BIOENGINEERING APPROACH TO UNDERSTANDING TMJ PATHOBIOLOGY

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Abstract

The temporomandibular joint (TMJ) is a load-bearing joint consisting of the condyle of the mandibular bone, the fossa eminence of the temporal bone, and a fibrocartilaginous disc held in between the bone surfaces by ligaments. The TMJ disc serves to distribute stress, lubricate movement, and protect the articular surfaces of the joint. Over ten million Americans suffer from TMJ disorders (TMD) that affect the movement and function of the joint, making everyday tasks like talking and eating difficult and painful. A wide variety of treatments and surgeries have been proposed and undertaken with limited success based on the varying degree of joint dysfunction. The fibrocartilage disc has become a major focus of study because disc displacement and degeneration are the primary causes of TMD, so a better understanding of the disc is required before more effective diagnostic techniques and treatment approaches can be developed. Some of these properties include the tissue biomechanical behavior under various loading conditions, the cellular composition of the disc, and basic cellular metabolic (energy) rates.

The TMJ disc has been found to be distinct from other cartilage types found in the body in regards to primary cell types, extracellular matrix components (ECM), and mechanical properties. These significant differences are attributed to the unique environment and loading conditions of the joint. It is generally believed that pathological mechanical loadings (e.g. sustained jaw clenching or traumatic impact) trigger a cascade of molecular events leading to TMJ disc degeneration and derangement, which are central to many TMJ disorders and pathophysiology. Therefore, the objective of this
research is to investigate the effect of sustained mechanical loading on nutrient transport and cell nutrition of the TMJ disc in order to better understand the biomechanical etiology of TMD. Our general hypothesis is that sustained mechanical loading can alter solute transport and nutrient concentrations in the TMJ disc, resulting in changes to the cellular metabolism, tissue composition, and mechanical function, ultimately leading to disc pathologies.

First, the biphasic mechanical properties of porcine and human TMJ discs were measured to characterize the complex mechanical environment of the joint. Compression and shear experiments were developed to validate the use of the porcine model and to correlate mechanical function with biochemical structure. Significant correlation between aggregate modulus and permeability with water content was found in human confined compression studies. Fluid pressurization was found to play a major role in the load support during dynamic compression and significant frequency dependence during dynamic testing was indicative of the viscoelastic nature of the tissue. These studies highlighted the unique biochemical and mechanical properties of the TMJ disc compared with other cartilage types.

Due to the avascular nature of TMJ disc tissue, transport of nutrients and removal of waste is a major difficulty. The rate of small nutrient (i.e., oxygen and glucose) transport in the TMJ disc is mainly governed by their diffusivities, which depends on solute size, matrix composition, and local mechanical strain. The transport of nutrients was investigated to develop new constitutive relationships between solute diffusivity and tissue hydration to establish strain-dependent transport properties. Our studies showed
that solute diffusivities in the TMJ disc were significantly lower than in other cartilaginous tissues and that compressive strain further impeded diffusion. These findings suggest that a steeper nutrient gradient exists in the TMJ disc and is likely vulnerable to pathological events such as sustained loading due to jaw clenching.

The nutrient gradients are dependent on the balance between the diffusion rates into the TMJ disc and the uptake and utilization by disc cells. TMJ disc cellular consumption rates of oxygen and glucose were measured in a variety of environmental conditions to develop functional relationships between nutrient consumption rates, oxygen tension, glucose concentration, and pH value. Consumption rates were found to be highly substrate dependent with increased concentrations resulting in increased consumption rates. Cell proliferation and matrix protein production were severely inhibited at low oxygen and glucose concentrations suggesting that nutrient environment heavily dictated cell responses and metabolism.

The objective of this project was to characterize the mechanical, biochemical, transport, and consumption properties of the TMJ disc in an effort to better understand TMJ disorders related to pathological loading and disc derangement. Future work for this research involves incorporating the TMJ disc properties into a predictive 3D finite element model of the in vivo TMJ environment. This model can be further developed into a TMD diagnostic tool based on patient specific magnetic resonance images (MRI) and jaw tracking data. This work will help to build new strategies for TMD treatment and can be applied to tissue engineering approaches in other cartilaginous tissues. Therefore, it is necessary to characterize the TMJ disc and its surrounding tissues via
experimental and theoretical research to accurately model the complex properties of native tissue before useful applications can be developed.
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Chapter 1 Introduction

1.1 Understanding TMJ Disorders

The temporomandibular joint (TMJ) is a load-bearing joint consisting of the condyle of the mandibular bone, the fossa eminence of the temporal bone, and a fibrocartilaginous disc held in between the bone surfaces by ligaments (Figure 1). The TMJ disc serves to distribute stress, lubricate movement, and protect the articular surfaces of the joint. The superior and inferior space of the joint is filled with synovial fluid which further lubricates movement during normal joint articulation. The TMJ disc is comprised mostly of water (65~85% wet weight) with a significant amount of collagen (75~90% dry weight) and proteoglycan (5~15% dry weight) [1-5]. Differences in biochemical composition and structure distinguish the disc into three regions: the anterior band, intermediate zone, and posterior band [6]. The fibrocartilage disc is avascular and aneural with intermixed collagen and elastic fibers forming the structure.

Over 10 million Americans are afflicted with TMJ disorders (TMD) that can cause noticeable pain and limited functionality of the jaw. Complications that are associated with TMD include limited jaw movement (sometimes characterized by joint clicking), damage or loss of teeth, as well as inflammation and swelling of the soft tissue. TMJ disc derangement is a common clinical finding (e.g., dislocation of disc) in patients with TMJ disorders [7, 8]. The primary cause of TMD is unknown due to the complexity of the joint and disorder, but numerous non-surgical treatments have been prescribed in an attempt to decrease forces experienced on joint surfaces, reduce pain sensations, or
lubricate movement. Many of the surgical techniques involve replacing or removing components of the TMJ which can either cause new problems or simply not treat the underlying disease.

