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UNIVERSITY OF MIAMI

EFFECTS OF GLUCOSE LEVELS ON GLUCOSE CONSUMPTION AND VIABILITY OF CHONDROCYTES AND INTERVERTEBRAL DISC CELLS

By

Garrett R. Waggoner

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

June 2013

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EFFECTS OF GLUCOSE LEVELS ON GLUCOSE CONSUMPTION AND VIABILITY OF CHONDROCYTES AND INTERVERTEBRAL DISC CELLS

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Lower back pain (LBP) and cartilage-related diseases such as osteoarthritis (OA) have a tremendous financial impact on the world today. It is well-understood that the provision of glucose to these avascular regions of the body plays a large role in the sustenance of health and the prevention of pathogenic processes. In order to undertake innovative approaches to allow for glucose-involved cell therapeutics and to enhance a wide range of stem cell therapies, we need to better understand avascular cell metabolism.

In this study, annulus fibrosus (AF), nucleus pulposus (NP), and cartilage cells were placed in agarose gel constructs and incubated in varying concentrations of glucose for up to 12 days. Live cell density was quantified using a viability staining technique at varying time points. The stained samples were imaged and cropped using a standard area, and the cells in each image were counted using ImageJ. Cell density results indicated that cell viability can be maintained at 0.5 mM glucose for all cell types. NP cells appeared to lose viability when incubated in high concentrations of glucose medium. There was a statistically significant drop in cell density of NP cells incubated in 3.2 g/L vs. those in 0.1 g/L glucose medium.

Glucose consumption was analyzed using a glucose assay kit in conjunction with absorbance readings from a spectrophotometer. AF cells consumed the most

glucose in the first 24 hours of culture, followed by NP cells and then chondrocytes. The amount of glucose consumed increased for all cell types when cultured in higher concentrations of glucose.

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CHAPTER 1: INTRODUCTION

1.1 Glucose

As a fundamental metabolite for every living cell, glucose provides freely obtainable energy to cells in the form of simple sugars, intermediary molecules, and macromolecular compounds. In addition to an energy source, glucose can be utilized as a building block of carbon skeletons for the synthesis of other molecules such as glycogen, proteins, lipids, and nucleic acids.[1] For example, hexose sugars are used as components of glycoproteins such as the proteoglycans that assist in providing the structure of certain extracellular matrices of cartilage. These proteoglycans also perform adhesive functions, transfer information in a paracrine-like fashion, and determine the microcirculation of their tissues. Due to its metabolic relevancy, the presence of glucose is key to the viability and healthy functioning of cells, particularly in avascular tissues.

1.2 Chondrocytes

Articular cartilage is a type of hyaline cartilage that covers the surfaces of diarthrodial joints, allowing for smooth mobility.[2,3] The connective tissue is classified as aneural, avascular, alymphatic, and hypocellular. Working along with the synovial fluid within the joint, it allows for nearly frictionless movement between the components within the joint. Specifically, the role of this cartilaginous tissue is to bear the load of the compressive forces involved in normal joint movement and to distribute this load to a broader surface area. It is able to accomplish these tasks due to its tensile strength and elasticity,[2]

Cartilage tissue contains chondrocytes that are responsible for the production of the macromolecular framework that allows for the flexible nature of the tissue.[4] This framework consists of collagens, proteoglycans, and other noncollagenous proteins. The matrix provides protection for the cells from day-to-day use of the joint, determines the permeability of the tissue, serves as a mechanical signal transducer, and sustains the phenotype of the chondrocytes. The chondrocytes perform tissue remodeling to replace degraded macromolecules and are responsible for the synthesis of each component of the extracellular matrix (ECM). Aging decreases chondrocytes' abilities to regenerate these matrix components, leading to articular cartilage degeneration.[3] The chondrocytes are able to detect changes in the articular cartilage matrix, queuing it to anabolically or catabolically alter the matrix. They use this structural remodeling technique to replace lost macromolecules during cartilage degeneration.[5] By receiving mechanical, electrical, and chemical signals from their environment, they control their production of collagens, proteoglycans, and noncollagenous proteins as they remodel the ECM.[2]

Articular cartilage is composed of horizontal layers, or zones, containing cells with varying characteristics. From the surface to interior, they are the superficial, transitional, deep, and calcified zones. The sizes, shapes and metabolic pathways within the cells vary according to their depth, and cell density is greatest near the surface of the tissue.[6] The contents comprising the extracellular matrix also vary according to tissue depth. Tissue fluid and the collagens, proteoglycans and other proteins make up the macromolecular cartilage matrix. The fluid, containing electrolytes, gases, small proteins, and metabolites, interacts with the macromolecules

to give the rigidity of the matrix to compressive forces.[2] The pericellular, territorial, and interterritorial regions surrounding each chondrocyte are used to describe the distribution of hyaluronic acid, linkage proteins, and proteoglycans.[7] These regions provide the structural properties to self-sustain during mechanical loading.[8] Through the interactions of these components, the ECM is able to provide structural integrity to the cartilage tissue.

A large contributor to this integrity is collagenous protein. Specifically, type II collagen makes up 80% to 90% of the total collagen in the matrix and interacts with other collagen types to form a sort of endoskeleton within the cartilage.[2] Microfibrillar type VI collagen[9], which is located in the pericellular matrix and stratifies the territorial and interterritorial regions[10], is thought to be responsible for chondrocyte attachment to the backbone of the matrix.[2] Aggrecans, or proteoglycan aggregates, make up 90% of the proteoglycan structure within cartilage tissue. They are durable and swell in solution, making them able to uphold under mechanical loading. They are bound to and stabilized by hyaluronic acid and link protein.[11] These linkages determine the level of incorporation of aggrecan in the ECM. Chondrocytes produce hyaluronic acid in response to environmental stimuli in order to determine aggrecan retention.[7] The ability of articular chondrocytes to undergo reversible deformation in response to compressive forces originates from the distribution of the collagenous network. Retention of the proteoglycan matrix in solution permits the pressure response involved in this deformation.[12] The collagen turnover rate is much slower than that of the proteoglycans and other components of the macromolecular framework. Macromolecular products of this turnover are continually released into

the synovial cavity, eventually reaching the cardiovascular system. For this reason, they may be used as a measuring tool for metabolic deviations.[13]

Along with lubricating the joint, the synovial fluid provides nutrition and eliminates waste in the area through diffusion. This is necessary due to the avascular and alymphatic nature of cartilaginous tissue. The synovial fluid, produced by synoviocytes, consists of electrolytes, nutrients such as glucose, small molecules, and metabolic waste products including carbon dioxide and oxygen.[2] Nutrients like glucose must diffuse through both the synovial membrane and the cartilage matrix to be used by the cells.[14] The size and charge of the molecules passing through the matrix, as well as the organization and composition of the matrix (especially the concentration, composition, and concentration of large proteoglycans), determine the rate diffusion.[15] Monovalent and divalent ions, glucose, amino acids, and water normally pass through the ECM. These nutritional factors play an integral role in the development, maintenance, repair, and remodeling of the cartilage by the chondrocytes.

Glucose is considered to be an essential metabolite and structural component of cartilage, and its sustained delivery and uptake are pertinent to the growth and proper functionality of the tissue.[16] Because nutrients must diffuse through the synovium and ECM before reaching the chondrocytes, the cells must be able to survive in moderately low glucose concentrations.[15] Nevertheless, chondrocytes are highly glycolytic and require a sustained supply of glucose for proper cellular homeostasis.[17] They consume glucose for the production of ATP and utilization of sulfated sugars such as glucosamine sulfate for the production of the ECM. There exist channels

within synovial joints to provide nutrients to metabolically active chondrocytes that are distant from blood circulation. The distance from chondrocytes to the nearest capillary can be over 1 cm, as in the human knee.[18] Fenestrations within capillaries that are oriented toward the synovial joint space to allow for more efficient diffusion are insufficient in supplying chondrocytes near the calcified layer with enough glucose. Therefore, spaces within the cartilage provide the microcirculation necessary for large substances such as glucose to reach the deeper chondrocytes in the tissue. This is generally aided by dynamic mechanical loading on the joint during physical activity. Interestingly, regular exercise has been shown to reduce progression of osteoarthritis (OA) in mice by breaking up cytokine clusters and improving overall glucose tolerance.[19]

It is common that OA occurs concurrently with endocrine dysfunction associated with glucose imbalance, such as diabetes mellitus. Studies have suggested that metabolic disorders involving glucose imbalance trigger cartilage degeneration.[1] Even minor changes in the concentration of glucose within the microenvironment immediately surrounding the chondrocytes can directly cause impairment of IGF-1-mediated anabolic activities. Poor management of insulin-dependent diabetes mellitus can lead to this glucose depletion or elevation and can progress to further pathologies in the cartilage tissue. Because of the role of glucose in the viability of chondrocytes and as a building block of the chondrocyte-generated macromolecular matrix, the importance of sustaining physiological levels of glucose should not be underrated with regard to cartilage dysfunction.

The main cause of joint-related disability in both human and animal OA is a general loss of cartilage.[20] OA is the most common non-inflammatory disorder of movable joints, specifically favoring large, weight-bearing joints. Characteristics of OA include atypical synthesis of cartilage ECM, gradual reduction of cellular density, subsequent fragmentation and degradation of the cartilage, peri-articular osteophytosis, an initial decrease followed by an increase in subchondral bone density, and inconstant synovial inflammation.[20]

Principally, OA is the clinical phenotype of various connective tissue function abnormalities coupled with abnormal chondrocyte behavior. Often observed in cartilage degeneration are the overwhelmed reparative capabilities of the chondrocytes, and the cellular activities in OA manifest in a convalescent and catabolic manner. Cartilage lesions are initiated by mechanical stress, shifting the interaction between chondrocytes and the surrounding matrix as well as the metabolic activity of the chondrocytes themselves.[21] In the early stages of OA, the water and proteoglycan content of the matrix increases during a transient proliferation of chondrocytes. The cellular topography of the chondrocytes changes according to their increasingly undifferentiated nature, causing them to cluster. Collagen type X, which is typically produced in fully differentiated chondrocytes, has been shown to surround these clusters in osteoarthritic cartilage. The up-regulated production of collagen type X in spite of their undifferentiated nature indicates a self-reparative approach within the damaged tissue.[22] Although generally recognized as a self-regulated attempt to minimize cartilage degeneration, chondrocyte proliferation is rarely sufficient to

combat degradation. This chondrocyte cloning is typically followed by apoptosis and reduced cell density.[23]

In early OA, chondrocytes and synoviocytes release catabolic coenzymes, including IL-1B, IL-6, and TNF-α which induce matrix-degrading enzymes, including matrix metalloproteinases (MMPs) and aggrecanase. These are responsible for articular cartilage matrix degradation.[24] As the MMPs begin to overpower its tissue inhibitors, the imbalance causes the activation of the MMPs, potentially contributing to cartilage degeneration. In addition, IL-1β may trigger depletion of the ECM by decreasing production of cartilaginous proteoglycans and collagen type II.[25] To note, elevated levels of IL-1β systemically stimulate glucose transport and metabolism, instigating hypoglycemia and diminishing glucose-induced insulin secretion.[26] Stimulated glucose metabolism has been reported as an effect of IL-1β in articular cartilage.[27] Once the matrix is degraded, a repair matrix is formed by the chondrocytes that is ineffective at withstanding mechanical loading. Especially in concentrated areas of proteoglycan depletion, the ECM is broken down in an accumulation of vertical, oblique, and tangential clefts.1 In addition, chondrocyte apoptosis intensifies tissue degeneration.

