Nutrient Related Mechanisms of Intervertebral Disc Degeneration

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NUTRIENT RELATED MECHANISMS OF INTERVERTEBRAL DISC DEGENERATION

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Sarah Elizabeth Cisewski
August 2016

Accepted by:
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The intervertebral disc (IVD) separates the vertebrae allowing flexibility, strength, as well as a wide range of mechanical motion in the spine. Millions of Americans are afflicted with IVD degeneration which can cause low back pain and limited functionality of the spine. Deviation from physiological nutrient levels due to abnormal mechanical loading and age is believed to be one of the main mechanisms for low back pain associated disc degeneration. Since the IVD is the largest avascular structure in the body, transport of nutrients (e.g., O₂ and glucose) is primarily done through the passive transport mechanism of diffusion. Transport of nutrients and solutes through the extracellular matrix is important in maintaining the normal function of tissues, so deviation from physiological levels can cause tissue necrosis and matrix degradation. The objective of this research is to investigate the effect of mechanical loading on nutrient transport and cell nutrition of the IVD in order to develop a 3D imaging based finite element model to better understand in vivo fluid and nutrient transport within the human IVD as well as the biomechanical etiology of disc degeneration. Therefore, our central hypothesis is that sustained mechanical loading can alter solute transport and nutrient concentrations in the IVD, resulting in changes to the cellular metabolism, tissue composition, and mechanical function, ultimately leading to disc degeneration in the human IVD disc. To address this hypothesis, this dissertation established a set of aims including;

Aim 1: Determine the metabolic phenotype of human IVD cells.
Aim 2: Examine the effect of mechanical strains on glucose and lactate diffusivity values of the cartilage endplate region of human IVDs in vitro.

Aim 3: Develop and validate a 3D multiphasic mechano-electrochemical finite element model of the human IVD to quantify and predict changes in nutrient levels under various loading conditions that occur in vivo.

The ultimate goal of this project is to characterize the nutrient diffusivities and metabolic phenotype of the IVD to develop and validate a 3D multiphasic mechano-electrochemical finite element model in an effort to quantify and predict changes in nutrient levels under various loading conditions that occur in vivo and better understand low back pain associated disc degeneration. The outcome of this study will yield 1) a new realistic anisotropic mechano-electrochemical theory and finite element model for investigating the transport of fluid and solutes in human IVDs under various loading conditions and 2) the first study to characterize the effect of mechanical strains on nutrient diffusivity values of the cartilage endplate region of human IVDs in vitro. Finally, the project will bring a human biomechanical model for improving clinical diagnosis of disc degeneration.
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significantly increased with an increase in glucose concentration (p=0.001). At 0.5mM and 1mM glucose, the average GCR significantly increased with a decrease in oxygen level (p=0.001). No significant regional tissue difference was observed at 2.5% and 5% oxygen level as well as all glucose concentrations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05. ................................................................. 151

**Figure 47.** The lactate production rate (LPR) [nmol/million cells/hr] of porcine TMJ explants versus oxygen level (2.5 and 5%) varied by glucose concentration (0.5, 1, 5, and 10mM). At 2.5 and 5% oxygen level, the average LPR significantly increased with an increase in glucose concentration (p=0.001). At 0.5, 1, and 5mM glucose concentrations, the average LPR significantly increased with a decrease in oxygen level (p=0.001). No significant regional tissue difference was observed at 2.5% and 5% oxygen level as well as all glucose concentrations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05. ................................................................. 153

**Figure 48.** The ratio of lactate production to glucose consumption of porcine TMJ explants versus oxygen level (2.5 and 5%) varied by glucose concentration (0.5, 1, 5, and 10mM). No significant differences were observed due to change in glucose concentration (p=0.30) The ratio at 10mM glucose concentration was significantly higher at 5% oxygen level compared to 2.5% oxygen level (p=0.035). There were no regional differences detected in respect to oxygen level and glucose concentration. * = oxygen effect = p < 0.05..................154
CHAPTER 1: INTRODUCTION

Understanding Intervertebral Disc Degeneration

The degeneration of the intervertebral disc (IVD) has been implicated as the primary etiological factor of low back pain. Since the IVD is the largest avascular tissue in the human body, the passive transport of essential nutrients is crucial to maintain disc health. The balance between nutrient transport rates through the matrix and the rate of consumption by disc cells establishes a concentration gradient inside the disc. Deviation from physiological nutrient levels, due to abnormal mechanical loading and age, are suggested to be the main mechanisms for disc degeneration which impedes tissue engineering or repair attempts.

Objective and Specific Aims

Due to the difficulty of measuring nutrient gradients in vivo, mathematical models have been used to predict the nutrient environment inside cartilaginous tissues. However, due to a unique and complex tissue environment, nutrient transport properties and metabolic rates of human IVDs are largely unknown and appropriate theoretical models for systematically predicting nutrient transport are not available. The goal of this research is to fill this gap by measuring transport properties and characterize the metabolic phenotype of human IVDs in vitro, and to develop a new mechano-electrochemical transport theory and finite element model for investigating the transport of fluids and solutes in vivo under mechanical stress in normal and degenerated IVDs.

Efforts to numerically simulate and diagnose human IVD degeneration in vivo are still limited by the lack of bridging multiple parameters together to develop an advanced
analytical model. The development of a multiphasic mechano-electrochemical finite element model of human IVDs with realistic tissue and cell properties will test the hypothesis that sustained mechanical loading can alter solute transport and nutrient levels in the human IVD, thereby altering the cellular metabolism, tissue composition, and mechanical function resulting in disc degeneration, and increased age will exacerbate the impact of mechanical loading on the human IVD disc, more readily leading to a pathological state. To address this hypothesis, this dissertation established a set of aims including:

**Aim 1: Determine the metabolic phenotype of human IVD cells.** Knowledge of the complex relationship between metabolite usage and production is crucial for understanding how IVD disc cells, at different ages as well as degeneration grades, maintain homeostasis by only receiving nutrients and removing wastes via diffusion. Therefore, the 1a) O\(_2\) consumption rate of human IVD cells will be determined using an O\(_2\) metabolism chamber; and 1b) the glucose consumption rate and lactate production rate will be determined using a biochemical analyzer.

**Aim 2: Examine the effect of mechanical strains on glucose and lactate diffusivity values of cartilage endplate region of human IVDs in vitro.** The rate of solute transport in tissue is governed by solute diffusivity. The IVD is a charged, hydrated soft cartilage tissue under mechanical loading. Due to the relationship between water volume fraction and tissue deformation, the measured solute diffusivity depends on tissue water content. Therefore, the 2a) strain-dependent glucose diffusivity and 2b) strain-dependent lactate diffusivity of human IVD cartilage endplates (CEP) will be determined, at various ages as
well as grades of degeneration, under various strain conditions using diffusion chambers to develop new constitutive relationships between solute diffusivity and tissue hydration to establish strain-dependent transport properties.

**Aim 3: Develop and validate a 3D multiphasic mechano-electrochemical finite element model of the human IVD to quantify and predict changes in nutrient levels under various loading conditions that occur *in vivo***. The IVD will be modeled as a fluid-saturated, inhomogeneous poroelastic medium consisting of charged solid, fluid, ions, and nutrients. Specifically, a **3a**) 2D axial symmetric finite element model of cylindrical IVD explants will be developed for model validation, and **3b**) a real geometry image based 3D inhomogeneous finite element model will be developed based on the multiphasic mechano-electrochemical transport theory with realistic metabolic rates (identified in **Aim 1**) and material properties (identified in **Aim 2**).

The focus of this thesis is on developing new knowledge in cartilaginous tissue degeneration that will drive the fields of soft tissue biomechanics and cell biology. Linking tissue mechanics and cell metabolism with an advanced finite element model will establish a solid foundation for understanding human IVD physiology and pathophysiology as well as improving clinical diagnosis of disc degeneration. Furthermore, the theories and experimental techniques developed in this study will have a significant impact on other areas of research as well, such as investigating the delivery of drugs and other biological factors, which may stimulate cellular metabolic activities in soft tissues. The ensuing findings of this thesis will promote the development of new methods for tissue engineering
in vitro and in vivo, since nutritional supply is a major consideration in engineering healthy tissues.

Similar to the IVD, the normal adult human TMJ disc is a large avascular fibrocartilage structure and the nutrients required by disc cells are supplied through diffusion by blood vessels and synovial fluid at the margins of the disc. Nutrient concentrations (i.e., oxygen/glucose) can profoundly affect TMJ disc cell viability, energy metabolism, matrix synthesis, and response to inflammatory factors. It is hypothesized that the nutrient concentrations and cell metabolic rates in TMJ discs are sensitive to the pattern and magnitude of the mechanical loading during jaw function and are therefore potential early bio-indicators for evaluating the impact of mechanical loading on TMJ disorders and degeneration. In order to test this hypothesis, the metabolic phenotype of porcine TMJ disc cells and fresh tissue explants were examined to characterize the complex relationship between oxygen level and glucose concentration to understand how TMJ disc cells maintain homeostasis by only receiving nutrients and removing wastes via diffusion. Specifically, the combined effects of oxygen level and glucose concentration TMJ disc cell viability, ATP production, radioactive proline and sulfate incorporation (i.e., collagen and proteoglycan synthesis), and rates of glucose consumption and lactate production. The resulting findings will further characterize the nutrient related mechanisms in fibrocartilaginous tissues.

**Organization of Dissertation**

This dissertation is organized in chapters of related studies that combine to form the overall aims of this project. In **Chapter 2**, a comprehensive overview of the anatomy,
biochemistry, and function of the IVD is presented. The symptoms and previous treatment approaches for low back pain associated disc degeneration are also outlined. In addition, the mechanical and transport properties are described as well as the finite element modeling methods compiled from the literature. In Chapter 3, a baseline comparison of oxygen consumption rates and mitochondrial membrane potential of nondegenerate and degenerate human IVD nucleus pulposus (NP), annulus fibrosus (AF), and cartilage endplate (CEP) cells was determined. In Chapter 4, the effects of oxygen level and glucose concentration of the glucose consumption and lactate production rates of nondegenerate and degenerate human IVD NP and AF cells were investigated. In Chapter 5, the diffusivities of nutrient/metabolite solutes in healthy CEP were assessed, and further correlated with tissue biochemical composition and structure. Chapter 6 outlines the overall conclusions of this project and identifies future directions of this work. In the Appendices, the metabolic phenotype of the temporomandibular joint disc, another type of cartilaginous tissue within the body was characterized. In Appendix A, the effects of oxygen level and glucose concentration on the metabolism of TMJ disc cells was determined, specifically the combined effect on TMJ disc cell viability, ATP production, and radioactive proline and sulfate incorporation (i.e., collagen and proteoglycan synthesis). In Appendix B, the effects of oxygen level and glucose concentration on the glucose consumption and lactate production rates of porcine TMJ disc explants were analyzed.
CHAPTER 2: BACKGROUND AND SIGNIFICANCE

Structure, composition, and charged nature of the intervertebral disc

The intervertebral disc (IVD) is a fibrocartilage structure separating the vertebrae that allows a wide range of mechanical motion in the spine. Differences in biochemical composition and structure distinguish three regions of the disc: annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplates (CEP), as shown in Figure 1.

Figure 1. Anatomy of the Intervertebral Disc.

Annulus Fibrosus

The annulus fibrosus is a thick ring of fibrous cartilage composed of about 15 to 25 layers of lamellae which contain collagen fiber bundles within each parallel layer. The collagen fibers alternate to the left and right at approximately 60° to the vertical axis within each parallel lamellae layer, as shown in Figure 2b. Elastin fibers lie between the lamellae, possibly helping the disc to return to its original arrangement following bending, whether it is flexion or extension. They may also bind the lamellae together as elastin fibers pass radially from one lamella to the next. The cells of the annulus, particularly in the outer region, tend to be fibroblast-like, elongated, thin, and aligned parallel to the collagen fibers.
Toward the inner annulus the cells can be more oval. The cell density is approximately 9000 cells/mm$^3$, which is slightly higher than in the NP. Cells of the disc, both in the annulus and nucleus, can have several long, thin cytoplasmic projections, which may be more than 30 mm long. Their function in the disc is unknown, but it has been suggested that they may act as sensors and communicators of mechanical strain within the tissue. 

*Nucleus Pulposus*

The central nucleus pulposus is a gelatinous structure that contains randomly arranged wavy, fibrous strands of cartilage and fibroblasts. The randomly organized collagen fibers and radially arranged elastin fibers (sometimes up to 150 mm in length) are embedded in a highly hydrated aggrecan-containing gel. Interspersed at a low density (approximately 5000 cells/mm$^3$) are chondrocyte-like cells, sometimes located in a capsule within the matrix. Surrounding the nucleus is the annulus fibrosus, with the boundary between the two regions being very distinct in the young individual (<10 years).

*Cartilage Endplates*

The cartilage endplates (CEP) are located superiorly and inferiorly to the nucleus pulposus and the annulus fibrosus. Each endplate is a thin horizontal layer of hyaline cartilage separating the disc from the vertebral body. The cartilage endplates are approximately 0.6 to 1.0 mm thick, being thinnest in the center. The endplate extends over the nucleus pulposus as well as the central part of the annulus fibrosus. Due to the interweaving of the annulus fibrosus fibers and the endplate’s cartilage fibers, the endplate has a strong attachment to the IVD. The collagen fibers run horizontal and parallel to the vertebral bodies, with the fibers continuing into the disc. The healthy adult disc has no
blood vessels, but it has some nerves, mainly restricted to the outer lamella. The cartilaginous endplate, like other hyaline cartilages, is normally totally avascular and aneural in the healthy adult.

Figure 2. Morphology of a nondegenerate intervertebral disc. **a:** Cross-section and sagittal sections through a nondegenerate adult human disc showing the size of the disc and the main morphological features; i.e., the soft hydrated nucleus surrounded by the lamellae of the annulus and the thin cartilaginous endplate interspersed between the disc matrix and the vertebral body. **b:** Schematic view of the collagen network of the disc showing the organization of the annulus lamellae together with details of the network revealed in scanning electron micrographs. (Modified from Reference)7

Histology of Thompson Grade I and II bovine IVD discs8 was analyzed and the lamellae structure in the AF region was visible and the NP was gelatinous. A thin hyaline cartilage layer called the CEP was found between the interface of the disc and bone. The CEP, as did NP and AF tissue, swelled immediately once exposed to the PBS solution.
Based on histological analysis, the cell density in the CEP layer was much higher than in both the NP and AF regions (Figure 3). The average thickness of the CEP was approximately 0.6 mm. The collagen fibers in the CEP from both the central and lateral regions were more compacted than those in the NP and AF tissues, respectively. The collagen fibers from the AF also seemed to continue into the lateral endplate.
(b) Figure 3. Sagittal and frontal slices taken from bovine IVD plugs, which were harvested from the central and lateral regions. The segments were rapidly fixed in 10% neutral buffered formalin, decalcified, and paraffin wax embedded. Ehrlich’s hematoxylin and eosin (H&E) was used with the wax sections (7 μm) in order to study morphological characteristics of the CEP. (Modified from Reference)\textsuperscript{8}

Biochemical Composition of the Intervertebral Disc and Its Function

The disc is comprised mostly of water with significant quantities of collagen, proteoglycan, and other matrix proteins, as shown in Table 1.\textsuperscript{4,9,10} The IVD has negatively fixed charges in the extracellular matrix which significantly contribute to the swelling behavior of the tissue. Electrostatic interactions between immobile, fixed charges on the solid matrix and mobile free ions in the interstitial fluid give rise to electrokinetic effects (e.g., Donnan osmotic pressure and swelling, streaming potential and current, negative osmosis, and electro-osmosis effects).\textsuperscript{11} Thus, the swelling behavior and transport of fluid and solutes within the IVD depend upon physicochemical factors, such as water content, fixed charge density, and the type and concentration of bathing solutions.\textsuperscript{12} The mechanical functions of the disc are served by the extracellular matrix; its composition and
organization govern the disc’s mechanical responses. The main mechanical role is provided by the two major macromolecular components—collagen fibers and aggrecan.

_Collagen Fibers_

The collagen network, formed mostly of type I, which makes up about 70% of the dry weight of the annulus, and collagen type II, which is found the nucleus and inner annulus, together with other collagens, mainly types III, V, VI, IX, XI, provides tensile strength to the disc and anchors the tissue to the bone.  

_Aggrecan_

Aggrecan, the major proteoglycan of the disc, is responsible for maintaining tissue hydration through osmotic influences as well as absorbing compressive forces. The nucleus contains large proportions of hyaluronan and proteoglycan, mainly aggrecan, although other proteoglycans such as biglycan, decorin, and fibromodulin are also present. The proteoglycan and water content of the nucleus are significantly greater than in the annulus.

_Extracellular Matrix_

The matrix is an extremely dynamic structure. The balance between synthesis, breakdown, and accumulation of matrix macromolecules determines the quality and integrity of the matrix, and thus the mechanical behavior of the disc itself. The integrity of the matrix is also important for maintaining the avascular and relatively aneural nature of the healthy disc.
Table 1. Human Intervertebral Disc Composition.4; 8; 14

<table>
<thead>
<tr>
<th>Components</th>
<th>IVD</th>
<th>Annulus Fibrosus</th>
<th>Nucleus Pulposus</th>
<th>Cartilage Endplate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (% dry wt.)</td>
<td>10-60%</td>
<td>50-70%</td>
<td>15-25%</td>
<td>25%</td>
</tr>
<tr>
<td>Proteoglycans (% dry wt.)</td>
<td>15-65%</td>
<td>10-20%</td>
<td>~50%</td>
<td>8%</td>
</tr>
<tr>
<td>Water (% wet wt.)</td>
<td>65-90%</td>
<td>60-70%</td>
<td>70-90%</td>
<td>72.6±6.9%</td>
</tr>
</tbody>
</table>

It has been determined that bovine IVD has very comparable matrix composition to known human IVD values. The water content (% wet weight), total collagen content (% dry weight), and total proteoglycan content (% dry weight) of bovine CEP as well as NP and AF are shown in Figure 4.8 A significant regional difference was found for water content in the CEP between the central and lateral regions (central region: 75.6±3.1% and lateral region: 70.2±1.8%; p=0.004). Compared with that in the NP region (79.4±2.0%), the mean water content in the CEPs from both central and lateral regions (72.9±3.7%) was significantly lower (p<0.0001). A significant regional difference was also found for proteoglycan content in the CEP between the central and lateral regions (central region: 20.4±3.1% and lateral region: 11.7±2.1%; p<0.0001). Compared with that in the NP region (26.7±5.8%), the mean proteoglycan content in the CEPs from both the central and lateral regions (16.1±5.1%) was significantly lower (p=0.0006). For collagen content, a significant regional difference was also found in the CEP between the central and lateral regions (central region: 41.1±9.5% and lateral region: 73.8±10.8%; p<0.0001). Compared with that in the NP region (28.5±4.6%), the mean collagen content in the CEPs from both central and lateral regions (57.5±19.2%) was significantly higher (p<0.0001).
Figure 4. Results of biochemical assays measuring (A) water content, (B) total collagen content, and (C) total proteoglycan content for each region of the bovine IVD. Significant differences were detected for water, collagen, and proteoglycan contents between the NP, AF, and CEP. Regional differences were also found for water, collagen, and proteoglycan contents in the CEP between the central and lateral regions (*p values are lower than 0.05 as shown in the biochemical composition section; n=8 each group). (Modified from Reference)8

**Mechanical Properties**

*Creep compression behavior of Bovine Cartilage Endplate*

Also, the creep compression behavior of bovine cartilage endplate were well fitted to the biphasic theory to determining the equilibrium compressive aggregate modulus and hydraulic permeability. A regional variation was found for the aggregate modulus between the central and lateral regions in the CEP, indicating that the lateral region of the bovine endplate is much stiffer and might share a greater portion of the load in the disc. The
average hydraulic permeability of bovine CEP was observed to be substantially different from that in the NP and AF (Figure 5B), although no significant regional variation was found for the hydraulic permeability of bovine CEP. The average hydraulic permeability of bovine CEP is $0.11 \times 10^{-15} \text{ m}^4/\text{Ns}$, which is about 1/6 of that in bovine NP, 1/2 of that in bovine and human AF, 1/20 of that in bovine AC, and 1/10 of that in human AC. It suggests that, due to the small hydraulic permeability, the cartilaginous endplate blocks rapid fluid exchange and allows pressurization of the interstitial fluid in response to loading. Furthermore, as shown in the literature, the cartilage endplate also plays a significant role in preventing the disc from swelling with its intrinsic tensile resistance (high stiffness of collagen fibrils and entangled GAGs) $^{15-17}$. The nucleus pulposus contains a high density of fixed negative charges which are bonded with proteoglycans in the ECM. It could cause a high swelling pressure which helps to sustain the compressive load in the spine $^{18;19}$. The AF is formed by a series of concentric encircling lamellae, which could provide a strong resistant tensile force to prevent the whole disc from swelling $^{20;21}$. 
Figure 5. Comparison of (A) aggregate modulus $H_A$ and (B) permeability $k$ of bovine CEP (CEP-Central and CEP-Lateral) and bovine IVD tissue found in the literature (NP and AF) \(^{22}\). For the aggregate modulus, a significant regional variation was detected in the CEP between the central and lateral regions (*$p<0.0001$; Central: $n=14$; Lateral: $n=18$). The aggregate modulus of the CEP in the central and lateral regions were comparable with that of the NP and AF, respectively (*$p < 0.05$ \(^{22}\)). For hydraulic permeability, no significant regional variations were detected in the CEP between the central and lateral regions ($p=0.071$). Compared with that in the NP and AF \(^{22}\), the permeability in the CEP was found to be much smaller. (Modified from Reference)\(^8\)
The cartilage endplates above and below the disc play a significant role in IVD load supporting mechanisms by allowing pressurization of the interstitial fluid and maintaining swelling pressure in the disc. The significantly lower hydraulic permeability found in the CEP could block the nutrient solutes or the proteoglycan fragment outflow caused by the rapid convection under mechanical loading. Together with the low solute diffusivities of the CEP found in the literature, it’s suggested that the CEP could act as a gateway for solute transport into the disc and help to maintain a constant physiological nutrient and ECM ionic environment inside the IVD. Furthermore, the CEP could play an important role in preventing angiogenesis progression into the human IVD by blocking the rapid diffusion of angiogenic molecules such as vascular endothelial growth factor (VEGF) into the disc.

The central region of the bovine CEP had a lower aggregate modulus and swelling pressure than that in the lateral region, which could be correlated with their different biochemical compositions (higher water content in the central region of the CEP). These results are consistent with the inverse relationship between aggregate modulus and the water content found in the literature. This observation supported the hypothesis that water content is generally a stronger determinant of material properties than the GAG content. In this study, hydraulic permeability in the central CEP is higher than that in the lateral CEP, although it is not statistically significant (p=0.071). Correspondingly, the water content is higher in the central CEP than in the lateral CEP. Similarly, the correlation between hydraulic permeability and water content was found in bovine NP and AF, and porcine AF in the literature. Due to the scarcity of human disc tissue, bovine IVD has
previously been used as an alternative source for the study of disc metabolism, function, and biomechanics.\textsuperscript{35-39}

\textbf{Low back pain associated disc degeneration}

Low back pain (LBP) is a major socio-economic concern in the U.S. The NIH reports that Americans spend at least $50 billion each year on LBP which makes it the second most common neurological ailment in the U.S.—the most common being headaches. Although the exact cause of LBP is unclear, degeneration of the IVD has been implicated as a possible primary etiological factor. Poor nutritional supply to the disc is believed to be a key initiating component of degeneration where changes in disc morphology, biochemistry, function, and material properties are observed. The most significant biochemical change seen in the degenerated disc is a loss of proteoglycan,\textsuperscript{40,41} which may be responsible for the decrease in osmotic pressure and tissue hydration,\textsuperscript{42} resulting in loss of the load-support capability of the disc,\textsuperscript{43} as well as affecting the movement of molecules into and out of the disc.\textsuperscript{3} Increasingly elevated levels of fibronectin have been observed within the progressing degenerate disc\textsuperscript{44} which may up-regulates some matrix degrading enzymes. Currently, the main therapeutic options for degenerative disc disease include physical therapy, rest, and medication. If the conservative methods do not provide relief than a spinal fusion or total disc replacement may be recommended. A spinal fusion alleviates pain but does not restore the mechanical function of the disc and may cause further degradation of adjacent IVDs by altering the biomechanics of the spine.\textsuperscript{45} Artificial
discs replace some motion but cannot sustain compressive loads and may cause stress shielding on the vertebrae leading to possible implant failure.\textsuperscript{46}

Pathology

\textit{Nutrient Pathways of Disc Degeneration}

Since IVDs are the largest avascular tissue in the human body, transport of essential nutrients is crucial to maintain disc health. The cellular metabolism of the IVD occurs mainly through glycolysis, as the disc cells require glucose for survival and produce lactic acid at high rates. Oxygen is also necessary for cellular activity, although not necessary for survival, as its consumption pathway is unclear. The balance between nutrient transport rates through the matrix and the rate of cellular consumption establishes a concentration gradient inside the disc. Deviation from physiological nutrient levels due to decreased blood supply is suggested to be one of the primary mechanisms for disc degeneration. Blood supply may be altered as a result of atherosclerosis \textsuperscript{47}, sickle cell anemia, Caisson disease, Gaucher’s disease \textsuperscript{4}, diabetes mellitus \textsuperscript{48}, increasing age \textsuperscript{49, 50}, etc.

