Papain (Worthington), 10 mM L-cysteine, 100 mM phosphate and 10 mM EDTA in DI water (pH 6.3) overnight at 60°C. GAG production was determined using Alcian blue precipitation, where 0.02% w/v Alcian blue was added to media and gel samples to bind and precipitate the negatively charged proteins. The precipitate was isolated by filtration (Multiscreen HTS Filter Plate, Fisher Scientific, Pittsburgh, PA) and was rinsed with a solution of 4.1 g/L sodium acetate, 10.2 g/L magnesium chloride, and 14.2 g/L sodium sulfate in DI water to remove unincorporated isotope. The precipitate was released by incubation with 1.34 g/ml guanidine HCl in a solution of DI water/isopropanol (2:1 v/v). Total proteoglycans produced were measured in counts per minute (cpm) on a Beckman LSC 6500 (MDA 80.3 pCi). All samples were normalized to the gel DNA content as measured by the PicoGreen® assay (Invitrogen). Briefly samples of the gel digest were assay per the manufacturers protocol. Two independent experiments were performed with a sample size of n=3 per experiment.

7.2.12 Immunohistochemistry

Hydrogels were collected 12 hours after dynamic loading for immunohistochemical analysis. Gels were fixed in 4% (v/v) paraformaldehyde (Sigma) in PBS for 24 hours, and then transferred to 30% sucrose for 3 days. Samples were dehydrated and embedded in paraffin before being sectioned

into 10 μm slices. The sections were then pretreated with 2080 U/ml hyaluronidase (Sigma) and 100 mU/ml chondroitinase ABC (Sigma) for 30 minutes at room temperature. The sections were washed in PBS and then with 1 ml of PBS with 1% BSA and 0.05% Triton X-100. The samples were rinsed in PBS and then blocked in PBS with 1% BSA for 5 minutes at room temperature. The samples were rinsed in PBS before application of mouse anti-human aggrecan IgG1 (1:2.5, US Biologicals, Swampscott, MA) or rabbit anti-human collagen II IgG (1:25, US Biologicals primary antibody in PBS with 1% BSA at 4°C overnight. The samples were rinsed in PBS before application of secondary antibody, AlexaFluor 546 (1:200, Invitrogen) for aggrecan and AlexaFluor 488 (1:200, Invitrogen) for collagen, in PBS at room temperature for 2 hours. The samples were rinsed in PBS and a solution of 50 μg/ml DAPI was applied for 5 minutes. The samples were rinsed and sealed between a slide and coverslip using flouramount (Southern Biotech, Birmingham, Alabama) before being imaged on a CLSM (Zeiss). Two independent experiments were performed with one gel per experiment (n=2), where samples from both experiments were stained and analyzed together. Two images were taken per sample with laser power, gain, offset and pinhole kept constant for all images. The average fluorescence for each image, where fluorescence for either AlexaFluor 546 or 488 was analyzed independently of the DAPI fluorescence, was measured using ImageJ software and normalized to the number of nuclei present in the image.

7.2.13 Statistical Analysis

Data are reported as the mean with a 95% confidence interval about the mean. To determine statistical significance an ANOVA with Tukeys post-hoc analysis was used with α=0.05. All data were from independent observations, followed a Gaussian distribution and exhibited a homogeneous variance.

7.3 Results

Hydrogels were fabricated with varying total macromer concentration for PEG-CHS hydrogels to identify a formulation that resulted in similar tangent modulus under compression for PEG-only

hydrogels (Figure 7.1A). The weight percent of total macromer giving an equivalent compressive modulus to that of PEG-Only gels (∼123 MPa) was 15% w/v with a PEG:CHS ratio of 80:20. Under these formulations, no significant difference in cellular strain was observed under a 5% gross strain (Figure 7.1B).

Intracellular pH (pH_i) in chondrocytes cultured in 2D (Figure 7.2A) was affected by the type of culture medium, which was supplemented with salts or sucrose to adjust medium osmolarity from 330 to 460 mOsm. The pH_i decreased significantly with increasing osmolarity for both salt and sucrose supplemented medium. This drop was greatest for the salts-supplemented media at lower osmolarities of 400 and 430 mOsm and greatest for the sucrose-supplemented media at high osmolarites of 460 mOsm. The pH_i in chondrocytes cultured in 3D in PEG-Only gels was also affected by salt-supplemented medium with varying osmolarity (Figure 7.2B). Similar to 2D cultures, the pH_i decreased with increasing osmolarity, however the pH_i inside the PEG gels appeared to be higher than in 2D culture. The pH_i of PEG-CHS gels was statistically similar to that of the PEG-Only gels in 400mOsm media.

PEG-only and PEG-CHS cell-laden hydrogels were subjected to dynamic compressive loading for 6 hours. During loading, proteoglycan synthesis was measured by $^{35}SO_4$ incorporation. Proteoglycan synthesis for the loaded constructs was normalized to the respective free swelling gels and the data are shown in Figure 7.3. Dynamic loading decreased proteoglycan synthesis by 26.3% over free swelling constructs in PEG-Only gels. Contrarily, in the PEG-CHS gels, loading increased proteoglycan synthesis by 26.0% over the free swelling constructs. The addition of BAPTA-AM to the free swelling samples decreased proteoglycan synthesis by 33% in the PEG-Only and PEG-CHS gels, while the addition of BAPTA during loading decreased proteoglycan synthesis by 46.4% in PEG-Only gels and by 32.3% in the PEG-CHS gels.