OA is both multifactorial and polygenic in nature. The anabolic and catabolic components arise from various mechanical, biochemical, and systemic origins.[1] Poor nutrition is generally regarded as a substantial contributing factor to the pathogenesis of joint disorders in both humans and animals.[28,29] Because nutrition plays a large role in the catabolic-anabolic balance of cartilage tissue, it is important to comprehend both systemic glucose metabolism and the pathophysiology of malnourished

chondrocytes to unveil the origins of articular cartilage disorders. Catabolic and proinflammatory cytokines, such as IL-1β, are responsible for the progression of the disease, while anabolic factors, such as IGF-1, are involved in the chondrocyte's mitosis and synthesis of the macromolecular matrix as well as the inhibition of catabolism. One direct connection between diet and cartilage remodeling is the reduction of certain IGF-1 binding proteins that carry the growth factor in circulation.[30] It has been shown that IGF-1 sensitivity declines with age and that osteoarthritic joints do not respond as well to IGF-1 stimulation.[31] This "IGF-1-resistant state" involves insulin/IGF-1 signaling and glucose metabolism in the development of OA.[32] Also, studies report that articular chondrocytes express the obese *OB-R gene*, forming the leptin receptor *in situ*.[33] Chondrocytes that are stimulated by leptin display enhanced proliferation and macromolecular components of the ECM. Elevated levels of leptin expression have been reported in chondrocytes near capillary blood vessels intruding upon hypertrophic cartilage.[34] It is believed that through regulation of angiogenesis, leptin affects cartilage formation and therefore remodeling directly.

Another articular cartilage disorder, osteochondritis dissecans (OCD), involves the fragmentation of cartilage tissue as pieces break off from the surface into the joint space.[35] Excess protein and carbohydrate consumption and excessive supplementation have been shown to be prominent contributors to the progression of the disorder in food-producing animals.[36] It stands to reason that proper nutritional management would suppress the development and progression of OCD.[37] Additionally, researchers are optimistically looking into the modulation of nutrient transporters as a means of

prevention for the treatment and cure of OCD, OA, as well as other joint diseases. This is an indication of the therapeutic potential in managing the glucose availability of chondrocytes.

In summary, an imbalance between cartilage repair and degradation causes the loss of components within the macromolecular matrix, including proteoglycans and collagen type II. Due to the importance of IGF-1 and other signals responsible for the regulation of this balance, and the association between these signals with dietary factors and diet-related pathologies, it is widely understood that proper nutrient delivery within cartilage tissue and its provision to the chondrocytes themselves is pertinent to the health of the tissue.

1.3 Intervertebral Disc Cells

Lying between vertebral bodies, intervertebral discs (IVDs) hold the spinal column together. They occupy about one third of the length of the spine, and they must efficiently transmit mechanical loads arising from body weight and other forces during movement. Functioning as joints in the spinal column, they allow for bending, torsion, and flexion.[38] The discs are usually 7-10 mm thick and about 4 cm in diameter**.** A thick outer ring of fibrous cartilage, known as the annulus fibrosus (AF), surrounds a gelatinous interior called the nucleus pulposus (NP). The NP is located in between cartilage end-plates above and below the disc.[38]

One difference between the AF and NP regions is the organization of the fibers that make up the tissue. The NP contains collagen fibers that are laid out randomly and elastin fibers aligned radially.[39] A highly hydrated matrix containing aggrecan surrounds the fibers. The cells in the region, similar to chondrocytes, are

scattered throughout at a low density of about 5000/mm^3.[40] The border between the NP and AF regions is distinctive in children under 10 years of age and gradually becomes more uniform during the aging process.

The annulus is composed of approximately 20 lamellae, which are rings that have collagenous fibers running parallel with each other.[41] The ring formations alternate in orientation in each adjacent lamellae. Elastin fibers that are positioned between the lamellae likely bind the lamellae to one another and serve to return the disc to its neutral arrangement after a flexion or extension.[39] AF cells tend to be more like fibroblasts in character with a thin, elongated shape. They are oriented parallel to the collagen fibers. The cells become more ovular toward the inner lamellae. Unlike chondrocytes located in articular cartilage, cells of both the AF and NP regions are apt to contain many long, slim projections that are typically 30 µm in length.[42,43] Many presume that they communicate within the tissue regarding mechanical strain.[42] Located above and below each IVD are cartilaginous end-plates. These layers are composed of hyaline cartilage and are usually thinner than 1 mm.[38]

Although healthy adult IVDs are considered almost completely aneural, they do contain some nerves in the outer AF lamellae that often adjoin to proprioceptors.[44] However, similar to other hyaline cartilage such as articular cartilage, the endplates are aneural and avascular in a typical healthy adult. Vessels extending from the spinal artery exist in longitudinal ligaments alongside the IVD. In children under 12 months of age, these vessels extend into the cartilage endplate.[38] Branches of the sinuvertebral nerve or divisions of the ventral rami or grey rami communicantes can

accompany the vessels or exist in the tissue independently. Many of these nerves are myelinated.[45]

The disc's mechanical functions are served by the organization and composition of the ECM. The two major macromolecules composing the matrix, similar to hyaline cartilage, are collagen and aggrecan**.** Type I and Type II collagen make up 90% of the dry weight of the AF and NP matrices, which gives the tissue tensile support and serves to anchor the tissue to the bone**.**[46] Aggrecan supplies osmotic pressure to the tissue through its chondroitin and keratan sulphate chains.[47] The nucleus contains a higher proteoglycan and water content than the annulus. As in other extracellular matrices, there exist many other collagen types and other small proteoglycans. The biochemical processes within IVD tissues are similar to that of articular cartilage; metalloproteinases and aggrecanases are responsible for the breakdown of the matrix components. Equilibrium between synthesis, breakdown, and buildup of matrix components give the tissue its physical and mechanical characteristics, as well as maintaining the avascular and aneural nature of a normal disc.

As mentioned, there exist many similarities between articular cartilage and IVD, particularly regarding the biochemical mechanisms. However, there exist some differences in the structure of the matrix, one of which is the aggrecan structure and composition. In IVDs, the aggrecan macromolecules are more heterogeneous, containing less aggrecan and more degraded remains. Whereas articular cartilage aggrecan is approximately 80% clustered, the IVD can be only 30% clustered in the same individual.[48] Because disc proteoglycans become more difficult to withdraw

from older individuals, crosslinking is thought to be tighter in the IVD matrix than in other connective tissues.[38]

The skeletal system develops with age, and the border between the annulus and nucleus becomes less discernible. Also, the nucleus becomes less gelatinous and more fibrotic in nature.[49] Both age and degeneration may contribute in the morphology of the disc becoming more disorganized. The annular lamellae may diverge and the collagen and elastin fibers may become disordered.[38]

Often, fissures may form as a cleft in the disc, especially in the NP region. As the process of degeneration progresses, more nerves and vessels find their way into the disc.[44] As in articular cartilage degeneration, cell proliferation occurs initially, leading to clusters of cells that are undifferentiated and fibroblast-like in nature, especially in the nucleus.[50,51] Necrotic and apoptotic processes advance, and large amounts of cell death follow.[52] These necrotic advances are surprisingly common, occurring in greater than half of cells in adult discs.[53] Mild cleft formation and granulation in discs of individuals as young as two years of age have been reported.[54] As the age of the individual increases, there is a higher prevalence of degenerative development, such as cell death, cell proliferation, granular change, concentric tears, and mucous degeneration.[38] The difference between aging and the degeneration processes that are pathological in nature are quite muddled.

Because the proteoglycan content of the disc is a major factor to its load-bearing properties, the loss of proteoglycan from the matrix often leads to pathogenicity.[55] Proteoglycan deficiency within the ECM can cause a decrease in osmotic pressure in the disc, as well as a loss of hydration during compression.[56] The

discs tend to bulge because they lose height and weight more rapidly than healthy discs.[57] Because of this deformation, uneven stress distributions develop in the AF region or endplates, and this disproportionate pressure may result in discogenic pain.[58] Alterations in disc dynamics affect other spinal structures and may accelerate further degeneration or injury. For instance, apophyseal joints next to the discs may potentially develop osteoarthritic variations when the discs lose height.[59] The ligamentum flavum may thicken, lose elasticity, and bulge into the spinal canal, resulting in stenosis.[60]

As in articular cartilage degeneration, the loss of proteoglycan matrix triggers a degenerative cascade. Aggrecan's charge and high concentration in the disc make it osmotically active, permitting the flow of only relatively small and uncharged molecules.[61] As the concentration of aggrecan decreases during degeneration, large molecules such as growth factors and cytokines may more easily penetrate the disc, allowing for angiogenesis and neurogenesis. These processes have been linked with chronic back pain.[62] Aggrecan loss is additionally problematic because of its capacity to inhibit neuronal disc ingrowth.[63]

IVDs are the largest avascular tissue in the human body. For this reason, a sufficient nutrient supply is deemed critical for sustaining proper tissue function and avoiding disc degeneration.[64,65] Oxygen and glucose, as well as amino acids, sulfate, and other substrates involved in matrix production are provided by blood circulation, located at the margins of the discs.[66] In order to reach the center of the nucleus of a human lumbar disc, nutrients must travel from the bordering capillaries through up to 8 mm of dense ECM that supports the IVD. Reversely, metabolic wastes such as

lactate are removed by diffusion out of the disc and into the blood supply. Oxygen and nutrient concentrations, as well as pH levels, drop nearer to the center of the disc while metabolite concentrations rise.[67]

Unlike articular cartilage, where nutrients must diffuse through the synovium and the matrix to be used by chondrocytes, two routes exist for nutrients to reach IVD cells. The molecules may pass through the cartilaginous endplate or the annulus periphery; however, most pass through the endplate. Changes in blood supply to the disc, made evident by MRI studies, significantly affect the solute supply to the disc. These studies also show that solutes move by means of diffusion, and disc pathology corresponds to a reduced nutrient supply.[66]

For nutrients to reach the disc matrix, they must first arrive by capillaries and nutrient channels and then pass through the dense hyaline cartilage endplate. The endplate is composed of cartilage that is similar to but less hydrated than articular cartilage.[68] As in the other cartilage tissues, the dense proteoglycan structure and diminished hydration functions to create a permeable barrier, restricting transport of many large and charged particles.[69] Calcification of the cartilage endplate can further restrict movement of particles such as glucose. Accompanying the development of calcification with age, the thickness of the endplate also decreases.[70] Solute and matrix properties such as charge and diameter define the ease with which transport occurs through the matrix. As in hyaline cartilage, collagen embedded in a polyanionic proteoglycan gel make up the IVD matrix, restricting the flow of large molecules, and to an extent, even glucose (MW 180) is restricted.[71]

It is widely believed that nutrient deprivation corresponds with disc degeneration. Particularly for NP cells, there are many steps at which the nutrients must travel that can be interrupted. Atherosclerosis of the abdominal aorta and other blood flow-related disorders have been linked to disc degeneration and back pain.[72,73] Research has also been done investigating the manner in which thrombophilic and hypofibrinolytic disorders such as sickle cell anemia[74] and Gaucher disease[75] block capillaries of the endplate, limiting nutrient flow. The progressions of these disorders are associated with a decline in the health and functionality of vertebral bodies. Capillary flow is also impeded by short-term exposure to vibration[76] and smoking[77] through their effects on muscarinic receptors.[78] In addition to blood flow blockage, calcification of the bordering cartilaginous endplate as seen in scoliosis or sclerosis of the subchondral bone may hinder nutrient availability, especially to NP cells.[79] Within degenerate discs, the permeability of subchondral bone and the cartilaginous endplate was significantly lower than in healthy discs, implicating a contribution toward further degeneration.[65]

Disturbances of transport in degenerate discs gain further evidence through findings associating a lower pH and higher lactic acid concentration in the affected discs.[80,81] Even in early degeneration, the transport of magnetic resonance imaging (MRI) contrast media is inhibited. In discs that were further degenerated, disturbances presented themselves in the cartilaginous endplate.[82] Diminished transport of a gaseous tracer corresponds to a reduced cell density in scoliotic discs.[83] Also in scoliotic discs, a drop in nutrient supply has been correlated with a loss of cell viability.[84] In addition, an *in vivo* study has reported that factors in disc degeneration

hinder nutrient transport.[85] Solutes are more easily transported from bone to disc in normal discs than in degenerate discs *in vitro.*[65] It is clear that many cases of IVD degeneration arise from nutrient deprivation.

An *in vitro* experiment of IVD cells grown in 3D culture portrays an increased growth of apoptotic and enucleated cells in accordance with greater cell seeding density.[86] They reported that a reduced glucose supply produced larger clusters with even higher tendencies of apoptosis and senescence. These results suggest a reduction in glucose may have more of an adverse effect on the survival of implanted cells than on their initial growth. Because the cells need glucose for the production of the surrounding ECM and not just for survival, the productivity of the cells also plays an important role in preventing or reversing degeneration.