\textit{Effect of Mechanical Load and Injury to the IVD}

Abnormal mechanical loading to the disc tissue is also suggested to lead to degeneration and therefore, clinical symptoms such as lower back pain. In the literature, there are two contrasting hypotheses, termed overload and immobilization, used to explain accelerated disc degeneration via mechanical conditions. The overload, or “wear and tear,” explanation, as depicted in Figure 6, suggests that excessive motion produces trauma to the disc, such as fiber damage, delamination, annulus tears, and associated proteoglycan loss, and may result in partial healing due to slow tissue turnover. Contrary, the
immobilization theory encompasses the under-use of mechanical motion, which actually is believed to cause similar tissue responses compared to the overload theory. The similar tissue responses include altered cell level signals (e.g., fluid flow, cell strain, altered nutrition, accumulation of waste products, and loss of cellularity), and matrix remodeling (e.g., alterations in gene expression, enzyme activity, composition, and structure).\textsuperscript{51} A healthy cartilage endplate is critical for maintaining disc health by helping to resist disc herniation or tears.\textsuperscript{29; 52; 53} Damage may be induced to the cartilage endplate by abnormal loading and therefore lead to internal disc disruption, which then may initiate disc degeneration or the herniation processes.\textsuperscript{15; 54; 55} Damage to the cartilage endplate was also found to be strongly associated with the onset of innervation in the interface between the vertebral body and cartilage endplate known as the bony endplate layer.\textsuperscript{31} The nerves may be further sensitized by mechanical and chemical stimuli due to cartilage endplate damage leading to low back pain.\textsuperscript{27; 30} Therefore, damage to the cartilage endplate may play an irreplaceable role in the progression of disc degeneration and associated low back pain.
Genetic Factors in Disc Degeneration

Recent findings in the literature suggest that genetic components may contribute to the development and progression of disc degeneration. There are a number of genes that have been associated with intervertebral disk degeneration in humans, including genes coding for collagen I, collagen IX (COL9A2 and COL9A3), collagen XI (COL11A2), IL-1, aggrecan, vitamin D receptor, IGF-1 receptor, and matrix degrading enzymes (MMP-2, -3, and -9). For specific genes and some environmental factors, gene—gene, gene—environment and gene—age interactions may exist.\textsuperscript{56-59}
Diagnosis and Mechanism of Disc Degeneration Associated Low Back Pain

Low back pain is characterized as a symptom of a medical condition, not a diagnosis itself. The mechanism of low back pain can be divided into mechanical causes (e.g., degenerative processes of disc and facets, herniated disc, spinal stenosis, osteoporotic compression fracture, spondylolisthesis, and traumatic fractures), non mechanical spinal causes (e.g., neoplasia such as spinal cord tumors, primary vertebral tumors, infection such as osteomyelitis and septic discitis, and inflammatory arthritis), visceral causes (e.g., pelvic organ involvement, renal involvement, and aortic aneurysm) and all others. Disc degeneration associated low back pain is found to be the most common mechanical cause of back pain. The mechanisms of disc degeneration associated low back pain include (1) pressure on the posterior nerves caused by bulging of the annulus fibrosus, (2) pain generators in the end-plates that may be provoked by endplate deflection, as well as (3) the flowing out of irritating substances from within the disc such as glycosaminoglycans and lactic acid due to internal disc disruption. Therefore, disc degeneration associated low back pain could be considered as the pain caused by chemical or mechanical irritation of the nerves surrounding the IVD. The pain pathways for disc degeneration associated low back pain are still very controversial. Traditionally, it is believed that pain signals originate in the nerve roots adjacent to the disc and move into the corresponding dorsal root ganglion (DRG) and the spinal cord. However, recent research suggests that pain signals from the lower lumbar discs (i.e., L4 and L5) are detoured up the sympathetic nerves (i.e., gray ramus communicans) and into the upper lumbar DRGs, especially at the L2 level, as shown in Figure 7. As a result, it would be possible for some patients with L4 and L5 disc
degeneration associated pain signals to have L1 or L2 dermatomal pain (i.e., groin and anterior thigh pain). Upon the onset of low back pain, approximately only 20-30% of patients have definitive pathology necessitating a more invasive procedure. The majority of individuals (i.e., 80%) experiencing low back pain will follow a conservative treatment regimen and return to work within 6 weeks either with or without pain.²⁶ Twenty percent, however, do not return to work within the 6-week period and typically need repeated diagnostic and medical treatment procedures.
Figure 7. Pain pathways for discogenic pain. A: Pain signals that generate from the disc traverse pass into the corresponding dorsal root ganglion (DRG) and into the spinal cord. B: Pain signals from the lower lumbar discs (L4-L5) detour up the sympathetic nerves (grey ramus communicans) into the upper lumbar DRG, especially at the L2 level. (Modified from Reference)
Classification of Disc Degeneration: IVD Grading Systems

Computed tomography (CT) and magnetic resonance imaging (MRI) techniques are most commonly used to collect information regarding the morphology and structure of the IVD, bone, and nerves, to identify the cause of low back pain and develop a suitable medical treatment plan. The use of IVD degeneration grading systems, such as the Thomson grading system and the Pfirrmann grading system, are essential to identifying the disc degeneration level during the diagnosis process. In the Thomson grading system, a five category grading scheme was developed using gross morphological information from the nucleus pulposus, annulus fibrosus, and endplates (cartilaginous and bony) of midsagittal sections, as well as the periphery of the vertebral body of the human lumbar intervertebral discs as shown in Table 2 and Figure 8. However, the application of this grading system is limited due to the fact it uses histology images from the midsagittal plane, and therefore, may only be used in vitro. In addition, the entire morphology of the disc cannot be assessed using this grading scheme.67
Table 2. Description of the Morphologic Grades within the Thompson Grading System. (Modified from Reference) 67

<table>
<thead>
<tr>
<th>Grade</th>
<th>Nucleus</th>
<th>Annulus</th>
<th>End-plate</th>
<th>Vertebral body</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Bulging gel</td>
<td>Discrete fibrous lamellas</td>
<td>Hyaline, uniformly thick</td>
<td>Margins rounded</td>
</tr>
<tr>
<td>II</td>
<td>White fibrous tissue peripherally</td>
<td>Mucinous material between lamellas Extensive mucinous infiltration; loss of anular-nuclear demarcation</td>
<td>Thickness irregular</td>
<td>Margins pointed</td>
</tr>
<tr>
<td>III</td>
<td>Consolidated fibrous tissue</td>
<td>Focal defects in cartilage</td>
<td>Early chondrophytes or osteophytes at margins</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Horizontal clefts parallel to endplate</td>
<td>Focal disruptions</td>
<td>Fibrocartilage extending from subchondral bone; irregularity and focal sclerosis in subchondral bone</td>
<td>Osteophytes less than 2 mm</td>
</tr>
<tr>
<td>V</td>
<td>Clefts extend through nucleus and annulus</td>
<td>Diffuse sclerosis</td>
<td>Osteophytes greater than 2 mm</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8. The appearance of discs (Grade I through IV) in the Thompson grading system. (Modified from Reference) 67
In the Pfirrmann grading system, MRI signal intensity, disc structure, distinction between the nucleus pulposus and annulus fibrosus, as well as disc height information from sagittal MRI T2-weighted images are used to identify IVD degeneration levels with a five category grading scheme as shown in Table 3 and Figure 9. This grading system detects variations in disc hydration or composition using noninvasive MRI techniques. This grading system provides a standardized and reliable assessment of disc morphology for research purposes and clinical diagnosis. However, use of this grading scheme has caused disagreement between different grades of IVD degeneration due to the fact that the system is based on the macroscopic morphology of the disc.68 Overall, quantitative IVD degeneration grading systems are needed to accurately identify disc degeneration levels in future clinical diagnosis.
Table 3. Classification of Disc Degeneration using the Pfirrmann Grading System. (Modified from Reference) ⁶⁸

<table>
<thead>
<tr>
<th>Grade</th>
<th>Structure</th>
<th>Distinction of NP and AF</th>
<th>Signal Intensity</th>
<th>Height of IVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Homogeneous, bright white</td>
<td>Clear</td>
<td>Hyperintense, isointense to cerebrospinal fluid</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Inhomogeneous with or without</td>
<td></td>
<td>Hyperintense, isointense to cerebrospinal fluid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>horizontal bands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Inhomogenous, gray</td>
<td>Unclear</td>
<td>Intermediate</td>
<td>Normal to slightly decreased</td>
</tr>
<tr>
<td>III</td>
<td>Inhomogenous, gray to black</td>
<td>Lost</td>
<td>Intermediate to hypointense</td>
<td>Normal to moderately decreased</td>
</tr>
<tr>
<td>IV</td>
<td>Inhomogenous, black</td>
<td>Lost</td>
<td>Hypointense</td>
<td>Collapsed disc space</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 9. Pfirrmann grading system for the assessment of lumbar disc degeneration; (A) Grade I, (B) Grade II, (C) Grade III, (D) Grade IV, and (E) Grade V. (Modified from Reference)⁶⁸

Treatment Options for Discogenic Pain

Currently, the main therapeutic options for degenerative disc disease include a combination of physical therapy, rest, and medication. If the more conservative methods
do not provide relief then a surgical procedure may be recommended such as a spinal fusion or total disc replacement. A spinal fusion alleviates pain but does not restore the mechanical function of the disc and may cause further degradation of adjacent IVDs by altering the biomechanics of the spine. The United States Food and Drug Administration (FDA) approved two artificial discs for total disc replacement in the lumbar spinal region in October 2004 and January 2006, respectively—the Charite III (DePuy Spine, Inc, Raynham, Massachusetts) and the ProDisc II (Synthes Spine, West Chester, Pennsylvania), as shown in Figure 10. These artificial discs replace some motion but cannot sustain compressive loads and may cause stress shielding on the vertebrae leading to possible implant failure.

Figure 10. The (A) CHARITÉ™ Artificial Disc and the (B) PRODISC®-L Total Disc Replacement. www.fda.gov.

The CHARITÉ™ Artificial Disc and PRODISC®-L Total Disc Replacement similarly consist of two parts: (1) two cobalt-chrome alloy endplates that are anchored to the top and bottom surfaces of the vertebrae, (2) a ultra-high molecular weight polyethylene (UHMWPE) core that fits between the two endplates. The UHMWPE core and endplates help restore the natural disc height between the two vertebrae. The endplates can slide over the domed parts of the core, which can allow movement at the level where it is implanted.
Initial fixation mechanisms, surgical technique and implantation, as well as device kinematic characterize the major differences between the CHARITÉ™ Artificial Disc and PRODISC®-L Total Disc Replacement. The CHARITÉ™ has 6 spikes on the endplates of the device and the PRODISC®-L has a central keel. These different fixation mechanisms revealed differences in initial fixation strength of the devices to the vertebral bodies and showed different device migration and failure rates. The surgical technique of the CHARITÉ™ device was relatively difficult compared with that of the PRODISC®-L because of the oblique handle of the insertion forceps. In regards to the device kinematics, the difference in constraint may result in different strain and loads on the facet joints. In turn, the facet joints of the replaced segments in the long-term may show different degrees of degeneration depending on the specific device implanted.  

Future Directions: New Therapies

Current efforts are attempting to develop new treatments for disc degeneration associated low back pain which address the weaknesses of traditional treatments. In order to avoid consequences of synthetic implants, such as metal implant fatigue and the immune reaction to wear debris, the use of cell based therapies is being investigated to regenerate IVD tissue.

Injection of Growth Factors

The direct injection of growth activators is an option to stimulate IVD regeneration due to the fact that matrix synthesis and IVD cell proliferation are influenced by several growth factors and enzymes. Members of the TGF-β superfamily, BMP7, and other
growth factors increased proteoglycan synthesis and stimulated IVD restoration in canine, rat, and rabbit models. In addition, IVD regeneration was stimulated for 13 months when a patient was treated with a mixture of matrix components and growth factors via direct injection. However, the success of this technique is limited due to the availability of viable cells as well as the fast diffusion of growth factors out of the IVD, which indicates that this treatment is primarily suitable for early to moderate stages of disc degeneration.

**Gene Therapy**

For more long term effects, integration of the growth activators directly into the genome of disc cells is being investigated. The *ex vivo* gene therapy (removal of the cells, transfection *in vitro*, and return of the transfected cells into the IVD) is preferred due to the safety reasons. Adeno-mediated therapy of human disc cells with transcription factors, growth factors, anabolic enzymes, and TIMP has led to some restoration of the IVD structure and increased proteoglycans and collagen synthesis. However, little is known about the influence on regulatory pathways caused by the release of growth factors and limited healthy disc cells are present in the degenerated IVD.

**Tissue Engineering and Regeneration**

In the case of Grade V disc degeneration, utilizing tissue engineering methods, such as cultivation of disc cells on a 3D scaffold, are being investigated. The application of pressure, and/or other physical forces, are being used for *in vitro* cultivation of disc cells on a 3D scaffold. Increased understanding of disc cell biology as well as the development of several biomaterials imitating IVD properties have indicated that tissue engineering may become a reality. However, inadequate biomechanical behavior of

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tissue scaffolds as well as complications involved with scaffold insertion prevents this method from clinical use.

Cell Transplantation

It has been shown that supplementing degenerate disc tissue with mesenchymal stem cells, NP cells, or chondrocytes, demonstrates a regenerative effect in rabbit models. Additionally, patients in a disc repair clinical study showed stimulated matrix regeneration and experienced pain relief after cell transplantation with autologous chondrocytes. Conversely, the injection of a pure cell solution has led to extensive cell leakage through the injection site, which may be caused by inner disc pressure. Currently, it is unclear whether this type of therapy would decrease disc degeneration associated low back pain.

Extracellular Matrix Assisted Cell Transfer

Due to limitations associated with pure cell solution injections, the injection of a biomaterial coupled with a cell solution may be an efficient treatment. Requirements for suitable IVD biomaterials include mechanical stability, biocompatibility, biodegradation, sterilizability, and a low viscosity for injection devices. In general, biomaterials which are injected as fluids will polymerize by crosslinking or through the addition of gel medium, such as agarose. Several biomaterials for IVD regeneration, such as silicones, chitosane, or components of the extracellular matrix (ECM) like hyaluron, fibrin, collagen or silk-elastin copolymers, have been analyzed in animal models. Since several ECM components have been found to interact with disc cells and influence their behavior, this method would benefit if the utilized biomaterials prevented the leakage of the cells.
Limitations of this method include maintaining disc cell phenotypes as well as lack of knowledge regarding the characteristic molecular profile of the AF and NP cells as a result of the resulting interactions between the matrix and cells.

Furthermore, IVD regeneration requires the restoration of the complex nutrient supply of the disc, which is supported by the cartilage endplates located superiorly and inferiorly to the IVD. Currently, more knowledge is necessary to refine and develop new strategies for IVD regeneration and the treatment of disc degeneration associated low back pain.

**Disc Nutrition**

Due to its large avascular nature, critical nutrients required for maintaining tissue health are supplied by blood vessels at the margins of the disc. Diffusion mechanism regulated the supply of nutrients and removal of metabolic wastes within the IVD. Therefore, solute transport plays a key role in disc cell nutrition. The balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells determines the resulting concentration gradient inside the disc. Disruptions to the nutrient supply of the disc are strongly correlated to the development of disc degeneration.

**General Cell Energy Metabolism**

A constant supply of energy stored in the chemical bonds of organic molecules is needed to generate and maintain cellular activity. The particular oxidation, or breakdown, of glucose and other sugars embodies the necessary fuel molecules required for cell energy metabolism. In animal cells, there are several key steps involved in the catabolism of sugars and as a result, the production of important activated carrier molecules.
Glycolysis

The first major metabolic process, which probably evolved early in the history of life, is glycolysis. During glycolysis, a glucose molecule with six carbon atoms is converted into two molecules of pyruvate, each of which contains three carbon atoms. For each glucose molecule, two molecules of adenosine triphosphate (ATP) are hydrolyzed to provide energy to drive the earlier steps, but four molecules of ATP are produced in the later steps. At the end of glycolysis, there is a resulting net gain of two ATP molecules for each molecule of glucose. This entire process occurs within the cytosol of most cells and requires no molecular oxygen (O₂) to produce ATP. Although no molecular oxygen is used in glycolysis, oxidation occurs via the nicotinamide adenine dinucleotide redox pair, in that electrons are removed by NAD⁺ to produce NADH, via some of the carbons derived from the initial glucose molecule. Two molecules of NADH are formed per glucose molecule during the course of glycolysis. For the majority of cells, glycolysis is a prelude to the final stage of the breakdown of organic molecules.

Aerobic Cell Metabolism

For aerobic organisms, which require molecular oxygen to survive, the two NADH molecules will supply electrons to the electron-transport chain, and the resulting NAD⁺ will be used again for glycolysis. The oxidation of pyruvate continues in the mitochondria of the cell, in a process known as the citric acid cycle, resulting in the major end products of carbon dioxide (CO₂) and high energy electrons carriers. Three molecules of NADH, one molecule of flavin adenine dinucleotide, reduced (FADH₂), and one molecule of guanosine triphosphate (GTP) include the electron carriers that subsequently drive the
mitochondrial located process of oxidative phosphorylation. The transferred high energy electrons coupled with oxygen gas and protons pass along the electron-transport chain to extract all available energy, resulting in the formation of approximately 36 molecules of ATP for cellular processes, as shown in Figure 11.

Anaerobic Cell Metabolism

For those organisms which do not utilize molecular oxygen and can grow and divide without it as well as certain tissues that can continue to function when oxygen is limited, glycolysis is the cell’s primary source of ATP. In these conditions, the pyruvate and high energy electrons (i.e., NADH) remain in the cytosol of the cell. Eventually, the pyruvate enters in one of two breakdown pathways, while the NADH gives up its electrons returning to its NAD⁺ for utilization in glycolysis. The pyruvate will enter into a type of fermentation pathway producing ethanol and CO₂ or lactate depending on a limited or complete lack of oxygen levels, as shown in Figure 11. Both fermentation pathways yield much less energy combined compared to complete oxidative phosphorylation.107
Figure 11. Glucose Metabolism in Mammalian Cells. Afferent blood delivers glucose and oxygen (via hemoglobin) to tissues, where it reaches cells by diffusion. Glucose is taken up by specific transporters, where it is converted first to glucose-6-phosphate by hexokinase and then to pyruvate, generating 2 ATP per glucose. In the presence of oxygen, pyruvate is oxidized to HCO₃⁻, generating 36 additional ATP per glucose. In the absence of oxygen, pyruvate is reduced to lactate, which is exported from the cell. Both metabolic processes produce hydrogen ions (H⁺), which cause acidification of the extracellular space. HbO₂, oxygenated hemoglobin. (Modified from Reference)¹⁰⁸

IVD Cell Metabolism

Intervertebral disc cells maintain normal energy supply primarily through glycolysis, even in the presence of oxygen.¹⁰⁹ The conversion of glucose to lactic acid at high rates produces adenosine triphosphate (ATP). The IVD cellular environment is very harsh. It includes conditions such as an acidic pH due to the high production of lactic acid, hypoxia, low levels of glucose, as well as constant mechanical stress.

Metabolite Influences on IVD Cell Activity

Disc cell activity is regulated by extracellular oxygen and glucose concentrations as well as extracellular pH. It has been shown in the literature that IVD cells utilize
glycolytic pathways, with or without the presence of oxygen, but require oxygen to carry out other cellular activities. It has been shown that at low oxygen tensions that the production rates of several macromolecules, such as sulfated glycosaminoglycans or proteins, are significantly inhibited. The exact pathways in which oxygen is utilized remains unclear. Some studies have reported that at low oxygen conditions the IVD cells exhibit a negative Pasteur effect in which the rate of glycolysis is diminished.\textsuperscript{109} Contrary to these reports, other studies have demonstrated the Pasteur effect—under low oxygen, glycolysis rates and lactate production rates increase.\textsuperscript{110; 111} Differences in these findings from the literature are unclear. However, it is widely supported in the literature that an acidic pH level significantly decreases the rates of glycolysis and the rate of oxygen uptake, and as a result, the production of ATP. In addition, the presence of growth factors, changes in the stresses in the surrounding matrix, and the osmotic environment have each been observed to alter the rates of energy metabolism.\textsuperscript{112}

Maintenance of the extracellular matrix is also influenced by local extracellular oxygen and pH levels. The rates of matrix synthesis seem to peak at 5\% oxygen compared to ambient oxygen levels as well as low oxygen tensions. At 1\% oxygen tension, rates of sulfate incorporation are only one-fifth of the rates seen at 5\% oxygen. In regards to extracellular pH, rates of matrix synthesis are 40\% higher at pH 7.0 compared at pH 7.4. However, the rates significantly decrease once the cellular environment becomes acidic. Rates of extracellular matrix degradation, however, appear to be less sensitive to pH. The production of contributing matrix metalloproteinases is comparable at pH 7.0 and pH 6.4.
As a result, acidic pH levels may increase the rate of matrix breakdown by inhibiting synthesis but not degradation.

For the IVD cells to retain their viability, the levels of extracellular nutrients and pH must remain above critical values. Glucose is a critical nutrient dictating the production of ATP as a result of glycolysis. The viability of IVD cells will plummet within twenty-four hours if the glucose concentration falls below 0.5 mM. As shown in Figure 12b, an acidic environment also will increase the rate of cell death. Even with adequate glucose at pH 6.1, the cell viability is severely compromised.

![Figure 12. Effect of nutrient-metabolite concentrations on IVD cell viability. (a) Cells were cultured at 0% oxygen under different glucose concentrations and pH values and after 24 hours of incubation, cells cultured at low glucose showed loss of viability, particularly under acid pH. (b) Cells were cultured at 0% oxygen. Cells at pH 7.4 and with adequate glucose showed no loss of viability after six days in culture; at low glucose or low pH, few cells were still alive at this point. (Modified from Reference) 105](image)

Previous studies focused on bovine, canine, and porcine models have shown that glucose and oxygen consumption rates of IVD cells and articular cartilage cells are
dependent on culture conditions. Examining nutrient consumption rates of human IVD cells under different culture conditions needs to be given special focus to develop functional relationships between O₂ level, glucose concentration, and pH.

In humans and other species, the nucleus pulposus frequently transitions from notochordal cells (NCs) to chondrocyte-like mature NP cells (MNPCs) with age. It has been shown that this transition coincides with the development of disc degeneration, contrary to other species that maintain NCs. The fate of the NCs is unknown and subject to a continuous debate in the literature. Some reports suggest that the adult nucleus pulposus cells derive from cells “invading” into the disc, while other studies indicate that these cells originate from involution of the notochord. It has been argued that nutritional demands of IVD cells have been the driving force behind the disappearance of NCs in the human nucleus pulposus and their consequent replacement with chondrocyte-like cells during disc maturation. Nevertheless, further understanding of these mechanisms may provide insight into the means of disc degeneration. The use of porcine NCs and bovine MNPCs to measure metabolic rates, as shown in Table 4, revealed that NCs demand more energy and are less resistant to nutritional stress than MNPCs. In addition, the lactate: glucose mole ratio at 5 hours was 1.7 for NCs and 2.1 for MNPCs. A production: consumption mole ratio of 2.0 would indicate the supply of energy from glycolysis alone. Thus, it can be inferred that NCs use glucose in reactions other than glycolysis, while the metabolism of MNPCs is almost purely glycolytic.
Table 4. Rates of energy production in NCs and MNPCs based on rates measured under standard conditions (pH 7.4, 21% oxygen, 5mM glucose)* (Modified from Reference 113)

<table>
<thead>
<tr>
<th></th>
<th>NCs, nmoles/10^6 cells/hour</th>
<th>MNPCs, nmoles/10^6 cells/hour</th>
<th>NC:MNPC (porcine:bovine) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial rates with no FBS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate production at 5 hours†</td>
<td>355 ± 21</td>
<td>204 ± 9</td>
<td>1.74</td>
</tr>
<tr>
<td>Glucose consumption at 5 hours†</td>
<td>205 ± 16</td>
<td>95 ± 8</td>
<td>2.2</td>
</tr>
<tr>
<td>Lactate production at 1 day</td>
<td>462 ± 23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactate production at 2 days</td>
<td>374 ± 23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactate production at 3 days</td>
<td>518 ± 32</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Rates in culture with 6% FBS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate production at 2 days</td>
<td>534 ± 22</td>
<td>356 ± 13</td>
<td>1.50</td>
</tr>
<tr>
<td>Lactate production at 8 days</td>
<td>455 ± 12</td>
<td>295 ± 19</td>
<td>1.54</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SEM. FBS = fetal bovine serum.
† The lactate:glucose mole ratio at 5 hours was 1.7 for notochordal cells (NCs) and 2.1 for mature nucleus pulposus cells (MNPCs).