Aggrecan deposition in PEG-Only and PEG-CHS gels 12 hours after the loading application is shown in Figure 7.4A with semi-quantitative analysis given in Figure 7.4B. Aggrecan deposition was similar in both PEG-only and PEG-CHS gels under free swelling conditions. Loading decreased aggrecan deposition in PEG-Only gels by 40% compared to free swelling, while PEG-CHS had 22-

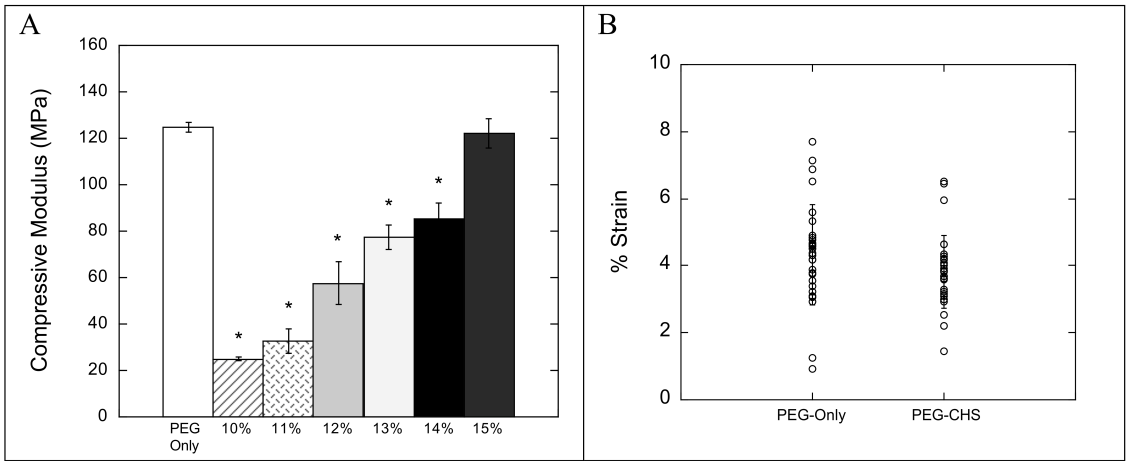

Figure 7.1: Compressive modulus (A) of PEG-CHS gels with a constant ratio of CHS to PEG (80:20) and increasing macromer weight percent and percent cell strain (B) at 5% applied strain to the gel. Compressive modulus data represents the mean over a sample size of n=3 with a 95% confidence interval. Cell strain data represents strain of individual cells (n=30) measured in 3 separate gels, with a 95% confidence interval about the mean.

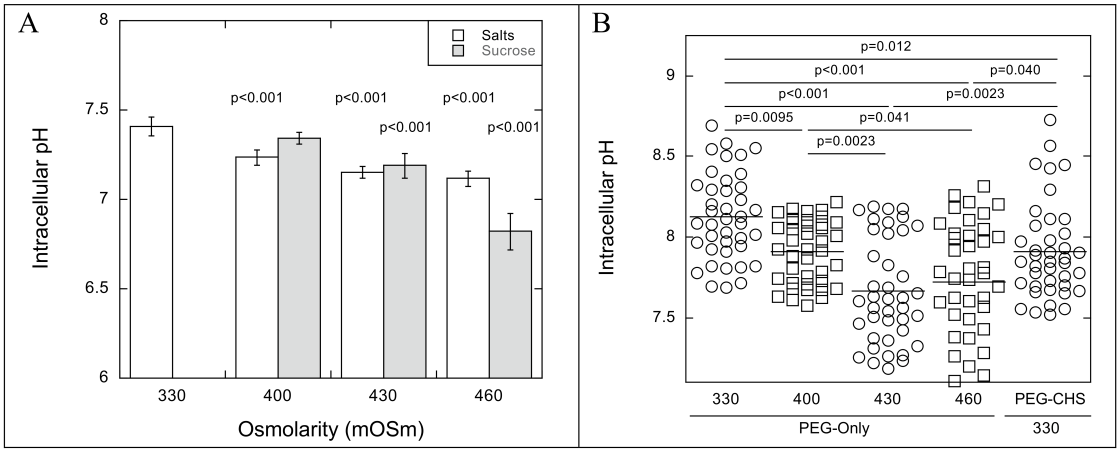

Figure 7.2: Fluorescence ratio of the intracellular pH dye BCECF-AM, converted to pH_i in chondrocytes in suspension (n=6) (A) and of chondrocytes encapsulated (B) in either PEG-Only gels with osmolarity adjusted using salts in the media or PEG-CHS gels (80:20) in 330mOsm media (n=8). Data in A represent the mean with a 95% confidence interval. Statistics in figure A refer to the p-value between 330mOsm and all other conditions.

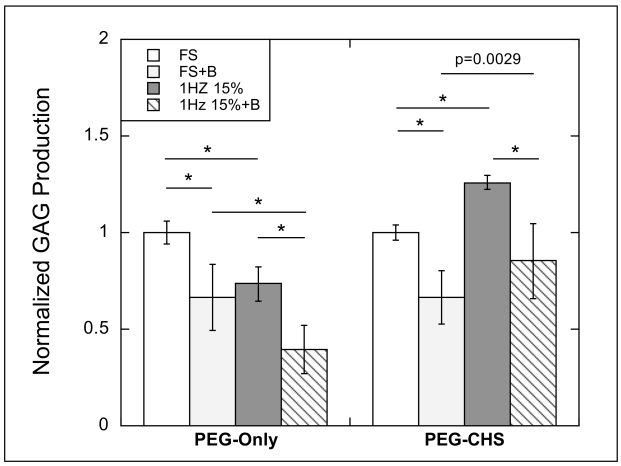

Figure 7.3: GAG production over 6 hours in PEG-Only and PEG-CHS gels normalized to free swelling (FS) controls for the respective gel type (A) or PEG-CHS gels normalized to the identical PEG-Only conditions (B). $*$ indicates a p-value <0.001.

33% more aggrecan with loading compared to either free swelling constructs. BAPTA-AM decreased aggrecan deposition in free swelling culture by ∼20% in both types of gels. BAPTA-AM reduced aggrecan levels in loaded PEG-CHS gels to levels that were similar to the free swelling PEG-CHS gels.

Collagen II deposition in PEG-Only and PEG-CHS gels 12 hours after the loading application is shown in Figure 7.5A with semi-quantitative analysis given in Figure 7.5B. Collagen II deposition was similar in both PEG-only and PEG-CHS gels under free swelling conditions. Treatment with BAPTA-AM resulted in significant down-regulations in collagen II deposition under free swelling for both gel types. Loading inhibited collagen II deposition by 58.6% in PEG-only and 77.3% in PEG-CHS. Treatment with BAPTA-AM led to a further down regulation in collagen II deposition.