With disc degeneration, changes in tissue morphology, biochemistry, and mechanical properties occur. Although the etiology of disc degeneration is multifactorial, pathological mechanical loading is believed to be a primary cause. The mechanical forces at the tissue level affect the physicochemical signals at the cellular level. The biological response of the disc cells to these stimuli initiates tissue remodeling, resulting in changes in ECM structure and tissue material properties. Both in vitro and in vivo animal studies have shown that mechanical loading affects the synthesis of matrix molecules, such as GAG, in the TMJ disc [2, 9-11]. It is generally believed that pathological loading, such as sustained mechanical loading during jaw clenching and traumatic impact is the leading cause of TMJ disc derangement. Sustained mechanical loading can cause decreases in fluid load support and increased friction that can result in excessive tissue deformation and wear. However, the mechanobiology of the TMJ disc is still poorly understood, therefore the biochemical and mechanical properties of the TMJ disc microenvironment during loading need to be characterized.

Due to the avascular nature of the disc, nutrients required by cells for maintaining disc health are supplied by blood vessels and synovial fluid at the margins of the disc. The nutrient (e.g., oxygen, glucose, and other small solutes) transport within the TMJ disc primarily depends on diffusion due to the lack of vasculature. Therefore, solute transport plays a key role in cell nutrition and ECM maintenance. Studies have shown
that mechanical stress decreases tissue hydration and the transport rate of water and solutes in fibrocartilage [12]. Consequently, the impact of mechanical loading on nutrient transport was investigated in this research.

Recent dynamic finite element simulation and in vitro measurements have shown that human TMJ discs may deform up to 30% [13-15]. Furthermore, our studies have shown that mechanical strains can not only affect the rate of solute transport, but also the pathway of nutrient transport in cartilaginous tissues. These cellular changes result in decreases in functionality at the tissue level which translate into degraded physical and biological properties of the disc. Due to the avascular properties of the disc, transport rates of solutes are governed by solute diffusivities which largely depend on solute size and material properties of the tissue [16, 17]. The baseline nutrient diffusivities and effect of mechanical strain on diffusivities have not been determined for TMJ discs. Transport of nutrients through the extracellular matrix (ECM) is important in maintaining the normal function of tissues, so deviation from physiological levels can cause tissue necrosis and matrix degradation. Our studies suggested that a steep nutrient concentration gradient could develop in the TMJ disc and this nutrient environment made the tissue susceptible to degeneration resulting from pathological mechanical loading. Our studies addressed this by measuring nutrient (i.e., oxygen, glucose, and small ions) diffusivities under various mechanical strains to determine the relationship of loading on nutritional transport.

The balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells within the tissue determines the resulting concentration
gradient inside the disc. To our knowledge, no study has been performed on TMJ discs to determine nutrient consumption rates, but similar approaches have been undertaken for intervertebral discs (IVD) and knee articular cartilage [18, 19]. The rates of consumption for nutrients (glucose, oxygen, etc.) and production of waste products (lactic acid) depends largely on the solute environment to which the cells are exposed. Previous studies showed that the TMJ was comprised of unique cell populations that were distinct from other fibrocartilage types. This suggested that nutrient requirements could be significantly different in TMJ discs and would need to be fully characterized in order to understand how disc cells maintained homeostasis. Due to the unique nutrient environment it is also necessary to determine the impact on cell proliferation and differentiation on disc cells. Therefore, this project will characterize the biomechanical, biochemical, cellular, and transport properties of TMJ discs to fully develop a model to better understand TMJ pathologies.

1.2 Objective and Specific Aims

The goal of this project was to investigate the effect of sustained mechanical loading on nutrient transport and cell nutrition in order to better understand the etiology of TMJ disorders. Our general hypothesis is that sustained mechanical loading can alter solute transport and nutrient concentrations in the TMJ disc, resulting in changes to the cellular metabolism, tissue composition, and mechanical function, ultimately leading to disc pathologies. Since the TMJ disc is a large avascular structure, transport of nutrients
(e.g., oxygen and glucose) is primarily done through the passive transport mechanism of diffusion. Transport of nutrients and solutes through the extracellular matrix (ECM) is important in maintaining the normal function of tissues, so deviation from physiological levels can cause tissue necrosis and matrix degradation. Therefore, we studied the mechanical environment of the disc by characterizing the viscoelastic properties of the disc tissue during static and dynamic loading. The effect of sustained mechanical loading on nutrient transport rates and nutrient environment were then investigated. The resulting changes in cell behavior, metabolism, and tissue composition were then studied to better understand the biological consequence. In order to achieve these goals, we propose the following specific aims.

**Aim 1:** Characterize the biphasic viscoelastic compression and shear mechanical properties of porcine and human TMJ discs.

**Rationale:** The primary function of the TMJ disc is to provide load support and prevent bone to bone contacts during jaw motion. As the cartilage is subjected to loading during movement, the microenvironment of the joint is significantly altered as fluid flows through the solid components of the ECM and the tissue begins to deform. In order to understand TMJ disc pathologies, it is necessary to characterize the mechanical properties and environment of the disc. Therefore, in this specific aim we will investigate the viscoelastic compression and shear properties of porcine and human TMJ discs utilizing the biphasic model.
Aim 2: Examine the effect of mechanical strain on nutrient transport rates in the TMJ disc.

Rationale: Transport of nutrients is a major difficulty for avascular tissues. The rate of nutrient (i.e. oxygen and glucose, small solutes) transport in the TMJ disc is mainly governed by the diffusivity of these molecules. Deformation of the tissue has been shown to affect the permeability and diffusivity through the cartilage, suggesting that loading can significantly impact the nutrient pathway. Therefore, in this specific aim, we will determine oxygen and glucose diffusivity values of porcine TMJ discs under various strain conditions and develop new constitutive relationships between solute diffusivity and tissue hydration to establish strain-dependent transport properties. It is our hypothesis that mechanical strains significantly impede the nutrient supply through TMJ disc tissues and may result in degenerative biological responses.