Nutrient Supply to the IVD cells

The IVD is the largest avascular structure in the human body. The critical nutrients (e.g., oxygen, glucose, and other small solutes) required for maintaining tissue health are supplied by blood vessels at the margins of the disc. The supply of nutrients and removal of metabolic wastes within the IVD mainly depends on diffusion mechanisms. Therefore, solute transport plays a key role in disc cell nutrition. The balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells determines the resulting concentration gradient inside the disc. Nutrients must diffuse as far as 10 mm from the capillaries originating in the vertebral bodies through the cartilage endplates and dense extracellular matrix of the nucleus to the cells, as shown in Figure 13.
Figure 13. Pathways for nutrient supply to the disc cells. (A) The avascular nature of the disc compared with the vertebral body and ligaments. Arrows demonstrate routes for solute transport into the disc. B. Schematic showing transport between the blood supply and the disc. (Modified from Reference)7

Nutritional Pathways

Nutrients, such as glucose and oxygen, are supplied by the capillary network at the boundary of the endplates, and then diffuse through the cartilage endplate to the cells and lactic acid is removed by the reverse route, as shown in Figure 14.

Figure 14. Nutritional Pathways of in the nondegenerate IVD. (Modified from Reference)123
Growth factors and other bioactive molecules also diffuse to the disc from the blood supply or are produced by the cells themselves. The local environment governs production matrix macromolecules, which are assembled within the matrix, and protease are also produced by the cells and degrade the macromolecules. Degraded macromolecules slowly escape from the disc by diffusing towards the endplate and are removed the capillaries.7

*Solute Transport and Nutrient Profiles*

The balance between nutrient transport rates through the matrix and the rate of cellular consumption establishes a concentration gradient inside the disc. Due to the difficulty of measuring nutrient gradients *in vivo*, mathematical models have been used to predict the nutrient environment inside cartilage tissues. Calculations have reported that oxygen, glucose, and pH levels decrease from the superior and inferior endplates to within the center of the disc, while the concentration of lactic acid is highest in the center of the disc, as shown in Figure 15. The varying nutrient gradients, as a result of diffusion mechanisms, cause the cells within the disc to function at different metabolic rates. Due to low concentrations of glucose, oxygen, and pH as well as a high lactic acid concentration, the center of the disc will most likely experience disc degeneration first as a result of limited nutrient supply conditions.105
Constitutive and finite element models of IVD

The fundamentals of the theory and development of mathematical models in the field of soft tissue biomechanics are present in the literature. One of them is the well-known, biophasic theory for cartilage developed by Mow et al. (1980)\textsuperscript{124}, which considers soft tissue as a mixture of a water phase and a solid phase. Another is the triphasic mechano-electrochemical theory, developed by Lai et al. (1991)\textsuperscript{125}, for describing swelling and deformational behaviors of charged hydrated soft tissues based on a tertiary-mixture theory and the laws of thermodynamics.\textsuperscript{126} In the triphasic theory, a charged hydrated tissue is modeled as a mixture consisting of three distinct phases (i.e., solid, water, and ion phases). This theory has been successfully used to study the swelling, transport of fluid and ions, and electrokinetic phenomena in charged hydrated tissues.\textsuperscript{127} Recently in our lab, Yao et al. (2007) developed a new multiphasic mechano-electrochemical transport theory, based on the triphasic theory, which includes multiple species of ions or neutral solutes.\textsuperscript{128}
This theory has been used to investigate electromechanical properties and to develop finite element models for the IVD.\textsuperscript{129}

\textit{Effect of diurnal cycle loading}

The human IVD was subjected to a diurnal cyclic loading, including a 16-hour compression and 8-hour recovery. The oxygen, glucose, and lactate concentration distributions in the normal IVD at the end of the day (i.e., the end of compression), as well as at the beginning of the day (i.e., the end of recovery), were shown in Figure 16. It was apparent that the nutrient concentration profiles remained almost identical during the day and night. The changes of the concentration profiles were less than 1\% under the diurnal cyclic loading. Although the nutrient concentration levels were different compared to the IVD with a normal CEP, the concentration profiles also remained almost identical during the day and night in the IVD with a calcified CEP (results not shown).

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{figure16.png}
    \caption{Nutrient solutes (oxygen, lactic acid, and glucose) concentration distribution inside human IVD in the morning and before sleep: (a) x radius from center to periphery and (b) y axis from posterior to anterior. (Modified from Reference)\textsuperscript{130}}
\end{figure}
The nutrition concentration distributions were not uniform in the human IVD (Figure 17). Generally, the oxygen and glucose concentrations decreased moving away from the blood supply at the margin of the disc. In contrast, the lactate concentration increased toward the center of the disc. Significant nutrient concentration gradients existed inside the disc. The posterolateral region of the disc within the AF possessed the lowest glucose concentration of 0.540 mM, compared to the concentration of 5mM at the lateral boundary of the AF. This region also had the highest lactate concentration of 5.207 mM, compared to the concentration of 0.9mM at the lateral boundary of the AF. The center region of the disc within the NP possessed the lowest oxygen concentration of 0.3 kPa, compared to the concentration of 5.1 kPa at the boundary of the CEP.
Figure 17. Typical 3D nutrient solutes (glucose, lactic acid, and oxygen) distribution inside human IVD: (a) (c) (e) normal CEP and (b) (d) (f) calcified CEP (Unit: mM). (Modified from Reference)\textsuperscript{130}

**Effect of CEP calcification**

The calcification of the CEP dramatically decreased the glucose and oxygen concentrations and increased the lactate concentration inside the IVD (Figure 17). There were 69.3% and 33.9% decreases in minimum glucose and oxygen concentrations in the disc with a calcified endplate, respectively. In contrast, there was a 7.3% increase in
maximum lactic acid concentration in the disc with the calcified endplate. Moreover, compared to the AF region, the nutrient concentration levels in the NP region, compared to the AF region, were more significantly affected by the calcified CEP (Figure 17). There were 23.0% and 23.7% decreases in the mean concentrations of oxygen and glucose in the NP region, while 5.1% and 8.2% decreases were found in the AF region. Meanwhile, a 16.5% percent increase of mean lactic acid concentration was found in the NP region while an 8.7% increase was found in the AF region.

**Effect of NP cell injection**

The increase in cell density within the NP region due to the NP cell injection significantly decreased the extreme oxygen and glucose concentrations (lowest concentration inside the disc), while increasing the extreme lactic acid concentration (highest concentration inside the disc), as shown in Figure 18. The magnitude of the change of the extreme concentrations due to the cell injection also depended on the conditions of the CEP (i.e., normal, calcified, and thin calcified). Moreover, the condition of the CEP had a more significant impact on the extreme glucose concentration, than on the extreme lactic acid and oxygen concentrations. Specifically, the extreme glucose concentration sharply reached zero (Figure 18) when the NP cell density increased only 50% with the calcified CEP. Glucose concentration is a limiting factor for disc cell viability \(^{36; 131; 132}\). A Critical Zone was defined as a disc region in which the glucose concentration is lower than 0.5mM \(^{132}\). With the normal CEP, a small volume Critical Zone (0.016 cm\(^3\)) appeared in the AF region when the NP cell density increased 50% (Figure 19a-b). With the calcified CEP, a 50% increase of the NP cell density caused a 105.6%
increase in the volume of the Critical Zone (Figure 19c-d). With the thin calcified CEP, the increase in the volume of the Critical zone was reduced to 75.8% (Figure 19c-f).

Figure 18. Effect of increase in cell density by 50% in NP region on glucose concentration distribution inside human IVD: (a) (b) normal CEP; (c) (d) calcified CEP; (e) (f) thin calcified CEP. (Modified from Reference) The grey color regions indicate ‘critical zones’ in which glucose concentration is lower than 0.5 mM.
Figure 19. Effect of increase in cell density on 3D glucose concentration distribution in the disc with a normal (a) (b), calcified CEP (c) (d), and thin calcified CEP (e) (f). (Modified from Reference) The grey color regions indicate ‘critical zones’ in which glucose concentration is lower than 0.5 mM.

Significance and clinical relevance

It’s unclear how mechanical stresses and swelling pressure affect nutrient transport mechanisms in the human IVD. With this dissertation, we intend to bridge this gap by profiling the metabolism and evaluating the effect of mechanical strain on transport
properties of human IVD tissues. This study will drive the development of a new realistic anisotropic mechano-electrochemical theory and finite element model for investigating the transport of fluid and solutes in human IVDs under various loading conditions. In addition, this study will provide information on the possible nutrition-related mechanism of low back pain associated disc degeneration, which, according to the NIH, affects 8 out of 10 people at some point in their lives. Furthermore, the theories and experimental techniques developed in this study will have a significant impact on other areas of research as well, such as investigating the delivery of drugs and other biological factors, which may stimulate cellular metabolic activities in soft tissues. The ensuing findings of this proposal will promote the development of new methods for tissue engineering in vitro and in vivo, since nutritional supply is a major consideration in engineering healthy tissues.
CHAPTER 3: COMPARISON OF OXYGEN CONSUMPTION RATES AND MITOCHONDRIAL MEMBRANE POTENTIAL OF NONDEGENERATE AND DEGENERATE HUMAN IVD CELLS

The avascular nature of the intervertebral disc (IVD) creates a delicate balance between rate of nutrient transport through the matrix and rate of disc cell consumption necessary to sustain tissue health. Poor nutritional supply is believed to be a key initiating constituent of disc degeneration. This study was commenced to test the hypothesis that IVD nutrient environment parameters including oxygen level and glucose concentration will have a significant impact on oxygen consumption rates (OCR) and mitochondrial membrane potential in nondegenerate and degenerate human IVD cells. The real-time dissolved oxygen concentrations were monitored by a fiber optic oxygen sensor in a sealed metabolism chamber and curve fit to the Michaelis-Menten enzyme kinetics model. Using confocal microscopy and tetramethylrhodamine methyl ester (TMRM), the mitochondrial membrane potential was assessed. For nondegenerate IVD cells, no significant difference in average OCR (annulus fibrosus (AF): 16.95±11.51 nmol/10^6 cells/hr; nucleus pulposus (NP): 18.13±11.48 nmol/10^6 cells/hr; cartilage endplate (CEP): 14.12±6.329 nmol/10^6 cells/hr) was observed due to glucose concentration (p=0.89). The degenerate IVD cells cultured in 1mM glucose concentration exhibited a significantly larger OCR (93.76±16.83 nmol/10^6 cells/hr) than those cultured in both 5mM (53.99±10.77 nmol/10^6 cells/hr) and 25mM (48.90±12.03 nmol/10^6 cells/hr) glucose concentrations (p<0.05). The TMRM intensity per cell was significantly higher in degenerate AF cells compared to nondegenerate AF cells (p=0.03). The results show that human degenerate IVD cells are very active metabolically and poorly resistant to nutritional stress and support the idea that
a fall in nutrient supply as a consequence of the onset and progression of disc degeneration could drive a metabolic change in IVD cell phenotype. The data reported in this study contributes to our understanding of the unique nutrient metabolism mechanisms in the human IVD which may shed light on the nutrient related mechanisms associated with the onset and progression of IVD degeneration.

Introduction

The intervertebral disc (IVD) is a fibrocartilage structure separating the vertebrae that allows a wide range of mechanical motion in the spine. Differences in biochemical composition and structure distinguish three regions of the disc including the annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplates (CEP). Due to its large avascular nature, critical nutrients required for maintaining disc tissue health are supplied by blood vessels at the margins of the disc.123; 133 Within the IVD, diffusion mechanisms regulate the supply of nutrients and removal of metabolic wastes. The balance between the rate of nutrient transport through the matrix and rate of consumption by disc cells determines the local nutrient concentration gradient within the IVD.106 Poor nutritional supply to the disc is believed to be a key initiating constituent of degeneration where changes in disc morphology, biochemistry, function, and material properties are observed.123 Low back pain is a major socio-economic health concern in western industrialized societies.134; 135 Although the exact cause of low back pain is unclear, degeneration of the IVD has been implicated as a possible primary etiological factor.136
The cellular metabolism of the nondegenerate IVD is believed to occur mainly through glycolysis, as the disc cells require glucose for survival\textsuperscript{132} and produce lactic acid at high rates under hypoxia. Oxygen is also necessary for cellular activity, although not necessary for survival, as its consumption pathway is unclear. Previous studies have shown a dependence of oxygen consumption rates for bovine and porcine IVD cells on oxygen level, pH level, and glucose concentration.\textsuperscript{109; 113; 114} In the porcine IVD model, the NP cells were shown to have a significantly higher absolute OCR compared to that of AF cells.\textsuperscript{114} The inhibition of cellular respiration due to the high rate of glycolysis under high glucose concentrations, described as the Crabtree effect, has been demonstrated in porcine AF,\textsuperscript{114} but not porcine NP\textsuperscript{114} as well as bovine NP\textsuperscript{109; 113}. To our knowledge, the oxygen consumption rates of human AF, NP, and CEP nondegenerate as well as degenerate disc cells has not been investigated.

The oxygen consumption rate of articular chondrocytes\textsuperscript{137; 138} and porcine temporomandibular joint disc cells\textsuperscript{139} have also been shown to depend on local oxygen levels. The consumption of oxygen decreases as oxygen level decreases and is regionally dependent. The deep zone articular chondrocytes had a higher OCR than superficial zone cells.\textsuperscript{137} Compared to articular chondrocytes and animal IVD cells, the OCR of porcine TMJ disc cells was found to be about three times higher.\textsuperscript{139} The glucose effect on oxygen consumption rates, consistent with the Crabtree effect has been also been reported in bovine articular chondrocytes.\textsuperscript{140} Differences in tissue region (AF, NP, and CEP), and glucose level and degeneration may contribute to changes in oxygen consumption in the human IVD cells.
Understanding these relationships can provide valuable knowledge for theoretically predicting the oxygen distribution within the human intervertebral disc. Due to the difficulty of measuring nutrient gradients \textit{in vivo}, mathematical models have been used to predict the nutrient environment inside cartilage tissues. However, the nutrient transport properties and cell metabolic rates of human IVDs are largely unknown. New methods must address continuing challenges regarding a lack of knowledge of the IVD cellular metabolic responses and the development of new theories and models that more accurately predict the \textit{in vivo} nutrient environment.

Differences have been established between the behaviors and gene expression profiles of degenerate versus nondegenerate human IVD cells in regards to extracellular matrix (ECM) genes, ECM proteolysis, cell proliferation, apoptosis, growth factors, inflammatory mediators, and other genes.\textsuperscript{141-151} Mitochondrial gene expression patterns have also indicated possible mitochondrial dysfunction in degenerate human AF cells.\textsuperscript{152} A change in mitochondrial function related to IVD degeneration could influence the metabolic disc cell phenotype considering the important role the mitochondria plays in regulating cellular respiration, where nutrients are oxidized to generate energy through a mechanism called oxidative phosphorylation, which occurs in the mitochondria. The mitochondrial membrane potential is an indicator of the activity of the electrochemical gradient across the internal membrane of mitochondria, which provides energy for the adenosine triphosphate (ATP)-synthase complex, ultimately producing ATP. Previously, the correlation between mitochondrial membrane potential and disc degeneration has been
assessed, but due to a limited sample size the change in mitochondrial membrane potential between degenerate and nondegenerate human IVD cells is largely unknown.

The hypothesis of this study is that intervertebral disc nutrient environment parameters including oxygen level and glucose concentration will have a significant impact on oxygen consumption rates and mitochondrial membrane potential due to the unique nutrient metabolic mechanisms of the nondegenerate as well as degenerate disc cells. To address the hypothesis, this study determined the oxygen consumption rates of nondegenerate and degenerate human IVD at various glucose concentrations and examined differences in OCR among the AF, NP, and CEP cells. Confocal microscopy and TMRM intensity was also used to investigate mitochondrial membrane potential. The results show that human degenerate IVD cells are very active metabolically and poorly resistant to nutritional stress and support the idea that a fall in nutrient supply as a consequence of the onset and progression of disc degeneration could drive a metabolic change in IVD cell phenotype; the results suggest that their nutritional demands should be considered in prospective tissue engineering and regeneration approaches.

### Materials and Methods

#### Cell Isolation and Culture

Human IVD tissues (total $n=8$ donors, aged 21-65 years old, Thompson Grade I or II [i.e., nondegenerate]) consisting of pooled L1 through S1 lumbar motion segments, were collected through an Organ Procurement Organization (LifePoint Inc., Charleston, SC) within 24 hours of time of death under institutional approval. In addition, to-be-
discarded human IVD surgical waste samples from patients undergoing surgery for degenerative disc disease (total $n=5$ patients, aged 43-63 years old, Thompson Grade III or IV [i.e., degenerate]) were immediately obtained. Under sterile conditions, the disc tissues were well rinsed with phosphate buffered saline (PBS) (Hyclone) and carefully separated into annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplate (CEP) according to anatomic appearance as shown in Figure 20A, B, and C. Cells were released from these 3 regions by collagenase II (Worthington Biochemical Corp., Lakewood, NJ) and protease (Sigma Chemical, St. Louis, MO) digestion (AF and CEP: 1mg/mL collagenase and 0.6 mg/mL protease; NP: 0.5 mg/mL collagenase and 0.3 mg/mL protease) overnight at 37°C. Digestions were strained through a 70µm filter, washed with PBS, and re-suspended in high glucose (25mM) Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (Gibco Brl), 25µg/mL ascorbic acid and the osmolality was measured within the range of 290–310 mosmol (Vapro Vapor Pressure Osmometer, Elitech Group). Isolated intervertebral disc (IVD) cells were plated in monolayer culture at 21% O$_2$ and 5% CO$_2$ at 37°C in DMEM. The AF, NP, and CEP cells had an average adherence time of 3 days. The media was changed every 3 days, and upon reaching 95% confluence typically within 3 weeks, first passage (P1) cells were detached with 0.25% trypsin (Invitrogen) and re-plated at a 1:2 ratio and monolayer cultured to second passage (P2) for use in experiments.
Figure 20. (A) Nondegenerate human IVD from lumbar spine with sagittal plug of disc tissue, CEP, and vertebral body. The thin hyaline layer of CEP (~0.7mm) was carefully separated from the NP and AF (relatively more transparent than the CEP tissue). (B) Degenerate human IVD from discarded degenerate disc surgery. (C) Degenerate human IVD. The disc tissue from the transition zone between AF and NP regions was discarded. The thin layer of calcified CEP on the surface of the disc was carefully separated from the NP and AF according to anatomic appearance. Light microscopic images of cultured degenerate human IVD cells from (D) AF, (E) NP, and (F) CEP regions. There were no morphological differences in cultured degenerate and nondegenerate human IVD cells.

Oxygen consumption rate measurement

Cells from each tissue region were separated into 3 groups and incubated in DMEM with glucose concentrations of 1, 5, or 25mM for 24 hours (media contained no FBS and was buffered to a pH of 7.4). After cell viability was quantified by trypan blue exclusion
(0.4% in PBS), the P2 cell suspensions of a million cells per mL were placed into a stirred, water jacketed 500 µL metabolic chamber (Instech Laboratories, Plymouth Meeting, PA) maintained at 37°C, as shown in Figure 21A. The medium used in the metabolic chamber had been preheated to 37°C and stirred in air for 15 minutes to establish constant initial dissolved oxygen concentration. The concentration of dissolved oxygen in the culture medium at 37°C and atmospheric pressure was 200 µmol/L (or 21% oxygen level). The chamber was sealed and real time dissolved oxygen concentration in the medium was recorded by a fiber optic oxygen sensor (Ocean Optics, Dunedin, FL, USA) until the oxygen concentration fell to 0.95 µmol/L (0.1% oxygen level). The decrease in glucose concentration, change in pH of the culture medium, as well as cell viability measured at the end of the experiments were found to be minimal.

Figure 21B shows a typical plot of dissolved oxygen concentration over time. The rate of oxygen consumption in the NP, AF, and CEP cells enclosed in the metabolic chamber can be calculated from the recorded decrease in oxygen concentration versus time. Previous IVD studies have shown the relationship between the oxygen consumption rate and oxygen concentration can be expressed using the Michaelis-Menten enzyme kinetics equation given by\textsuperscript{113; 114}:

\[
R = \frac{V_{max} \times C}{K_m + C}
\]  

(1)

where \( R \) is oxygen consumption rate (nmol/10\(^6\) cells/hr), \( V_{max} \) is the maximum oxygen consumption rate (nmol/10\(^6\) cells/hr), \( K_m \) is the Michaelis-Menten constant (µmol/L), and \( C \) is the oxygen concentration in the chamber (µmol/L). Based on the conservation of mass,
the time rate of oxygen concentration change \((dC/dt)\) in the sealed chamber is given by\(^{114}\),

\[
\frac{dC}{dt} = \frac{V_{\text{max}}C}{K_m + C} \cdot \frac{N_{\text{cells}}}{\text{Vol}_{\text{chamber}}}
\]  

(2)

where \(N_{\text{cells}}\) is the number of cells in the chamber \((10^6 \text{ cells})\), \(\text{Vol}_{\text{chamber}}\) is the volume of the metabolism chamber equal to 0.5 mL. Integrating Equation 2, we can determine the oxygen concentration in the chamber over time:

\[
t = \frac{K_m}{0.001V_{\text{max}}N_{\text{cells}}/\text{Vol}_{\text{chamber}}} \ln \frac{C_0}{C} + \frac{C_0 - C}{0.001V_{\text{max}}N_{\text{cells}}/\text{Vol}_{\text{chamber}}}
\]  

(3)

where \(C_0\) is the initial \((t=0)\) oxygen concentration in the chamber. By curve fitting the recorded oxygen concentration data to Equation 3, the Michaelis-Menten kinetic coefficients of \(V_{\text{max}}\) and \(K_m\) were used to show the functional relationship between the oxygen consumption rate \(R\) and the oxygen concentration \(C\). The \(V_{\text{max}}\) is the maximum oxygen consumption rate achieved by the system at a high oxygen level, and the \(K_m\) is the oxygen concentration at which the oxygen concentration rate decreases to 50% of the \(V_{\text{max}}\).
Figure 21. (A) Schematic of the experimental setup for oxygen consumption rate experiments. AF, NP, and CEP IVD cells were sealed in a water-jacketed metabolism chamber. (B) Characteristic curve of dissolved oxygen concentration (µmol/L) in the
metabolism chamber over time (h). The experimental data were curve fit to Equation 3. 

\[ V = \frac{V_{max} \cdot [S]}{K_m + [S]} \]

The oxygen consumption rate (nmol/10^6 cells/hr) was plotted against dissolved oxygen concentration (µmol/L) based on the Michaelis-Menten equation with calculated coefficients of \( V_{max} \) and \( K_m \).

**Determination of mitochondrial membrane potential (\( \Psi_m \))**

Mitochondrial membrane potential (\( \Psi_m \)) in live cells (rat cardiac fibroblasts, nondegenerate human AF, and degenerate human AF cells) was measured under basal conditions \((t=0)\) using tetramethylrhodamine methyl ester (50nM; TMRM) by confocal microscopy (Zeiss LSM 880 NLO; 561nm He-Ne laser). Cells were preconditioned with 1mM glucose medium for 2 hours and loaded for 30 minutes at 37°C in the dark. Images were analyzed using Fiji (ImageJ) analysis software (Wayne Rasband; NIH). Results were reported as relative TMRM Intensity per cell. Surface area measurements were obtained from image analysis of confocal microscopy as well as transmission light microscopy.