To determine the effects of dynamic changes in ionic and osmotic environment on intracellular calcium signaling, calcium was imaged in individual chondrocytes cultured in 2D while the ionic and osmotic environment were changed. Figure 7.6A shows representative fluorescence ratio data collected over time for a randomly selected view of cells cultured in 2D in 330mOsm media. After osmotic perturbation, Ca^{2+} transients can be seen by increases in fluorescence, followed by decreases to the basal level occurring over ∼20-80 seconds. Treatment with BAPTA-AM led to overall

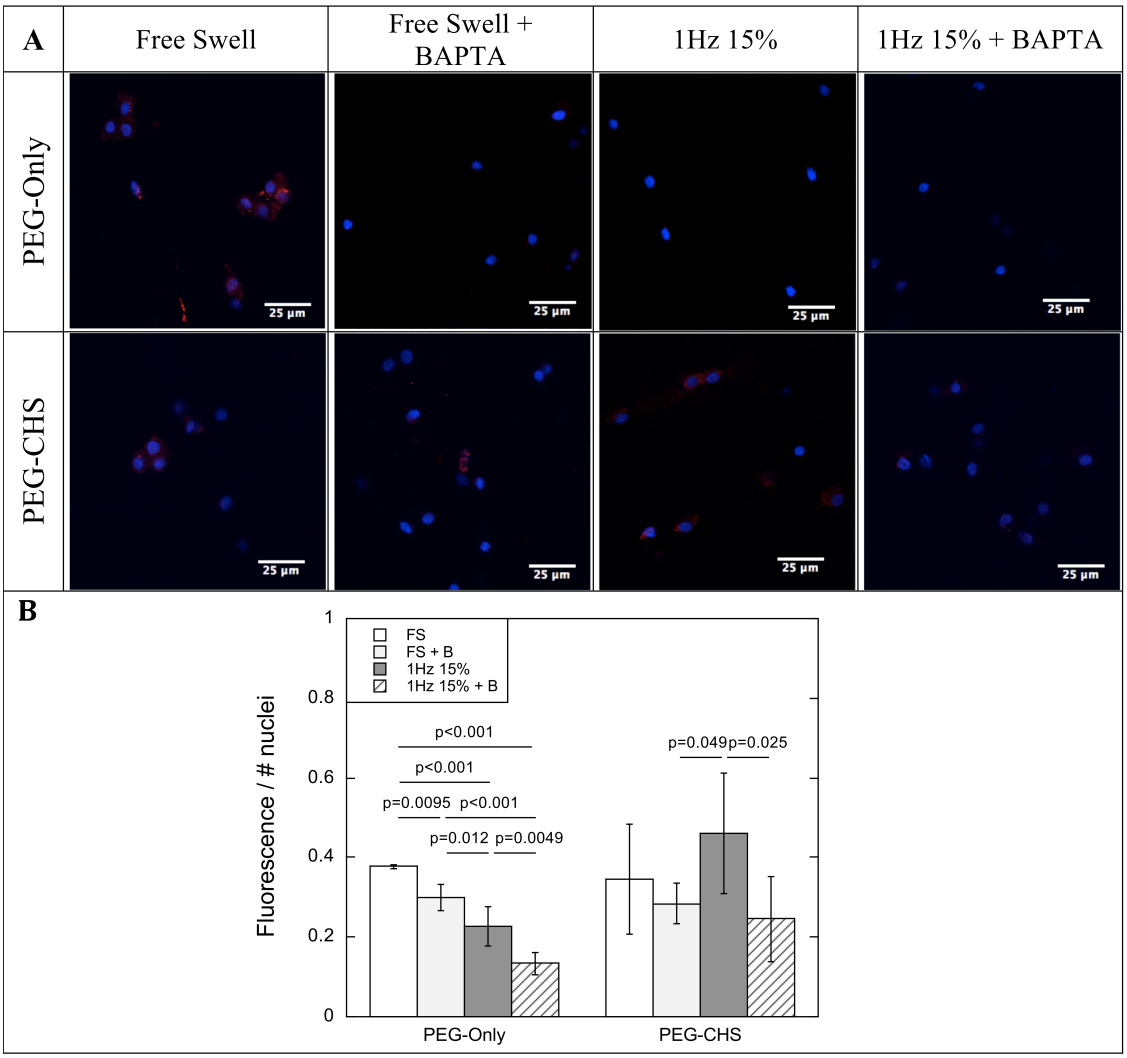

Figure 7.4: Immunohistochemistry images (A) and semiquantitative analysis (B) of aggrecan deposition in PEG-Only or PEG-CHS hydrogels with or without dynamic loading and with or without BAPTA-AM.

decreases in intracellular calcium and inhibited all calcium transients caused by changes in culture medium. After perturbation, the cells were treated with EGTA to chelate the intracellular calcium confirming the absence of a response followed by treatment with calcium chloride to confirm the ability of cells to uptake calcium. Any cell not exhibiting these characteristics was excluded from the analysis. Addition of EGTA in concert with BAPTA-AM was sufficient to cause instant necrosis, therefore the chondrocytes with BAPTA-AM were not calibrated with EGTA or calcium chloride solutions. Representative images of cells with a basal calcium level, indicated by a dark blue color,