Aim 3: Determine TMJ disc cell nutrient consumption rates and cell behavior under various culture conditions.

Rationale: The nutrient gradients are dependent on the balance between the rates of oxygen and glucose transport through the TMJ disc as well as the rates of consumption by the cells within the disc. TMJ disc cells require delivery of nutrients to maintain healthy cell proliferation and differentiation. This study will characterize the cellular consumption rates of TMJ disc cells in order to understand the nutrient supply requirements. It is our hypothesis that the nutrient environment that TMJ disc cells are exposed to has a significant effect on cell behavior and metabolism.
1.3 Organization of Dissertation

The following manuscript is organized in chapters of related studies that combine to form the overall aims of this project. In Chapter 2 we present a comprehensive overview of the anatomy, biochemistry, and function of the TMJ. The symptoms and previous treatment approaches for temporomandibular joint disorders are also outlined. This chapter will also describe the mechanical and transport properties as well as some modeling methods of the disc in the literature. In Chapter 3, the biphasic mechanical properties of porcine and human TMJ discs were measured to characterize the mechanical behavior and environment of the TMJ. These studies were also important in validating the use of the porcine model and to correlating mechanical function with biochemical structure. The results of this study indicated that the TMJ disc was unique in regards to structure and function, suggesting the need for further research to better understand the microenvironment of the tissue. In Chapter 4, the viscoelastic shear properties of porcine TMJ discs were measured to correlate with frequency, strain, and region. These experiments focused on the fluid-flow independent material properties of the disc occurring at small shear strains. The results of dynamic testing indicated the necessity for characterizing the viscoelastic properties of the TMJ disc. In Chapter 5, we measured the regional porcine and human TMJ disc small ion transport properties. These studies indicated that nutrient transport was significantly coupled with mechanical strain and tissue water content. In Chapter 6, we measured the one dimensional regional
glucose and lactate diffusivities under compressive strains. These studies showed that solute diffusivities in the TMJ disc were much lower than the values in other cartilaginous tissues and that compressive mechanical strain can further impede solute diffusion in the TMJ disc. In Chapter 7, the oxygen consumption rates of TMJ disc cells were measured in different environmental conditions and seeded on various substrates to determine the optimum testing protocol. These conditions were used to measure the glucose consumption rates of TMJ disc explants in Chapter 8. These studies showed that consumption rates were significantly dependent on substrate and nutrient environment. Cell behavior including proliferation, differentiation, and energy metabolism were also found to be nutrient concentration dependent. Chapter 9 outlines the overall conclusions of this project and identifies future directions of this work.
Chapter 2 TMJ Disorders, Structure, and Function

2.1 Anatomy and Biochemistry of the TMJ

As with other joints in vertebrate animals, the articulating bony surfaces are protected by thin cartilage layers that prevent bone to bone contacts which can be easily damaged by friction and impact. The superior and inferior space of the joint is filled with synovial fluid which further lubricates movement of the surfaces during normal joint function. The TMJ disc is a large avascular fibrocartilage disc that has connective tissue along the sides that allows for movement of the disc over the bone surfaces in a controlled manner. The lower portion of the disc is composed of the upper part of the mandible bone which is known as the condylar surface or condyle (Figure 1A). The cartilage covering the top of this bone is approximately 0.2-0.5mm thick and forms an ovoid dome shape with fibers directly entering the bone to anchor it to the condyle bone [20]. The upper surface includes the fossa of the cranium and has an even thinner cartilage layer of approximate thickness 0.1-0.3mm. The presence of the disc in the joint capsule prevents the bone-on-bone contact thus decreasing the wear of the condylar head and the articular fossa [4, 15, 19, 21]. The bones are connected with ligaments that completely surround the TMJ and form the joint capsule [11]. The posterior band of the TMJ disc is attached to the surrounding ligaments via thick fibrous tissues with interspersed adipose cells while the medial and lateral portions of the disc attach to the poles of the condyle [22]. Separating the joint cavity into two compartments, the TMJ disc is vital for normal joint function by providing unique support to the movement and
loading in the TMJ. Thickness of the disc varies between 2-4mm and in the literature the disc is separated into various regions based on morphological differences and variation in ECM distribution (Figure 1B). The TMJ disc is at the very center of the joint and the complex biomechanical, biochemical, and cellular properties of this cartilage need to be better understood before suitable replacement discs and therapeutic approaches can be developed to treat TMD.

![Figure 1](image1.png)

**Figure 1** A) Cross-sectional view of temporomandibular joint \[^{[23]}\]. B) Overhead view of TMJ disc outlining specific regions \[^{[24]}\].

### 2.1.1 Fibrocartilage

Fibrocartilage is mainly characterized as avascular and aneural cartilage with intermixed visible collagen and elastic fibers. It has been suggested that these properties are crucial for the compression tolerance of the tissue since applied forces of physiological magnitude would normally close the lumen of blood vessels and could initiate pain responses in innervated tissues. The difference from normal dense fibrous connective tissue is the small amount of amorphous matrix that stains metachromatically with toluidine blue. Fibers may not be normally apparent in hyaline cartilage because the fiber staining is masked by other ECM components \[^{[25]}\]. Fibrocartilage primarily differs
from hyaline cartilage due to the high concentration of collagen type I rather than type II. Another major distinction is potential for hyaline cartilage to form bone through endochondral ossification [26]. Fibrocartilage has been referred to as a transitional or secondary cartilage because it cannot be distinguished from connective fibrous tissue and hyaline cartilage as well as its origins from periosteum or endosteum [26, 27].