**Statistical analysis**

The oxygen consumption rate outcomes were presented as the mean and standard deviation (SD) from at least 5 separate oxygen consumption rate experimental runs from at least 3 separate cell isolations. The mitochondrial membrane potential outcomes were presented as the mean and SD from 23 different cells from each group. Using SPSS statistics software (SPSS 16.0, IBM, NY), two-way analysis of variance (ANOVA) and Tukey’s post hoc tests were performed to determine if significant differences existed between glucose concentration, disc tissue regions, and level of degeneration. Statistical differences were reported at \( P \)-values < 0.05.
Results

**Vital Cell Imaging.** There was no morphological difference between cultured degenerate and nondegenerate human IVD cells under a transmission light as well as confocal microscope. Degenerate human IVD cells from three tissue regions can be morphologically distinguished under a transmission light microscope, as shown in Figure 20E, F, and G. For both degenerate and nondegenerate disc tissue, the cells from the annulus fibrosus (AF) region are elongated fibrochondrocyte-like cells, while the cells from cartilage endplate (CEP) region are more rounded chondrocyte-like cells. The cells from the nucleus pulposus (NP) region are uniformly circular and grow slower than cells from the CEP and AF. As depicted in Figure 24A and B, confocal microscopy confirms both degenerate and nondegenerate IVD AF cells have no difference in surface area measurements.

**Oxygen consumption rates.** For degenerate and nondegenerate disc cells, oxygen consumption rates in human AF, NP, and CEP decreased with the decrease in oxygen concentration in accordance with the Michaelis-Menten model of enzyme kinetics. Figure 22 and Table 5 show the average $V_{max}$ and $K_m$ for nondegenerate and degenerate AF, NP, and CEP disc cells at 1mM, 5mM, and 25mM glucose mediums. For nondegenerate IVD cells, there was no significant difference in average $V_{max}$ (~10-23 nmol/10⁶ cells/hr) due to glucose medium concentration (p=0.89). Significant effects due to tissue region were found for $V_{max}$ in the nondegenerate NP and CEP tissue regions, as shown in Figure 22A with the nondegenerate NP cells exhibiting a larger average $V_{max}$ than that of the nondegenerate CEP cells, 18.13±11.48 nmol/10⁶ cells/hr and 14.12±6.329 nmol/10⁶
cells/hr, respectively (p=0.038). There was no significant difference in average $V_{max}$ between degenerate tissue regions (p=0.171). Significant effects due to glucose level were found for $V_{max}$ in all three degenerate cells as shown in Figure 22B. The degenerate IVD cells cultured in the 1mM glucose medium (93.77±16.83 nmol/10^6 cells/hr) exhibited a larger $V_{max}$ than those cultured in both 5mM (53.99±10.77 nmol/10^6 cells/hr) and 25mM (48.90±12.025 nmol/10^6 cells/hr) glucose mediums (p=0.0001). For the degenerate and nondegenerate IVD cells, there were no significant differences in $K_m$ between tissue regions (p=0.99; 0.90), glucose medium concentrations (p=0.31; 0.42), as well as grade of degeneration (p=0.49), as presented in Figure 22C and 22D. For the nondegenerate disc cells, the average OCR was significantly lower, approximately 3 times lower, than that from the degenerate disc cells (p=0.0001), and comparable to porcine^{113; 114} and bovine^{113} IVD cells data from the literature at 5mM glucose concentration, as shown in Figure 23. The two-way ANOVA interaction between glucose medium, tissue region, or grade of degeneration was significant (p=0.0001).
Figure 22. Comparison of (A) $V_{\text{max}}$ and (C) $K_m$ among human nondegenerate AF, NP, and CEP cells cultured in DMEM with varying glucose concentrations (n= at least 5 separate oxygen consumption rate experimental runs for each group from three separate cell isolations). Significant effects due to tissue region were found for $V_{\text{max}}$ (p=0.024), specifically comparing NP to CEP (p=0.038), while no significant differences were found for $K_m$. * = p<0.05. Comparison of (B) $V_{\text{max}}$ and (D) $K_m$ among human degenerate AF, NP, and CEP cells cultured in DMEM with varying glucose concentrations (n= at least 5 separate oxygen consumption rate experimental runs for each group from three separate cell isolations). Significant effects due to the glucose level were found for $V_{\text{max}}$ in all three regions, while no significant differences were found for $K_m$. * = p<0.05.
Table 5. Comparison of oxygen consumption rates between intervertebral disc (IVD) cells and articular chondrocytes (AC). * = Crabtree effect observed.

<table>
<thead>
<tr>
<th>Type of Joint and Species</th>
<th>Subpopulation</th>
<th>Glucose (mM)</th>
<th>$V_{max}$ (nmol/10$^6$ cells/h)</th>
<th>$K_m$ (µmol/L)</th>
<th>References</th>
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<tbody>
<tr>
<td>Bovine AC</td>
<td>* Superficial/Deep</td>
<td>0.5-5</td>
<td>3.2/6.6</td>
<td>68/63</td>
<td>154</td>
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<td>22.3</td>
<td>n/a</td>
<td>113</td>
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<tr>
<td>Bovine IVD</td>
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<td>5-10</td>
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<td>109</td>
</tr>
<tr>
<td>Bovine IVD</td>
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<td>14.7</td>
<td>n/a</td>
<td>113</td>
</tr>
<tr>
<td>Porcine IVD</td>
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<td>17.0</td>
<td>n/a</td>
<td>113</td>
</tr>
<tr>
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<td>Notochord cells</td>
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<td>90.9</td>
<td>n/a</td>
<td>113</td>
</tr>
<tr>
<td>Porcine IVD</td>
<td>* AF</td>
<td>1-25</td>
<td>6.0</td>
<td>35.7</td>
<td>114</td>
</tr>
<tr>
<td>Porcine IVD</td>
<td>NP</td>
<td>1-25</td>
<td>11.5</td>
<td>6.8</td>
<td>114</td>
</tr>
<tr>
<td>Nondegenerate Human IVD</td>
<td>AF, NP, CEP</td>
<td>1-25</td>
<td>11-23</td>
<td>9-16</td>
<td>Present study</td>
</tr>
<tr>
<td>Degenerate Human IVD</td>
<td>* AF, NP, CEP</td>
<td>1-25</td>
<td>40-103</td>
<td>8-16</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Figure 23. Comparison of the oxygen consumption rate (nmol/106 cells/hr) against dissolved oxygen concentration (µmol/L) based on the Michaelis-Menten equation with calculated coefficients of $V_{max}$ and $K_m$. $V_{max}$ is the maximum oxygen consumption rate.
(nmol/106 cells/hr) achieved by the system and $K_m$ is the dissolved oxygen concentration ($\mu$mol/L) at $\frac{1}{2}V_{max}$. The oxygen consumption rate of degenerate human IVD cells from the present study are about 3 times higher than nondegenerate human, porcine$^{113;114}$, and bovine$^{113}$ IVD cells.

**Mitochondrial Membrane Potential.** Using confocal microscopy and TMRM staining, the mitochondrial membrane potential of nondegenerate human AF cells, degenerate human AF cells, and rat cardiac fibroblasts (control) was determined. As shown in Figure 24, the nondegenerate human AF cells had a significantly lower mean relative TMRM intensity per cell (=465.8±163.7; p=0.03) compared to the degenerate human AF cells (=614.1±186.8) as well as the rat cardiac fibroblasts (=744.1±232.0). There was no significant difference between degenerate human AF cells and rat cardiac fibroblasts (p=0.068). The interaction between cell type and TMRM intensity is significantly different within these three groups (p=0.0001).
Figure 24. Mitochondrial studies at basal conditions (time 0) included confocal fluorescent imaging of (A) nondegenerate human AF cells, (B) degenerate human AF cells, and (C) rat cardiac fibroblasts preconditioned with 1mM glucose concentration (n = 23 cells for each cell type) and stained with 50mM tetramethylrhodamine methyl ester (TMRM). Relative TMRM Intensity per cell significantly increased with increased degeneration, * = p<0.05.

Discussion

In order to gain an understanding of the role of a diminished nutrient supply associated with degeneration on the IVD cell metabolic phenotype, human degenerate and
nondegenerate IVD AF, NP, and CEP cells were compared. Rates of oxygen consumption were measured since these are essential in regulating local oxygen concentrations around the cells. Mitochondrial membrane potential was determined, since this is critical for maintaining the physiological function of the electron transport chain to generate ATP in oxidative phosphorylation. It was observed that that degenerate IVD cells are more active metabolically; their rates of oxygen consumption and mitochondrial membrane potential were considerably higher than those in nondegenerate IVD cells under conditions tested. Furthermore, degenerate IVD cells were more sensitive to glucose deprivation and demonstrated the Crabtree effect unlike the nondegenerate cells.

During the dissection of the human lumbar spine segments obtained from a local tissue bank, the samples were very carefully separated and pooled into annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplate (CEP) according to anatomic appearance. The transition zone between the AF and NP region was not included to avoid mixed cell culture populations. All three tissue regions were easily distinguishable due to differences in tissue structure and composition in both the nondegenerate and degenerate tissues samples. The thick lamellae fibrous rings of the AF were a stark contrast to the gelatinous core of the NP. In the degenerate samples, the calcified cartilage endplates were very distinct from the other tissue regions. In the nondegenerate samples, the cartilage endplate was observed to be a thin hyaline cartilage layer approximately 0.6 to 1.0 mm thick.6 For the surgical IVD samples, repeated saline washes ensured the minimal amount of blood cells introduced in culture. The first media change was done less than 72 hours after plating the cells so any residual blood cells were washed away with the medium.
changes during P1 primary cell culture, as previously shown in the literature. Degenerate and nondegenerate cells were found to be comparable in morphological structure based on light microscopy imaging as well as area measurements from confocal microscopy.

Within the intervertebral disc, diffusion mechanisms regulate the supply of nutrients and removal of metabolic wastes. The delicate balance between the rate of nutrient transport through the matrix and rate of consumption by disc cells determines the local nutrient concentration gradient within the IVD. An impaired nutrient supply to the disc as a consequence of decreased nutrient transport due to degenerative changes results in decreasing glucose and oxygen concentrations, as well as increased lactate concentrations in the disc center. Our results indicate that degenerate IVD cells may be particularly sensitive to such stresses; for example, their response to glucose deprivation is to push an oxidative phenotype if oxygen is available, which could be induced by a fall in glucose concentration in the disc center. Degenerate disc cells exhibited a Crabtree effect (i.e., an increase in cellular respiration under low glucose concentrations). Although, a Crabtree effect provides a pathway for maintaining ATP production under low glucose conditions, its effects in the disc could be harmful rather than beneficial, since in the center of the avascular disc both oxygen and glucose concentrations are low. Therefore, a Crabtree effect combined will increase oxygen and glucose demand and could push glucose levels below those necessary for survival, while in addition, increased lactate production could lead to a fall in pH, also contributing to an already present degenerative tissue environment.

Due to the large avascular nature of the IVD, oxygen as well as other nutrients required for maintaining disc tissue health are supplied by blood vessels at the disc
It is suggested that both articular chondrocytes and IVD cells obtain their energy primarily through the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis, even in the presence of a high oxygen level. Present findings support a glycolytic metabolic phenotype with nondegenerate human IVD cells, while the degenerate cells are significantly more metabolically active and may push oxidative phosphorylation. In addition, a higher mitochondrial membrane potential in the degenerate human IVD AF cells suggests an activation of cellular respiration. This difference in metabolic phenotype may be indicative of a changing IVD cellular metabolic phenotype survival response associated with the onset and progression of disc degeneration.

The oxygen consumption rate of the human IVD cells was measured in a sealed metabolism chamber. This approach has been used to investigate the effect of oxygen level on the oxygen consumption rate of isolated articular chondrocytes, bovine and porcine IVD cells, and porcine TMJ explants. Those studies have shown that the relationship between the oxygen consumption rate and oxygen level can be modeled by the Michaelis-Menten equation with the two enzyme kinetic coefficients of $V_{max}$ and $K_m$. Our results showed that the kinetics of the oxygen consumption rate of nondegenerate and degenerate human IVD cells can also be well expressed by this equation. The oxygen consumption rates of nondegenerate human IVD cells from this present study, 18-20 month bovine IVD NP cells, 10-15 month porcine AF, and 4-5 month old NP cells were comparable, indicating the animal IVD cells are suitable models of nondegenerate human IVD cell type in terms of oxygen consumption. The oxygen consumption rates from 10-15 month old porcine NP cells (notochordal cells) were significantly higher which
supports the age associated changing IVD cell phenotype. It is known that before maturity in humans as well as some other animal models including the bovine and canine, the notochordal cells of the NP transition to chondrocyte-like cells that constitute a different phenotype.\textsuperscript{157-159} Glucose had no significant effect on oxygen consumption on both human nondegenerate IVD cells as well as the bovine NP cells, while heightened oxygen consumption rates when glucose is limited, described as the Crabtree effect, was observed for hyaline articular chondrocytes,\textsuperscript{140; 154; 160} porcine AF cells,\textsuperscript{114} and degenerate human IVD cells. While the present study provides valuable information regarding the oxygen consumption rates of nondegenerate and degenerate human IVD cells, it will be valuable to study the coupling of oxygen and glucose consumption, and lactate production to fully understand the energy metabolism.

The importance of defects in the mitochondria bioenergetics, mass, and morphology in the human degenerated annulus fibrosus has been highlighted in the literature. Specifically, a decline in mitochondrial mass and therefore bioenergetics defects, as well as increased rates of mitochondrial respiration were observed in degenerate human AF cells.\textsuperscript{161} An increase in mitochondrial respiratory rates seen in degenerate human AF cells supports our finding of increased rates of oxygen consumption in human degenerate IVD cells. These observations may reflect a higher metabolic requirement of these cells. While we observed a higher mitochondrial membrane potential associated with IVD degeneration, it was previously shown to decrease with degeneration.\textsuperscript{153} Further studies are needed to confirm an IVD degeneration associated change in mitochondrial membrane potential.
Possible limitations to this study include the loss of phenotype that *in vitro* IVD cells may experience when extracted from their extracellular matrix as well as during P1 and P2 monolayer culture. This culture method was used due to the low human IVD cell yield obtained after tissue digestion. Previous literature has examined the effect of high oxygen monolayer expanded *in vitro* culture of articular chondrocytes and found a cellular induction to an oxidative phenotype and metabolism.\textsuperscript{162} Therefore, it is necessary to investigate the effect of glucose concentration and oxygen level on metabolite rates (i.e., oxygen/glucose consumption, lactate production, ATP levels) in human IVD cells cultured in a 3D hypoxic environment to offer a more accurate *in vivo* representation of metabolite rates as well as preserving the IVD cell phenotype under cell culture passaging.\textsuperscript{114, 163-165}

In addition, it is of interest to investigate the effect of different inflammatory factor treatments the metabolic response of nondegenerate human IVD cells. Such inflammatory factors such as tumor necrosis factor alpha (TNF\textalpha{}), interleukin-6 (IL-6), and interleukin-1 (IL-1), should be considered because they are known mediators of inflammation and pain associated with IVD degeneration.\textsuperscript{146, 166-169} Osmolarity ranging from 316 to 600 mOsm was shown to exhibit minimal effects on the oxygen consumption rate of bovine articular chondrocytes.\textsuperscript{140} In our study, the osmolarity range of the medium with varying glucose concentrations was within 290 to 310 mOsm. This does create a slight variation and thus, in future studies, it would be beneficial to examine a more physiological hypertonic effect by utilizing osmotic balancing through the addition of mannitol.

In summary, the oxygen consumption rates at various glucose concentrations and the mitochondrial membrane potential of nondegenerate and degenerate human IVD cells
was investigated. The impact of oxygen level on the oxygen consumption rate was examined and a quantitative relationship was determined. The oxygen consumption rate was dependent on oxygen level. At a high oxygen level, the oxygen consumption rate remained constant while significantly decreasing as oxygen level dropped below 5%. Below 5% oxygen level, the oxygen consumption rate decreased in a highly concentration dependent manner. Based on the Michaelis-Menten equation, the relationship between oxygen consumption rate and oxygen level is solely depicted by the parameter $K_m$. Considering the avascular nature of the IVD, as well as the significantly higher oxygen consumption as well as mitochondrial membrane potential of the degenerate IVD cells compared the nondegenerate IVD cells, not only does a delicate oxygen gradient potentially exist in the degenerate IVD, but these cells are very active metabolically and poorly resistant to nutritional stress. Such an environment may be vulnerable to any continuing nutrient changes or pathological event which may further disrupt the nutrient supply and encourage the progression of IVD degeneration while driving a metabolic change in IVD cell phenotype.

Recently, there has been increased interest in tissue engineering and regenerative approaches for treatment of the degenerated IVD. However, in the light of current findings, the heightened nutritional requirements and increased sensitivity to nutrient deprivation of degenerate IVD cells may affect approaches. Such an environment may be vulnerable to any continuing nutrient changes or pathological event which may further disrupt the nutrient supply and encourage the progression of IVD degeneration. Those investigating
prospective tissue engineering methods in disc repair should consider these possible limitations.
CHAPTER 4: THE EFFECTS OF OXYGEN LEVEL AND GLUCOSE CONCENTRATION ON THE GLUCOSE CONSUMPTION AND LACTATE PRODUCTION RATES OF HUMAN IVD NP AND AF CELLS

The avascular nature of the intervertebral disc (IVD) creates a delicate balance between rate of nutrient transport through the matrix and rate of disc cell consumption necessary to sustain tissue health. Poor nutritional supply is believed to be a key initiating constituent of disc degeneration. This study was initiated to test the hypothesis that IVD nutrient environment parameters including oxygen level and glucose concentration will have a significant impact on glucose consumption and lactate production rates in nondegenerate and degenerate human IVD cells. Seventeen human lumbar spines, consisting of pooled L1 through S1 motion segments per spine, were collected through an Organ Procurement Organization (LifePoint Inc., Charleston, SC) within 24 hours of time of death (aged 22-65 years old, Thompson Grade I through IV) under institutional approval. IVD annulus fibrosis (AF) and nucleus pulposus (NP) cells were isolated, cultured, and suspended in well plates at 1 million cells/mL with 1, 5, or 25mM glucose DMEM under 1%, 5%, or 21% oxygen level, respectively. The glucose and lactate concentrations were measured at 4 hours. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration difference, cell densities, and time. The GCR was more sensitive to change in glucose concentration while the LPR, indicative of rate of glycolysis, was more dependent on oxygen level. The human IVD NP cells appear to display a negative Pasteur effect while the LPR of AF cells was not dependent on change in oxygen level. These rates were tissue dependent in that the NP had higher consumption and production rates compared to that of AF cells. Overall, the ratio of LPR to GCR was
higher in nondegenerate NP and AF cells compared to that of degenerate. The data reported in this study contributes to our understanding of the unique nutrient metabolism mechanisms in the human IVD which may shed light on the nutrient related mechanisms associated with the onset and progression of IVD degeneration.

Introduction

The intervertebral disc (IVD) is a fibrocartilage structure separating the vertebrae that allows a wide range of mechanical motion in the spine. Differences in biochemical composition and structure distinguish three regions of the disc including the annulus fibrosus (AF), nucleus pulposus (NP), and cartilage endplates (CEP). Due to its large avascular nature, critical nutrients required for maintaining disc tissue health are supplied by blood vessels at the margins of the disc. Within the IVD, diffusion mechanisms regulate the supply of nutrients and removal of metabolic wastes. The balance between the rate of nutrient transport through the matrix and rate of consumption by disc cells determines the local nutrient concentration gradient within the IVD. Poor nutritional supply to the disc is believed to be a key initiating constituent of degeneration where changes in disc morphology, biochemistry, function, and material properties are observed. Low back pain is a major socio-economic health concern in western industrialized societies. Although the exact cause of low back pain is unclear, degeneration of the IVD has been implicated as a possible primary etiological factor.

The cellular metabolism of the nondegenerate IVD is believed to occur mainly through glycolysis, as the disc cells require glucose for survival and produce lactic acid
at high rates under hypoxia. Oxygen is also necessary for cellular activity, although not necessary for survival, as its consumption pathway is unclear. Previous studies have shown a dependence of oxygen consumption rates for human, bovine, and porcine IVD cells on oxygen level, pH level, and glucose concentration.\textsuperscript{109; 113; 114} The inhibition of cellular respiration due to the high rate of glycolysis under high glucose concentrations, described as the Crabtree effect, has been demonstrated in human degenerate IVD cells (see Chapter 5) and porcine AF,\textsuperscript{114} but not porcine NP,\textsuperscript{114} bovine NP,\textsuperscript{109; 113} as well as human nondegenerate IVD cells (see Chapter 5). Glucose consumption and lactate production rates also support a glycolytic phenotype and have also shown to be depend on glucose concentration and pH level in bovine and porcine IVD cells.\textsuperscript{109; 113} Temporomandibular joint (TMJ) disc explants exhibited at positive Pasteur effect (i.e., an increase in glycolysis rate and hence in the rate of lactate production under low oxygen level). Previously in the literature, articular chondrocytes have been shown to demonstrate a negative Pasteur effect, while it is unclear the effect displayed by IVD cells, due to conflicting reports.\textsuperscript{109; 111; 113; 122; 156}

To our knowledge, the rate of glucose consumption and lactate production of nondegenerate and degenerate human IVD NP and AF cells has not been determined. Glucose consumption and lactate production rates in cell suspensions of human degenerate and nondegenerate IVD NP and AF cells were measured. The results show that human degenerate IVD cells are very active metabolically and poorly resistant to nutritional stress and support the idea that a fall in nutrient supply as a consequence of the onset and progression of disc degeneration could drive a metabolic change in IVD cell phenotype;
the results suggest that their nutritional demands should be considered in prospective tissue engineering and regeneration approaches.

Materials and Methods

Cell Isolation and Culture

Seventeen human lumbar spines, as described in Table 6, consisting of pooled L1 through S1 motion segments per spine, were collected through an Organ Procurement Organization (LifePoint Inc., Charleston, SC) within 24 hours of time of death (aged 22-65 years old, Thompson Grade I through IV [Grade I and II considered nondegenerate; Grade III and IV considered degenerate] under institutional approval. Under sterile conditions, the disc tissues were well rinsed with phosphate buffered saline (PBS) (Hyclone) and carefully separated into annulus fibrosus (AF) and nucleus pulposus (NP) according to anatomic appearance. Cells were released from these 2 regions by collagenase II (Worthington Biochemical Corp., Lakewood, NJ) and protease (Sigma Chemical, St. Louis, MO) digestion (AF: 1mg/mL collagenase and 0.6 mg/mL protease; NP: 0.5 mg/mL collagenase and 0.3 mg/mL protease) overnight at 37°C. Digestions were strained through a 70µm filter, washed with PBS, and re-suspended in high glucose (25mM) Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin (Gibco Brl), 25µg/mL ascorbic acid and the osmolality was measured within the range of 290–310 mosmol (Vapro Vapor Pressure Osmometer, Elitech Group).
Isolated intervertebral disc (IVD) cells were plated in monolayer culture at 21% O2 and 5% CO2 at 37°C in DMEM. The AF and NP cells had an average adherence time of 5-7 days. The media was changed every 3 days, and upon reaching 95% confluence typically within 3 weeks, first passage (P1) cells were detached with 0.25% trypsin (Invitrogen) for use in experiments.

**Table 6.** Demographic Data on Donor Lumbar Spine IVD Tissues Used in GCR and LPR Studies.

<table>
<thead>
<tr>
<th>Spine Specimen</th>
<th>Thompson Grade</th>
<th>Age (yr)/Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>50/F</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>22/M</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>57/M</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>50/F</td>
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<td>5</td>
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<td>7</td>
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<td>9</td>
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<td>22/F</td>
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<tr>
<td>10</td>
<td>3</td>
<td>46/M</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>64/F</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>54/F</td>
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<td>13</td>
<td>3</td>
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<tr>
<td>15</td>
<td>4</td>
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<tr>
<td>16</td>
<td>4</td>
<td>64/F</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>33/M</td>
</tr>
</tbody>
</table>

**Glucose and Lactate Concentrations Measurement**

Human AF and NP cells were preconditioned for 12 hours and then suspended in well plates at 1 million cell/mL with 1, 5, or 25mM glucose DMEM under 1%, 5%, or 21% oxygen level. Glucose and lactate concentrations were measured with an YSI 2700 Select Biochemistry Analyzer (YSI Inc., OH). The instrument is capable of measuring glucose and lactate values within the ranges of 0-50mM and 0-29mM, respectively. These
measurements were taken at basal conditions and 4 hours. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration difference, cell densities, and time. At the end of the experiments, a minimal change in pH was measured and the cell viability was examined via trypan blue exclusion to confirm greater than 90% viability.