Back pain affects approximately 12% to 35% of the population in Western industrialized societies, and about 10% are chronically impaired.[87] Along with a strong relationship between back pain and the IVD, there is also an association with degeneration and other maladies, such as sciatica and disc herniation.[88] Disc herniation and prolapse are among the most encountered IVD disorders. These situations involve a bulge or rupture either posteriorly or posterolaterally, pressing on the roots of the nerve in the canal.[38] Inappropriate load-bearing is thought to be the final step before rupture. It may be caused *in vitro* in healthy discs by forces larger than those that naturally occur. However, in Adam's experiment involving disc rupture, the vertebral body typically fails before the disc,[89] indicating that nutritional deprivation precedes the disorder.

The stepwise process involving degeneration and injury suggests degeneration is necessary before the disc herniates. Autopsies have revealed herniation may originate from NP fragment migration through tears that are previously formed in the AF.[90] Because structural failure does not always correspond with pain, the mechanism of pain is not fully understood.[91] It is believed that back pain results from not only pressure on the nerve root from herniation, but from an inflammatory cascade involving molecules such as arachidonic acid, interleukins, prostaglandins, and MMPs.[92] This biochemical shift in the disc itself is thought to make the nerve root more sensitive. Support for this model has shown tumor necrosis factor-α antagonists were beneficial to sciatic patients. Many components of this mechanism play dual roles, such as MMPs that are not only produced in prolapsed discs, but also believed to allow for the resorption of the herniation. Because of the complexity of the inflammatory cascade, precaution should be taken in regard to therapeutic strategies that interrupt these mechanisms, and emphasis has shifted to nutritional approaches in modern literature.

To conclude, the nutritional availability to IVD cells is critical because of the narrow physiological range allowed for cell viability. The components and functionality of the ECM, as well as mechanisms of pathogenicity, are quite similar to that of articular cartilage. Disc degeneration typically occurs more rapidly than articular cartilage degeneration, and its concomitant disorders affect a wider proportion of the population. IVD degeneration is prevalent in part because solutes and nutrients must diffuse even further from the circulatory system to be used by the cells, compared to articular cartilage. For this reason, there is a growing interest in

therapeutic techniques for degeneration of avascular tissues that encourage nutrient transport.[86]

1.4 Therapeutics

As previously discussed, OA is the broad term given to various mechanical irregularities involved with degeneration of articular cartilage and sometimes the underlying bone.[93] Usually in a late stage in the progression of OA, symptoms such as pain and stiffness are manifested in the affected joints, particularly after strenuous activity or lack of activity. Sufferers complain of a grating sensation felt during movements. In its late stages, the disease can be intensely debilitating, potentially affecting one's lifestyle and employment.[94] It affects nearly 27 million Americans, with an impact of $128 billion in lost wages and productivity.[95,96] Although we are aware of several risk factors, including genetics, obesity, and fractures, the direct cause of OA is still unknown.[93] Moreover, there is no cure for OA, and common treatments include analgesics, exercise, lifestyle changes, and physical therapy, none of which halt the progression of the disease significantly.[97]

When lifestyle changes are not sufficient to ease OA symptoms, many resort to microfracture surgery or total knee replacement surgery.[93] Because microfracture surgery causes damage to the injury site, and because an artificial joint typically fails within 10 to 15 years after implantation, these treatments are avoided if at all possible.[98,99] The inability of degenerative cartilage to self-repair, which was previously examined in detail, is perhaps the largest obstacle in OA treatment. Specifically, the chondrocytes' anabolic mechanism of producing components of the ECM demands the large consumption of nutrients such as glucose.[100] Nutrient flow is

reduced in degenerated cartilage, and this may not only contribute to the pathogenesis of OA but is also an obstacle in the transplantation of healthy chondrocytes.[101] This metabolic defectiveness has led researchers to approach tissue engineering in a way that accounts for the self-reparative shortcomings of degenerated articular cartilage.

Tissue constructs in various combinations of autologous cells, scaffolds, bioreactors, mechanical stimulations, and growth factors have been proposed for the replacement of damaged or nonfunctional cartilage.[102] Autologous chondrocyte implantation (ACI) involves the removal of a small fragment of articular cartilage from a non-load bearing site, such as the femoral chondyle, and inserting its isolated chondrocytes into the defected joint, covered with periosteum harvested from the tibia of the patient.[103] The procedure has had some positive outcomes, although the invasiveness of the procedure is a substantial drawback.[104] ACI, microfracture surgery, and total knee replacement aim to repair the cause of the disease rather than treat the symptoms and, therefore, are regarded as more comprehensive approaches. Tissue engineering looks into the possibility of a non-autogenic source, providing the benefits of these approaches while limiting surgical invasiveness.[105]

Common cartilage tissue engineering approaches involve the integration of autologous or allogeneic stem cells or chondrocytes into man-made scaffolds that imitate *in vivo* conditions.[105,106] These scaffolds may consist of natural materials derived from native cartilage or synthetic materials similar to ECM components.[107] Materials typically utilized include type 1 and type II collagen, alginate, chitosan, fibrin, agarose, chondroitin sulfate and hyaluronan.[108] Polymers, such as polyethylene glycol (PEG) and polycaprolactone (PCL), are under investigation for scaffolding

design.[109] The majority of approaches involve either the development of a porous composite scaffold through processes such as freeze-drying or electrospinning or the formation of a cross-linked network into a hydrogel.[110,111] Although there has not been a biomaterial regarded as the ideal choice for tissue engineering, many show the ability to support cell viability and matrix production.[106] However, there is a wide range of conditions these tissue engineering systems must uphold. Hydrogels lack the necessary mechanical strength to support cells and the surrounding tissue,[112] and other more mechanically sound, synthetic scaffolding constructs often lead to poor integration into the defect site or rejection of the implant from the host.[107]

In the search for an ideal stem cell-based therapeutic approach in avascular tissues like articular cartilage, the challenge of the provision of adequate nutrition to the affected area remains at the crux of the pathophysiological dilemma.[1,113] Attention should be brought to less invasive methods in the reversal of pathogenesis, as opposed to aggressive surgeries or the mere treatment of symptoms. Methods such as growth factor delivery and cell transplantation would likely be more beneficial if coupled with nutrient augmentation to the operation site.[1,114] While it is conceivable that supplementary nutritional availability will assist the reversal of articular cartilage degeneration, the incorporation of such a therapeutic approach has been lacking in the literature. With a better understanding of the specific nutritional need of chondrocytes, the incorporation of such an approach will be a realistic endeavor.

Treatment for lower back pain (LBP) has generally involved a conservative approach, with the goal of increasing day-to-day functionality. LBP affects as much as 80% of people in their lifetimes.[115] It is found not only in the elderly populations

but also the working populations, causing a drastic economic burden in western society. Sufferers of LBP in the age range of 25-60 years generally face disc herniation.[116] Practitioners no longer recommend bed-rest to patients. Instead, analgesics, muscle relaxants, oral and locally injected corticosteroids, anesthetics, and manipulation therapies are commonly used to ease the pain.[38] Such interventions as intradiscal electrotherapy have generally been based on anecdotal evidence in the past, and some trials have brought to question its efficacy for long-term relief.[117] More invasive procedures include discectomy and immobilization of the ailing vertebrae. The latter, referred to as spinal fusion, is currently regarded as the “gold standard” of treatment. In the operation, the IVD is excised, and natural bone growth processes, such as an autograft or allograft, are used to fuse the vertebrae. In the current therapy, patients that receive these mechanical prostheses have a high probability of reoperation.[115] Non-biological prostheses are available for use in artificial total disc replacement; however, the invasive surgery induces trauma, and the inanimate prosthesis eventually deteriorates.

The above operations are performed on about one out of every 400 Americans, as compared to one out of every 2000 British patients.[118] The rates of success of these various procedures are similar. The outcomes of surgery in well-selected patients are generally positive[119]; however, 70%-80% of patients who qualify for surgery regarding disc herniation or back pain ultimately recuperate with or without surgery.[120,121] Disc degeneration is believed to affect other tissues and eventually lead to spinal stenosis.[38] For this reason, cell-based therapies have come into focus, aiming at the restoration of height and biomechanical function of the disc.

Many therapies utilize the cell's own capability for self-repair in the restoration of the disc matrix. One approach is to increase the natural capacity for matrix production by the insertion of growth factors such as IGF-1[122,123], and these factors have provided as much as a five-fold increase. Because injection has provided only short-lived improvements, gene therapies and scaffolding systems have been assessed for the gradual release of these growth factors. Additionally, analysis of glucose consumption following intradiscal injection of IGF-1 has revealed a reduced glucose concentration, increased lactate accumulation, and a resultant pH drop at the site of injection.[114] Because of the complexity of catabolic and anabolic processes that maintain normal matrix biomechanics, it is impossible to propose an optimal therapeutic model for growth factor delivery without further establishing a deeper understanding of pathogenesis.[124] In the future, an ideal treatment for degenerative disc disease must achieve not only pain relief but also the biological reversal of the degenerative cascade.

Current research has pursued the practical implementation of functional cells in conjunction with scaffolding systems for IVD therapy. NP replacement hydrogels have been widely tested *in vivo* and *in vitro.*[115] Structures that are currently available for testing generally lack either a high load-bearing capacity or biocompatibility. Less has been studied regarding AF repair, and the objective of affixing the hydrogels within AF tissue has not yet been refined. Progenitor cells have shown promise for a potential therapeutic resolution due to their ability to regenerate tissue and activate cells in the IVD. While the field has potential, there is still much to uncover in regard to the biological mechanisms of the IVD and engineering approaches.

A goal of NP repair is the restoration of the disc matrix. Common strategies include the introduction of matrix-producing cells, molecules that encourage matrix regeneration, and shock-absorbing hydrogels.[115] The therapeutic approach is dictated by severity of degeneration. Many criteria exist for a proper NP hydrogel. These include allowing for a minimally invasive surgery, solidification after implantation to deter leakage, the ability to bear heavy loads, the prevention of degradation and biological response, the ability to hold water and maintain swelling pressure, and allowing the growth of cells and the surrounding matrix within the gel.

Interventions that hold promise include a thermo-reversible hydrogel, that can solidify from room temperature into a gel upon exposure to body temperatures. Also, chitosan and hyaluronan[125] have potential to satisfy many of the above criteria, due to their optimal biocompatibility. Photo-crosslinking of natural polymers such as alginate[126] or cellulose[127] with functional groups like methacrylates or N-vinylpyrrolidone through exposure to UV rays can cause a sol/gel transition of the hydrogel *in vivo.* Such photo-crosslinking hydrogels provide manipulation time before solidification and cytocompatibility.[126] Recent studies have shown that chemical crosslinking of polymers containing human disc cells also has the potential for high cytocompatibility and injectability for NP generation, in spite of the considerable amount of heat generated in the process.[128]

Multipotent and pluripotent stem cells could provide a suitable replacement for young and healthy NP cells that are more difficult to obtain. These cells can originate in the synovium,[129] bone marrow,[130] and adipose tissue.[131] One recent clinical trial utilized autologous bone-marrow-derived MSCs with results showing the

alleviation of pain and disability.[132] Conditions in the IVD are relatively harsh for MSCs, and low survival of injected cells is of concern.[133,134] Pre-conditioning and the use of various hydrogel carriers may provide a higher yield of injected MSCs that survive *in vivo*.[135]

Although there lies promise in the unveiling of the phenotypic character of NP and AF cells, as well as chondrocytes, the signaling cascades remain unclear. While IGF-1, basis fibroblast growth factor (FGF-2), platelet derived growth factor (PDGF), and growth and differentiation factor -5 (GDF-5)[136] could encourage discogenesis, they may also prevent discogenic differentiation.[137] Growth factors within the TGF and BMP families can lead to chondrogenic differentiation of MSCs.[138] As mentioned, growth factors such as IGF-1 may stimulate stem cell proliferation and processes but may in turn siphon the nutrient supply, potentially instigating further degeneration.

Pre-conditioning MSCs in hypoxia,[136] in a 3D environment, with mechanical stimulation, by co-culturing them with NP cells[139] or in media conditioned with notochordal cells[140] have all shown to induce both chondrogenesis and discogenesis. In addition to the problem of reduced glucose and pH after injection of such growth factors as IGF-1, there lies the obstacle of containing the cells and growth factors from outside the tissue of interest in the prevention of osteogenesis.[141] The multipotency of the cells is a cause of concern because of the potential for leakage, as this can lead to the development of osteophytes.[142] A carrier can therefore function to not only provide a framework for the attachment and growth of MSCs but also to protect them from leaking.