2.1.2 Chondrocytes

Cartilage is extensively distributed in fetuses and serves as the template for the skeleton to develop through endochondral ossification, but in adults cartilage mainly provides support for mechanical loads [28]. Cartilage is capable of this task by absorbing shocks and allowing articulation of joints, and failure to accomplish these functions results in pain and restricted mobility of the joint. Cartilage is primarily formed by the production and secretion of proteins from chondrocyte cells. Chondrocytes are capable of producing a high matrix to cell volume ratio with chondrocytes occupying only 10% of articular cartilage volume [25]. The extracellular matrix is composed of collagen, proteoglycans, glycoproteins, and hyaluronan which help to grant the tissue its supportive mechanical properties. Chondrocytes are capable of increasing cartilage tissue volume by proliferating to produce more cells, secreting extracellular matrix components, or increasing in cell volume which normally occurs during hypertrophy (terminal differentiation) [28]. The avascular nature of cartilage tissue means that these cells rely on diffusion to obtain the nutrients to maintain this growth, so the metabolism of the cells is capable of operating at low concentrations of oxygen and glucose at low local pH [1]. All cartilage shares the characteristic of being avascular, so transport of nutrients, waste,
and growth factors has been a major research focus in understanding cartilage development and degeneration.

Using light microscopy techniques, Detamore et al. reported that the TMJ disc is primarily composed of chondrocytes and fibroblasts in a ratio of 2.35 to 1 [24]. While hyaline cartilage is primarily made of chondrocytes and collagen type II, TMJ discs and intervertebral disc (IVD) are comprised of mixed cell types and mainly collagen type I. Characterizing chondrocytes only by shape with light microscopy is not necessarily easy or accurate, so many groups have attempted to detect specific ultrastructure traits to better distinguish cell types in fibrocartilage. Chondrocytes of hyaline cartilage have a clear pericellular matrix and an extensive organelle network with large amounts of rough and smooth endoplasmic reticulum (Figure 2B) [24, 29]. This separate pericellular capsule is not as visible for the chondrocyte-like cells of TMJ discs and other fibrocartilages [16, 27, 30]. The pericellular matrix was found in marmosets and rats, but not in human samples. Berkovitz et al. described the human TMJ disc as a dense fibrous connective tissue with cells found scattered sparsely. The cells found had visible cytoplasm and a moderate amount of organelles for protein synthesis and secretion (endoplasmic reticulum, mitochondria, golgi bodies, and vesicles) (Figure 2C). Collagen profiles were not found in TMJ disc cells as they are normally visible in connective tissues with rapid collagen turnover [31]. This indicates that collagen production and degradation may occur at a slower rate in TMJ cells which is usually more a characteristic of fibrocytes or fibroblasts. The result of this study suggests that the definition of fibrochondrocytes and
fibrocartilage needs to be more clearly defined before cell types can be accurately distinguished in the TMJ disc.

2.1.3 Fibroblasts

Early studies of the TMJ disc discovered non-uniform distributions of distinct cell populations, but without specific cell markers it was difficult to accurately qualify different cell types. Porcine discs serve as the best animal model to simulate human discs due to similar dimensions, cell characteristics, and comparable omnivorous diet. Histological studies by Detamore et al showed that the average cell density was 681 ± 197 cells/mm² with 70% ± 11% being fibroblasts (mean ± standard deviation) [24]. Fibroblasts in the TMJ disc were distinguished by having large nuclei with few organelles; rough endoplasmic reticulum was found in minimal amounts, and smooth ER was observed in even lower quantities (Figure 2A). The fibroblasts were found to be aligned with the collagen fibrils along the periphery while chondrocytes were vastly more abundant in the center of the TMJ disc [24]. Chondrocyte-like cells were also found in abundant concentrations in the center of the TMJ disc corresponding with the greater amounts of chondroitin sulfate and higher compressive modulus found for this region [32, 33]. Increased levels of these types of cells have been found in the inferior joint space which may indicate a more mechanically demanding environment as evidenced by increased levels of degeneration occurring here in some cases of TMJ disorders [34].

Fibroblasts were found primarily at the periphery of the disc and were in the largest quantities at the anterior and posterior attachment sites [35, 36]. This elevated density of fibroblasts may grant the disc significant tensile strength in the anteroposterior
direction which results from the disc being stretched taut and pulling surrounding attachment ligaments [14]. Collagen fibers can be found to run along the periphery of the disc and anteroposteriorly through the center of the disc. This structure may support the notion that the TMJ disc is primarily anchored at the anterior and posterior bands which correlates well with the anteroposterior movement of the disc that occurs during normal joint opening and closing.

2.1.4 Fibrochondrocytes

Cells of the TMJ disc are sometimes referred to as fibrochondrocytes because these cells exhibit chondrocytic markers but lack a well developed pericellular matrix [13]. Cell populations of the disc appear to be more fibroblast-like in nature because the proteinase and proteinase inhibitors are similar to those found in synovial fibroblasts [37]. Immunohistological staining has shown that fibroblasts, chondrocytes, and fibrochondrocytes express different markers in normal and dysfunctional discs. Currently no studies have isolated the various TMJ disc cell types and studied the response of each to various stimuli [13].

Fibrochondrocytes or fibrocartilage cells closely resemble chondrocytes in having visible rough endoplasmic reticulum, glycogen granules, lipid droplets, and intermediate filaments with actin fibers that help organize the cell structure. These cells are capable of synthesizing a variety of ECM components including collagens, proteoglycans, and noncollagenous proteins. The specific types of collagen found include: the fibrillar collagens (types I, II, III, V, and XI), fibril assisted collagens with interrupted triple helices (types IX and XII), and network-forming collagens (types VI and X).
Fibrocartilage cells are also capable of producing various proteoglycans including aggrecan, versican, decorin, biglycan, lumican, and fibromodulin [27].