Statistical analysis

The measurements of each outcome (i.e., glucose and lactate concentration) were performed in triplicate and repeated in three independent experiments (n=9). The results were presented as the mean with standard deviation. Two-way analysis of variance (ANOVA) and Tukey’s post hoc tests were performed to determine the singular and combined effect of oxygen level (2.5% or 5%) and glucose concentration (0.5, 1, 5, or 10mM) on IVD glucose consumption and lactate production rates. SPSS 16.0 software (SPSS Inc., Chicago, IL) was used for examining all statistical analyses and significant differences were reported at P-values < 0.05.

Results

Glucose Consumption Rate (GCR)

The results, as shown in Figure 25, indicate a glucose substrate dependence in nondegenerate NP cells as increasing glucose concentration increased GCR at low levels of oxygen. The effect on oxygen level and glucose concentration on nondegenerate NP cells is significant (p=0.0001). At 1 mM glucose, there was no change in GCR at the different oxygen levels. At 5 mM glucose, the GCR is significantly lower at 1% oxygen.
level compared to 5% and 21% oxygen levels (p=0.0001 and p=0.03, respectively). At 25mM glucose, the GCR trend due to oxygen level is significant and unclear. At 1% and 5% oxygen levels, the GCR significantly increased with increasing glucose concentrations (p=0.0001).

![Nondegenerate NP Cells](image)

**Figure 25.** The glucose consumption rate (GCR) [nmol/million cells/hr] of human nondegenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

In human degenerate IVD NP cells, the effect of glucose concentration and oxygen level is significant at 1mM glucose and 1% oxygen level. As shown in **Figure 26**, the GCR of degenerate NP cells significantly increased with an increase in oxygen level (p=0.001) at 1mM glucose concentration. The GCR significantly decreased at 1% oxygen level and
1mM glucose concentration compared to the higher glucose concentrations (p=0.007 and p=0.0001, respectively).

![Figure 26](image)

**Figure 26.** The glucose consumption rate (GCR) [nmol/million cells/hr] of human degenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

The results, as shown in **Figure 27**, indicate a constant GCR in nondegenerate AF cells at 1mM glucose concentration with a change in oxygen level (p=0.624). At 5mM glucose, a significant decrease in GCR was shown with an increase in oxygen level. From 1% to 21% oxygen level, the GCR significantly decreased from 84.68 ± 57.06 nmol/million cells/hr to 39.84 ± 38.73 nmol/million cells/hr (p=0.01). **Figure 27** shows a significant decrease in the GCR of nondegenerate AF cells at 1% oxygen level with a decrease in glucose concentration (p=0.002).
Figure 27. The glucose consumption rate (GCR) [nmol/million cells/hr] of human nondegenerate AF cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM or 5mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

In degenerate AF cells, oxygen level was found to have no effect on GCR at low levels of glucose concentration (p=0.07), as shown in Figure 28. At 1% oxygen level, a decrease in glucose concentration from 5mM to 1mM resulted in a significant decrease in GCR for the degenerate AF cells (p=0.0001), as shown in Figure 28.
Figure 28. The glucose consumption rate (GCR) [nmol/million cells/hr] of human degenerate AF cells with varying oxygen levels (1% or 21%) and glucose concentration (1mM or 5mM). n=9 for all combinations. # = glucose effect = p < 0.05.

As shown in Table 7, the average GCR of nondegenerate NP cells (140.36 ± 176.2 nmol/million cells/hr) was significantly higher than that of nondegenerate AF cells (87.85 ± 101.5 nmol/million cells/hr), p=0.01, respectively. There was no significant difference in the average GCR of degenerate NP and AF cells (144.3 ± 125.6 nmol/million cells/hr and 110.1 ± 164.3 nmol/million cells/hr, respectively, p=0.254). There was no change in average GCR between degenerate and nondegenerate NP cells (p=0.614) as well as that between AF cells (p=0.67).

Lactate Production Rate (LPR)

The results, as shown in Figure 29, indicate an oxygen dependence in nondegenerate NP cells at low oxygen levels on the LPR. At 1mM glucose, the decrease
in oxygen level significantly decreased LPR (p=0.0001). At 5mM glucose, LPR was significantly higher at 5% oxygen level compared to 1% and 21% (p=0.0001). At 25mM glucose, the LPR significantly increased with a decrease in oxygen level (p=0.0001). As shown in Figure 29, a glucose concentration effect was observed to be significant at low oxygen levels. At 1% oxygen level, the LPR significantly increased at 25mM glucose compared to 1mM and 5mM glucose (p=0.0001). In contrast at 5% oxygen level, the LPR was significantly decreased at 25mM glucose compared to 5mM glucose (p=0.004).

![Nondegenerate NP Cells](image)

**Figure 29.** The lactate production rate (LPR) [nmol/million cells/hr] of human nondegenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

In degenerate human NP cells, the LPR significantly increased from 1% oxygen to 21% oxygen at 1mM glucose (p=0.005), as shown in Figure 30. In contrast at 5mM
glucose concentration, the LPR was significantly higher at 1% oxygen level compared to the higher oxygen levels (p=0.001). At 25mM glucose, no oxygen effect on LPR was observed (p=0.053). In Figure 30, at 1% oxygen level, the LPR was significantly lower at 1mM glucose compared to 5mM and 25mM (p=0.01). At 5% oxygen level, the LPR was unchanging with a change in glucose concentration (p=0.062). At 21% oxygen level, the LPR at 1mM glucose was significantly higher compared to that at 5mM (p=0.017).

![Figure 30. The lactate production rate (LPR) [nmol/million cells/hr] of human degenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.](image)

The LPR of nondegenerate AF cells was shown to be constant at 1mM glucose and all oxygen levels in Figure 31 (p=0.653). At significant increase in LPR is observed at 5mM glucose and 1% oxygen level compared to the other oxygen level (p=0.0001). The
LPR trend at 25mM glucose is significant and unclear as oxygen level changes (p=0.0001). As shown in Figure 31, the LPR significantly increases with an increase in glucose concentration at 1% oxygen level (p=0.0001). In contrast, at 5% oxygen level, the LPR significantly decreased at 25mM glucose compared to 1mM and 5mM glucose concentration (p=0.0001). A slightly significant increase in LPR is observed at 21% oxygen level due to increasing glucose concentration (p=0.047).

Figure 31. The lactate production rate (LPR) [nmol/million cells/hr] of human nondegenerate AF cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

The LPR results, as shown in Figure 32, indicate a glucose substrate dependence in degenerate AF cells as increasing glucose concentration increased LPR at 1% and 21% oxygen levels.
Figure 32. The lactate production rate (LPR) [nmol/million cells/hr] of human degenerate AF cells with varying oxygen levels (1% or 21%) and glucose concentration (1mM or 5mM). n=9 for all combinations. # = glucose effect = p < 0.05.

As shown in Table 7, the average LPR of nondegenerate NP cells (131.60 ± 88.29 nmol/million cells/hr) was significantly higher than that of nondegenerate AF cells (79.41 ± 71.85 nmol/million cells/hr), p=0.0001, respectively. The average LPR of degenerate NP (222.78 ± 128.7 nmol/million cells/hr) was significantly higher than that of degenerate AF cells (169.63 ± 130.27 nmol/million cells/hr), p=0.026. The average LPR of degenerate NP and AF cells was significantly higher than that of nondegenerate NP and AF cells (p=0.0001).

Ratio of LPR to GCR

As shown in Figure 33, the ratio of LPR to GCR of nondegenerate NP cells is constant at 1mM glucose and all oxygen levels at an average of 0.71 (p=0.167). At 5mM
glucose, the ratio significantly increases to $1.10 \pm 0.63$ at 21% oxygen level compared to $0.73 \pm 0.36$ at 1% oxygen level ($p=0.008$). At 25mM glucose, the ratio is significantly higher at 5% oxygen compared to the other oxygen levels. The ratio at 5% oxygen and 25mM was significantly higher compared to all other glucose levels as well.

![Graph](image)

**Figure 33.** The ratio of lactate production rate (LPR) [nmol/million cells/hr] to glucose consumption rate (GCR) [nmol/million cells/hr] of human nondegenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = $p < 0.05$. # = glucose effect = $p < 0.05$.

As shown in **Figure 34**, the ratio of LPR to GCR of degenerate NP cells is constant at 5mM and 25mM glucose for all oxygen levels at an average of $0.55 \pm 0.29$ ($p=0.217$ and $p=0.187$, respectively). At 1mM glucose, the ratio is significantly higher at 21% oxygen compared to 1% and 5% oxygen levels ($p=0.0001$ and $p=0.002$, respectively). The ratio of
LPR to GCR was shown to significantly increase with an increase in glucose concentration at 1% oxygen level (p=0.0001).

**Figure 34.** The ratio of lactate production rate (LPR) [nmol/million cells/hr] to glucose consumption rate (GCR) [nmol/million cells/hr] of human degenerate NP cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

As shown in **Figure 35**, the ratio of LPR to GCR of nondegenerate AF cells is constant at 1mM glucose for all oxygen levels at an average of 0.71 ± 0.30 (p=0.874). At 5mM glucose, the ratio is significantly higher at 5% oxygen level (p=0.008) compared to 21% oxygen level. At 25mM glucose, the ratio is significantly higher at 5% oxygen level (p=0.003) compared to 1% oxygen level. At 1% oxygen level, there was a significantly higher ratio at 25mM compared to 5mM glucose concentration (p=0.006). For both 5% and 21% oxygen level, the ratio of LPR to GCR was highest at 25mM glucose.
Figure 35. The ratio of lactate production rate (LPR) [nmol/million cells/hr] to glucose consumption rate (GCR) [nmol/million cells/hr] of human nondegenerate AF cells with varying oxygen levels (1%, 5%, or 21%) and glucose concentration (1mM, 5mM, or 25mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

As shown in Figure 36, the ratio of LPR to GCR of degenerate AF cells is shown to increase to $0.87 \pm 0.35$ at 1mM glucose with an increase in oxygen level ($p=0.004$). In contrast, at 5mM glucose the ratio decreases to $0.40 \pm 0.06$ with an increase in oxygen level ($p=0.002$). At 1% oxygen level, the ratio increases from $0.22 \pm 0.06$ to $0.55 \pm 0.01$ with an increase in glucose concentration ($p=0.0001$). In contrast, at 21% oxygen level, the ratio decreases from $0.87 \pm 0.35$ to $0.40 \pm 0.06$ with an increase in glucose concentration ($p=0.027$).
Figure 36. The ratio of lactate production rate (LPR) [nmol/million cells/hr] to glucose consumption rate (GCR) [nmol/million cells/hr] of human degenerate AF cells with varying oxygen levels (1% or 21%) and glucose concentration (1mM or 5mM). n=9 for all combinations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

As shown in Table 7, there was no significant difference between the average ratio of LPR to GCR of nondegenerate NP cells (1.54 ± 0.92) to nondegenerate AF cells (1.03 ± 0.52), p=0.825, respectively. There was no significant difference between the average ratio of LPR to GCR of degenerate NP cells (2.22 ± 0.46) to degenerate AF cells (2.27 ± 0.64), p=0.838, respectively. The average ratio of LPR to GCR of degenerate NP was significantly higher than that of nondegenerate NP cells (p=0.012). The average ratio of LPR to GCR of degenerate AF was significantly higher than that of nondegenerate AF cells (p=0.028).
Table 7. Comparison of glucose consumption rate (GCR) and lactate production rate (LPR) in the Human IVD and other cartilaginous tissues at pH 7.4.

<table>
<thead>
<tr>
<th>Type of Joint and Species</th>
<th>Glucose (mM)</th>
<th>Oxygen Level (%)</th>
<th>GCR (nmol/million cells/hr)</th>
<th>LPR (nmol/million cells/hr)</th>
<th>LPR:GCR Ratio</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine NP</td>
<td>5</td>
<td>21</td>
<td>205 ± 16</td>
<td>355 ± 21</td>
<td>1.7</td>
<td>113</td>
</tr>
<tr>
<td>Bovine NP</td>
<td>5</td>
<td>21</td>
<td>95 ± 8</td>
<td>204 ± 9</td>
<td>2.1</td>
<td>113</td>
</tr>
<tr>
<td>Bovine NP</td>
<td>0.4-0.7</td>
<td>n/a</td>
<td>10-120</td>
<td>-</td>
<td>2.01 ± 0.07</td>
<td>109</td>
</tr>
<tr>
<td>Bovine NP</td>
<td>1-5</td>
<td>2.5-17.5</td>
<td>-</td>
<td>75-200</td>
<td>2.01 ± 0.07</td>
<td>109</td>
</tr>
<tr>
<td>Porcine TMJ disc explants</td>
<td>0.5, 1, 5, 10</td>
<td>2.5, 5</td>
<td>5-20</td>
<td>7-33</td>
<td>~0.5</td>
<td>See Appendix B</td>
</tr>
<tr>
<td>Nondegenerate Human NP</td>
<td>1, 5, 10</td>
<td>1, 5, 21</td>
<td>140.36 ± 176.2</td>
<td>131.60 ± 88.29</td>
<td>1.54 ± 0.92</td>
<td>Present study</td>
</tr>
<tr>
<td>Nondegenerate Human AF</td>
<td>1, 5, 10</td>
<td>1, 5, 21</td>
<td>87.85 ± 101.5</td>
<td>79.41 ± 71.85</td>
<td>1.03 ± 0.52</td>
<td>Present study</td>
</tr>
<tr>
<td>Degenerate Human NP</td>
<td>1, 5, 10</td>
<td>1, 5, 21</td>
<td>144.3 ± 125.6</td>
<td>222.78 ± 128.7</td>
<td>2.22 ± 0.46</td>
<td>Present study</td>
</tr>
<tr>
<td>Degenerate Human AF</td>
<td>1, 5, 10</td>
<td>1, 5, 21</td>
<td>110.1 ± 164.3</td>
<td>169.63 ± 130.27</td>
<td>2.27 ± 0.64</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Discussion

Due to the large avascular nature of the IVD, oxygen as well as other nutrients required for maintaining disc tissue health are supplied by blood vessels at the disc periphery.\textsuperscript{133} It is suggested that both articular chondrocytes and IVD cells obtain their energy primarily through the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis, even in the presence of a high oxygen level.\textsuperscript{122, 156} In order to gain some understanding of the role of a diminished nutrient supply associated with degeneration on the IVD cell
metabolic phenotype, we compared nondegenerate and degenerate IVD NP and AF cells. Specifically, rates of glucose consumption and lactate production were measured since these are essential in regulating local nutrient and metabolite concentrations around the cells.

Present findings support a glycolytic metabolic phenotype with nondegenerate and degenerate human IVD cells, while the degenerate cells are significantly more metabolically active. Rate of lactate production, as a marker of a glycolytic metabolism, was observed to decrease at low glucose levels and 1% oxygen level. This is expected considering glucose is the driving substrate for glycolysis and therefore, lactate production. The rate of lactate production for nondegenerate nor degenerate human AF cells was unaffected by oxygen level at 1mM glucose concentrations. A negative Pasteur effect, i.e., a diminished LPR at low oxygen levels, was observed in both nondegenerate and degenerate human NP cells. A negative Pasteur effect has been reported for isolated bovine NP cells and articular cartilage.\textsuperscript{109, 122} Overall, there was no change in GCR between nondegenerate and degenerate cell types but the LPR was significantly higher in the degenerate NP and AF cells. In combination with previous oxygen consumption rate data, the higher LPR and oxygen consumption rate of the degenerate human IVD cells also supports a more active metabolism in these cells.

This difference in effect of oxygen level between tissue regions may be attributed to the difference in embryonic origins of the tissue types. The NP develops from the notochord contrary to the AF which arises from the mesenchyme.\textsuperscript{159} It is known that before maturity in humans as well as some other animal models including the bovine and canine,
the notochordal cells of the NP transition to chondrocyte-like cells that constitute a different phenotype.\textsuperscript{157-159} This NP transition does not occur in the porcine and rodent animal models.\textsuperscript{157} The higher glucose consumption and lactate production of the nondegenerate human NP cells could be indicative of a more active metabolic phenotype in the NP cells compared to AF cells.

For glycolysis alone, the lactate production: glucose consumption mole ratio should be 2.0.\textsuperscript{122} For the nondegenerate NP and AF cells, the mole ratio was less than 2.0; and closer to 1, therefore, glucose was consumed in reactions other than those leading to the reduction of pyruvate to lactate. The degenerate NP and AF ratio was higher than that of the nondegenerate cells indicating they may have higher nutritional demands. This difference in metabolic phenotype may be indicative of a changing IVD cellular metabolic phenotype survival response associated with the onset and progression of disc degeneration.

The GCR of the human IVD cells was found to be higher than those values reported in the literature for bovine NP cells\textsuperscript{109; 113} and porcine TMJ disc explants (see Appendix B), and lower compared to porcine NP cells.\textsuperscript{113} Such differences may be attributed to intrinsic differences between species as well as experimental culture conditions and methodology. The LPR of the human IVD cells was comparable to those values reported in the literature for bovine NP cells, lower than the LPR for porcine NP cells, and higher than porcine TMJ disc explants. The ratio of LPR to GCR of nondegenerate human IVD cells is most comparable to that of porcine NP cells. These cells have a ratio less than 2.0 indicating they are primarily glycolytic but consume glucose for reactions other than those
leading to glycolysis. Degenerate human IVD cells have a comparable ratio of LPR to GCR to that of porcine TMJ disc explants, indicating these cell types may consume significantly more glucose relative to their lactate production. The glucose may be used for storage purposes and other metabolic pathways such as oxidative phosphorylation. High oxygen consumption rates and high mitochondrial membrane potential of degenerate human IVD cells support the possible change in metabolic shift towards an oxidative phenotype of these cells. Further examination of the ATP production rates as well as specific activity of the mitochondria is of particular interest in future studies to confirm the oxidative phenotype of degenerate human IVD cells.

In summary, the effect of glucose concentration and oxygen level on glucose consumption and lactate production rates in nondegenerate and degenerate human IVD NP and AF cells using cell suspensions was examined. Overall, the rate of glucose consumption was more sensitive to change in glucose concentration while the rate of lactate production, indicative of rate of glycolysis, was more dependent on oxygen level. The human IVD NP cells appear to display a negative Pasteur effect while the LPR of AF cells was not dependent on change in oxygen level. These rates were tissue dependent in that human IVD NP overall had higher consumption and production rates compared to that of AF cells. Overall, the ratio of rate of lactate production to glucose consumption was less than 2 and higher in nondegenerate NP and AF cells compared to that of degenerate NP and AF cells. Considering the nutrient consumption and metabolite production trends, a delicate nutrient gradient and metabolic profile potentially exists in the nondegenerate human IVD. Such an environment will likely be very vulnerable to any pathological event.
that can impede nutrient supply, and ultimately result in the onset and progression of IVD tissue degeneration.
CHAPTER 5: REGION AND STRAIN-DEPENDENT DIFFUSIVITIES OF GLUCOSE AND LACTATE IN NONDEGENERATE HUMAN CARTILAGE ENDPLATE

The cartilage endplate (CEP) is implicated as the main pathway of nutrient supply to the healthy human intervertebral disc (IVD). In this study, the diffusivities of nutrient/metabolite solutes in healthy CEP were assessed, and further correlated with tissue biochemical composition and structure. The CEPs from non-degenerated human IVD were divided into four regions: central, lateral, anterior, and posterior. The diffusivities of glucose and lactate were measured with a custom diffusion cell apparatus under 0%, 10%, and 20% compressive strains. Biochemical assays were conducted to quantify the water and glycosaminoglycan (GAG) contents. The Safranin-O and Ehrlich’s hematoxylin and eosin staining and scanning electron microscopy (SEM) were performed to reveal the tissue structure of the CEP. Average diffusivities of glucose and lactate in healthy CEP were $2.68\pm0.93\times10^{-7}$ cm$^2$/s and $4.52\pm1.47\times10^{-7}$ cm$^2$/s, respectively. Solute diffusivities were region-dependent ($p<0.0001$) with the highest values in the central region, and mechanical strains impeded solute diffusion in the CEP ($p<0.0001$). The solute diffusivities were significantly correlated with the tissue porosities (glucose: $p<0.0001$, $r=0.581$; lactate: $p<0.0001$, $r=0.534$). Histological and SEM studies further revealed that the collagen fibers in healthy CEP are more compacted than those in the nucleus pulposus (NP) and annulus fibrosus (AF) and show no clear orientation. Compared to human AF and NP, much smaller solute diffusivities in human CEP suggested that it acts as a gateway for solute diffusion through the disc, maintaining the balance of nutritional environment in healthy human disc under mechanical loading and preventing the progression of disc degeneration.
Introduction

The cartilage endplate (CEP), a thin layer of hyaline cartilage at the cranial and caudal surfaces of human intervertebral disc (IVD), was found to be the main pathway of nutrient supply to the disc through *in vivo* and *in vitro* studies. Due to the avascular nature of the disc, the nutrients from the capillaries in the subchondral plates diffuse into the disc through the CEPs, while the metabolites diffuse out through a reversed direction. Pathological change, such as CEP calcification at the early stage of disc degeneration, could break down the precautious nutritional balance inside the disc by impeding nutrient/metabolite diffusion through the disc. By contrast, fractured or degenerated CEPs have been found to co-occur in severely degenerated discs. The lesions in the CEP can open up channels and hasten the inflow of cytokines, enzymes or angiogenic molecules which have deleterious effects on disc cells and further accelerate disc degeneration. The differential effects of the CEP on solute transport through the disc at different degeneration stages suggested that healthy CEP is a critical disc component for maintaining the unique disc nutritional environment under the physiological condition. The collapse of the balance between nutrient supply and intrinsic cellular demand inside the disc is considered one of the major factors for disc degeneration.

The CEP has unique biomechanical properties from other disc components [annulus fibrosus (AF) and nucleus proposes (NP)] and articular cartilage. Although the solute diffusion behaviors in human CEP were previously studied using the fluorescein-labeled markers and contrast agents, the diffusivity values of basic
nutrient/metabolite (i.e., glucose/lactate) in healthy or degenerated human CEP are largely unknown. Glycolysis is believed to be the major energy metabolism pathway for disc cells \textit{in vivo} by consuming glucose to generate adenosine triphosphate (ATP) and producing lactic acid as a waste product \textsuperscript{176}. Therefore, the knowledge about the diffusion rates of glucose and lactate in human CEP is crucial for understanding disc nutrition.

The rate of solute diffusion in cartilaginous tissue is governed by solute diffusivities which are affected by the composition and structure of the tissue matrix, as well as mechanical strains on the tissue \textsuperscript{177}. Therefore, the objective of this study was to measure the nutrient/metabolite diffusivities of healthy human CEPs in four regions (central, lateral, anterior, and posterior) under three compressive strains (0\%, 10\%, and 20\%). Specifically, the effect of mechanical strain on the glucose/lactate diffusivities in the CEP was determined using the diffusion cell method \textsuperscript{178}. Biochemical compositions of the CEP were further characterized and correlated with the diffusion properties. In addition, the microstructures of healthy human CEP were revealed using histological staining techniques and scanning electron microscopy (SEM). We hypothesized that the diffusivities of glucose and lactate in healthy human CEP were regional-dependent due to its unique tissue composition and structure; and mechanical loading impacts the rates of solute diffusion in this tissue by changing the tissue hydration. The goal of this study was to establish a baseline measurement of nutrient/metabolites diffusivities in healthy human CEPs. The results of this study may facilitate the understanding of the role of human CEP in IVD nutrition and provide new insights into nutrition-related mechanisms of disc degeneration and regeneration.
Materials and Methods

Specimen Preparation

Twelve human lumbar spines (age 33-65 years old) obtained from an Organ Procurement Organization (LifePoint Inc., Charleston, SC) were screened based on the Thompson grading system under an institutional approval. To establish a quality baseline measurement in healthy CEPs, only spines without degenerated discs (Grade III-V) and related diseases were selected. To limit the tissue variation, only L2-L3 and L3-L4 discs were used. Further, only healthy CEPs without artifacts, such as fissures and calcification, were included for the measurements. Considering these criteria, six disc motion segments were harvested from three lumbar spines within 24 hours after death (58 year old female, 42 year old male, and 54 year old female). Three L3-L4 disc motion segments were used for diffusion cell experiments, while three L2-L3 disc motion segments were used for histological and SEM studies. Healthy human CEPs were harvested and tested within three days after receiving the spine.