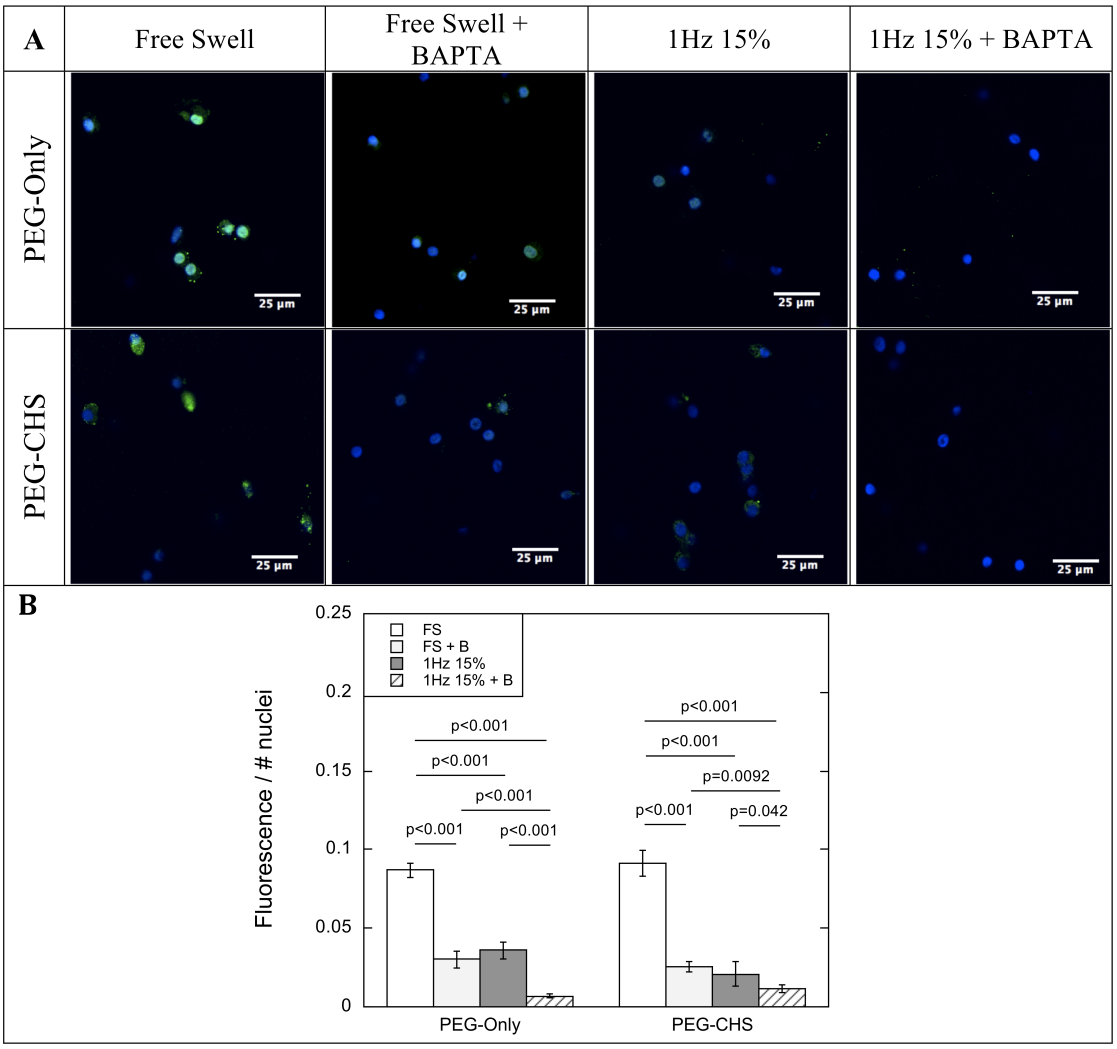

Figure 7.5: Immunohistochemistry images (A) and semiquantitative analysis (B) of collagen II deposition in PEG-Only or PEG-CHS hydrogels with or without dynamic loading and with or without BAPTA-AM.

and cells exhibiting calcium transients, indicated by a blue-green color, are shown in Figure 7.6B.

The percentage of cells responding to increases or decreases in osmolarity with calcium transients is shown in Figure 7.7A for media adjusted with salts. Increasing osmolarity from 330 to 400mOsm increased the percentage of cells responding by 27%, while increases up to 430 and 460mOsm decreased the percent of cells responding by ∼17% compared to the 330mOsm condition. Decreasing osmolarity seemed to have a greater effect on calcium transients, where all conditions of decreasing osmolarity resulted in significantly greater number of cells responding when compared

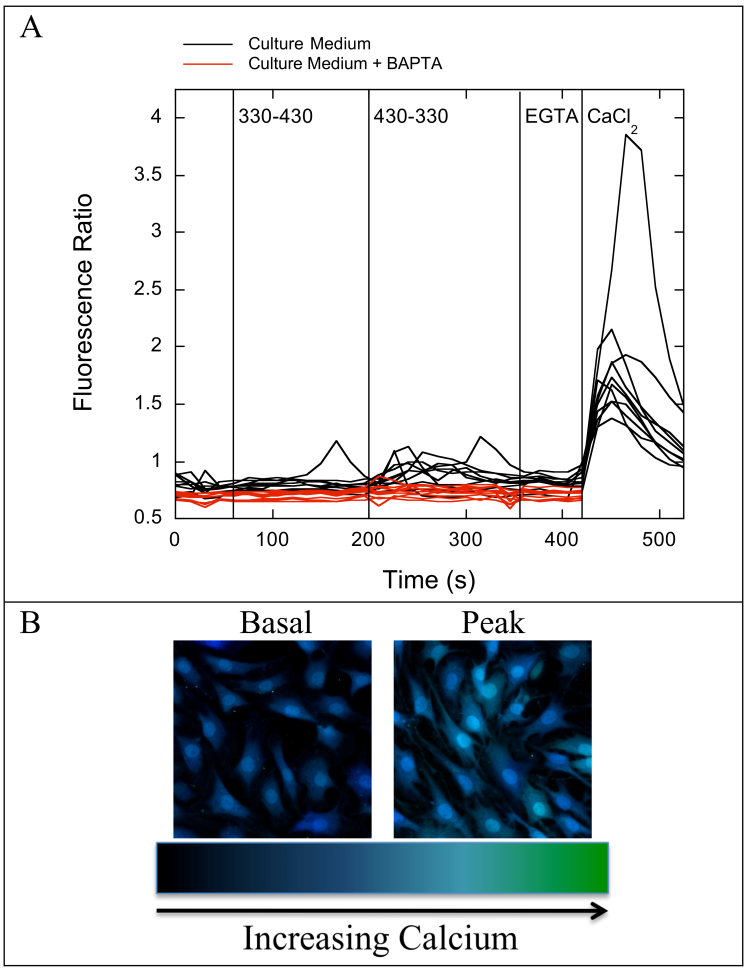

Figure 7.6: Representative graph (A) of fluorescence ratio indicating, intracellular calcium, over time in one set of cells with an osmolarity change from 330-430 and 430-330 either with or without BAPTA-AM and with EGTA and calcium chloride calibrations, where media osmolarity was adjusted with salts. Representative images (B) of basal and peak calcium levels in plated chondrocytes.

to the 330mOsm base condition. Decreasing osmolarity from 430-330mOsm showed the greatest increase in cells responding, with a 49.5% increase over the 330 mOsm condition, while 400-330mOsm and 460-330mOsm were slightly lower with only 44.1% and 39.1% increases over the base condition, respectively. The percentage of cells responding to increases or decreases in osmolarity with calcium transients is shown in Figure 7.7B for media adjusted with sucrose. Increasing osmolarity from 330-400mOsm decreased the percent of cells responding by 24.8% compared to the 330mOsm base condition, while no other conditions had a significant effect. Deceasing osmolarity from 460-