Figure 2 Transmission electron micrographs of various cell types.  
A) Fibroblast found in TMJ disc showing aligned collagen fibrils and lack of obvious pericelluar matrix.  (R-ER) denotes rough endoplasmic reticulum, (CM) is the cell membrane, and (NM) is the nuclear membrane.  
B) Chondrocyte-like cell from the TMJ disc with very few mitochondria (MC) and organelles. Notable characteristics are the large nucleus, lack of pseudopodia, and indistinguishable pericellular matrix.  
C) Hyaline cartilage chondrocyte with a variety of organelles, extensive smooth (S-ER) and rough endoplasmic reticulum, as well as small amounts of Golgi bodies (G). Pseudopodia are apparent and pericellular matrix (PM) is distinct from extracellular matrix[24].
2.2 Biochemistry

2.2.1 Collagen

Collagens are usually separated into fibrillar and nonfibrillar types of collagen; the fibrillar type aggregates together while the nonfibrillar includes a much more diverse group of collagens that do not form clusters. The fibrillar types include I, II, III, V, and XI and form highly ordered overlapping fibrils that contribute to the tensile strength of tissue [27]. Non-fibrillar collagens serve a variety of functions including stabilization of membranes, assisting in angiogenesis, and interactions with other ECM components [17].

Collagen comprises approximately 30% of the TMJ disc wet weight, 83 to 96% of the dry weight and roughly 50% of the volume [10, 38-40]. Random collagen fiber orientation was found on the surface of the disc while deeper layers showed directionality and regional variation. The average collagen fiber diameter was 18 μm with a standard deviation of 9 μm [9]. Collagen fiber diameter varies between regions and species, but it is assumed that larger fibers are more characteristic of tissues primarily subjected to tensile forces [41]. Collagen forms in bundles of fibers that are aligned in a ring-like fashion around the periphery of the disc and primarily run anteroposteriorly in the central regions of the disc [13]. These fibers exhibit a wavy and crimped pattern that can be observed across the full thickness of the disc [3, 5, 42, 43]. Scapino et al. found that only minor changes in the crimp and alignment of the collagen fibers occurred during stress relaxation and strain. They hypothesized that the drag against water flow and the solid
matrix (collagen and GAGs) resulted in the unique viscoelastic mechanical properties of the disc [31].

The TMJ disc is primarily composed of collagen type I with only minute amounts of collagen type II, the primary collagen found in hyaline cartilage. Collagen content has been found to vary significantly by region and species (Table 1) [6, 18, 35, 36]. For primate TMJ discs, the collagen type II that is detected, is mainly localized at the periphery of the disc or near chondrocyte-like cells [36]. The anterior and posterior bands were primarily composed of collagen type I with very weak staining of collagen II. In the intermediate zone collagen type I staining was abundant, but clusters of collagen type II were intermixed that measured a couple hundred microns in diameter. In porcine discs, collagen type II was found in significantly lower amounts than type I with the largest concentration found in the intermediate region [9]. Collagen types III, VI, IX, and XII have been detected, but collagen type I is the dominate ECM component of TMJ discs [2, 6, 7].

**Table 1** Regional collagen content of various animal models [23].

<table>
<thead>
<tr>
<th>Authors</th>
<th>Location</th>
<th>Content (mg/g)</th>
<th>Species</th>
<th>Dry/wet weight</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior band</td>
<td>342 ± 10</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Snorum attachment</td>
<td>365 ± 18</td>
<td></td>
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<tr>
<td></td>
<td>Medial attachment</td>
<td>372 ± 18</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Lateral attachment</td>
<td>360 ± 17</td>
<td></td>
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<tr>
<td></td>
<td>Posterior attachment</td>
<td>368 ± 21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berkovitz and Robertshaw (1993)</td>
<td>Periphery (anterior)</td>
<td>52.3 ± 6.7% (v/v)</td>
<td>Rabbit</td>
<td>Dry</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td></td>
<td>Center</td>
<td>58.0 ± 6.6% (v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gage <em>et al.</em> (1990)</td>
<td>Posterior attachment</td>
<td>377 ± 21</td>
<td>Human</td>
<td>Wet</td>
<td>Electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Lateral attachment</td>
<td>372 ± 18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disc</td>
<td>304 ± 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nakano and Scott (1989)</td>
<td>Disc</td>
<td>830</td>
<td>Bovine</td>
<td>Dry</td>
<td>Hydroxyproline</td>
</tr>
</tbody>
</table>
2.2.2 Elastin

The elastin content of TMJ discs varies between species, but in general is a very low percentage of the dry weight. Studies found that bovine discs contained 3-7% total mass and only 0.339% total volume in humans [8, 44]. Elastin was found in greater amounts on the superior surface and periphery of the disc indicating significant heterogeneity [12, 44]. Two studies specifically found more elastin fibers in the posterior region and attachments of the disc [9, 12]. Elastin is sometimes found along side collagen fibers and is assumed to help the disc retain and restore form after loading [45]. The low number of elastin fibers is unlikely to contribute significantly to the tensile or compressive properties of the disc [36, 42]. The elastic fibers have also been found to marginally resist tensile force for strains up to 50% which are beyond the normal range in discs [42]. While the elastin fibers do not directly contribute to the mechanical properties many groups have suggested the distribution of these fibers is an indicator of the regions subjected to the most stretching and recovery [45].