Just as the disc is naturally reliant upon sufficient nutrient supply, therapeutic interventions discussed also depend on nutritional availability. In the case that either the cartilaginous endplates have calcified[143,113] or the abdominal aorta or lumbar arteries have occluded,[144] matrix-producing cell therapy will likely have no benefit, because the lack of nutrient supply will reduce the population of existing and inserted cells. In addition, reduced pH and glucose concentration can cause elevated matrix breakdown and decreased matrix production.[145] One method of increasing nutrient supply to the disc has been the insertion of 5-hydroxytryptamine (5-HT), a receptor antagonist which has been shown to induce greater blood flow to the nerve roots near the disc in patients with disc herniation.[146] A calcium channel antagonist, nimodipine, may also increase blood supply to the disc through vascularization, which may improve the cells' nutrient supply.[147]

A limitation in the transplantation of various stem cells, especially MSCs, is the high death rate upon implantation.[148,149] On examining the main cause of cellular death of transplanted cells, Deschepper performed an experiment involving the transplantation of human MSCs.[150] Two factors were compared: oxygen tension and nutrient deprivation. The article reasons that the two factors are related and refutes the presumption that oxygen concentration is solely important. The *in vivo* study was performed looking at hMSC survival through ectopic transplantation into mice. After placing the cells into a cylindrical poly-acrylonitrile-sodium methallyl sulfonate "PASM" scaffold with fibrin hydrogels and glucose, it was shown that after 14 days, the number of viable cells was 5 times higher than of the same scaffolds without glucose (15% viability). In addition to the better viability, the peripheral

vascularization of implanted tissue constructs were greatly improved with the presence of glucose, potentially because of the overexpression of hypoxia-inducible factor 1-α and its relation to angiogenesis. The idea that anoxia is the most responsible factor of cell death was challenged by continuous near-anoxic conditions for 21 days at 5g/L glucose. The cells were still viable, continued to proliferate, and showed appropriate phenotypic markers. Because NP cells require more ATP for the production of matrix, it is likely that NP cells respond more quickly to changes in oxygen than AF cells or chondrocytes,[151] and it has been shown that oxygen, pH, and nutrient supply are interrelated with respect to cellular metabolism.[152]

These obstacles involved in therapeutic transplantation establish the need for the proper selection of cell type. Although transplantation of somatic stem cells has attracted global attention, the practical usage of these cells is still under development. MSCs have been viewed as a popular alternative[148] because they are easier to expand in large quantities. Although studies performed on smaller animals such as rabbits[153] and canines[154] have reported MSCs to be a viable option for cellular transplantation, studies on larger animals[149] have failed to implement them successfully. Small animal models have been considered unrepresentative of clinical use because of anatomical and physiological restraints related to body size.[155] Nutrients and other molecules must travel a larger distance to reach implanted cells in avascular environments in humans than in our smaller animal counterparts. It is plausible that MSCs are not adequately equipped to survive in the “harsh” conditions associated with such an avascular environment.

While it is a well-established principle that nutrition is pivotal to the survival of endogenous and transplanted cells in such avascular environments,[156] there has - to date - been no clinical solution to remedy this underlying dilemma. A primary issue in implementation is the invasiveness of injections. A needle with a diameter greater than 40% of the disc height significantly alters mechanical properties of cadaver discs, regardless of animal body size.[157] Puncturing of the AF tissue leads to NP depressurization, and in live models, this can provoke degenerative changes. However, a bovine disc organ culture revealed maintained cell viability, excluding cells localized at the injection site, in addition to a possible remodeling response for damaged areas within a week.[158] Such outcomes leave potential for nutrient augmentation by a minimally invasive discogram procedure. Additionally, it is conceivable in the future to supply glucose within a hydrogel or cell-scaffolding system in the treatment of LBP.

1.5 Rationale of Current Study

Cellular biochemistry and metabolism within avascular tissues are only partially understood, particularly in regard to the etiology of degenerative processes. Cigarette smoking, metabolic disorders such as diabetes mellitus, and blood aneurisms affect cell physiology and capillary flow. For this reason, they are all risk factors for OA and IVD degeneration,[1,77,159] due largely to an interference of glucose supply.[83] It is evident that nutrition and metabolites are a vital factor in the prevention and reversal of many forms of disease progression that afflict avascular tissues, and there has been a wide investigation on the effect of such factors as glucose and oxygen supply, as well as pH on cell viability. Bibby and Urban investigated the interactions of these

three components on cell viability,[160] indicating they are likely to exist concurrently *in vivo*. It was determined while lower oxygen and pH levels were not ideal for cell viability, glucose deprivation is significantly more detrimental. Using a profusion chamber, Urban and Horner determined to what extent cell populations in agarose discs were reduced by the distance glucose must diffuse to reach the cells.[156] While these experiments help to unveil the nutritional need for IVD tissues, they lack the precision to be used for biomedical engineering approaches. To my understanding, it remains unclear in the literature what avascular cell populations can be sustained in specific levels of glucose, the range of surrounding glucose concentration that is optimal for survival, and how much glucose these cell types consume in varying levels of glucose. These are valuable tools that can be used in a wide range of biomedical engineering faculties.

In accordance with the current limited understanding of cellular nutritional need in avascular tissues, survivorship curves have transiently related cell density at varying glucose concentrations. The results of the study were used in the pursuit of a constitutive model for further computer modeling and numerical methods. Such methods in the past have been utilized for the analysis of cell viability in relation to nutrient transport and other elements, such as growth factors.[113,114,152,161] In addition to a transient response of sustained nutrient supply at various concentrations, an equilibrium response for chondrocytes exhibited the stabilization of cell populations for each level of glucose. Additionally, the relation between cell density and glucose consumption was analyzed to provide an investigation of the consumption of glucose per individual cell per day. Since the experiment was performed with chondrocytes,

AF, and NP cells, a broader understanding of cellular nutritional need has been provided for the therapeutic strategies targeted at many different disorders, including OA and LBP. This fundamental investigation will lead to more thorough and precise investigations of IVD and chondrocyte metabolism and eventually to therapeutic interventions that can be used for the determination of proper nutrient delivery.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Isolation and Expansion

Porcine articular chondrocytes from the proximal head of the porcine humerus, as well as AF and NP cells from the porcine IVD, were isolated.[162] In short, the humeri and discs were removed from the 4-to 6-month-old pigs within approximately 2 hours following sacrifice. Cartilage tissue located on the articulating surfaces of the humeri were removed with a scalpel under a sterile hood and were finely chopped, incubated in an enzyme solution containing Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Cor.), 1% antibiotic-antimycotic (Atlanta Biologicals, Inc., Lawrenceville, GA), collagenase type II (1.5 mg/mL; Worthington Biochemical Corp. Lakewood, NJ), and protease (0.6 mg/mL; Sigma Aldrich, St. Louis, MO). The AF and NP cells were isolated and cultured in the same solution. After the tissues were cultured in the described enzyme solution at 37°C and 5% CO_2 for no longer than 48 hours, remaining undigested tissue was removed using a 70 µm cell strainer (BD Biosciences, San Jose, CA). The cartilage, AF, and NP cells were then cultured and expanded at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic.

The chondrocytes were dyed in trypan blue at a 1:1 ratio to be counted with a hemacytometer. Approximately 10 million cells were harvested and plated into a P175 flask, and the cells were passaged a week later into two separate culture flasks. One flask was passaged once more into three separate flasks, and the cells were allowed to proliferate until approximately 20 million cells were available. The AF

and NP cells were expanded in their first passage and used when they reached confluency.

2.2 Chondrocyte Experiment

2.2.1 Dilutions and Culture

An initial, twelve-day experiment was designed for the observation of a transient and equilibrium response to varying glucose concentrations. After cell isolation and expansion, the chondrocytes were placed into 2% agarose constructs, using a mold with a diameter of 8 mm and height of 1.5 mm. Gel samples were formed, and each gel contained approximately 700,000 cells. They were counted using a hemacytometer and were resuspended at 20 million cells/mL. The final cell seeding density was 10 million cells/mL. 70 μL of 50% gel and 50% of the resuspended medium were put in the wells to harden. Six additional gel constructs were created for calibration of the cell-counting software. These six gels were divided into three groups based on a cytometer count: two with 10 million cells/mL, two with 1 million cells/mL, and the last two with 0.1 million cells/mL.

All samples were cultured overnight in 0.5 mL of high glucose DMEM (4.5 g/L) as described above. Separate DMEM solutions with 10% FBS and 1% antibiotic-antimycotic were created with six varying glucose dilutions (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 g/L). Following overnight culture, the old medium was removed, and the gel constructs were sliced into quarters using a trimming knife (Sakura Finetek, Torrance, CA) and then divided into 6 groups in 24 well culture plates. The new glucose dilutions were added at a volume of 0.5 mL to each well. For the next 12 days, a medium change was performed each day, and during days 1 and 2, the old medium

was preserved for analysis of glucose consumption. To account for the change in glucose concentration due to evaporation in the incubator, a negative control was applied for each dilution (i.e. 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 g/L) in the days of the experiment that glucose consumption was analyzed.

2.2.2 Viability Staining and Imaging

On day 3, 5, 7, 9, and 12, samples from each dilution group were put in separate well plates and the live and dead cells within the gels were stained using a viability/cytotoxicity kit (Biotium, Inc. Hayward, CA). After the gel sections were added to a 24 well plate, 0.5 mL Dulbecco's phosphate buffered saline (PBS, Gibco, Grand Island, NY), 1 μL ethidium homodimer-1 (EthD-1), and .25 μL calcein were added to each well, and the samples were covered from light and stirred for at least 30 minutes.

The samples were imaged using an Olympus IX70 microscope, and images were taken of the slices at 10x magnification using fluorescein isothiocyanate (FITC) and Texas Red (TR) for the observation of live and dead, or apoptotic, cells. Q Capture Pro 6.0 (Media Cybernetics, Inc. and QImaging, Inc.) was used to capture the images. Two captures of the live cell stain and one capture of the dead cell stain were saved and cropped. An area of interest (AOI) was designated using the Q Capture Pro imaging software for the provision of a standardized method of cropping the images for cell counting, and the same dimensions of the AOI were used consistently throughout every experiment.

The six calibration gels were sliced, stained, and imaged correspondingly on day one of the experiment, when the populations within the gels were analogous to

the hemacytometer counts. A standard curve was created from the calibration gels to determine cell population in each of the samples.

2.2.3 Cell Counting

The samples were double-blinded so as to objectify the counting process and to eliminate bias. The cells in each AOI of both the live and dead stained images were quantified using ImageJ (Broken Symmetry Software, Inc.) Settings for detection of length and width of the cells, as well as threshold, were modified depending on the quality of each picture, and if the software could not detect cells due to low image quality, the remaining cells were counted by hand.

2.3 AF and NP Cell Experiment

In conjunction with the transient chondrocyte experiment, both AF and NP cells were put into 70 μL gels and were sliced into halves and quarters, respectively. Gels were diluted with DMEM in order to generate standards for calibration of the counting software in similar fashion to the chondrocyte study. The six calibration gels were sliced, stained, and imaged correspondingly on day 0 of the experiment (24 hours after cell seeding). The standard curve was generated from the calibration gels as described in the chondrocyte study to determine cell population in each of the samples. Glucose dilutions (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 g/L) at 0.5 mL per sample were changed daily for the duration of each 12-day experiment, and old medium was collected from the wells on the first day for glucose consumption.

Six to seven gels were produced for each dilution per cell type. After overnight culture in high glucose medium, the gels were placed into their respective dilutions for comparison with chondrocyte viability. They were cultured for 12 days,

in accordance with the equilibrium response determined in the chondrocyte experiment. The cells were stained and imaged on day 12. Two FITC images and one TR image were taken and cropped using the same AOI as the chondrocyte study. The cropped images were double-blinded, and live and dead cells were counted using the same procedure as for the chondrocytes.