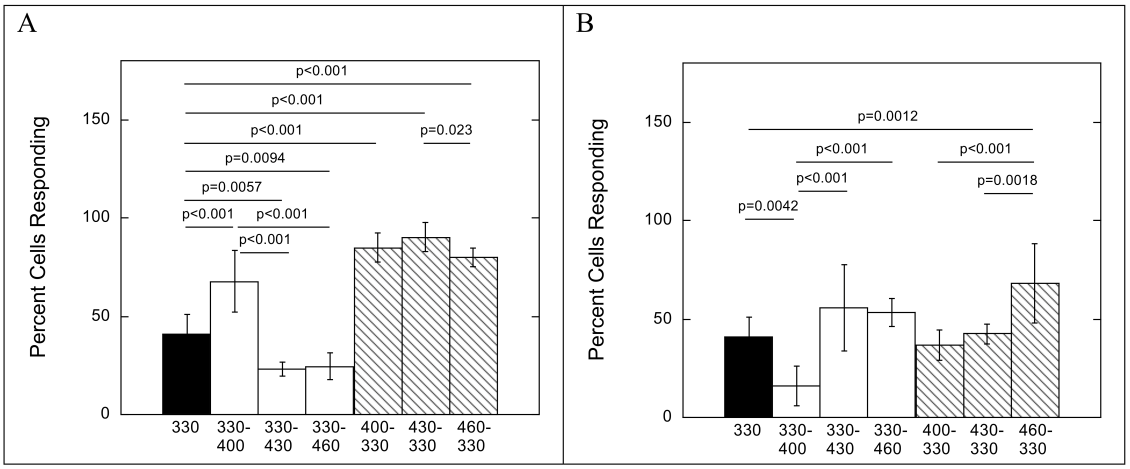

Figure 7.7: Percentage of cells responding to increases or decreases in solution osmolarity with calcium transients in media adjusted to the specified osmolarities with salts (A) or sucrose (B). Data represent the mean of n=3 samples with a 95% confidence interval.

330mOsm increased the percentage of cell responding by 27.4%, while all other conditions had no significant effect.

GAG and collagen production in chondrocytes encapsulated in PEG-Only gels and cultured in free swelling conditions with media at 330, 400, 430 or 460mOsm adjusted with salts or sucrose are shown in Figure 7.8A and 7.8B, respectively. GAG production was highest with 430mOsm medium where osmolarity adjusted with salts gave a 47% increase and osmolarity adjusted with sucrose gave a 192% increase in GAG over the 330mOsm medium constructs. Constructs cultured at 400 and 460mOsm were not significantly different from each other regardless of the type of supplemented medium. On average, GAG production in samples adjusted with sucrose was 1.92-fold higher than samples adjusted with salts. Collagen production was highest in 430mOsm samples adjusted with salts (97.1% increase) and in 460mOsm samples adjusted with sucrose (84.9% increase) compared to the 330mOsm samples. On average the collagen production was similar in samples with media adjusted with salts and sucrose.

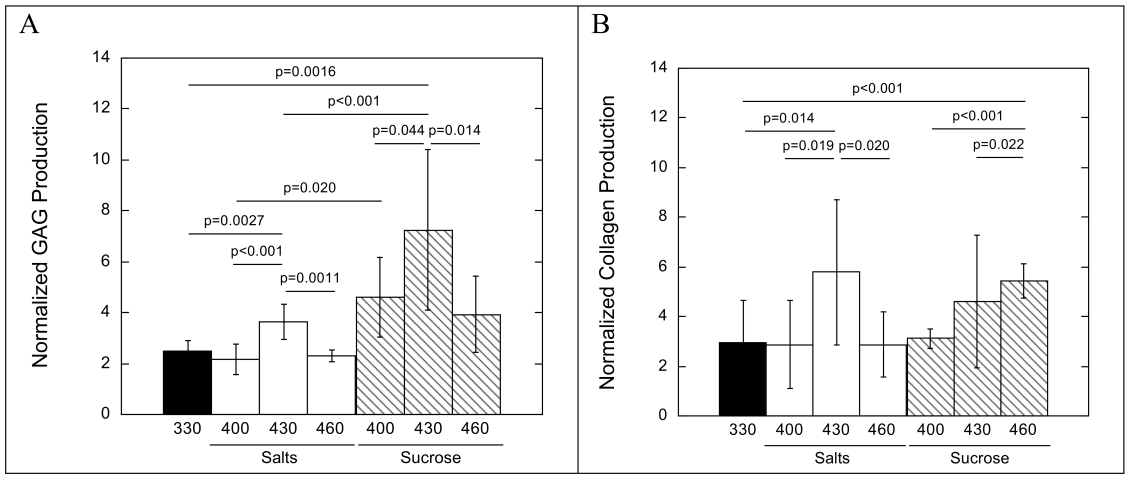

Figure 7.8: GAG (A) and collagen (B) production in chondrocytes encapsulated in PEG hydrogels and cultured for 7 days in media with osmolarity adjusted using salts or sucrose. Data are normalized to DNA content and day 0 time points for the respective conditions. Data represent the mean of n=4 samples with 95% confidence intervals.

7.4 Discussion

This study confirms that a negatively charged environment, dynamic loading, osmolarity, and ionic strength are all factors that influence tissue production by chondrocytes encapsulated in 3D hydrogels. Our data indicate that GAG and collagen productions by chondrocytes in neutral or charged hydrogels are mediated in part by intracellular calcium. In particular, we show that dynamic changes in osmolarity lead to changes in intracellular calcium transients suggesting that dynamic loading of a charged environment may influence intracellular calcium and subsequently could influence tissue production. Our findings point towards calcium as being an important signaling molecule in how chondrocytes sense and respond to a charged environment under loading, thus warranting further study into Ca^{2+}-mediated chondrocyte mechanotransduction.