2.2.3 GAG/Proteoglycans

The compressive properties of the disc are primarily due to the proteoglycan and water content while collagen imparts the tensile characteristics. Proteoglycans are composed of a protein backbone with glycosaminoglycan side chains that are extremely hydrophilic. This attribute slows the flow of water out of the ECM and allows the tissue to support relatively large instantaneous forces due to the incompressibility of water [13].
Other less hydrophilic proteoglycans with fewer GAG chains act as guides for the alignment of collagen fibers which leads to the anisotropy of the tensile properties. Collagen fibers confine aggregan molecules within the ECM to create a meshwork that helps trap water and resists compression of the tissue [46]. The gel-like properties of aggregan may also help protect ECM proteins from degradative enzymes and serve as a slow-release reservoir for other molecules during deformation [27]. In TMJ discs, hyaluronic acid concentrations are much lower than chondroitin-6-sulfate, chondroitin-4-sulfate, dermatan sulfate, and keratan sulfate concentrations which make the composition distinct from hyaline cartilage [9]. Studies on GAG content of the disc have found it to vary between 1-10% throughout the disc with an average at or below 5% [9, 36, 47-49]. Chondroitin sulfate and dermatan sulfate account for 75-93% of the GAG content of the disc, while keratan sulfate and heparin sulfate have only been found in trace amounts [9, 33, 47, 49]. Detamore et al., (2005) found that the overall GAG content of discs was 5.3±1.2% of the total dry weight and only 1.5±0.3% of the wet weight. Chondroitin sulfate was 4.4 times more abundant than dermatan sulfate, 8.2 greater than keratan sulfate, and 164 times more prevalent than hyaluronic acid. No significant differences between inferior and superior layers were found for GAG content [9].

The exact GAG distribution is unknown because each group that has studied GAG content used different quantification approaches and animal models which resulted in several contrasting results (Figure 3). Almarza et al., (2006) reported high GAG content in the medial region compared to the anterior and lateral regions with the lowest concentrations in the posterior region. This study used dimethylmethylene blue
colorimetric staining to quantify the total GAG content per dry weight of porcine discs [48]. Detamore et al., (2005) reported similar results of low GAG levels in the posterior region and increasing content in the lateral to medial direction. The trends may have been the similar, but the actual quantities reported varied by as much 6% [9, 48]. A study on the bovine discs showed greater amounts of GAG in the central regions compared to the periphery of the disc and these results were similar to the distribution found with human discs [47, 50]. Mills et al. found directly contrasting results in primate discs with the anterior and posterior regions containing the largest GAG concentrations [36]. Although the exact distribution is heavily debated, most groups agree that the overall GAG content is relatively low in TMJ discs compared to the amounts found in hyaline cartilage [51].

With the wide range of results for the regional concentration of GAGs it is difficult to determine the relationship between biochemical makeup and mechanical properties. The primary hypothesis is that the side chains on large GAG molecules maintain a large negative charge that attracts and holds water impeding fluid flow, thus increasing stiffness and compressive modulus of the disc [52]. Although some groups have suggested that since GAGs comprise less than 10% of the total dry weight of the disc that they may only play a minor role in contributing to the compressive properties [13, 48, 53].
2.2.4 Water Content

Water content is a large percentage of the total weight of the TMJ disc and is believed to contribute significantly to the compressive strength when fluid flow is impeded by the solid components of the ECM. The incompressibility of water and attraction to hydrophilic ECM components has been hypothesized to be a major component of TMJ mechanical function. The overall water content is around 71±2% of the total mass of the disc and some regional differences were observed (Figure 4). In the mediolateral direction, the medial region showed the highest water content (75.3±2.1%) while significantly less water was found in the central and lateral regions (71.3±3.7% and 71.3±4.1%, respectively). In the anteroposterior direction, the anterior band (74.5±2.9%) and intermediate region (73.7±3.1%) had significantly higher water content than the posterior band (70.1±4.0%). No significant differences were found between the superior (73.0±4.1%) and inferior surfaces (72.6±3.6%). Regions of higher chondroitin sulfate correlated with higher water content likely a result of the highly charged side chains. Studies also found significant regional variation in compressive stiffness as a result of
water content distribution linking biochemical composition with mechanical property anisotropy [9, 45].

![Regional water content on the superior surface of the TMJ disc](image)

**Figure 4** Regional water content on the superior surface of the TMJ disc [9].

### 2.3 Function-Correlation between Structure and Composition

Studies have found collagen type II and chondroitin sulfate proteoglycans primarily localized near chondrocyte-like cells suggesting that these cells maintain small regions of hyaline-like ECM within the disc [9, 36, 48]. The fibroblast-like cells have mainly been found near collagen type I fibers and dermatan sulfate proteoglycans [24, 35, 36]. Since these types of cells make up the majority of the cellular components of the disc (~70%), they are most likely responsible for producing and maintaining the ring-like collagen structure found in the disc periphery [13, 24]. Little direct correlation has been found between cell distribution and mechanical properties as some regions of high chondrocyte-like cell density show significant tensile strength [54-56]. The posterior and lateral regions showed significant resistance to shear and compressive stresses, but lack high levels of chondroitin sulfated GAGs that are normally present in regions that support this loading type [24, 48]. The intermediate zone was found to have greater levels of
fibrochondrocytes, total collagen (specifically type II), and GAGs than the anterior and posterior bands. The intermediate zone was an order of magnitude softer than the anterior and posterior bands when stretched in tension mediolaterally [9]. These results show that cell type is strongly correlated with the ECM composition, but does not correlate well with the mechanical properties of the region [13].

Many groups theorize that chondroitin sulfate distribution is responsible for the tissue’s capacity to support compressive loading due to the large negatively charged GAG chains that are very hydrophilic [13]. Although many studies have attempted to correlate total GAG content or more specifically chondroitin sulfated proteoglycan content with the compressive properties of TMJ discs, no significant conclusions have been achieved [57-59]. The low percentage of proteoglycans and high concentration of collagen led some researchers to hypothesize that stretched collagen fibers provided the compressive loading support as they were strained transversely [13, 42, 52, 57].

2.4 Age Effect on Biochemistry

Studies on the changes to biochemical composition with age have shown that calcium, collagen, and sulfate levels increase during the developmental stages of many animals [33, 47, 60, 61]. Chondroitin sulfate and keratan sulfate levels increase dramatically [33]. Just as with the controversy associated with the composition and distribution of ECM components, dissenting opinions on the age impact on elastic fiber content exists as well. Minarelli et al. found elastic fibers to decrease with age while
Nagy et al. observed a build-up over time [6, 62]. A study on the relation between relaxation time and age found that increases in age increased the relaxation time [63]. Also Lai et al. found that the ratio of collagen to water content increased with age and resulted in increased shear modulus [64]. These studies in animal models suggest that age significantly impacts biochemical composition resulting in changes in tissue mechanical properties.