The disc motion segments were opened through the median plane of the disc with a scalpel. Cylindrical plugs of NP or AF/endplate/bone were extracted from four regions (center, lateral, anterior, and posterior) from both superior and inferior surfaces of the disc with an 8 mm diameter corneal trephine (Figure 37). The plugs harvested from the left lateral region were used for diffusion, histological staining and SEM protocol development, while those from the right lateral region were used for data collection. The plug was microtomed to carefully remove the overlying NP/AF (relatively more transparent than
CEP tissue) and the vertebral bone. The final disc-shape CEP specimens were punched out with a 6 mm diameter trephine for the diffusion cell experiment. The specimen preparation was conducted in a moisturized hood to prevent tissue dehydration. The prepared CEP specimens (n=24, from 3 L3-4 discs of 3 spines) had an average initial thickness of 0.734±0.103 mm, which is in the same range as stated in previous studies.\textsuperscript{179}
Figure 37. (A) Schematic of specimen preparation. Human VB/CEP/Disc tissue plugs were harvested from various regions of the disc motion segment (VB: vertebral body). Disc-shape human CEP specimens were prepared using a microtome and corneal trephine for diffusion and biochemical measurements. (B) Schematic of diffusion cell for glucose/lactate diffusivity measurements.

Glucose and Lactate Diffusivity Measurements

A previously established custom diffusion cell was used to measure the strain-dependent diffusivities of glucose and lactate in the CEP specimens. It consisted of two non-conductive acrylic solution chambers with a channel separated by the specimen holder (Figure 37B). The specimen was held between two rigid porous plates (hydrophilic polyethylene, 50-90 µm pore size, Small Parts, Inc., Miami Lakes, FL) to inhibit swelling and sealed with an O-ring. The compressive strains were applied to the CEP specimens by changing of the spacers placed between the two chamber halves.
The CEP specimen was first held at its initial thickness (0% strain level). 500 µL of 20 mg/mL glucose with 10 mg/mL lactate mixed into normal phosphate-buffered saline (PBS) was pipetted into the upstream chamber while 200 µL of concentrated PBS solution was pipetted into the downstream chamber. The concentrated PBS solution was used to balance the osmolarity of the glucose/lactate solution in the upstream chamber (530 mOsm/L) to prevent any convection through the specimen due to osmosis. The diffusion cell was placed in an incubator with 37°C. The stir bars were utilized to maintain constant solute distribution within the solution. After glucose and lactate were allowed to diffuse through the tissue specimen for a 15-minute time interval, the contents of the downstream chamber were emptied and glucose and lactate concentrations were measured with YSI 2700 Select Biochemistry Analyzer (YSI Inc., Yellow Springs, OH). Following each 15-minute time interval, the downstream chamber was refilled with 200 µL of fresh PBS solution, while the upstream chamber was refilled with 500 µL of fresh glucose/lactate solution. The experiment was repeated until the same concentration (within 5%) in the downstream chamber was obtained for 2-3 consecutive readings, suggesting that steady state had been reached. An average of 2.5 hours (10 intervals of 15 minutes each) was necessary to reach steady state. Once steady state was achieved at 0% compression, the experiment was repeated for 10% and 20% compressive strains. The apparent diffusivity \( (D_{\text{app}}) \) was calculated based on the one-dimensional steady state diffusion theory:

\[
D_{\text{app}} = \ln \frac{C_{\text{up}} - C_{\text{down}(t_0)}}{C_{\text{up}} - C_{\text{down}(t)}} \frac{v_{\text{down}} h}{A}
\] (1)

where \( C_{\text{up}} \) is the concentration in the upstream chamber which is assumed to be constant. \( C_{\text{down}(t_0)} \) is the downstream concentration at initial diffusion time \( t_0 \) and \( C_{\text{down}(t)} \) is the
concentration at time $t$. $h$ is the thickness of the specimen, and $A$ is the cross section area through which the diffusive flux occurs. This area was calculated as 50% of the area of the porous plates confining the specimen, as the porous material has a 50% open area. $V_{down}$ is the volume in the downstream well. Due to solution replacement at the start of each 15-minute interval, the value of $C_{down}(t_0)$ is always equal to zero in Equation (1); the value of $C_{down}(t)$ is the averaged value of the 2-3 consecutive readings of downstream concentration at steady state.

**Histological and SEM Studies**

Bone/CEP/disc tissue plugs were taken at the four regions from both superior and inferior surfaces of two disc motion segments at L2-L3 level (Figure 37A). The plugs were rapidly fixed in 10% neutral buffered formalin, decalcified, and paraffin wax embedded. Ehrlich’s hematoxylin and eosin (H&E) and Safranin-O & Fast Green were used with the wax sections (7 μm). For the SEM study, bone/CEP/disc tissue plugs were taken from one disc motion segment at L2-L3 level. The plugs from the inferior surface were microtomed to remove both bone and disc tissue to reveal the collagen fibers of the CEP on the horizontal plane (X-Y plane; Figure 38). Meanwhile, the plugs from superior surface were cut anteroposteriorly to show the microstructures of bone, CEP, and disc tissue on the vertical plane (Y-Z plane; Figure 38). The specimens were then fixed in PBS solution with 2.5% glutaraldehyde, dehydrated with a series of ethanol, and dried in hexamethyldisilazane (HMDS) solution. A gold layer with 20 μm thickness was coated on the specimens to enhance contrast. Images were taken under a JEOL JSM-5600LV SEM (JEOL USA, Inc., Pleasanton, CA) at 35x, 50x, and 500x magnifications.
Figure 38. (A) Histological images for the sandwich structure between the human VB, CEP, and disc tissue (NP or AF) at superior surface of the disc motion segment under 4x and 10x magnifications (10x images with blue outlines were enlarged from the corresponding marked areas in 4x images). The two rows of images on the top were from Safranin-O slices, while the two rows on the bottom were from H&E slices (CENT-Central; LAT-Lateral; ANT-Anterior; POST-Posterior). (B) SEM images for CEP and disc tissue at central region under 35x, 50x, and 500x magnifications.
Porosity and GAG Measurements

A buoyancy method was used to determine the initial porosity (ratio of water volume to wet tissue volume) of the CEP specimens at 0% strain level$^{181}$:

$$
\phi_0^w = \frac{W_{wet} - W_{dry}}{W_{wet} - W_{PBS}} \frac{\rho_{PBS}}{\rho_w}
$$

(2)

where $\phi_0^w$ is initial porosity, $W_{wet}$, $W_{PBS}$, and $W_{dry}$ are weights of specimens in the air, in PBS solution and after lyophilized. $\rho_{PBS}$ and $\rho_w$ are the densities of the PBS solution and water. The porosities of the CEPs under 10% and 20% strain levels were calculated based on the relationship between tissue porosity and dilatation $e$ ($e = J^{-1}$ where $J$ is the tissue deformation)$^{125}$,

$$
\phi^w = \frac{\phi_0^w + e}{1 + e}
$$

(3)

The lyophilized tissues were then assayed for glycosaminoglycan (GAG) content. The Blyscan Glycosaminoglycan Assay kit (Biocolor Ltd., Newtonabbey, Northern Ireland) was used to determine the GAG content based on 1,9-dimethylmethylene blue dye binding, with standards provided by the manufacturer.

Statistical Analysis

The measurements were reported using the means and standard deviations (SD). The glucose and lactate diffusivities were examined for significant differences by mechanical strain and disc region using two-way ANOVA that allowed for correlation among measurements from the same spine and also incorporated error heterogeneity by disc region. The porosity and GAG content were similarly examined for differences by disc region. Correlations between solute diffusivities and porosity were determined.

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marginally using the linear mixed effects model with a random effect for spine. Due to the limited sample size, the sex and age effects were not considered in this study. The statistical analysis was conducted in R (R Core Team, 2015) using the package nlme.

**Results**

*Glucose and Lactate Diffusivities*

The apparent glucose and lactate diffusivities were measured for healthy human CEP at 0%, 10%, and 20% compressive strains (Figure 39A and B). A significant strain effect was found for the diffusivities of both solutes (p<0.0001). The cross-region glucose diffusivity at 0% strain was $2.68\pm0.93\times10^{-7}$ cm$^2$/sec, at 10% strain it decreased to $1.96\pm0.81\times10^{-7}$ cm$^2$/sec (-27%), and at 20% strain was $1.44\pm0.68\times10^{-7}$ cm$^2$/sec (-46%). The lactate diffusivity was $4.52\pm1.47\times10^{-7}$ cm$^2$/sec, $3.56\pm1.20\times10^{-7}$ cm$^2$/sec (-21%) and $2.76\pm1.08\times10^{-7}$ cm$^2$/sec (-39%) for 0%, 10%, and 20% strains, respectively. A significant regional effect was also found for the diffusivities of both solutes (p<0.0001). The glucose diffusivity in the central region at 0% strain (Central: $3.44\pm0.97\times10^{-7}$ cm$^2$/sec) was significantly higher than in the lateral and posterior regions (Lateral: $2.46\pm0.86\times10^{-7}$ cm$^2$/sec, p=0.020; Posterior: $1.91\pm0.39\times10^{-7}$ cm$^2$/sec, p<0.0001). The lactate diffusivity in the central region at 0% strain (Central: $5.52\pm1.52\times10^{-7}$ cm$^2$/sec) was also significantly higher than in the lateral and posterior regions (Lateral: $4.11\pm1.48\times10^{-7}$ cm$^2$/sec, p=0.036; Posterior: $3.36\pm0.64\times10^{-7}$ cm$^2$/sec, p<0.0001). No significant difference was detected for both glucose and lactate diffusivities between central and anterior regions (Glucose: $2.78\pm0.83\times10^{-7}$ cm$^2$/sec, p=0.085; Lactate: $4.88\pm1.34\times10^{-7}$ cm$^2$/sec, p=0.391). There was
no evidence for an interaction between region and strain for either glucose (p=0.983) or lactate (p=0.986).

Figure 39. Effect of compressive strains on the regional distribution of (A) glucose diffusivity and (B) lactate diffusivity of human CEP. (Sample size n=6).
Porosity and GAG content

The cross-region porosity in CEP was 0.667±0.049 (at 0% mechanical strain) and significant variation among the four regions was detected (p=0.034) with the central region having higher values (p<0.04) than the anterior and posterior regions, but not differing from the lateral region (p=0.31) (Table 8). The average GAG content in CEP was 90.08±17.77 µg/mg dry tissue and also differed among the four regions (p=0.001) with the central region significantly higher than the lateral, anterior, and posterior regions (p<0.009).

Table 8. Porosity ($\phi^w$) at 0% strain and GAG content (mean ± standard deviation) of human CEP in four disc regions.

<table>
<thead>
<tr>
<th></th>
<th>Central (n=6)</th>
<th>Lateral (n=6)</th>
<th>Anterior (n=6)</th>
<th>Posterior (n=6)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\phi^w$</td>
<td>0.706±0.030</td>
<td>0.680±0.016</td>
<td>0.632±0.061</td>
<td>0.648±0.050</td>
<td>0.667±0.049</td>
</tr>
<tr>
<td>GAG</td>
<td>109.49±6.99</td>
<td>72.65±16.50</td>
<td>89.18±13.67</td>
<td>84.08±6.34</td>
<td>90.08±17.77</td>
</tr>
</tbody>
</table>

Histological Appearance and Collagen Fiber Microstructure

Histological images (H&E; Safranin-O&Fast Green) showed that there was a thin layer of cartilage endplate between the interface of the human vertebral body and disc tissue (Figure 38A). In the Safranin-O images, the CEP region appeared to be bright red. The thickness of the CEP varied by region, with a value between 0.6mm and 1.2mm. SEM images further revealed the unique sandwich structure consisted of bone, CEP, and disc tissue (Figure 38B). It is apparent that collagen fibers in CEP layer were more compacted compared to NP and AF tissues. There is no clear fiber orientation in both horizontal and vertical planes for the CEP.
Correlation between Material Properties and Tissue Biochemical Composition

The correlations between solute diffusivities (glucose and lactate) and porosities were found to be statistically significant, as shown in Figure 40A and B (Glucose: \( r=0.581 \), \( p<0.0001 \), \( n=72 \); Lactate: \( r=0.534 \), \( p<0.0001 \), \( n=72 \)). No statistical significant correlations were observed between solute diffusivities and GAG contents (\( p>0.11 \)).
Figure 40. Correlation between tissue porosity and the (A) glucose diffusivity and the (B) lactate diffusivity. (Sample size \( n = 74 \)).

Discussion

This study was commenced to determine the baseline nutrient/metabolite solute (glucose and lactate) diffusivities in healthy human CEP and further investigate the effects of disc region and mechanical strain on the tissue transport properties. The results showed that the solute diffusivities in human CEP were much smaller than those in other disc components (e.g., AF) and articular cartilage (Table 9). This suggested that healthy human CEP may act as a gateway for nutrient/metabolite solute inflow/outflow through the disc 23; 24. The mechanical strain-dependent solute diffusivities further suggested that CEP may facilitate the maintenance of a stable extracellular nutrient environment by impeding the solute transport through the disc under mechanical loading conditions. These results
correspond to recent findings in the literature that the CEP could act as a mechanical barrier by facilitating interstitial fluid pressurization and resisting disc herniation under abnormal loadings \(^8; ^{17}; ^{53}\). In addition, the results of this study further supported the previous notion that healthy CEP may play an important role in ECM homeostasis and the progression of angiogenesis, which is associated with disc degeneration, by blocking the rapid diffusion of cytokines, enzymes, or angiogenic molecules such as vascular endothelial growth factor (VEGF) into the disc \(^{24}; ^{27}; ^{30}; ^{42}; ^{174}\).

### Table 9. Glucose and lactate diffusivities (mean ± standard deviation) in human CEP and other cartilaginous tissues. (CEP: cartilage endplate; AF: annulus fibrosus; AC: articular cartilage).

<table>
<thead>
<tr>
<th>Species</th>
<th>(D_{\text{Glucose}}) (10^{-7} \text{ cm}^2/\text{s})</th>
<th>(D_{\text{Lactate}}) (10^{-7} \text{ cm}^2/\text{s})</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CEP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.44±0.97-Central</td>
<td>5.52±1.52-Central</td>
<td>Present study (0% strain)</td>
<td></td>
</tr>
<tr>
<td>2.46±0.86-Lateral</td>
<td>4.11±1.48-Lateral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.78±0.83-Anterior</td>
<td>4.88±1.34-Anterior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.91±0.39-Posterior</td>
<td>3.36±0.64-Posterior</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CEP</td>
<td>5.8±2.1-Central</td>
<td>----</td>
<td>(^3)</td>
</tr>
<tr>
<td>1.8±0.3-Peripheral</td>
<td>----</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human AF</td>
<td>17±1.5</td>
<td>----</td>
<td>(^3)</td>
</tr>
<tr>
<td>7.56±0.75</td>
<td>----</td>
<td></td>
<td>(^{180})</td>
</tr>
<tr>
<td>Bovine AF</td>
<td>13.8±0.15</td>
<td>----</td>
<td>(^{178})</td>
</tr>
<tr>
<td>Human AC</td>
<td>13.5-14.6</td>
<td>----</td>
<td>(^{183})</td>
</tr>
</tbody>
</table>

Conversely, due to the avascular nature of human IVD, the main pathway for nutrient supply to the disc is diffusion through the CEP. The low solute diffusivities in the CEP can lead to a critical nutrient environment in human IVD (i.e., a steeper nutrient/metabolite gradient with low oxygen and glucose, and high lactate concentrations
at the center of the disc)\(^{171}\). Such delicate nutrient environment may be vulnerable to any pathological changes of the CEP, such as calcification \(^{24; 123}\). The deterioration of the extracellular nutrient environment will change the metabolism and synthesis behaviors of disc cells and affect the cell viability, leading to initiate/accelerate disc degeneration \(^{111; 113; 132; 176}\).

The diffusivities of nutrient/metabolite solutes were found to be region-dependent with significantly higher values in the central region, which is consistent with a previous study of glucose diffusivities in human CEP \(^3\). This can be associated with the regionally dependent biochemical composition in the CEP with the highest porosity being in the central region. Our results showed that the glucose and lactate diffusivities in healthy human CEP were significantly correlated with the tissue porosities instead of the GAG contents. This finding further supported the previous hypothesis that water content is generally a dominated determinant for the diffusion properties of small solutes (molecular weight < 5000 Dalton) in cartilaginous tissues \(^{184-186}\). Compared to other disc components and articular cartilage, the smaller solute diffusivities in healthy human CEP can also be attributed to its ECM structure with compacted collagen fibers, as shown in our histological and SEM studies (Figure 38). This was in agreement with the high collagen content and tensile modulus of human CEP in a recent study \(^{17}\).

Due to the technical challenges of in vivo measurements, finite element (FE) models were commonly used to predict the extracellular mechano-electrochemical environment in the human IVD \(^{121; 187-189}\). Although there were a variety of characterizations on material properties of AF and NP tissues \(^3; 23; 24; 190; 191\), the tissue properties, especially transport
properties, of human CEP are rare. Consequently, the CEP were either excluded or modeled with material properties of other disc components or articular cartilage in current FE models. Our results clearly showed that the diffusion properties of human CEP were significantly different from other disc components (Table 9). Therefore, the region and strain dependent solute diffusivities, as well as the porosities, determined in this study should be incorporated into the FE models to better predict the physiological nutrient environment in human IVD, which can serve as a baseline for future analysis on disc degeneration and regeneration.

Several limitations of this study should be noted. To establish a quality baseline measurement in healthy human CEP, the disc motion segments were screened by age (30-65 years old), disc level (L2-L4), and degeneration conditions (Grade I-II). Although only twenty four healthy CEP specimens obtained from three fresh, healthy, and mature lumbar spines were eligible for the diffusion study, the results successfully demonstrated the statistical significance for testing the hypothesis. Due to the scarcity of human disc samples, a future study with a larger sample size needs to be conducted to fully understand how other factors (e.g. age, sex, disc level, and CEP calcification) affect the diffusion properties in the CEP and gain a further understanding on the role of CEP in the progression of disc degeneration.

The rigid porous plates in the diffusion cell to compress the specimens may cause a stagnant layer formation between the tissue and the solution, although they were much more permeable than the CEP tissue. The stirring rod could minimize the effect of boundary layer formation, but it may not be eliminated entirely 192. As shown in the
previous studies, a 7% less of the apparent glucose diffusivity was found in porcine articular cartilage which was measured with porous plate than that without porous plate\textsuperscript{178}.\textsuperscript{180} However, the porous plates were necessary in this study to confine the CEP specimens due to significant tissue swelling and provide the means to control the strain levels of the specimens. In addition, the apparent diffusivities ($D_{app}$) measured in this study represent the coupling effect of the intrinsic diffusivity ($D$) and partition coefficient ($K$) ($D_{app}=KD$).

A previous study has shown that the partition coefficient was dependent on the mechanical compression in cartilage tissues\textsuperscript{193}. To characterize the intrinsic diffusivity which is more essential for the FE modeling of the IVD, further experiments are necessary to determine the strain-dependent solute partition coefficients in the CEP.

In summary, this study measured the baseline nutrient/metabolite solute (glucose and lactate) diffusivities in healthy human CEP and further studied the effects of disc region and mechanical strain on the tissue transport properties. The diffusivities of glucose and lactate in healthy human CEP were regional-dependent due to its unique tissue composition and structure; and mechanical loading impedes the rates of solute diffusion by changing the tissue porosity. Compared to the AF and NP, human CEP has much smaller solute diffusivities and acts as a gateway for solute diffusion through the disc. The results of this study may facilitate the understanding of the role of human CEP in IVD nutrition and provide new insights into nutrition-related mechanisms of disc degeneration and regeneration.
CHAPTER 6: OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The major impact of this dissertation is the characterization of the metabolic phenotype of human intervertebral disc (IVD) cells and evaluation of the effect of mechanical strain on transport properties of human IVD tissues. This knowledge will be useful in quantifying and predicting changes in nutrient levels under conditions that occur in vivo and better understanding pain associated disc degeneration. These studies underlined the significant impact of the local nutrient environment on the biological metabolic responses in regards to cell proliferation, differentiation, and maintenance. These biological responses therefore affected the intrinsic properties of the disc tissue resulting in a cascade of changes possibly associated with the onset and progression of degeneration. These studies supported our general hypothesis that sustained mechanical loading can alter solute transport and nutrient concentrations in the IVD, resulting in changes to the cellular metabolism, tissue composition, and mechanical function, ultimately leading to disc degeneration in the human IVD disc.

Chapter 3: In this study, a baseline comparison of oxygen consumption rates and mitochondrial membrane potential of nondegenerate and degenerate human IVD nucleus pulposus (NP), annulus fibrosus (AF), and cartilage endplate (CEP) cells was determined. These data are the first report of oxygen consumption rates of nondegenerate and degenerate human IVD samples used to correlate local nutrient concentrations with cellular metabolism. As hypothesized, the IVD nutrient environment parameters including oxygen
level and glucose concentration had a significant impact on oxygen consumption rates and mitochondrial membrane potential due to the unique nutrient metabolic mechanisms of the nondegenerate and degenerate disc cells. The results show that human degenerate IVD cells are very active metabolically and poorly resistant to nutritional stress and support the idea that a fall in nutrient supply as a consequence of the onset and progression of disc degeneration could drive a metabolic change in IVD cell phenotype. The results suggest that their nutritional demands should be considered in prospective tissue engineering and regeneration approaches.

Chapter 4: This series of experiments investigated the effects of oxygen level and glucose concentration on the glucose consumption and lactate production rates of nondegenerate and degenerate human IVD NP and AF cells. These data are the first report of glucose consumption and lactate production rates of nondegenerate and degenerate human IVD NP and AF cells used to correlate local nutrient concentrations with cellular metabolism. As hypothesized, the oxygen level and glucose concentration had a significant impact on the glucose consumption and lactate production rates of nondegenerate and degenerate human IVD NP and AF cells due to the unique nutrient metabolic mechanisms. The results support the idea that human degenerate IVD cells are very active metabolically and poorly resistant to nutritional stress and therefore, a fall in nutrient supply as a consequence of the onset and progression of disc degeneration could drive a metabolic change in IVD cell phenotype. The results further emphasize that the nutritional demands of degenerative IVD cells should be considered in prospective tissue engineering and regeneration approaches.
Chapter 5: In this study, the diffusivities of nutrient/metabolite solutes in healthy cartilage endplates were assessed, and further correlated with tissue biochemical composition and structure. The results of this study are the first report of strain-dependent glucose and lactate diffusivities of healthy human IVD cartilage endplates, at various ages, under various strain conditions using diffusion chambers to develop new constitutive relationships between solute diffusivity and tissue hydration to establish strain-dependent transport properties. As hypothesized, the diffusivities of glucose and lactate in healthy human CEP were regional-dependent due to its unique tissue composition and structure; and mechanical loading impacted the rates of solute diffusion in this tissue by changing the tissue hydration. Histological and SEM studies revealed more compact collagen fibers and no clear orientation in healthy CEP compared to those in the nucleus pulposus (NP) and annulus fibrosus (AF). Compared to human AF and NP, much smaller solute diffusivities in human CEP suggested that it acts as a gateway for solute diffusion through the disc, maintaining the balance of nutritional environment in healthy human disc under mechanical loading and preventing the progression of disc degeneration.

Challenges

Chapter 3 and 4: Human IVD cells were cultured in monolayer and expanded through 2 passages with the assumption that cell phenotype was unchanging. Previous studies have shown a loss in phenotype that in vitro articular chondrocytes experience when extracted from their ECM as well as monolayer cell passaging. They are induced to an oxidative
phenotype and metabolism. Therefore, it is necessary to investigate the effect of glucose concentration and oxygen level on metabolite rates (i.e., oxygen/glucose consumption, lactate production, ATP levels) in human IVD cells cultured in a 3D hypoxic environment to offer a more accurate \textit{in vivo} representation of metabolite rates as well as preserving the IVD cell phenotype under cell culture passaging in a future study. In addition, this study was carried out with the assumption that the range of osmolality measured was similar to the physiological osmolality of the IVD. This may create variation in the results, therefore, it would be beneficial to examine this possible effect by utilizing osmotic balancing through the addition of mannitol.