One of the primary goals of this work was to isolate the effects of a charged matrix and dynamic loading on chondrocytes. To accomplish this goal, neutral PEG-Only and negatively charged PEG-CHS gels were developed to exhibit similar mechanical properties and similar levels of cellular deformation. In addition, it was important to assess the local osmotic environment in the PEG-CHS gels resulting from the presence of chondroitin sulfate. The extracellular osmotic

environment has been shown to lead to changes in intracellular pH, which is thought to occur because water, ions and protons are transported across the cell membrane [23, 38]. Therefore to estimate the local osmotic environment within the PEG-CHS gels, intracellular pH was measured in chondrocytes encapsulated in PEG-CHS and compared to chondrocytes encapsulated in PEG-Only gels cultured in medium of known osmolarity. We first confirmed that increasing osmolarity led to decreases in pH_i with either salts or sucrose, which follows with the shrinkage of cells at higher osmolarities resulting in the concentration of protons in the cell and thus a decrease in pH [8]. By comparing PEG-CHS gels to PEG-Only gels in medium of varying osmolarity, the pH_i in the PEG-CHS gels was estimated to be 400mOsm. This osmolarity was subsequently selected for the PEG-Only gels such that the osmotic environment inside the gels would be similar for each gel type under free swelling culture conditions. Interestingly, the pH_i in chondrocytes in suspension appeared to be lower than that of chondrocytes encapsulated in PEG hydrogels. This could be due to differences in osmolarity in the media, and inside the gel, possibly due to limited transport of larger molecules, such as components of the FBS, which could contribute to the osmolarity.

The application of dynamic loading significantly increased proteoglycan synthesis in the PEG-CHS gels, but inhibited proteoglycan synthesis in PEG-only gels. This finding is similar to previous results from our group [34] indicating that the presence of fixed negative charges provides an important cue to the cells, which regulates how chondrocytes sense and respond to dynamic loading. Aggrecan deposition correlated with the proteoglycan synthesis results showing an overall mean increase in aggrecan fluorescence in response to loading in the PEG-CHS gels, but an inhibition in the PEG-only gels. Under free swelling conditions, proteoglycan synthesis and aggrecan deposition were similar for both hydrogel environments. Because the hydrogel macroscopic properties and the free swelling osmolarities were similar between PEG-only and PEG-CHS, the increase is proteoglycan/aggrecan synthesis is attributed to the dynamic changes in osmolarity that arose during to loading. Interestingly, for collagen II deposition, the charged environment was not sufficient to overcome its inhibition in response to dynamic loading.

Results from other groups have shown that a dynamic osmotic environment can initiate in-

tracellular calcium signaling [2, 39], and calcium signaling in response to dynamic loading regulates tissue production [1]. Therefore, we hypothesized that intracellular calcium may be involved in mediating these responses to loading in charged gels. In general, chelation of intracellular calcium inhibited proteoglycan synthesis, aggrecan deposition and collagen II deposition regardless of culture condition (gel type or loading). Our findings suggest that calcium is involved in the regulation of tissue production. The changes in tissue production as a result of charge also appear to be mediated by intracellular calcium whereby chelating calcium in the loaded PEG-CHS gels resulted in proteoglycan synthesis and aggrecan deposition, which was similar to that of the untreated loaded neutral PEG-only hydrogels. Some studies suggest that K^+ and Cl^- ion channels may be activated by osmotic stress and changes in ionic environment, such as slow conductance calcium-potassium channels, L-type calcium channels, and stretch activated ion channels, all of which are known to influence intracellular calcium signaling [18]. Because cellular strains were similar in the PEG-only and PEG-CHS hydrogels at 5% strain, we hypothesized that the cellular strains would be similar at 15% strain and that the increase in proteoglycan synthesis and aggrecan deposition in the PEG-CHS gels may be regulated by dynamic changes in the ionic and osmotic environment via calcium events [16].

To test this hypothesis, experiments were performed to assess whether chondrocytes respond to dynamic changes in osmolarity by initiating calcium transients. Indeed our data show that dynamic changes in osmolarity alter calcium transients, but in a manner that was dependent on whether the osmolarity increased or decreased. Interestingly, nearly all of the cells responded with calcium transients when the osmolarity was decreased to 330 mOsm regardless of the initial starting osmolarity. This could be an artifact of how the studies were conducted, as the studies were all conducted in the same sequence, by increasing then decreasing osmolarity, which could precondition the cells and affect the results, however further study is required to investigate preconditioning of chondrocytes to intracellular calcium signaling. Taken together, findings from this study have led us to develop the following hypothesis. Intracellular calcium transients induced by dynamic changes in osmolarity, which results from a charged environment, enhances proteoglycan/aggrecan synthesis

in chondrocytes in 3D cultures.

Calcium transients have been observed and characterized in chondrocytes in steady state culture and with compressive loading, and have a characteristic shape, where a fast increase in calcium is observed with a slightly slower decrease to basal levels, occurring on a time scale of seconds to minutes, as was observed in our studies [40]. Calcium signaling that occurs on this time scale has been associated with regulation of cell metabolism and transcription, with one transient being sufficient to effect downstream changes in cellular response [19, 20]. Intracellular calcium signaling initiated by dynamic loading in cartilage, where the negative charges lead to changes in ionic and osmotic environment with loading, has been reported in other studies [17]. Regulatory volume decrease (RVD), initiated by an influx of Ca^{2+} ions, is one mechanism that has received much attention as a potential pathway for regulation of tissue production [41, 42, 43]. RVD has been shown to activate many surface ion channels, such as transient receptor potential vanilloid 4 (TRPV4), which is a stretch activated ion channel permeable to calcium and has been characterized as an osmotically sensitive channel [44, 45, 46]. TRPV4 deficient mice were shown to develop osteoarthritis, suggesting a role for this channel in regulating tissue homeostasis through osmotic environment [44]. Studies utilizing specific inhibitors of TRPV4 in a dynamic osmotic environment could provide further evidence of the role of loading in a charged matrix in regulating tissue production. One other pathway that is activated by RVD and could potentially regulate calcium signaling in response to ionic changes is the Na^{+}-Ca^{2+} exchanger (NCE) of the cell surface. *Sanchez et al.* [46] found that the NCE is partially responsible for the increase in intracellular calcium seen with hypotonic challenge, however the connection between NCE and tissue production is not clear. Further study is needed to understand the role of these channels in chondrocyte response to compression-induced changes in ionic and osmotic environment.