2.4 Glucose Assay

During medium change, the remaining DMEM solutions were collected from samples on the first day of each experiment and frozen at -4° C. A glucose assay kit (Sigma-Aldrich, Co.) was used to quantify glucose consumption of the samples. The reagent is a solution of 1.5mM NAD, 1.0 unit/mL of hexokinase, 1.0 unit/mL ATP, 1.0 unit/mL of glucose-6-phosphate dehydrogenase. Glucose is catalyzed in the final step to 6-phosphogluconate. The consequent increase in absorbance at 340 nm is directly proportional to the concentration of glucose in each sample. Samples were vortexed, added in a 1 to 4 ratio (25 μL to 100 μL) with the reconstituted reagent, vortexed again, and then incubated for 15 minutes. After that time, 100 μL of each sample was transferred to a 96 well plate for spectrophotometer reading (DTX 880 Multimode Detector, Beckman-Coulter).

The absorbance of four samples at each concentration of medium was measured at 340 nm with the spectrophotometer to determine glucose concentrations. A standard curve was formed in order to control for ambient changes in temperature, humidity, reagent activity, etc. The standard curve included points with an absorbance between 0.275 and 0.791, and samples that fell out of this range were diluted at a 1 to 10 ratio and were immediately measured again. All samples of higher concentrations

fell into the operable range when measured again. Controls were used in culture without samples to account for change in glucose concentration in medium resulting from evaporation. The variances in glucose concentrations of the controls versus the medium after one day of culture represents consumption of glucose by cells in the sample. In order to account for uneven slicing, the quartered samples were weighed after imaging to find weight ratios in the determination of cell number for each quarter. Glucose consumption data was compiled with cell per sample so that consumption per cell could be calculated. The final volume of medium per well was estimated, assuming it would change inversely with concentration. Using the estimated final volume, the quantity of glucose (nm per million cells per hour) involved in cell metabolism was calculated for each sample.

CHAPTER 3: RESULTS

3.1 Preliminary Measurements

3.1.1 Cell Count Calibration

A pilot study using SHED cells (NOVA Southeastern University, Fort Lauderdale, FL) was performed to calibrate cell counts within a specified AOI with previous hemacytometer counts. Gels with a volume of 100 μL and 2% agarose were constructed at 10 million cells/mL and were plated for a day to calibrate the cell-counting software for future experiments. The images were gelled, sliced, stained, and imaged on the same day. The cell-counting technique was justified as an appropriate measuring tool for the study.

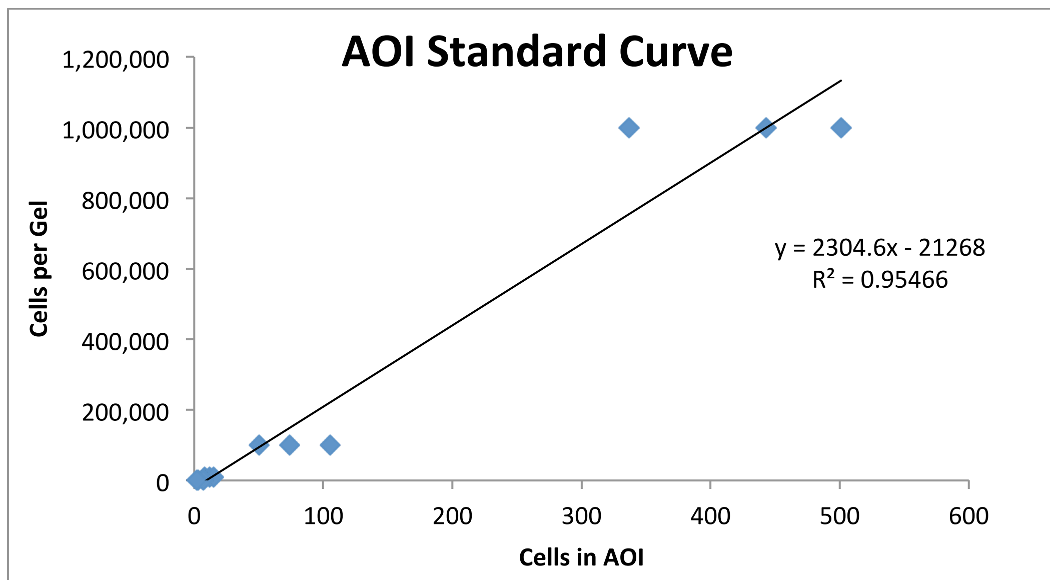


Figure 1: Standard Curve for determination of a cell counting method using a predetermined AOI.

3.1.2 SHED Survivorship Curve

A study was performed using SHED cells to determine the feasibility of cell modeling in a population study. Cell counts were recorded after four days at five separate concentrations (0.2, 0.4, 0.8, 1.6, and 4.5 g/L). The data shows an obvious

trend in population difference between those cultured in lower concentrations versus those in moderate or higher concentrations. The previous standard curve was used to determine an approximate cell count for each imaged sample. A logarithmic curve can be observed in the survival of SHED cells after a four day period.

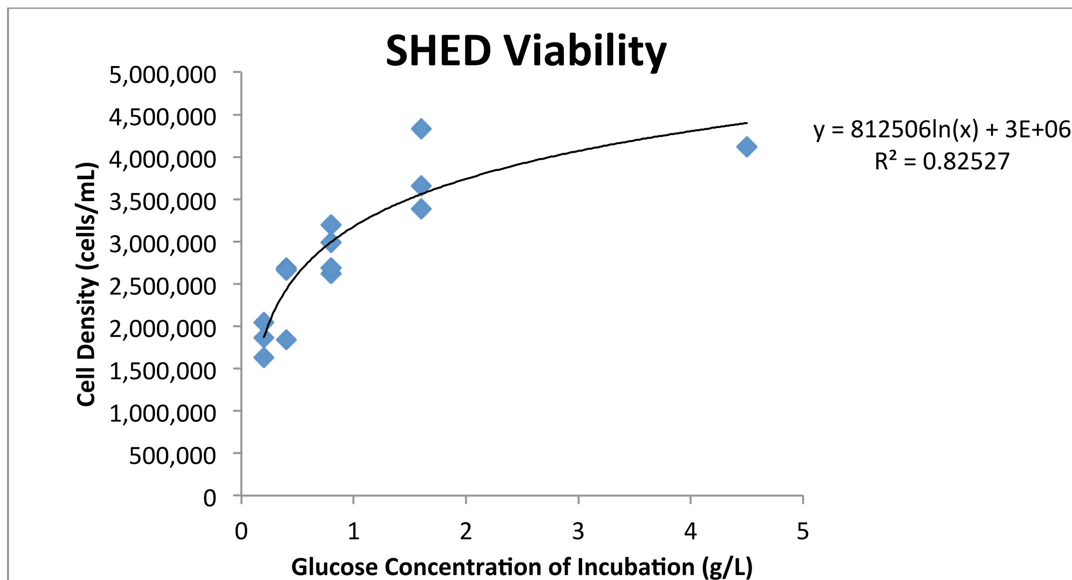


Figure 2: SHED cell survivorship curves in various concentrations of glucose DMEM.

3.1.3 Temporal Analysis of Survival

As a pilot study, an analysis of SHED cell populations in 2% agarose gels was performed for determination of a survivorship curve. Logarithmic curves can be observed in the cell density of the samples, and a considerable decrease in live cell density can be seen on day four.

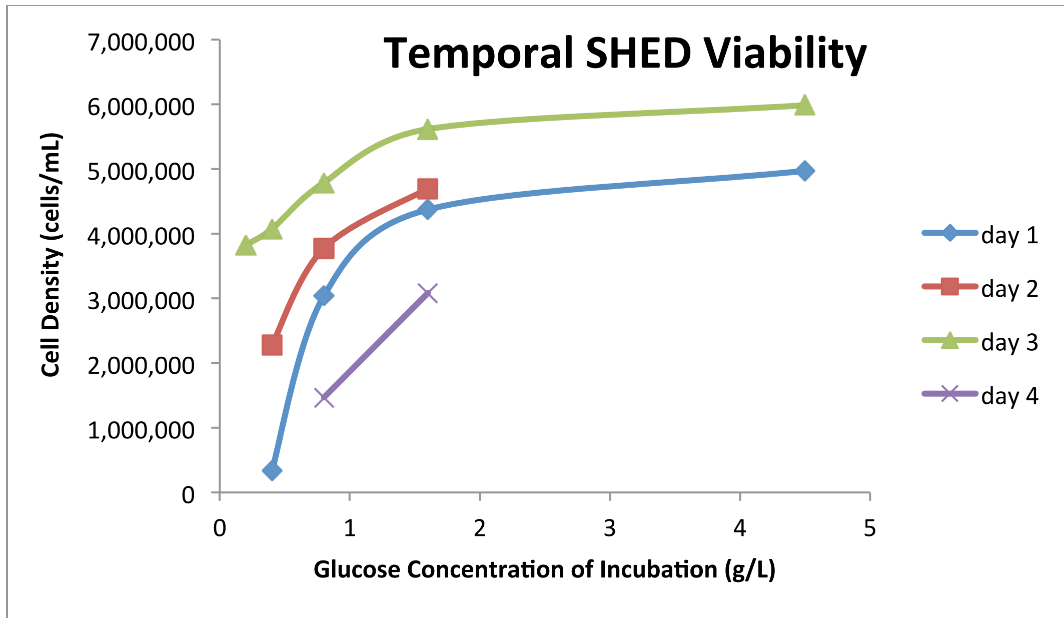


Figure 3: Survivorship curve for cells cultured in various concentrations of glucose DMEM.

3.1.4 Equilibrium Response

An experiment was conducted using porcine chondrocytes from articular cartilage to determine protocol for an experiment that displays changes in cell density over time. The study was performed to determine the possibility of reducing gel volume to 50μL. Samples were incubated in glucose concentrations of 0.1 and 0.2 g/L and were sliced, stained, and imaged on days 3, 6, and 9 of the experiment.

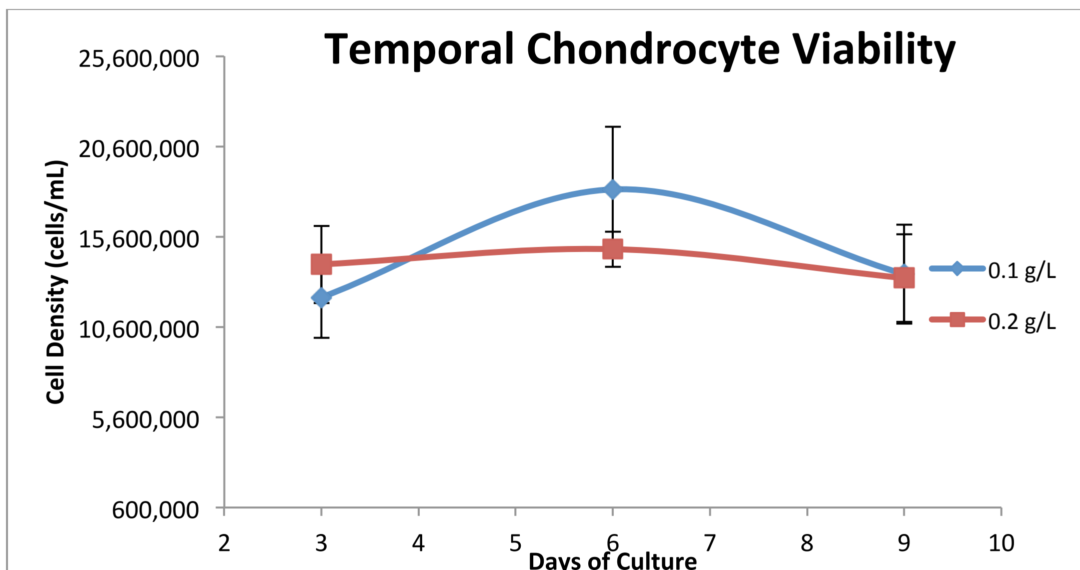


Figure 4: A temporal analysis of chondrocyte cell density shows sustained cell populations after 9 days of culture.

3.1.5 Glutamine Deprivation

A pilot study was conducted using 0.1 and 0.2 g/L glucose medium dilutions to eliminate the possibility of glutamine metabolism affecting cell density. Based on the results of this pilot study, glutamine deprivation seemed to affect cell survival.