\textbf{Chapter 5:} Several limitations of this study should be noted. To establish a quality baseline measurement in healthy human CEP, the disc motion segments were screened by age, disc level, and degeneration conditions. Although the results were statistically significant, only twenty four healthy CEP specimens from three human lumbar spines were eligible for the diffusion study. Due to the scarcity of human disc samples, a future study with a larger sample size needs to be conducted to fully understand how other factors (e.g. age, sex, disc level, and CEP calcification) affect the diffusion properties in the CEP and gain a further understanding on the role of CEP in the progression of disc degeneration.

\textbf{Future Goals}

The goal of this dissertation was to quantitatively characterize the effect of mechanical loading on human IVD nutrition and mechanical function, which truly bridges
IVD biomechanics and biology. By incorporating realistic cell and transport properties, a predictive 3D finite element model can be developed to investigate the in vivo effect of local nutrient and metabolite concentrations on nutrient transport and cell metabolic behavior. This model can also be used as a degenerative diagnostic tool based on patient specific data. This direction will help to build new strategies for intervertebral disc degeneration treatment and tissue engineering approaches in other cartilaginous tissues.

**Chapter 3 and 4:** It is of interest to investigate the effect of different inflammatory factor treatments on the metabolic response of nondegenerate human IVD cells. Such inflammatory factors such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and interleukin-1 (IL-1), should be considered because they are known mediators of inflammation and pain associated with IVD degeneration. In addition, the effect of age and gender on metabolic nondegenerate and degenerate IVD cellular responses to changes in the local nutrient environment can be investigated in the future. It is also of interest to determine the effect of local nutrient environment parameters on the nutrient consumption and metabolite production rates of nondegenerate and degenerate human CEP cells.

**Chapter 5:** Future characterization of human CEP to fully understand how other factors (e.g. age, sex, disc level, and CEP calcification) affect the diffusion properties within the tissue is of interest. This direction will gain a further understanding on the role of CEP in the progression of disc degeneration. Our results clearly showed that the diffusion properties of human CEP were significantly different from other disc components.
Therefore, the region and strain dependent solute diffusivities, as well as the porosities, determined in this study should be incorporated into a finite element model to better predict the physiological nutrient environment in human IVD, which can serve as a baseline for future analysis on disc degeneration and regeneration.

Future work for this research involves incorporating the human IVD cell metabolic and transport properties into a 3D finite element model of the *in vivo* IVD environment. Recently in our lab, Yao et al. (2007) developed a new multiphasic mechano-electrochemical transport theory, based on the triphasic theory, which includes multiple species of ions or neutral solutes. This theory has been used to investigate electromechanical properties and to develop finite element models for the IVD. Incorporation of the cell metabolic and transport properties into the model can be further developed in an IVD degeneration diagnostic tool based on patient specific magnetic resonance images (MRI). The work described in this dissertation represents progress towards understanding the mechanisms of IVD pathobiology in order to develop new strategies for diagnosis and treatment from a bioengineering modeling perspective. The techniques and approaches defined in this dissertation will also be useful in developing new strategies for IVD tissue regeneration related to the unique nutrient environment and can be translated into tissue engineering applications in other cartilage tissue types.
APPENDICES
APPENDIX A1: THE EFFECTS OF OXYGEN LEVEL AND GLUCOSE CONCENTRATION ON THE METABOLISM OF PORCINE TMJ DISC CELLS

The objective of this study was to determine the combined effect of oxygen level and glucose concentration on cell viability, ATP production, and matrix synthesis of temporomandibular joint (TMJ) disc cells. TMJ disc cells were isolated from pigs aged 6-8 months and cultured in a monolayer. Cell cultures were preconditioned for 48 hours with 0, 1.5, 5, or 25mM glucose DMEM under 1%, 5%, 10%, or 21% O2 level, respectively. The cell viability was measured using the WST-1 assay. ATP production was determined using the Luciferin-Luciferase assay. Collagen and proteoglycan synthesis were determined by measuring the incorporation of [2, 3-3H]proline and [35S]sulfate into the cells, respectively. TMJ disc cell viability significantly decreased (\(P<0.0001\)) without glucose. With glucose present, decreased oxygen levels significantly increased viability (\(P<0.0001\)), while a decrease in glucose concentration significantly decreased viability (\(P<0.0001\)). With glucose present, decreasing oxygen levels significantly reduced ATP production (\(P<0.0001\)) and matrix synthesis (\(P<0.0001\)). A decreased glucose concentration significantly decreased collagen synthesis (\(P<0.0001\)). The interaction between glucose and oxygen was significant in regards to cell viability (\(P<0.0001\)), ATP production (\(P=0.00015\)), and collagen (\(P=0.0002\)) and proteoglycan synthesis (\(P<0.0001\)). Although both glucose and oxygen are important, glucose is the limiting nutrient for TMJ disc cell survival. At low oxygen levels, the production of ATP, collagen, and proteoglycan are severely inhibited. These results suggest that steeper nutrient gradients may exist in the TMJ disc and it may be vulnerable to pathological events that impede nutrient supply.
Introduction

The temporomandibular joint (TMJ) is a load-bearing joint, consisting of the condyle of the mandibular bone and the fossa eminence of the temporal bone, separated by a fibrocartilaginous disc. Temporomandibular joint disorders (TMJD) affect approximately 35 million people in the United States with tremendous morbidity and financial cost, yet its etiology remains poorly understood. In approximately 30% of TMJD patients, mechanical dysfunction of the TMJ disc, especially displacement due to tissue degeneration, is a common event. The mean age of onset of degenerative changes in the TMJ is between 18 and 44 years, which for unknown reasons is a decade earlier than in post-cranial joints. In contrast to other joints, attempts to surgically reconstruct the TMJ is often unsuccessful and may result in severe disabilities. Thus, research to understand the pathophysiology of TMJ disc degeneration for earlier diagnosis and management are essential.

The mechanical function of the TMJ disc is determined by the composition and structure of its extracellular matrix (ECM). The TMJ disc has a distinctive ECM composition when compared to hyaline cartilage and other fibrocartilaginous tissues. The TMJ disc is comprised primarily of water with a significant amount of collagen type I and a small amount of proteoglycan. The normal human TMJ disc is a large avascular structure, so the nutrients required by the disc cells for maintaining a healthy matrix are supplied by synovial fluid at the margins of the disc as well as through nearby blood vessels at the connection to the posterior bilaminar
The balance between the rate of nutrient transport through the matrix and the rate of consumption by disc cells establishes a concentration gradient across the TMJ disc. In articular cartilage, these gradients of essential nutrients can profoundly affect chondrocyte viability, energy metabolism, matrix synthesis, and the response to inflammatory factors. Studies have shown that oxygen and glucose play critical roles in the metabolism of chondrocytes and are essential for both adenosine triphosphate (ATP) production and matrix synthesis. In the IVD, cellular energy metabolism is dominated by anaerobic glycolysis, thus glucose levels play a significant role in ATP production and matrix protein synthesis. A disrupted nutrient supply has long been implicated in the development of IVD disc degeneration, including cartilage end-plate calcification and a further decrease in oxygen and glucose levels. In TMJ disc cells, recent studies have shown that hypoxia with inflammation modulates the gene expression of tenascin-C and matrix metalloproteinases. However, unlike the chondrocytes and IVD cells, the effect of essential nutrients (e.g., oxygen and glucose) on the energy metabolism and matrix synthesis of TMJ disc cells is still largely unknown.

Our recent studies have shown that solute diffusivities in the TMJ disc are much lower than the values in articular cartilage and the IVD, and compressive mechanical strain can further impede solute diffusion in the TMJ disc. Moreover, our cell metabolic studies have shown that the TMJ disc has a higher cell density and higher oxygen consumption rates compared to articular cartilage and the IVD. Therefore, it is likely that a steeper nutrient gradient may exist in TMJ discs and thus, it is more vulnerable to pathological events which impede nutrient supply, including sustained joint loading due to
jaw clenching and bruxism. To understand the biological consequence of a limited nutrient supply, it is necessary to examine the impact of nutrient levels on TMJ disc cells.

The objective of this study was to examine the combined effect of oxygen level and glucose concentration on TMJ disc cell viability, energy metabolism, and matrix protein synthesis. Specifically, the cell viability, ATP production, and radioactive proline and sulfate incorporation (i.e., collagen and proteoglycan synthesis) were measured in porcine TMJ disc cells under defined oxygen levels and glucose concentrations.

Materials and Methods

Cell isolation and culture

A total of nine porcine heads (American Yorkshire, male, aged 6-8 months) were collected from a local abattoir within 2 hours of slaughter (i.e., three porcine heads on three independent experimental days). Both left and right TMJs were removed en bloc with the capsule intact from each porcine head. The six TMJ discs were pooled together and harvested under sterile conditions and then digested overnight at 37°C with 0.1% (w/v) collagenase II (Worthington Biochemical Corp., Lakewood, NJ) in standard 25 mM glucose DMEM (HyClone) containing 10% fetal bovine serum (FBS) (Invitrogen). Digestions were strained through a 70µm filter, washed with PBS, and re-suspended in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco Brl) and 25 µg/mL ascorbic acid. Isolated TMJ disc cells were plated at 1×10⁴ cells/cm² at 21% O₂ and 5% CO₂ at 37°C in 25 mM glucose DMEM. The media was changed every 2 days, and upon reaching confluence typically within 2 weeks, first-passage (P1) cells were detached.
with trypsin-EDTA (Invitrogen). Cells were re-plated at a 1:2 ratio and cultured in a monolayer to second passage (P2) for use in experiments. After cell viability was quantified by trypan blue exclusion (0.4% in buffered saline solution), the P2 cells were seeded at $1 \times 10^4$ cells into 96 wells. At 90% confluence, the culture medium was replaced by DMEM plus 10% FBS at 4 different glucose concentrations. These mediums were prepared by the supplementation of glucose-free DMEM with 0, 1.5, 5, or 25 mM glucose and the osmolality was measured within the range of 290 - 310 mosmol (Vapro Vapor Pressure Osmometer, Elitech Group). The FBS (Invitrogen) was filtered by the manufacturer until glucose levels were < 5 mg/dL, which equates to approximately 0.27 mM glucose, therefore, the presence of glucose due to the presence of FBS in the testing medium is minimal. A 25 mM glucose concentration is normally adopted for in vitro cell culture, and the typical glucose concentration in plasma is 5 mM. Although the exact glucose environment has not been determined, it can be expected that the glucose concentration in TMJ disc tissue can range from 0 to 5 mM. Studies on intervertebral discs have shown that the oxygen level and glucose concentration can be as low as almost 0 inside the tissue \cite{132, 156}. For each glucose concentration, cells were further cultured under various oxygen levels (1%, 5%, 10%, and 21% O$_2$) for 48 hours in a triple gas incubator in which N$_2$ was used to reduce O$_2$ levels \cite{207}.

**WST-1 assay for examining metabolically active cell viability**

Cell viability of the preconditioned experimental groups was measured after 48 hours using the WST-1 kit (Roche Molecular Biochemicals, Mannheim, Germany). Water-soluble tetrazolium salt, 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1, 3-
benzen disulfonate (WST-1), has been demonstrated to be a simple and rapid measurement of cell viability with extremely low cytotoxicity. A ten percent working solution was made by mixing one part volume of the cell viability reagent WST-1 with nine parts volume of media. Quantification of the formazan dye produced by metabolically active cells was done via a scanning multi-well spectrophotometer (420-480nm)\textsuperscript{211}. Absorbance values collected from cells cultured at 25 mM glucose and 21% oxygen level were considered the control measurement due to the initial \textit{in vitro} expansion culture conditions. All other absorbance values from other cell culture conditions were normalized to the control.

\textit{ATP measurement}

Levels of intra- and extra- cellular ATP of the preconditioned experimental groups after 48 hours were determined using the Luciferin-Luciferase kit (PerkinElmer, Wellesley, MA). At the end of an oxygenated or hypoxic incubation period, 100 µL of the cell suspension was mixed with 50 µL of a mammalian cell lysis solution (0.1M alkaline solution to inactivate endogenous ATPases and to stabilize the released ATP) in a 96-well microplate and mixed for 5 minutes. The mixture was then combined with 50 µL of the substrate (Luciferase/Luciferin) solution and mixed for an additional 5 minutes. The plate was allowed to dark-adapt for 10 minutes in the luminometer before luminescence counting was initiated. The total per viable cell based ATP production was then calculated by normalizing to the WST-1 absorbance values.\textsuperscript{212} The total ATP production per viable cell was then normalized to the control (25 mM glucose and 21% oxygen level).
**[2, 3-3H] Proline Incorporation Assay**

Cellular synthesis of collagen was determined by measuring the incorporation of radioactivity (derived from [2, 3-3H] proline) into collagen. The TMJ disc cells were exposed to 20 μCi/mL (2, 3-3H) proline in 2 mL of medium for the final 24 hours of the total 48 hour incubation. The cell layer was washed three times with PBS and homogenized with a polytron in 0.2% Triton-X 100 and 50 mM Tris/HCl. The cell homogenate was digested with 0.02% collagenase and incubated for 4 hours at 37°C. After incubation, 10% TCA/0.5% tannic acid was added to each sample, centrifuged at 4,000 rpm at 4°C for 10 minutes and washed three times with 10% TCA/0.5% tannic acid. The precipitates were each solubilized in 50 mM Tris/HCl and the radioactivity was measured in a scintillation counter. The per viable cell based proline incorporation was calculated by normalizing to the WST-1 absorbance values. The radioactive proline incorporation per viable cell (i.e. collagen synthesis per viable cell) was then normalized to the control (25 mM glucose and 21% oxygen level).

**[35S] Sulfate Incorporation Assay**

Cellular synthesis of proteoglycan was determined by measuring the incorporation of radioactive [35S]sulfate into the GAGs. The matrix-forming TMJ disc cells were exposed to 5.0 μCi/mL [35S]sulfate in 2 mL of medium for the final 4 hours of the total 48 hour incubation. The cell layer was washed three times with PBS and solubilized with 2 mg/mL of Pronase E in 5 mM CaCl2 and 0.2 M Tris/HCl at 56°C for 3 hours. The precipitates incorporating [35S]sulfate were collected on glass-fiber filters and washed three times with cetyl pyrinium chloride (CPC). The radioactivity of the cells precipitated with CPC was
measured in a scintillation counter. The per viable cell based sulfate incorporation was calculated by normalizing to the WST-1 absorbance values. The radioactive sulfate incorporation per viable cell (i.e. proteoglycan synthesis per viable cell) was then normalized to the control (25 mM glucose and 21% oxygen level).

Statistical analysis

The measurements of each outcome (i.e., cell viability, ATP production, collagen synthesis, and proteoglycan synthesis) were performed in triplicate and repeated in three independent experiments (n=9) under a defined combination of oxygen level and glucose concentration. The results were presented as the mean with 95% confidence intervals. One-way and two-way analysis of variance (ANOVA) and Tukey’s post hoc tests were performed to determine the singular and combined effect of oxygen level (1%, 5%, 10%, or 21%) and glucose concentration (0, 1.5, 5, or 25 mM) on TMJ cell viability, ATP production, collagen synthesis, and proteoglycan synthesis. SPSS 16.0 software (SPSS Inc., Chicago, IL) was used for examining all statistical analyses and significant differences were reported at P-values < 0.05.

Results

Metabolically active cell viability

The relationship between plated porcine TMJ disc cell number and WST-1 reagent absorbance was determined to be linear, as shown in Figure 41. Glucose and oxygen concentrations had a significant impact on cellular viability (Figure 42). In the presence of glucose, an increase of oxygen level significantly decreased cell viability (P < 0.0001) with
the average viability at 21% oxygen being approximately 2.1 times lower than that at 1% oxygen. In contrast, an increase of glucose concentration significantly increased TMJ disc cell viability ($P < 0.0001$), although the magnitude of impact is smaller compared to that of the oxygen level. The average viability in 25 mM glucose was about 1.5 times higher than that in 1.5 mM. In the absence of glucose, cell viability significantly dropped ($P < 0.0001$). Therefore, the following measurements of ATP production and radioactive [2, 3-3H] proline and [35S] sulfate incorporation were conducted in the presence of glucose at concentrations of 1.5 mM, 5 mM, and 25 mM. The interaction between oxygen and glucose was significant ($P < 0.0001$).

![Figure 41](image-url)  

**Figure 41.** WST-1 standard curve showing the linear relationship between plated porcine TMJ disc cell number and absorbance. R = 0.9228.
Figure 42. The cell viability of porcine TMJ disc cells. The data shown were means and 95% confidence intervals. Experiments were performed in triplicate on three independent experimental days (n=9). Absorbance values were normalized to the control (25 mM glucose and 21% oxygen level). The effects of oxygen levels (1%, 5%, 10%, and 21%) and glucose concentrations (0, 1.5, 5, and 25 mM) were assessed. An increase of glucose concentration significantly increased TMJ disc cell viability (P < 0.0001). In the presence of glucose, an increase of oxygen level significantly decreased cell viability (P < 0.0001). The interaction between oxygen and glucose was significant (P < 0.0001). Significance (P < 0.05) of oxygen level effect and glucose level effect are denoted by a * and #, respectively.

ATP measurement

The effects of oxygen level and glucose concentration on intra- and extra-cellular ATP production are shown in Figure 43. In the presence of each of the glucose concentrations, an increase of oxygen level significantly increased the measurement of intra- and extra-cellular ATP (P < 0.0001). The average ATP measurement at 21% oxygen was about 2.3 times higher than that at 1% oxygen. However, an increase of glucose
concentration from 1.5 mM to 25 mM had no significant impact on the average ATP measurement ($P = 0.1388$). The interaction between oxygen and glucose was significant ($P = 0.00015$).

Figure 43. The measurement of ATP per viable cell of porcine TMJ disc cells. The data shown were means and 95% confidence intervals. Experiments were performed in triplicate on three independent experimental days (n=9). The total per viable cell based ATP production was calculated by normalizing to the WST-1 absorbance values. The total ATP production per viable cell was then normalized to the control (25 mM glucose and 21% oxygen level). The effects of oxygen levels (1%, 5%, 10%, and 21%) and glucose concentrations (1.5, 5, and 25 mM) were assessed. An increase of glucose concentration from 1.5 mM to 25 mM had no significant impact on the average ATP measurement ($P = 0.1388$). In the presence of each of the glucose concentrations, an increase of oxygen level significantly increased intra- and extra-cellular ATP production ($P < 0.0001$). The interaction between oxygen and glucose was significant ($P = 0.00015$). Significance ($P < 0.05$) of oxygen level effect is denoted by a *.
[2, 3-3H] Proline Incorporation

The effects of oxygen level and glucose concentration on radioactive proline incorporation, and thus collagen synthesis, are shown in Figure 44. In the presence of glucose, a reduced oxygen level resulted in a significant reduction of collagen synthesis within all glucose concentrations ($P < 0.0001$). The average collagen synthesis dropped 6 fold from 21% oxygen to 1% oxygen. Although the effect of glucose concentration on collagen synthesis was statistically significant ($P < 0.0001$), the magnitude of impact was much smaller compared to that of the oxygen level. At the 21% oxygen level, an increase of glucose concentration from 1.5 mM to 5 mM significantly increased average collagen synthesis by 27% ($P = 0.033$) and further increase of the glucose concentration had no significant effect ($P = 0.187$). The interaction between oxygen and glucose was significant ($P = 0.0002$).
Figure 44. The [2, 3-3H] proline incorporation per viable cell (i.e. collagen synthesis per viable cell) of porcine TMJ disc cells. The data shown were means and 95% confidence intervals. Experiments were performed in triplicate on three independent experimental days (n=9). The per viable cell based proline incorporation was calculated by normalizing to the WST-1 absorbance values. The [2, 3-3H] proline incorporation per viable cell was then normalized to the control (25 mM glucose and 21% oxygen level). The effects of oxygen levels (1%, 5%, 10%, and 21%) and glucose concentrations (1.5, 5, and 25 mM) were assessed. Although the effect of glucose concentration on collagen synthesis was statistically significant (P < 0.0001), the magnitude of impact was much smaller compared to that of the oxygen level. At the 21% oxygen level, an increase in glucose concentration from 1.5 mM to 5 mM significantly increased collagen synthesis (P = 0.033), while further increase of the glucose concentration had no significant effect (P = 0.187). In the presence of glucose, a reduced oxygen level resulted in a significant reduction of collagen synthesis within all glucose concentrations (P < 0.0001). The interaction between oxygen and glucose was significant (P = 0.0002). Significance (P < 0.05) of oxygen level effect is denoted by a *.

[^35]S Sulfate Incorporation

The effects of oxygen level and glucose concentration on radioactive sulfate incorporation, and thus proteoglycan synthesis, are shown in Figure 45. In the presence of
all glucose mediums, reduced oxygen level resulted in a significant reduction of proteoglycan synthesis ($P < 0.0001$). The average proteoglycan synthesis dropped 5 fold from 21% oxygen to 1% oxygen. However, an increase in glucose concentration from 1.5 mM to 25 mM, had no significant effect on average proteoglycan synthesis ($P = 0.0821$). The interaction between oxygen and glucose was significant ($P < 0.0001$).

Figure 45. The $^{35}$S sulfate incorporation per viable cell (i.e. proteoglycan synthesis per viable cell) of porcine TMJ disc cells. The data shown were means and 95% confidence intervals. Experiments were performed in triplicate on three independent experimental days (n=9). The per viable cell based sulfate incorporation was calculated by normalizing to the WST-1 absorbance values. The $^{35}$S sulfate incorporation per viable cell was then normalized to the control (25 mM glucose and 21% oxygen level). The effects of oxygen levels (1%, 5%, 10%, and 21%) and glucose concentrations (1.5, 5, and 25 mM) were assessed. An increase in glucose concentration from 1.5 mM to 25 mM had no significant effect on average proteoglycan synthesis ($P = 0.0821$). In the presence of all glucose mediums, reduced oxygen level resulted in a significant reduction of proteoglycan synthesis ($P < 0.0001$). The interaction between oxygen and glucose was significant ($P < 0.0001$). Significance ($P < 0.05$) of oxygen level effect is denoted by a *.
Discussion

The present findings show that different oxygen levels and glucose concentrations affect the viability of porcine TMJ disc cells. In the absence of glucose, cell viability significantly decreased. In the presence of glucose, decreased oxygen levels increased porcine TMJ disc cell viability. In agreement with other studies on cartilaginous tissues, specifically using bovine nucleus pulposus cells, the viability of the intervertebral disc cells began to decrease when glucose levels fell below 0.5 mM \(^{132; 214}\). It thus appears that glucose may be the limiting nutrient for the survival of TMJ disc cells, rather than oxygen, as has previously been theoretically proposed for intervertebral disc cells \(^{215}\). Articular cartilage chondrocytes have been shown to have higher proliferation rates at decreased oxygen levels. Proliferating chondrocytes are well adapted to hypoxic conditions within their avascular environment with glycolysis as the main energy source \(^{216}\). Inside the chondrocyte, a molecule of glucose is converted into glucose 6-phosphate, which is the building block for the synthesis of N-acetylglucosamine, chondroitin sulphate, and hyaluronan \(^{156}\) as well as maintaining cell viability. Due to its avascular nature, articular cartilage obtains its critical nutrients, including oxygen and glucose, from the surrounding synovial fluid by diffusion mechanisms \(^{217}\). Articular cartilage consumes very little oxygen \(^{138; 218}\), both because it has very few cells and because those cells utilize a predominantly glycolytic metabolism. Similar to articular cartilage chondrocytes, the porcine TMJ disc cells live in an avascular tissue environment and rely on diffusion mechanisms for nutrient supply. Accordingly, as shown in this study, TMJ disc cells appear to be able to survive in the extracellular matrix with limited nutrients and a low oxygen level. In regards to \textit{in vitro}
culture, isolated cells of the TMJ disc were previously observed to have an order of magnitude faster proliferation time than chondrocytes obtained from hyaline cartilage, suggesting TMJ disc cells are more active than chondrocytes in monolayer. Such differences may potentially be due to differences in cell type and maturity, as well as culture conditions.