To gain more insight into the mechanisms involved in chondrocyte response to dynamic loading in charged hydrogels, differentiating between the ionic and osmotic effects of osmolarity may provide useful information as to the role of ionic environment in cellular response to dynamic loading and how that response influences calcium signaling. Through osmolarity adjustments with sucrose,

we can alter the osmotic pressure of the media without changing the ionic environment, while adjustments with salts will alter both ionic and osmotic properties of the media. Ionic strength and osmotic pressure appeared to differentially regulate intracellular calcium signaling, where decreasing ionic strength appears to have the greatest effect on calcium signaling. Interestingly, the condition with the most cells responding with calcium transients was decreasing osmolarity from 430mOsm, which is the closest to the physiological osmolarity *in vivo* and would suggest that ionic strength is a greater regulator of calcium signaling than osmotic pressure [8]. Increases in ionic strength of the extracellular media have been shown to increase activity of ion co-transporters, such as the Na^+/Ca^{2+} transporter, which could explain why the calcium response is greater in media combining ionic strength and osmotic pressure, rather than just osmotic pressure [47]. As intracellular pH was different in media with sucrose and salts (Figure 7.2A), this suggests that transfer of protons is regulated more by ionic effects; potentially through activation of K^+/H^+ exchangers. Intracellular pH has been shown to regulate tissue production, therefore any differences in pH between samples cultured in sucrose and salts will presumably lead to differences in tissue production [38, 47, 23].

GAG production with increasing osmolarity adjusted with salts and sucrose showed similar trends in that the most physiological osmolarity produced the most GAG, which has been shown for media adjusted with salts in previous studies [48]. Samples cultured in sucrose produced more GAG than samples cultured in salts, suggesting that GAG production is regulated more by osmotic effects, rather than ionic. This is supported by studies that have shown increases in GAG synthesis with application of osmotic pressure [21]. Previous studies have shown that increasing CHS concentration increases osmotic pressure with constant ionic strength [4], and given that GAG production has been found to be regulated by GAG concentration [48], taken with our data this suggests that GAG production may be a feedback mechanism by which cells control their osmotic environment. When chondrocytes sense a change in osmotic pressure they appear to respond with changes in GAG synthesis, which may be a mechanism to adjust the extracellular osmotic pressure to maintain homeostasis.

Collagen production appeared to be differentially regulated with ionic versus osmotic effects,

where samples cultured in sucrose had similar collagen production levels compared to salts, however the trends of tissue production with osmotic environment were not similar. The ionic environment, which produced the most GAG, also produced the most collagen, however when osmotic effects were isolated, collagen production appeared to increase with increasing osmolarity. The differential regulation of collagen synthesis by ionic and osmotic has not previously been reported, however it could be related to osmotically induced changes in SOX9 gene expression, which is a transcriptional factor that regulates collagen II synthesis [49]. While total collagen production is clearly regulated by the osmolarity of the culture medium and collagen II deposition is highly regulated by calcium signaling, a dynamic osmotic environment may not regulate collagen synthesis or higher osmolarities may be required to have an affect on collagen deposition.

Interestingly increases in the number of cells exhibiting calcium transients with decreases in osmolarity in media with salts corresponds to increases in GAG production in chondrocytes cultured in salts and sucrose, as well as collagen production in salts, while increases in the number of cells exhibiting calcium transients with decreases in osmolarity in media with sucrose corresponds to increases in collagen production in sucrose. This supports the hypothesis that the presence of charge in hydrogels may regulate tissue production through calcium signaling, however the exact mechanisms for this need to be investigated further.

To summarize, this study has determined that GAG and collagen production are mediated by intracellular calcium signaling, and intracellular calcium signaling may also regulate GAG production with dynamic loading in a charged environment. Findings from this study show that ionic and osmotic effects give differential responses with respect to intracellular calcium signaling as well as tissue production. However, it is not clear how calcium is involved in mediating tissue production induced by ionic and osmotic effects. Taken together this suggests that calcium dependent pathways may be activated in response to loading in a charged environment; however further study is required to elucidate these pathways. Future studies to determine the mechanisms behind the differential regulation of calcium signaling and tissue production will give insight into how osmotic homeostasis is maintained in cartilage and will provide insight into better tissue engineering

strategies that focus on controlling osmotic environment to guide tissue production.

7.5 References

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Chapter 8

Conclusions and Recommendations

8.1 Conclusions

This thesis has provided a greater understanding of the role of age-associated changes in how chondrocytes sense their environment with respect to a charged matrix and physiological and injurious loading. This research has made several key contributions: i) defining improved methods for the photoencapsulation of chondrocytes, ii) identification of loading regimes, which lead enhance tissue synthesis and which are age-dependent, and iii) laid the ground work for future studies to investigate the differential effects of ionic and osmotic environment on calcium signaling and tissue production, such that the specific cues responsible for regulation of tissue production can be isolated towards developing better tissue engineering strategies.

The first objective optimized photoencapsulation methods for chondrocytes in PEG hydrogels, where the combination of antioxidants, physiological osmolarity, and the development of some PCM, resulted in an improved robustness against free-radical damage during photoencapsulation. Our findings indicate primary isolated chondrocytes are susceptible to free radical damage during photoencapsulation. These findings may be important in other tissue engineering applications where freshly isolated cells are employed with photoencapsulation strategies. The identified encapsulation and culture conditions maintain chondrocyte survival and function for several weeks post-encapsulation and present significant progress towards developing strategies that employ adult chondrocytes at low encapsulation densities with photopolymerization.

The photoencapsulation process is known to lead to the generation of photoinitiator radicals

and macroradicals on propagating chains and to extracellular ROS when oxygen is present, which induce oxidative stress in chondrocytes. This oxidative stress appears to have longer-term negative effects on chondrocyte anabolism. The presence of a PCM, however, reduced chondrocyte death during photoencapsulation. We have shown that this increase in viability directly correlated with decreased levels of oxidative stress, which subsequently improved chondrocyte anabolic activity. Our findings suggest that photoencapsulation may actually inhibit the capacity for tissue production in chondrocytes due to radical damage and that minimizing oxidative stress, by allowing time for formation of PCM, may have long-term beneficial effects on tissue elaboration when employing photopolymerizations to encapsulate chondrocytes for cartilage tissue engineering applications.