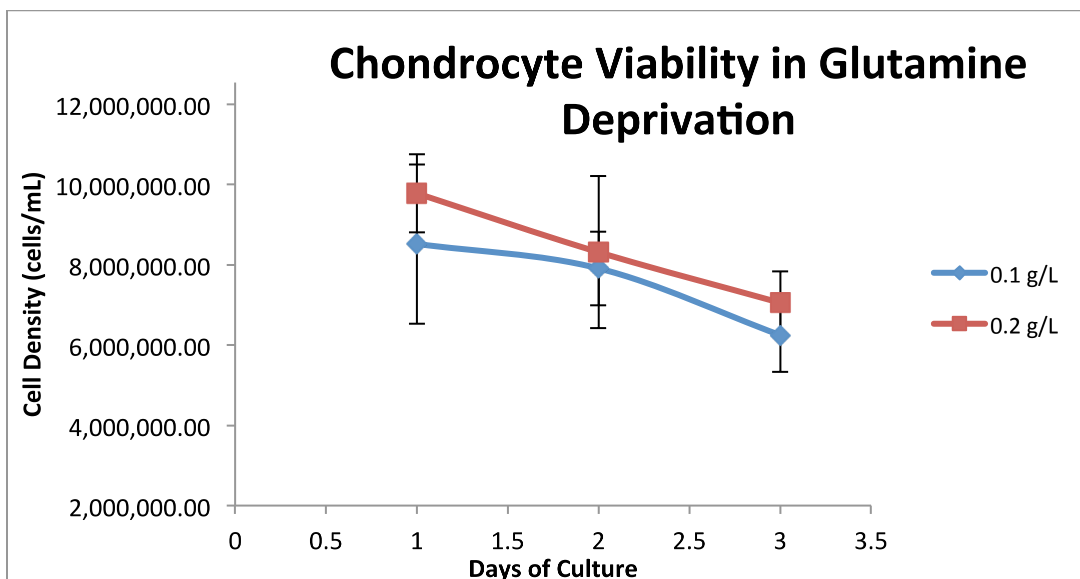
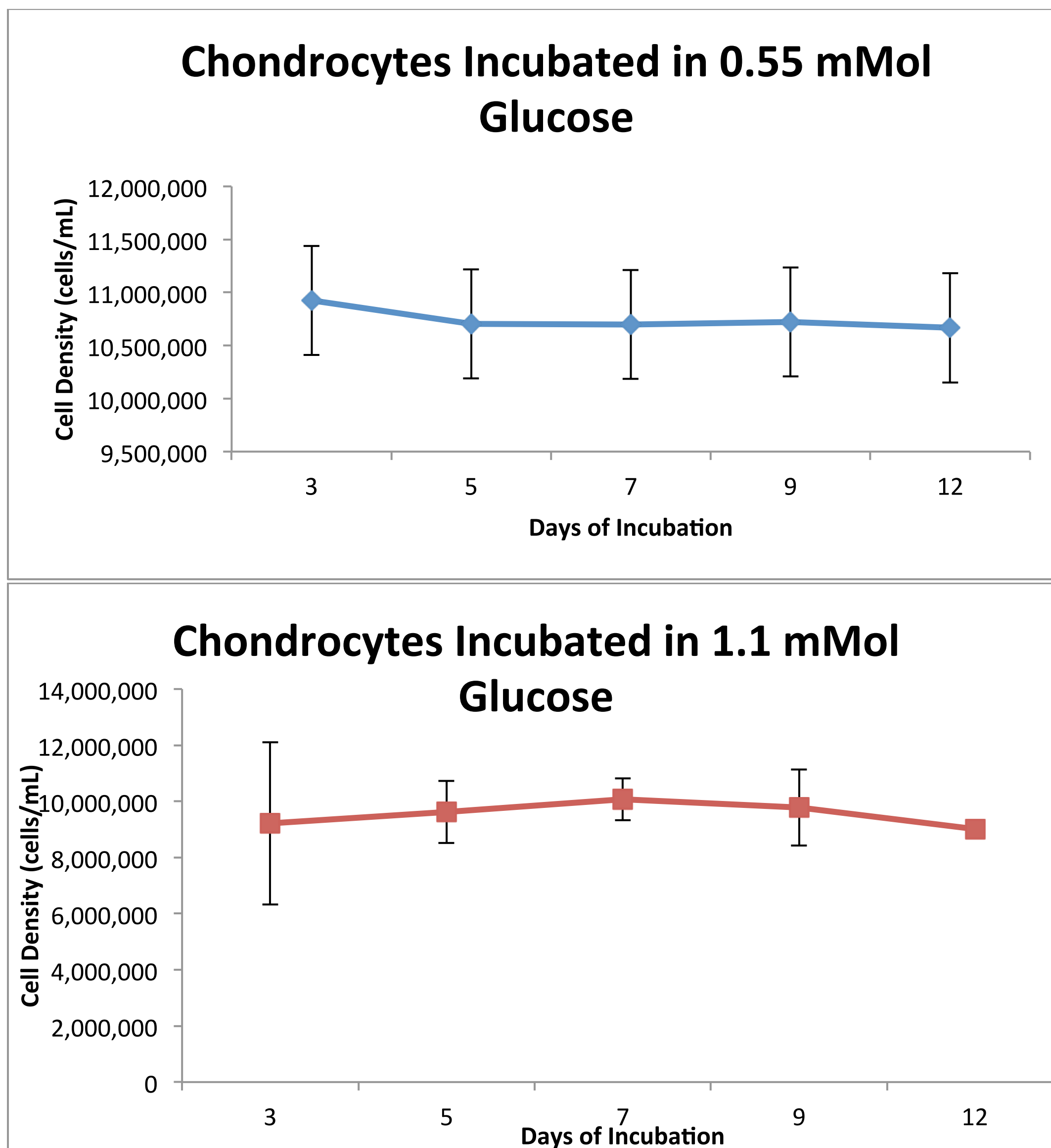


Figure 5: Chondrocyte density was analyzed after culture without the presence of glutamine to determine its role in cell survival.

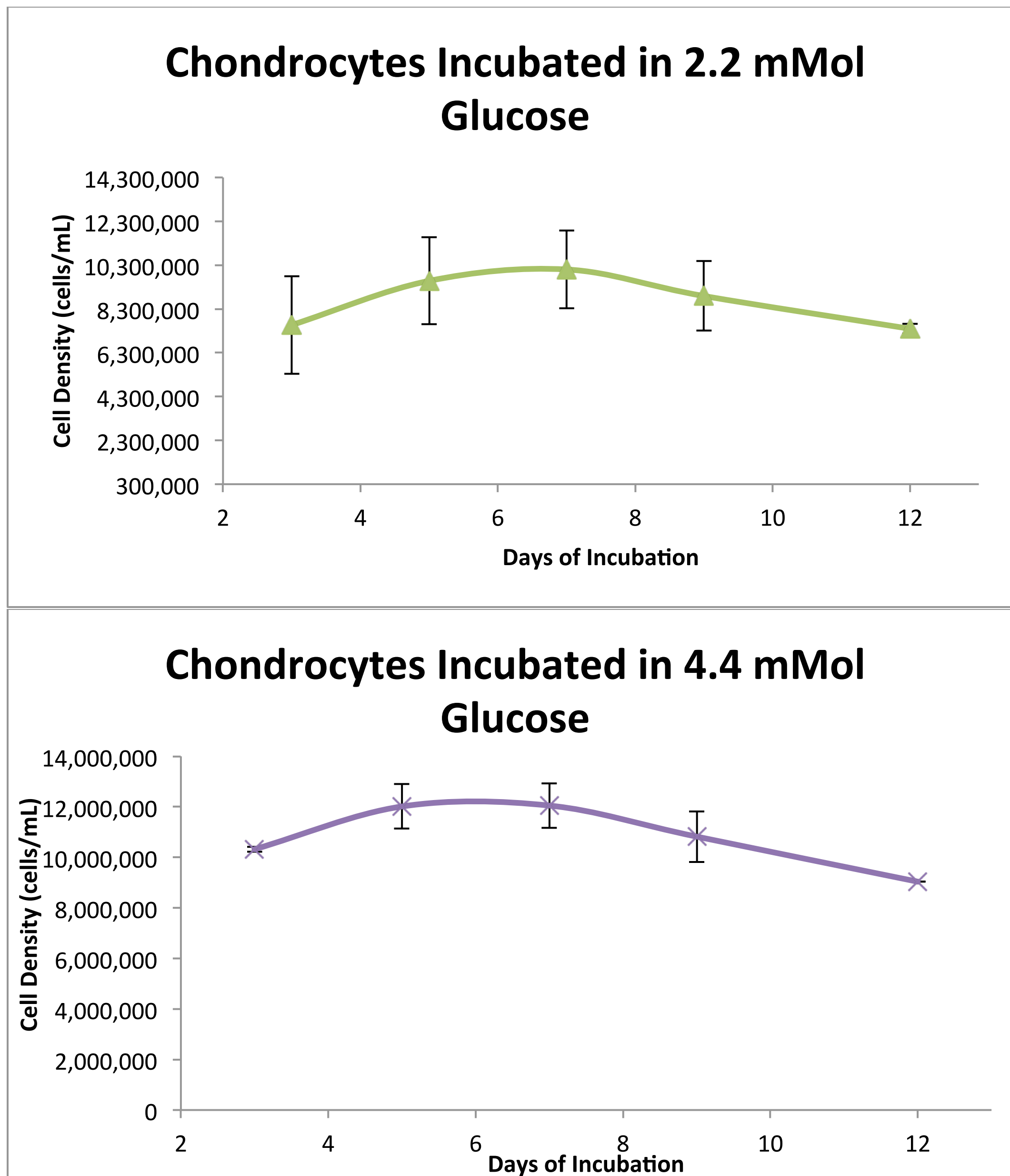
3.2 Cell Viability

3.2.1 Chondrocyte Viability

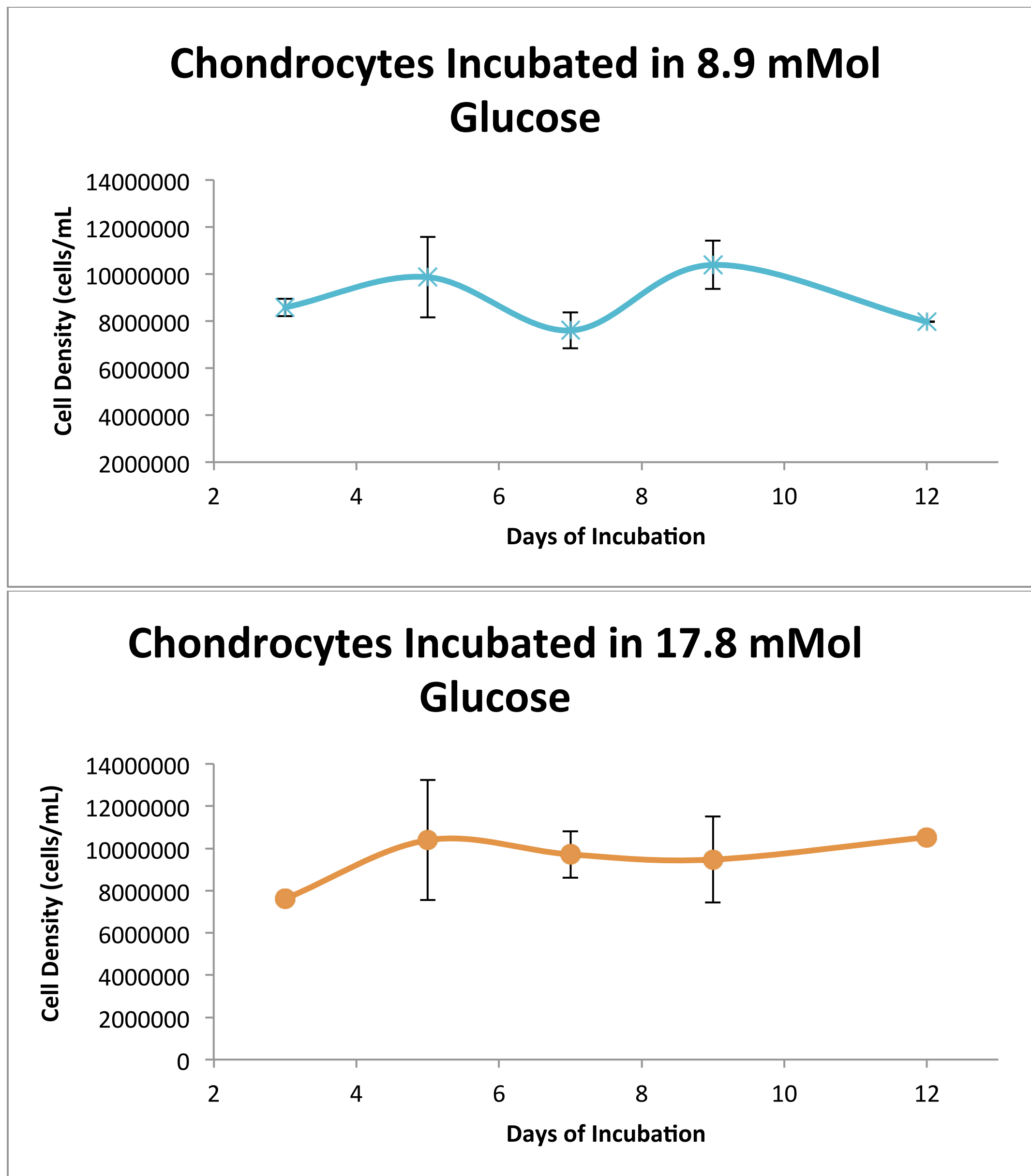
Chondrocyte samples were incubated in glucose dilutions without glutamine at about 3 samples per concentration per time point. Cell density was analyzed at days 3, 5, 7, 9, and 12. Even at low glucose concentrations of 0.1 and 0.2 g/L (i.e. 0.56 and 1.11 mM), the samples were able to maintain cell density.