2.5 TMJ Disorders

Temporomandibular joint disorder (TMD) is a term that describes over 20 pathological conditions. The most common form, which affects roughly 70% of TMD patients, involves internal derangement or disc displacement [65]. TMJ disc displacement can cause erosion of the condyle cartilage resulting in the degenerative process of arthritis. Osteoarthritis can cause further deterioration of the joint cartilage and lead to pain and loss of jaw function [66, 67]. Non-surgical treatments have shown poor results for patients suffering from severe osteoarthritis leaving the only options as partial or total TMJ implants [68]. At the current time, these implants have suffered catastrophic failures as a result of wear, loosening, and immune responses [69-71]. As a result, numerous implant replacements and corrective surgeries are required over the course of the patient’s life [69].

There are a number of hypothesized causes for TMD including: direct injury to the joint or muscles of the jaw, grinding or clenching of the teeth, disc displacement,
aging, and/or development of arthritis [19, 72]. TMJ disorders are primarily treated in female patients between the ages of 20 and 40 years with the ratio of female to male patients being reported anywhere from 3:1 to 8:1 [73, 74]. This discrepancy in treatment by gender has been attributed to hormonal differences because these symptoms primarily appear during childbearing ages in females. Sex hormones have already been proven to affect differentiation, proliferation, and metabolism in connective tissues including cartilage [74, 75]. One study that directly sought to characterize the impact of sex hormones found that female rats produced significantly lower levels of collagen in controls, and this difference disappeared for ovariectomized groups [76]. This suggests that female sex hormone concentrations directly affect the biochemical composition of the TMJ which has been hypothesized to alter the biomechanical properties of the disc. At the present time, it is unclear the exact pathway and receptors responsible for the TMD gender paradox, but its existence clinically and experimentally in animal studies suggests that further research is necessary [77].

2.5.1 Disc Displacement and Other Derangement

Of the estimated 10 million Americans that receive treatment for TMJ disorders, roughly 70% of these patients are believed to suffer from disc displacement [24]. For normal function of the TMJ, the disc and condyle must maintain coordinated movements or pathological loading may occur in the joint [65]. During disc displacement, the disc shifts from the normal conformation and can cause bone to bone contacts that may permanently damage the cartilage surfaces. The initial symptom of disc displacement is sometimes a clicking or popping sound that is caused by the disc leaving alignment as the
mouth opens and then again when the disc is forced back into the joint cavity during jaw closing [72]. Disc displacement is sometimes categorized into two types: 1) with reduction, where the displaced disc returns to its normal position while the mouth is open and 2) without reduction, where the disc remains in its abnormal conformation and even impedes jaw opening sometimes resulting in joint locking (Figure 5) [78]. A study performed by Tanaka et al. found that stress distributions were significantly different between normal and anteriorly displaced discs [72]. Changes to internal joint stress distribution have been suggested to increase friction between articular surfaces causing permanent tissue damage which may be related to the high frequency of osteoarthritis after diagnosis of internal disc derangement [19, 72, 79, 80].

Wilkes et al., (1978) developed a system of classification for TMJ derangement or displacement that utilized clinical and radiological findings as well as anatomic pathology of the joint. Early stage TMD was classified as slight displacement of the disc with healthy tissue and possible clicking during movement. The early intermediate stage is where pain is noticeable, and the disc begins to be visibly deformed in radiograms. The intermediate stage is qualified by recurring pain, possible joint locking, and formation of hard disc tissue. The late intermediate derangement of the disc shows increased pain, flattening of bone surfaces, and greater remodeling of the disc. The last stage of degeneration shows significant loss of joint function, disc perforations, and gross degeneration of cartilage and bone surfaces of the joint [81, 82].
Figure 5 Schematic of anterior disc displacement with reduction (ADDWR) and without (ADDWOR) [78].

2.5.2 Fibrocartilage Degeneration

Cartilage degeneration in most cases is known as arthritis and can be divided into two classes: inflammatory rheumatoid arthritis (RA) and non-inflammatory osteoarthritis (OA). The common symptoms of these diseases are loss of cartilage ECM and large presence of matrix metalloproteinases (MMPs) and disintegrins. These enzymes cleave sections of collagen and proteoglycans of the ECM, so treatments have previously focused on blocking these pathways [1]. Arthritis of the TMJ can also result in tissue inflammation in a condition known as synovitis [53]. Degenerative diseases of the TMJ cause the slow breakdown of joint tissues over the course of several years, and the tissues affected are not capable of rapid regeneration, so early detection methods are very important in the treatment of arthritis [83]. Symptoms of degeneration are similar to those experienced during disc displacement and include: pain, stiffness, joint noise, and restricted movement [84]. Distinguishing features of arthritis must be determined via radiographic, histological, or biochemical techniques [85].
Osteoarthritis is the most common form of arthritis, and is characterized by the degeneration of the cartilage and tissue remodeling of adjacent bone surfaces [86, 87]. The pathophysiology of the disease can be somewhat determined by the molecular and cellular components found in the synovial fluid of the joint. These include cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor generated by monocytes and macrophages arriving in the damaged tissue. Inflamed synovial membrane tissue has been found to produce abnormal synovial fluid that lowers viscosity which detrimentally affects normal lubrication of the joint [88, 89]. Since there are no biomarkers to detect osteoarthritis of the TMJ currently, diagnosis is usually done with imaging techniques that can only detect irreversible damage of the tissue and bone. Earlier detection methods and treatments are currently being researched in animal models due to the impracticality of acquiring pre-osteoarthritic samples from humans [90].