In an effort to characterize and understand chondrocyte response to physiological loads and the role of age in this response, utilizing the improved encapsulation strategies determined in the first objective, we have demonstrated that chondrocytes respond differently to physiological loading of varying strain and frequency with respect to age and that regulation of tissue homeostasis is also altered with age. This study has identified optimal loading conditions for developing a more functional engineered tissue in PEG hydrogels with respect to patient age. As PEG hydrogels provide a basis for isolating the effects of cell age on response to dynamic compression, this study has also provided a basis for further study into changes in mechanotransduction with aging. The insights gained from this could potentially be used to develop therapies that prevent age related changes in tissue composition, which are thought to be a factor contributing to development of osteoarthritis, as regulated by mechanical cues.

Investigations into chondrocytes response to impact injury with age have demonstrated that given similar injurious mechanical stimuli, adult and juvenile chondrocytes did respond with increases in cell death partially through apoptosis, increased in ROS, increases in catabolic activity and decreases in anabolic activity. Juvenile chondrocytes responded with more apoptosis and intracellular ROS than the adult cells, suggesting an underlying connection between ROS and apoptosis. Our data suggests that chondrocytes respond to injury through changes in anabolic and catabolic activity differentially with respect to age, where juvenile cells decreased aggrecan synthesis and in-

creased collagen degradation, while adult chondrocytes decreased collagen synthesis and increased aggrecan catabolism. The results of this study provide insight into the potential mechanisms for development of post-traumatic joint disease and the associated increase in risk with age. Specifically, the observed changes in mechanotransduction with age, in concert with factors such as inflammation after injury, could be responsible for the increased progression of OA with increasing age after impact injury. Further study into the mechanisms of the cells responses could provide valuable insight that will lead to improved post-injury therapies targeted to preventing the onset of osteoarthritis.

The investigation into chondrocyte response in a charged matrix with dynamic loading has confirmed that addition of charge to PEG hydrogels led to changes in GAG and collagen production that appear to be mediated by intracellular calcium signaling. Incorporation of negative charges in the cell culture matrix has emerged as a tool to bolster tissue production in synthetic PEG hydrogels, along with physiological loading. However, many of the mechanisms involved in transfer of extracellular cues into biomechanical signals that regulate tissue production still remain to be determined. We have shown that ionic and osmotic effects of culture media have differential effects on intracellular calcium signaling as well as tissue production, suggesting that multiple pathways may be involved in chondrocytes response to a charged environment. Future studies to determine the mechanisms behind the differential regulation of calcium signaling and tissue production with ionic and osmotic environment will give insight into how osmotic homeostasis is maintained in cartilage and will provide improved tissue engineering strategies that focus on mimicking the charged matrix of cartilage to guide tissue production.

Taken altogether, thesis has shown that chondrocyte age is a major factor in cellular response to physiological cues and that tissue engineering strategies utilizing PEG hydrogels will require specific cues based on the cell age. When applied in a clinical setting, the results and implications of this thesis will enable tissue engineering strategies to be more robust, making therapies tailorable to patient age. The presence of a charged environment has proven to be a potentially useful tool in guiding cartilage tissue production, however specific mechanisms of tissue regulation through

calcium signaling remain to be elucidated. These results will have significant implications in the future design of tissue engineering strategies that utilize autologous chondrocytes and will further the general knowledge of chondrocyte biology towards developing treatments for joint disease, such as osteoarthritis.

8.2 Recommendations

Although this thesis has provided significant insight into alterations in chondrocyte response to physiological cues with age, further study is required to elucidate the specific mechanisms altered by aging that lead to the differential responses observed in this study. The differential regulation of tissue production with physiological loading is a significant finding, but with loading creating changes in chondrocyte deformation, fluid flow and nutrient diffusion, it is difficult to differentiate between these effects to hypothesize potential mechanisms of tissue regulation and age-related changes. Studies that isolate these effects could provide valuable insight into the mechanotransduction pathways activated with compressive loading and the changes in response with age could be more clearly characterized. Utilization of microfluidic devices, such that fluid flow, cell shear, and medium osmolarity could be tightly controlled would allow for isolation of these stimuli and the corresponding cellular responses. Theoretical modeling of fluid and nutrient flow could also provide insight into why particular loading regimes, with respect to strain and frequency, are preferential for tissue production, particularly with age.

Interestingly, with injurious loading, the response of cartilage appears to be mostly related to cellular response with respect to age, rather than changes in mechanical properties of the tissue with age, based on comparison to previous studies that have utilized cartilage tissue from juvenile and adult donors. This study has shown differential regulation of aggrecan and collagen anabolic and catabolic activity with mechanical injury, however further study is needed to understand the mechanotransduction pathways involved in response to cartilage injury and how these pathways are affected by age. Our studies have suggested that increased ROS in adult chondrocytes may play a role in the altered response to injury with age, however the identity of these ROS is a subject of

debate. Elucidation of the specific ROS increased in aging and injury could provide insights into the observed changes in tissue homeostasis through oxidative stress mechanisms.

Finally, this thesis has suggested a role for intracellular calcium signaling in regulation of tissue production in a charged environment with dynamic loading. However, there are many different pathways associated with changes in intracellular calcium signaling that could contribute to the observed regulation of aggrecan and collagen. Studies that utilize specific inhibitors of intracellular calcium stores or membrane transporters of calcium could help to elucidate the specific mechanisms that are activated by addition of these charges with load, and will lead to potential targets for cues to regulate cartilage production. Our studies have indicated that ionic and osmotic effects of culture media can differentially regulated intracellular calcium signaling and tissue production, suggesting that they contribute to the overall effect of a charged environment on chondrocytes function through separate pathways within the cell. Future studies to determine the mechanisms behind the differential regulation of calcium signaling and tissue production will give insight into how osmotic homeostasis is maintained in cartilage and will provide insight into better tissue engineering strategies that focus on mimicking the charged environment of the native tissue to guide tissue production.

In conclusion, this thesis has laid much of the ground work necessary to understand how chondrocytes sense and respond to physiological cues, such as mechanical loading and osmolarity, and the age-related changes in chondrocyte function that alter these responses. Many of the mechanisms behind the observed responses remain to be elucidated, however we have presented strong evidence of age-related changes in mechanotransduction in chondrocytes and regulation of tissue production by introduction of negative charges in a dynamically loaded system through intracellular calcium signaling, which will guide future tissue engineering strategies towards development of functional cartilage tissue and robust therapies for patients suffering from joint disease.

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