Figures 6 and 7: Chondrocyte cell populations incubated in low glucose DMEM concentrations were counted at five separate time points.



Figures 8 and 9: Chondrocyte cell populations incubated in moderate glucose DMEM concentrations were counted at five separate time points.



Figures 10 and 11: Chondrocyte cell populations incubated in high glucose DMEM concentrations were counted at five separate time points.

3.2.2 Nucleus Pulposus Viability

NP cells were cultured at 10 million cells/mL with 4 samples for lower concentrations (0.1, 0,2, and 0.4 g/L glucose) and 2 samples for each higher concentration (0.8, 1.6, and 3.2 g/L glucose). An observance of cell viability on day 12 revealed a reduction in cell density at higher glucose concentrations ($P<0.05$).

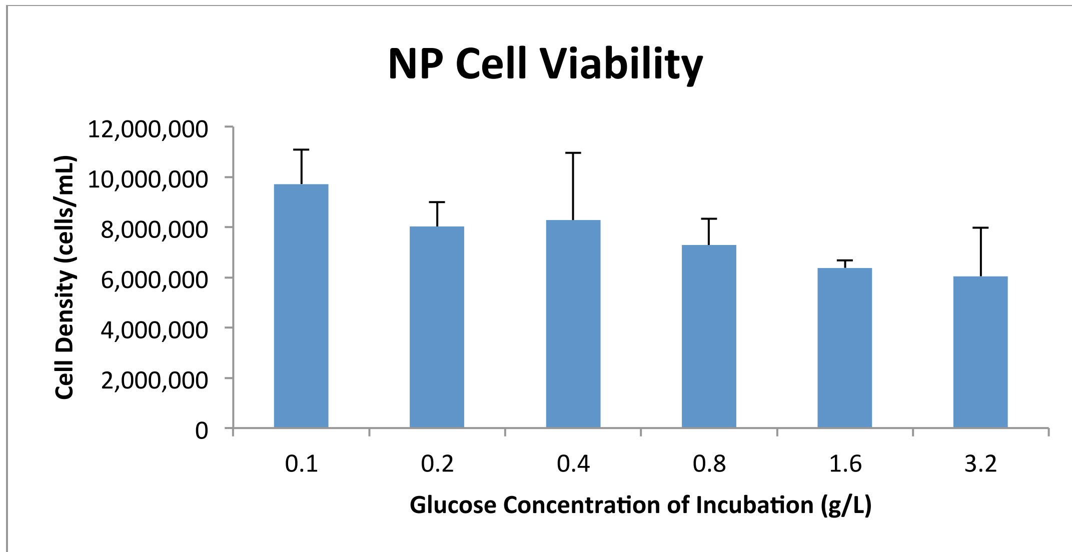


Figure 12: NP cell populations incubated with DMEM at various glucose concentrations were counted on day 12. Differences in cell density between samples cultured in 0.1 g/L and 3.2 g/L are statistically significant ($P<0.05$).

3.2.3 Annulus Fibrosus Cell Viability

AF cells were cultured at 10 million cells/mL with 3-4 samples per concentration per time point. An observance of cell viability at day 12 revealed a sustained population. Samples cultured at all glucose concentrations were able to sustain cell density after twelve days.

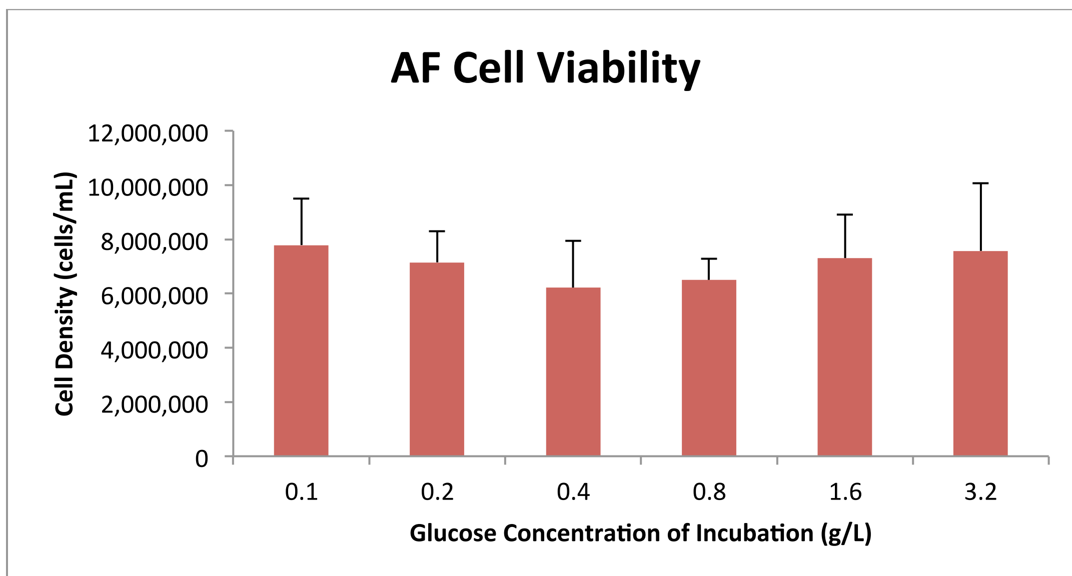


Figure 13: AF cell populations incubated with DMEM at various glucose concentrations were counted at day 12.3.3 Glucose Consumption

3.3 Glucose Consumption

3.3.1 Chondrocyte Consumption

Upon measuring the absorbance of each concentration of medium after the first day of culture, consumption of glucose per million cells per hour was determined. Based on the data of the cell's consumption of glucose in lower concentrations, cells cultured in higher amounts of glucose consumed larger amounts than cells in lower concentrations.

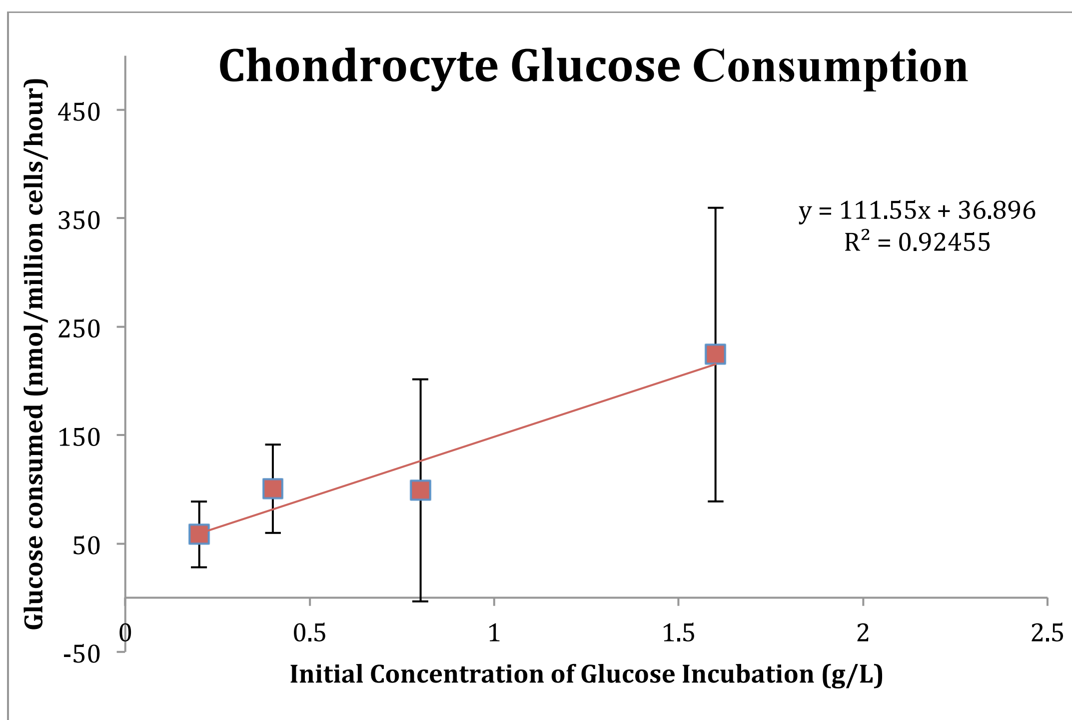


Figure 14: Glucose consumption of chondrocytes cultured in various DMEM glucose dilutions after the first day.

3.3.2 Nucleus Pulposus Cell Consumption

The medium used to culture gels containing the NP cells was collected after the first 24 hours of the experiment for determination of the new concentration of glucose after consumption. Similarly to the chondrocyte experiment, a control was

used to account for evaporation. Like the chondrocytes, NP cells consumed more glucose when exposed to an environment of higher glucose concentration.

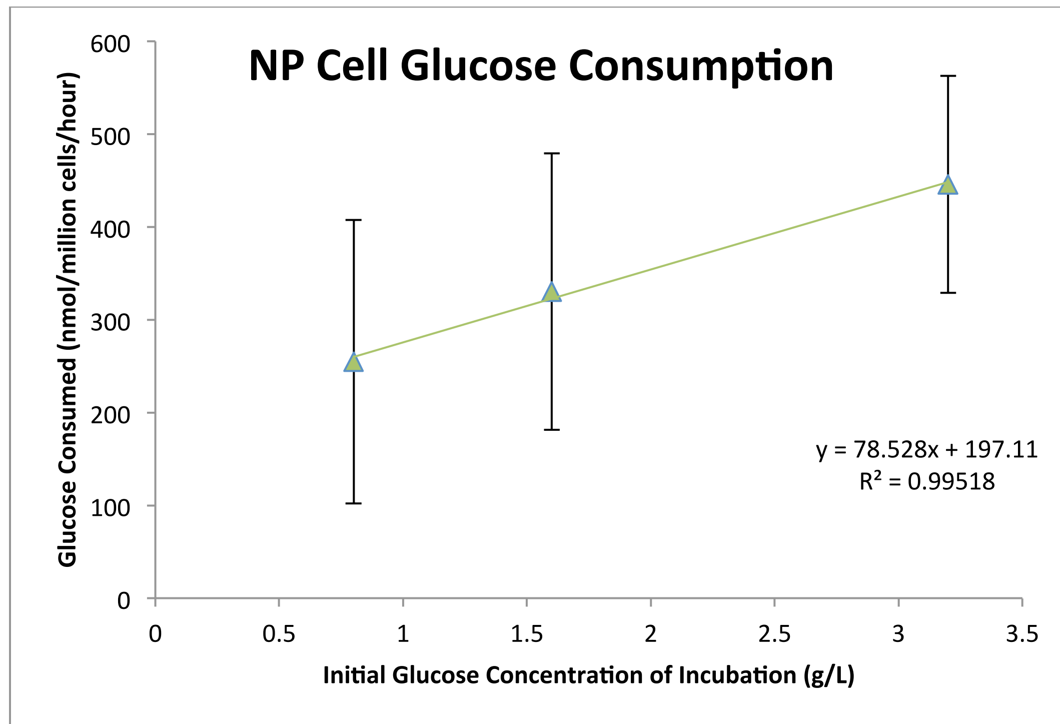


Figure 15: Glucose consumption of NP cells cultured in various DMEM glucose dilutions after the first day.