In our present findings in porcine TMJ disc cells, a decrease in oxygen level resulted in a decrease in the measurement of ATP in all glucose concentrations. Similar oxygen dependent results have been observed in bovine articular cartilage chondrocytes, whereby, the rate of glycolysis falls as oxygen levels drop, contributing to the fall in ATP and, hence, matrix synthesis. Both articular cartilage chondrocytes and IVD cells obtain their energy primarily through the Embden-Meyerhof-Parnas (EMP) pathway glycolysis, even in the presence of high oxygen. The switch between the two forms of respiration, aerobic versus anaerobic, utilized by animal cells was first noted by Pasteur in the late 19th century. In mammalian cells, glucose and glutamine are two major energy sources. Glucose is either converted anaerobically to lactate by glycolysis, yielding 2 moles of ATP per molecule of glucose, or aerobically to carbon dioxide and water by the tricarboxylic acid cycle (TCA), also known as oxidative phosphorylation, yielding 36 moles of ATP per molecule of glucose. Both articular cartilage chondrocytes and IVD cells, which utilize glycolytic metabolism in both high and low levels of oxygen, have extremely low oxygen consumption rates. In contrast, Kuo et al. reported a significantly higher oxygen consumption rate of porcine TMJ disc cells, compared to articular cartilage chondrocytes and IVD cells, which in combination with our present findings, may
be related to oxidative phosphorylation, and thus, a significant Pasteur effect for TMJ disc cells, but further investigation into the energy metabolism is necessary to confirm. Future studies to determine the effects of hypoxia and glucose concentrations in media on other key metabolites, such as glucose consumption and lactic acid production, in the TMJ disc cells may provide more information. Therefore, it appears that an aerobic to an anaerobic environmental shift would push porcine TMJ disc cells to utilize both glycolysis and oxidative phosphorylation pathways, respectively to maintain their metabolism.

Cell morphological studies have shown that the porcine TMJ disc contains an inhomogeneous distribution of a mixed cell population of fibroblast-like cells and chondrocyte-like cells, which are distinct from hyaline cartilage chondrocytes. The chondrocyte-like cells in the TMJ disc do not appear to exhibit the distinct pericellular capsule typical of articular cartilage chondrocytes. In addition, there are significant differences in organelle content between articular cartilage chondrocytes and chondrocyte-like cells in the TMJ disc, which likely suggests differences in cell behavior. The chondrocyte-like cells in TMJ discs have a greater number of mitochondria, both suggesting a higher metabolic activity than articular cartilage chondrocytes and supporting our finding that ATP production may be higher in the TMJ disc cells.

In our present studies, a decrease in oxygen level resulted in a decrease in collagen and proteoglycan synthesis rates in all glucose concentrations, suggesting that the porcine TMJ cells begin to decrease cellular differentiation in the presence of low oxygen as long as sufficient glucose was present. Previous studies have investigated the effects of hypoxia
on cellular metabolism in the IVD and articular cartilage and have found that it leads to a dramatic fall in the synthesis of the extracellular matrix. As matrix synthesis is closely coupled to intracellular ATP levels, it thus appears that an increase in glycolysis under hypoxia is insufficient to prevent a fall in intracellular ATP concentrations and hence cannot prevent a fall in matrix synthesis. When no Pasteur effect was observed in bovine nucleus pulposus cells, the synthesis of sulfated GAGs was reduced as oxygen level decreased. The Pasteur effect may even be harmful in a sense, whereby an increase in the rate of glycolysis under hypoxic conditions may lead to the depletion of an already limited supply of glucose in avascular tissues. If an absence of glucose does indeed cause cell death as indicated in this study, increasing the rate of glycolysis in cells may lead to more widespread cell death. Moreover, as a result of increased glycolysis, lactic acid production will increase in the hypoxic regions in the center of the TMJ disc. This fall in pH may cause a further decrease in cell viability, which will be detrimental to the TMJ disc.

In the avascular TMJ disc, the oxygen and glucose gradient may be steeper than that of articular cartilage, because of its higher cell density and rates of nutrient consumption and lower solute diffusivities through the extracellular matrix. This suggests that TMJ disc cells are more susceptible to pathological changes which impede nutrient supply, such as sustained joint loading due to jaw clenching or bruxism. In the TMJ disc, it is unlikely that hypoxia occurs alone, being that local concentrations of oxygen, glucose, and lactic acid are determined by the balance between solute transport and cellular metabolism. Therefore, changes in the concentrations of these solutes cannot
occur independently in vivo. In pathological conditions such as degenerate osteoarthritis, a further decrease in oxygen level may occur. Because of the role of hypoxia in modulating metabolic pathways, which in turn affects growth rates and the production of free ATP during hypoxia, these metabolic alterations will have important consequences for extracellular matrix turnover.

Possible limitations to this study include the loss of phenotype that in vitro inhomogeneous TMJ disc cells may experience when extracted from their ECM as well as during P1 and P2 culture. Previous literature has investigated the effect of high oxygen monolayer-expanded in vitro culture of articular chondrocytes and has found a cellular induction to an oxidative phenotype and metabolism. Therefore, it is necessary to investigate the effect of glucose and oxygen on cellular viability, proliferation, and metabolism in TMJ explants in a future study. The TMJ disc has been shown to have a significantly lower proteoglycan content and thus, a much lower fixed charge density than articular cartilage. As a result, we expect the physiological osmolality of the TMJ disc to be around 300 mosmol. In regards to the osmolality of the medium with varying glucose concentrations, the range of measured osmolality from 290 - 310 mosmol does create a slight variation and thus, in future studies, it would be beneficial to examine this possible effect by utilizing osmotic balancing through the addition of mannitol. In addition, while the pig is the best experimental model of the TMJ after comparison to sheep, cows, dogs, cats, rabbits, rats, and goats, it is necessary to investigate human TMJ discs in a future study.
In summary, we investigated the effects of glucose and oxygen on cell viability and cellular metabolism. Our results showed that in the absence of glucose, cell viability significantly decreased, suggesting that glucose may be the limiting nutrient for the survival of TMJ disc cells. In the presence of glucose, a decrease of oxygen level significantly increased cell viability. In contrast, an increase of glucose concentration significantly increased TMJ disc cell viability, although the magnitude of impact is smaller compared to that of the oxygen level. In addition, in the presence of glucose, a decrease of oxygen level significantly decreased intra- and extra-cellular ATP production and matrix synthesis. Our results suggest that TMJ disc cells utilize different metabolic mechanisms compared to other cartilage types, such as the IVD and articular cartilage. A possible reason is that the TMJ disc has multiple cell phenotypes that contribute to their cell behavior. The maintenance of oxygen and glucose homeostasis in the TMJ disc is essential for many vital cellular functions including viability and differentiation. Sustained mechanical loading on the TMJ disc will induce both oxygen and glucose concentrations to decrease towards the center of the TMJ disc. The results of this study therefore support the idea that a fall in nutrient supply due to pathological joint loading might be one pathway to disc degeneration. Future studies will determine the effects of hypoxia and glucose concentrations in media on other key metabolites, such as glucose consumption and lactic acid production, and energy metabolic pathways in the TMJ disc cells as well as explants in health and disease.
APPENDIX A2: NUTRIENT CONSUMPTION AND METABOLITE PRODUCTION RATES IN PORCINE TMJ DISCS: AN EXPLANT STUDY

Objective: To determine the combined effect of oxygen level and glucose concentration on the glucose consumption and lactate consumption rates of temporomandibular joint (TMJ) disc explants.

Design: TMJ disc explants were isolated from pigs aged 6-8 months and immediately placed in well plates with 0.5, 1, 5, or 10mM glucose DMEM under 2.5% or 5% oxygen level, respectively. The glucose and lactate concentrations were measured at 4 and 18 hours. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration difference, cell densities, and time.

Results: At 5% oxygen level and low glucose concentrations, a glucose substrate dependence is shown as increasing glucose concentration and decreasing oxygen levels increased the average GCR which ranged from 3-15 nmol/million cells/hr. Increasing glucose concentrations and decreasing oxygen levels resulted in a significantly increasing lactate production rate at low glucose levels. At 5mM glucose concentration, the lactate production rate increased from 16.40 ± 6.05 nmol/million cells/hr to 24.17 ± 3.899 nmol/million cells/hr with a decrease in oxygen level (5% to 2.5% oxygen). The average ratio of LPR:GCR (approximately 0.61 ± 0.28) was constant with increasing glucose concentrations for all oxygen levels. For low glucose concentrations, the ratio of LPR:GCR was constant with change in oxygen level. No significant regional tissue difference was observed for GCR, LPR, and LPR:GCR mole ratio at 2.5% and 5% oxygen levels for all glucose concentrations.
Conclusions: The trend of increasing GCR and LPR with increasing glucose concentrations and decreasing oxygen concentration, in combination with previously determined oxygen consumption rates, is indicative of a positive Pasteur Effect. Steeper nutrient gradients may exist in the TMJ disc and therefore, it may be vulnerable to pathological events that impede nutrient supply.

Introduction

The temporomandibular joint (TMJ) consists of a fibrocartilaginous disc which separates the condyle of the mandibular bone and the fossa eminence of the temporal bone. While the etiology of temporomandibular joint disorders (TMJD) remains largely unknown, approximately 35 million people in the United States are affected with tremendous morbidity and financial cost\(^1\). Mechanical dysfunction of the TMJ disc, especially displacement due to tissue degeneration occurs in approximately 30% of TMJD patients\(^2\). The onset of degenerative changes in the TMJ is between 18 and 44 years, which for unknown reasons is a decade earlier than in post-cranial joints\(^3\). Significant changes in disc morphology, biochemistry, material properties, and function are believed to accompany the degenerative process\(^4\). In contrast to other joints, efforts to surgically rebuild the TMJ is often ineffective and may result in severe disabilities\(^5\). Therefore, it is essential to understand the pathophysiology of TMJ disc degeneration for earlier diagnosis and management.

The nondegenerate human TMJ disc is a large avascular structure\(^6\). The nutrients required by the disc cells for maintaining disc health are supplied by nearby blood vessels
at the connection to the posterior bilaminar zone as well as synovial fluid at the margins of the disc. The transport of small nutrients within the TMJ disc mainly depends on diffusion. The balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells establishes a concentration gradient across the TMJ disc. These gradients of essential nutrients have been shown to profoundly affect articular chondrocyte viability, energy metabolism, matrix synthesis, and the response to inflammatory factors within articular cartilage. It has been shown that oxygen and glucose not only play critical roles in the metabolism of chondrocytes and are essential for both adenosine triphosphate (ATP) production and matrix synthesis. In the intervertebral disc (IVD), anaerobic glycolysis is the primary cellular energy metabolic pathway, thus glucose levels play a significant role in ATP production and matrix protein synthesis. A disrupted nutrient supply has long been implicated in the development of IVD degeneration, including cartilage endplate calcification and a further decrease in oxygen and glucose levels. In TMJ disc cells, recent studies have shown that hypoxia with inflammation modulates the gene expression of tenascin-C and matrix metalloproteinases. However, in comparison to articular chondrocytes and IVD cells, the effect of essential nutrients on the energy metabolism and matrix synthesis of TMJ disc cells is still largely unknown.

Although convection due to interstitial fluid flow, induced by mechanical loading, may affect large solute transport, the transport of small solutes (e.g., ions, oxygen, and glucose) within avascular cartilaginous tissues mainly depends on diffusion. Our recent studies have shown that solute diffusivities in the TMJ disc are much lower than the
values in articular cartilage and the IVD; and compressive mechanical strain can further impede solute diffusion in the TMJ disc\textsuperscript{208-210}. Moreover, our cell metabolic studies have shown that the TMJ disc has a higher cell density and higher oxygen consumption rates compared to articular cartilage and the IVD\textsuperscript{139}. Additionally, while glucose may be the limiting nutrient for the survival of TMJ disc cells, decreased oxygen levels were shown to increase TMJ cell proliferation, and decrease ATP production and matrix synthesis rates.\textsuperscript{233} Therefore, steeper nutrient gradients may exist in TMJ discs and thus, making the tissue more vulnerable to pathological events which impede nutrient supply, including sustained joint loading due to jaw clenching and bruxism. To understand the biological consequence of a limited nutrient supply, it is necessary to examine the impact of key metabolites and energy metabolic pathways on TMJ disc explants. The objective of this study was to examine the combined effect of oxygen level, glucose concentration, and tissue region on TMJ disc explant energy metabolism. Specifically, the glucose consumption and lactate consumption rates were determined in porcine TMJ disc explants under various oxygen levels, glucose concentrations, and explant regions.

\textbf{Materials and Methods}

\textit{TMJ Explant Isolation}

A total of thirty-six porcine heads (American Yorkshire, male, aged 6-8 months) were collected from a local abattoir within 2 hours of slaughter. The entire TMJ with capsule intact was removed \textit{en bloc}. The joints were opened under a sterile dissection hood and both TMJ discs were then removed and washed with 5-6 changes of phosphate buffered
saline (PBS). Fresh TMJ disc explants (~0.1 g wet weight/explant) from five different regions (i.e., Anterior, Intermediate, Lateral, Medial, and Posterior) were harvested and the tissue volume of each explant was determined in PBS based on the Archimedes’ principle. Each disc region was normalized by cell density values determined from confocal measurements. Explants were immediately diced into small pieces to minimize the concentration gradient of glucose within the explant and then placed into 24-well plates. These wells were filled with fetal bovine serum free DMEM glucose solutions at 0.5, 1, 5, or 10mM. The incubator had been preset to 2.5% or 5% oxygen level prior to plating of the explants.

Glucose and Lactate Concentrations Measurement

Glucose and lactate concentrations were measured with an YSI 2700 Select Biochemistry Analyzer (YSI Inc., OH). The instrument is capable of measuring glucose and lactate values within the ranges of 0-50mM and 0-29mM, respectively. These measurements were taken at 4 and 18 hours. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration difference, cell densities, and time. At the end of the experiments, the explant pieces were fully digested and the cell viability was examined via trypan blue exclusion to confirm greater than 90% viability.

Statistical analysis

For one glucose consumption rate (GCR) experiment, three punch explants from separate porcine TMJ discs were analyzed. Three separate GCR experiments were performed for each of the five unique regions (i.e., Anterior, Intermediate, Lateral, Medial,
and Posterior) of the disc that were investigated. Therefore, when analyzing one region of the TMJ disc at one oxygen level and glucose concentration, a total of nine samples were examined; when the data from all 5 regions were combined, a total of 45 samples were examined. This is also true of the lactate production rate (LPR) experiments under a defined combination of oxygen level and glucose concentration.

The measurements of each outcome (i.e., glucose consumption and lactate production rates) were performed in triplicate and repeated in three independent experiments (n=9). The results were presented as the mean with standard deviation. Two-way analysis of variance (ANOVA) and Tukey’s post hoc tests were performed to determine the singular and combined effect of oxygen level (2.5% or 5%), glucose concentration (0.5, 1, 5, or 10mM), and explant region (Anterior, Intermediate, Lateral, Medial, and Posterior) on TMJ explant glucose consumption and lactate production rates. SPSS 16.0 software (SPSS Inc., Chicago, IL) was used for examining all statistical analyses and significant differences were reported at \( P \)-values < 0.05.

Results

Glucose Consumption Rate (GCR)

The results, as shown in Figure 46, indicate a substrate dependence as increasing glucose concentration and decreasing oxygen levels increased the average GCR. No significant regional tissue difference was observed at 2.5% and 5% oxygen levels for all glucose concentrations. At 0.5mM glucose, the average GCR significantly increased with a decrease in oxygen level (\( p=0.001 \)). The GCR at 0.5mM glucose and 2.5% and 5%
oxygen levels was 8.311±5.522 nmol/million cells/hr and 3.843±2.382 nmol/million cells/hr, respectively. At 1mM glucose, the GCR was significantly higher at 2.5% oxygen level compared to 5% oxygen level (p=0.002). The GCR at 1mM glucose and 2.5% and 5% oxygen levels was 9.938±4.161 nmol/million cells/hr and 6.622±2.511 nmol/million cells/hr, respectively. At 5mM glucose, there was no significant change in GCR due to change in oxygen level (p=0.052). The GCR at 5mM glucose and 2.5% and 5% oxygen levels was 12.717±3.898 nmol/million cells/hr and 9.184±5.241 nmol/million cells/hr, respectively. At 10mM glucose, there was no significant change in GCR due to change in oxygen level (p=0.056). The GCR at 10mM glucose and 2.5% 5% oxygen levels was 19.55±7.807 nmol/million cells/hr and 16.45±15.48 nmol/million cells/hr, respectively.

**Figure 46.** The glucose consumption rate (GCR) [nmol/million cells/hr] of porcine TMJ explants versus oxygen level (2.5 and 5%) varied by glucose concentration (0.5, 1, 5, and 10mM). At 5% oxygen level, the average GCR significantly increased with an increase in glucose concentration (p=0.001). At 0.5mM and 1mM glucose, the average GCR significantly increased with a decrease in oxygen level (p=0.001). No significant regional
tissue difference was observed at 2.5% and 5% oxygen level as well as all glucose concentrations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

**Lactate Production Rate (LPR)**

Increasing glucose concentrations resulted in a significantly increasing lactate production rate, as shown in Figure 47. At 2.5% oxygen level, lactate production remained around 20 nmol/million cells/hour for low glucose concentrations of 0.5, 1, and 5mM. The LPR significantly increased to 33.68 ± 7.81 nmol/million cell/hr at 10mM glucose concentration (p=0.001). At 5% oxygen level, the lactate production was approximately 10 nmol/million cells/hr at low glucose concentrations and 17 nmol/million cells/hr at higher glucose concentrations. Decreasing oxygen levels resulted in a significantly increasing lactate production rate for 0.5, 1, and 5mM glucose concentrations, as shown in Figure 479. No significant regional tissue differences were observed at all oxygen levels as well as all glucose concentrations.
Figure 47. The lactate production rate (LPR) [nmol/million cells/hr] of porcine TMJ explants versus oxygen level (2.5 and 5%) varied by glucose concentration (0.5, 1, 5, and 10mM). At 2.5 and 5% oxygen level, the average LPR significantly increased with an increase in glucose concentration (p=0.001). At 0.5, 1, and 5mM glucose concentrations, the average LPR significantly increased with a decrease in oxygen level (p=0.001). No significant regional tissue difference was observed at 2.5% and 5% oxygen level as well as all glucose concentrations. * = oxygen effect = p < 0.05. # = glucose effect = p < 0.05.

Ratio of LPR to GCR

The ratio of lactate production to glucose consumption was constant with increasing glucose concentrations for all oxygen levels, as shown in Figure 48. The average ratio at 2.5% and 5% oxygen levels was 0.544 ± 0.159 and 0.671 ± 0.401, approximately. For low glucose concentrations, the ratio of lactate production to glucose consumption was constant with change in oxygen level, as shown in Figure 48. At 10mM glucose concentration, the average ratio of 0.918 ± 0.711 at 5% oxygen level was significantly greater than the average.
The ratio of lactate production to glucose consumption of porcine TMJ explants versus oxygen level (2.5 and 5%) varied by glucose concentration (0.5, 1, 5, and 10mM). No significant differences were observed due to change in glucose concentration (p=0.30). The ratio at 10mM glucose concentration was significantly higher at 5% oxygen level compared to 2.5% oxygen level (p=0.035). There were no regional differences detected in respect to oxygen level and glucose concentration. * = oxygen effect = p < 0.05.

**Discussion**

Since the nondegenerate fibrocartilage human TMJ disc is a large avascular structure, the nutrient consumption and metabolite production rates of the embedded cell population will be a key determinant of nutrient concentrations within the tissue. The objective of this study was to determine the glucose consumption and lactate production rates in porcine TMJ disc explants and further examine the effects of glucose concentration,
oxygen level, and disc region on those rates. Recent studies on the fibrocartilage intervertebral disc (IVD) cells have shown that the glucose consumption and lactate production rates depend on glucose concentration and pH level and support a glycolytic phenotype in bovine and porcine IVD cells. \(^{109; 113}\) In addition, the oxygen consumption of fresh TMJ disc explants at the physiological glucose concentration of 5mM was found to be regionally dependent and very high on a tissue volume as well as per-cell basis compared to articular cartilage and the IVD. \(^{132; 139; 156; 215}\) Further characterization of the basal nutrient consumption and metabolite production rates is necessary to obtain a realistic prediction of \textit{in vivo} nutrition distribution and cellular metabolic rates in the TMJ disc. It is believed that 2.5\% and 5\% oxygen levels fall within the physiological range of nondegenerate cartilage. \(^{137}\) Therefore, in this study, porcine TMJ disc explants were used to determine glucose consumption and lactate production rates at 0.5, 1, 5, and 10mM glucose concentration and 2.5\% and 5\% oxygen level.

Using tissue explants, it was necessary to incorporate the distribution of volume based cell density to determine the glucose consumption and lactate production rates on a per-cell basis. Previous cell density values measured using a confocal microscopy based technique to determine the in situ surface-regional cell distribution of the TMJ disc were used to calculate the cell based glucose consumption and lactate production rates. \(^{139}\) The change in glucose and lactate concentrations were measured with an YSI 2700 Select Biochemistry Analyzer (YSI Inc., OH), which is capable of measuring glucose and lactate values within the ranges of 0-50mM and 0-29mM, respectively. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration
difference, previously reported cell densities, and time. The GCR of TMJ explants was observed to increase with increasing glucose concentration and decreasing oxygen level across all disc regions. This trend was found to be more dependent on change in glucose substrate concentration which confirms that glucose may be the critical nutrient in TMJ disc cell metabolism, as previously described in the literature. Rates of glucose consumption ranged from 5-20 nmol/million cells/hr which were lower compared to porcine and bovine IVD cells which ranged from 10-205 nmol/million cells/hr. Increasing LPR was also observed in combination with increasing glucose concentration and decreasing oxygen level across all disc regions. The LPR trends were more dependent on change in oxygen level indicating a glycolytic cell phenotype sensitive to the availability of oxygen. Rates of lactate production ranged 7-33 nmol/million cells/hr which were lower compared to porcine and bovine IVD cells which ranged from 75-355 nmol/million cells/hr.

Both nondegenerate chondrocytes and IVD cells obtain their energy primarily through the Embden-Meyerhof-Parnas (EMP) pathway glycolysis, even in the presence of high oxygen. For glycolysis alone, the lactate production:glucose consumption mole ratio should be 2.0. For the porcine TMJ disc explants, the mole ratio was around 2.0; indicating the metabolism of the cells is almost purely glycolytic. Electron microscopy examining cell morphology has revealed an inhomogeneous distribution of a mixed cell population of fibroblast-like cells and chondrocyte-like cells in the porcine TMJ disc which is distinct from hyaline cartilage chondrocytes. It appears that the distinct pericellular capsule typical of articular chondrocytes is not present in the chondrocyte-like cells in the
Moreover, it has been shown that the TMJ discs have a greater number of mitochondria compared to articular chondrocytes. The trends of GCR, LPR, and the LPR:GCR mole ratio determined in this study may be related to some extent of oxidative phosphorylation in TMJ disc cells. This is supported by previously determined high oxygen consumption rates in TMJ disc explants as well as the inhibition of the production of ATP, collagen, and proteoglycan at low oxygen levels. TMJ disc explants exhibited at positive Pasteur effect (i.e., an increase in glycolysis rate and hence in the rate of lactate production under low oxygen level). Previously in the literature, articular chondrocytes have been shown to demonstrate a negative Pasteur effect, while it is unclear the effect displayed by IVD cells, due to conflicting reports. Although a positive Pasteur effect provides a pathway for maintaining ATP production under low levels of oxygen, its effects in the disc could be harmful rather than beneficial, since nutrient concentrations may be low in the avascular TMJ disc. As a result, a positive Pasteur effect will increase glucose demand and may push glucose concentrations below those necessary for survival, while in addition, increased lactate production could lead to a fall in pH, causing further disruption to a delicate nutrient and metabolite gradient within the TMJ disc tissue.

In summary, the effect of glucose concentration and oxygen level on glucose consumption and lactate production rates in five TMJ disc regions was determined using porcine tissue explants. The rate of glucose consumption was more sensitive to change in glucose concentration while the rate of lactate production, indicative of rate of glycolysis, was more dependent on oxygen level. The glucose consumption and lactate production
rates increased with an increase in glucose concentration and decrease in oxygen level in agreement with a positive Pasteur effect. These rates were not region dependent in contrast to porcine TMJ disc explant oxygen consumption rates. The rates of glucose consumption and lactate production of porcine TMJ disc explants were lower compared to IVD cells. Overall, at all glucose concentrations and oxygen levels, the lactate production: glucose consumption mole ratio remained constant, only increasing significantly at higher conditions than physiological glucose levels. Considering the nutrient consumption and metabolite production trends, a delicate nutrient gradient and metabolic profile potentially exists in the nondegenerate TMJ disc. Such an environment will likely be very vulnerable to any pathological event that can impede nutrient supply, and ultimately result in tissue degeneration.
REFERENCES


62. Cox JM. 1990. Low Back Pain: Mechanism, Diagnosis and Treatment. Maryland: Williams & Wilkins;