3.3.3 Annulus Fibrosus Cell Consumption

Similarly to the NP and chondrocyte studies, four samples of medium were collected from the wells after 24 hours of AF cell culture. After accounting for a rise in concentration of glucose after medium evaporation and for the reduction in volume, the amounts of glucose involved in metabolism for each category of cells in the various concentrations were determined. A significant rise in consumption can be seen in AF cells cultured in higher concentrations of glucose.

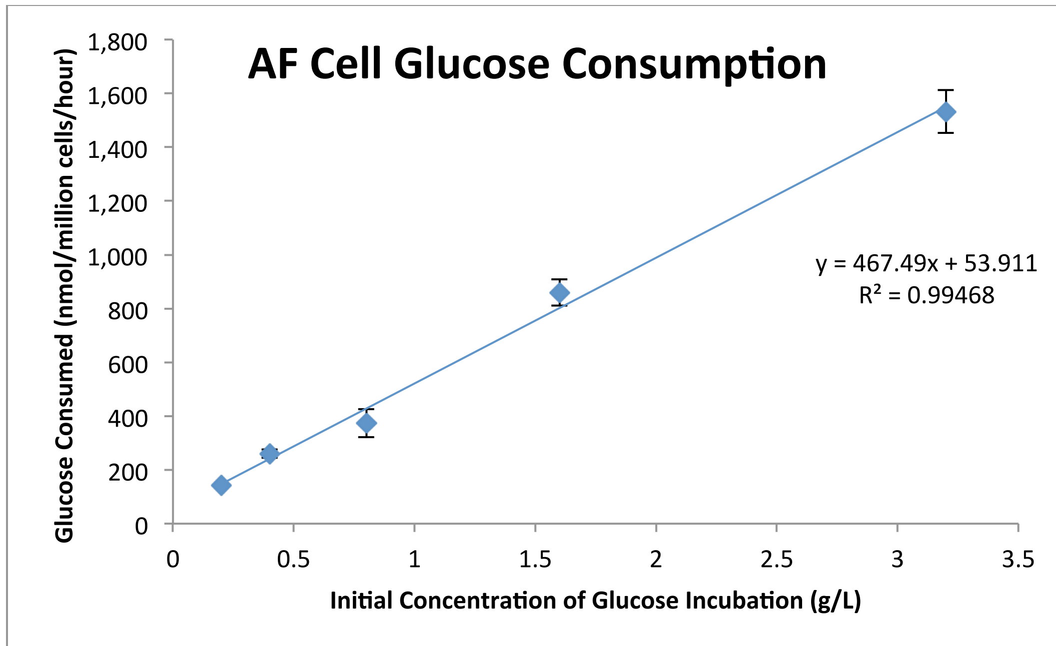


Figure 16: Glucose consumption of NP cells cultured in various DMEM glucose dilutions after the first day.

3.3.4 Cell Type Comparison

According to the glucose consumption data, AF cells more readily consumed glucose than NP or chondrocyte cells when exposed to higher concentrations of glucose, chondrocytes consumed the lowest amount of glucose, and NP cells consumed glucose at a slightly higher rate than did the chondrocytes.

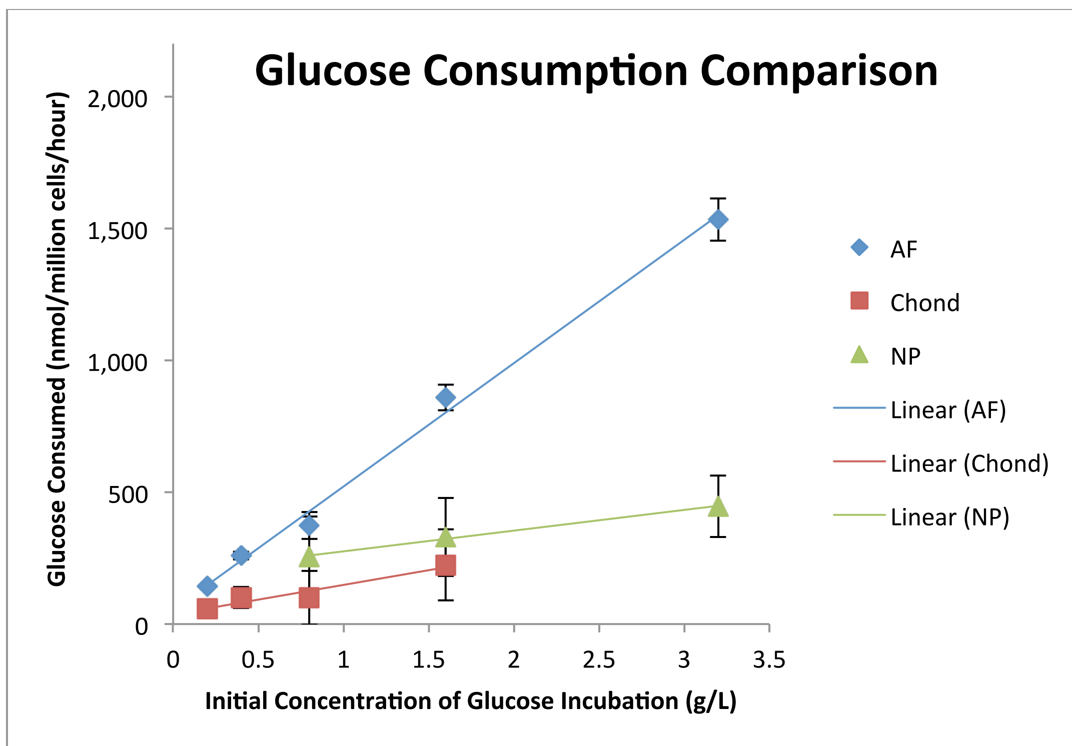


Figure 17: A comparison of glucose consumption between AF, NP, and chondrocyte cells.

CHAPTER 4: DISCUSSION

4.1 Cell Viability

For each avascular cell type, it was observed through viability staining and ImageJ counting that living cell populations persisted at even very low concentrations of glucose (as low as 0.5 mM). In gels that were conditioned in low glucose medium, the cell densities fluctuated in minor amounts due to cell death and mitosis. During the imaging process, substantial amounts of cell division could be seen, particularly in days 9 and 12. For NP cells, there was a drop in cell density in samples cultured at higher concentrations of glucose. It is possible that glucotoxicity might play a role in cell death through glycation reactions with proteins that ultimately form reactive oxygen species, which cause oxidative stress at the cellular level.[163] The same was not observed for chondrocytes or AF cells, indicating that NP cells might be particularly susceptible to apoptosis at higher glucose concentrations. NP cells' potential susceptibility to glucotoxicity could be involved in the pathogenesis of IVD degeneration.

According to Bibby and Urban[160], lactic acid production is significantly decreased in IVD cells cultured in 0.5 mM glucose compared to those in 5 mM glucose (about 0.1 and 1.0 g/L glucose, respectively). While their study examined the combined effect hypoxia and low pH on the viability of IVD cells, their data on lactic acid production is representative of the rate of glucose metabolism. While IVD cells are essentially stressed at around 0.5 mM, they are unable to survive at concentrations lower than 0.2 mM. It is reasonable to determine that glutamine deprivation has an effect on cell viability, however, its role in metabolism and the biochemical pathways

with which it is involved are somewhat deviant from that of glucose metabolism. The results from AF, NP, and chondrocyte cell viability experiments support previous research done by Bibby and Urban that cells are able to maintain viability when they are exposed to a medium of 0.5 mM glucose.

4.2 Glucose Consumption

For each examined cell type, a pattern was seen in the increase in consumption of glucose when cultured in higher glucose concentrations. The pattern could especially be seen in the AF data, where consumption was significantly greater than that of either NP cells or chondrocytes. Although it could be expected to have Michaelis-Menten kinetics in the curves of glucose consumption, it appears from the above data that no equilibrium was reached for the AF cell consumption, even at as high as 17 mM glucose. The NP cell and chondrocyte consumption data is more representative of Michaelis-Menten behavior.

Based on the glucose consumption data above, a considerable discrepancy is apparent between the consumption of various cell types, particularly the AF cells. The AF graph shows the tightest linear regression and smallest standard deviation of any of the consumption data. For the AF and NP cells, the quartered samples were assumed to include 175,000 cells per sample, as each full gel contained approximately 700,000 at 10 million cells/mL. This allowed for the determination of glucose consumption per cell in a given time frame. For the chondrocytes, each quarter was weighed and ratios were taken to determine the approximate cell count in each sample, and glucose consumption was calculated accordingly.

Based on the data from both cell viability experiments and from the NP glucose consumption curve, it is quite possible that NP glucose consumption, in relation to initial medium glucose concentration, would display a sharp drop in consumption. Because a substantial amount of dead NP cells were witnessed during viability staining in the two or three lowest concentrated dilutions (0.1, 0.2, and 0.4 g/L), and also because the concentrations of glucose in these three dilutions were undetectable in the range of the glucose assay (meaning significant amounts of glucose were utilized in cellular metabolism), it would be reasonable to infer that NP cells are dependent on a level of glucose for optimal functionality that is between the range of 0.2 g/L and 2 g/L.

According to Guehring *et al.*[164], chondrocyte-like mature NP cells (MNPCs) absorb 95 ± 8 nMol/10^6 cells/hour when cultured in 5 mM (~1.0g/L) glucose and no FBS. Given the differences in culture medium composition, the relatively small variance in the above glucose consumption data for NP cells and Guehring *et al.*'s experiments are reasonable. Additionally, because MNPCs would be expected to consume less glucose, based on the results above and their probable likeness to chondrocyte absorption, the lower amount of glucose consumption by MNPCs in comparison to NP cells is logical. One might also consider the possibility of glucose consumption being elevated because of higher concentrations of O_2 in the incubator. According to Schneider *et al.,* chondrocytes cultured in 5% and 21% O_2 had the same rate of glucose intake for both 1 and 4.5 g/L glucose medium cultures.[165]

4.3 Conclusion

In summary, the viability of certain avascular cell types, namely AF, NP, and chondrocyte cells, can be maintained in culture at or above 0.5 mM glucose. In addition, glucose consumption was shown to be greatest in AF cells, especially when incubated within a higher concentration of glucose medium. Chondrocytes were reported to consume the lowest amount of glucose, just below that of NP cells.

4.4 Limitations of Current Study

The main limitation of this study was the range of dilutions that were examined to display cell death and corresponding drop in cellular density in the agarose gels. This can be modified in future studies to show a fall in density with even lower concentration of glucose. Although samples with NP cells cultured in higher levels of glucose exhibited a statistically significant drop in cell density over a twelve day period, further studies are needed to confirm this finding.

The glucose assay used to measure consumption was able to detect concentrations only between 0.05 and 5 mg/mL. The spectrophotometer was therefore unable to detect meaningful changes in absorbance for solutions that had dropped in concentration below 0.05 mg/mL due to metabolism, including solutions that had started at 0.1 g/L for all cell types and 0.1, 0.2, and 0.4 g/L for NP cells. In addition, because glucose was measured 24 hours after the onset of incubation, more frequent measurements would allow for a more accurate representation of the ratio between initial vs. consumed glucose. A glucose meter, in addition to the potential of greater accuracy, would also allow for more frequent and numerous measurements of final glucose concentration.

4.5 Future Studies

In order to properly see substantial cell death due to nutrient deprivation, cells in a gel scaffold should be incubated in a range of glucose dilutions between 0.1 and 2 mM (~.018 and .36 g/L respectively) for at least 5 to 7 days. Additionally, a larger sample size (5 samples per data point) would help support findings of cell death due to glucose deprivation or glucotoxicity. A study showing the drop in NP cell density in higher concentrations should be repeated to display consistent results.

Additionally, the previously mentioned glucose meter should be used for the determination of consumption at points below 2 mM glucose (~.4 g/L) in the understanding of cell metabolism in a stressing and depraved environment. The glucose consumption curves, once enhanced by more data, can be used for molecular modeling purposes in the near future.

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