2.5.3 Causes

Studies on animal TMJ discs have shown that changing biomechanical stresses can cause tissues to quickly adapt to maintain efficiency of the joint. Loss of this ability can be due to age, tissue injury, hormones, or a pathological joint environment (i.e. abnormal loading or poor cellular nutrition) [91]. Normal physiological loading has been shown to help maintain normal growth and development of the TMJ while excessive hydrostatic stresses result in catabolic effects [92]. One interesting result of mechanical stresses in the cartilage tissue is the production and accumulation of free radicals [93-95]. These molecules have unpaired electrons in their outer orbits that can cause free-radical mediated reactions that damage ECM and cellular components through
activation of degenerative cell processes [93, 94, 96]. Appearance of free radicals has been seen during normal masticatory function as well as during abnormal movements such as clenching, and if cells are unable to keep these potentially harmful molecules in check then pathological outcomes may occur [94].

Another possible cause of TMD is the loss of physiological nutrition transport within the disc. The living cells of the TMJ disc require nutrients to maintain growth and development and waste products must be removed to keep the environment from becoming toxic. Since the TMJ disc is avascular, solutes must be transported passively via diffusion to all of the cells. Studies have shown that sustained loading decreases the rate of diffusion and transport properties of the cartilage significantly, thus decreasing the nutrient levels deep within the tissue. The local pH can also significantly drop as lactic acid builds up and the cells begin to die at low nutrient levels (i.e. oxygen and glucose) within 24 hours [97].

2.5.4 Treatments

Usually the treatment begins with conservative, non-surgical therapies first, with surgery left as the last option. The majority of TMD patients can be successfully treated by non-surgical therapies and surgical interventions may be required for only a small portion of the TMD population. Some of the ways that patients can personally alleviate pain and keep symptoms from worsening are performing jaw exercises, avoiding extreme or rapid movements, applying hot and cold compresses to the region, and eating soft foods. Patients may also be given splint devices to fit over the teeth to prevent the effects of clenching and grinding. Grinding of the teeth or bruxism is believed to cause TMD
through tooth erosion, muscular strain, and inflammation of the tissue within the joint space [11]. Splints are therefore prescribed in the hopes of controlling the consequences of bruxism and hopefully slow the onset of TMDs [98, 99].

2.5.4a Arthrocentesis

The simplest type of surgical procedure to treat symptomatic TMD is arthrocentesis, during which a needle is inserted into the damaged joint and fluid is used to wash the cartilage surfaces. The surgery only requires local anesthetic and is primarily used to treat patients with sudden restrictions in jaw movement with no prior TMJ problems [100]. Another similar method involving the use of a blunt instrument to physically move a stuck disc from abnormal conformation has also been prescribed [11, 100]. These techniques are mainly used to treat the earliest and least severe symptoms of TMD.

2.5.4b Arthroscopy

Another procedure for treating TMD is arthroscopy, which involves making a small incision in front of the patient’s ear and inserting thin instruments to manipulate the displaced or damaged disc. The instruments are capable of being connected to a video screen and enable the surgeon to examine the joint in vivo without making large incisions. The physician can then make the decision to remove inflamed tissue or simply push the disc back into place, but if the ligaments have been stretched beyond their elastic range then the results may only be temporary [11]. To help treat this problem, physicians sometimes implant a device known as a mini anchor into the bone of the condyle [101,
This device showed a success rate of 90% in reducing pain, providing occlusal stability, and increased jaw opening [102]. The anchor has also shown osseointegration and has been reported to be biocompatible for up to 59 months [103]. The combination of arthroscopy and anchors has been shown to significantly reduce pain and disability as well as improve jaw function and motion.

2.5.4c Joint Replacements

Patients suffering from severe TMD sometimes undergo the removal of the diseased disc through a discectomy procedure. This method has shown poor outcomes due to the increased loading on the bone surfaces without the disc to help reduce friction and impacts [19, 104]. When all other treatments fail to treat the symptoms of TMD, patients may receive either a partial or total joint replacement. If only the condyle or fossa is replaced then the surgery is considered partial, but if both are replaced then it is a total joint replacement [19]. Some particular disease cases are usually treated with joint replacement including: bony ankylosis, necrosis of the condyle, multiple previous surgeries, tumor formation, and advanced rheumatoid arthritis [70, 105-107]. Long-term studies on joint replacements have supported the safety and effectiveness of the treatment when patients have exhausted all other forms of treatments [11, 108].

2.5.4d Disc Implants

When discussing implantable devices, it becomes necessary to outline the required properties of the material to be safely used on patients. Specifically for the TMJ the material must be biocompatible, show low wear properties, be capable of fitting
anatomical structures, remain stable, and be corrosion resistant [102, 107]. These issues were not fully addressed in the Proplast implant of the 1980s that created wear particles and caused severe foreign body cell reactions. The Proplast-Teflon TMJ implant caused extensive foreign-body cell reactions that were significantly worse than prior to implantation [109]. The cells that accumulated in the region produced an immune response that caused the destruction of the surrounding bone and decreased the stability of the implant. Eventually the FDA issued safety alerts urging against the use of the device and sought yearly evaluations of current Proplast patients to monitor if implants needed removal [110]. Ultra-high molecular weight polyethylene (UHMWPE) has recently been used as a TMJ disc replacement because of its low wear properties and excellent biocompatibility [111, 112]. Testing by van Loon et al. determined that UHMWPE wear rates were 100 times lower than for Proplast/Teflon disc replacements when used in conjunction with a stainless-steel condyle surface [112]. The results of these experiments suggest that synthetic materials can be found to replace damaged tissue with temporary success, but a biomaterial that reintroduces normal, healthy tissue is the ultimate solution for TMD [113].

2.6 Mechanical Properties

TMJ discs are subjected to a variety of loading forces during normal jaw movements which can be categorized into compression, tension, and shear. Compressive loading decreases the height in the loading direction, while tension stretches the tissue,
and shear stresses one surface in relation to the stationary parallel surface. As the disc deforms, internal forces are created that act on the ECM components and cells of the tissue which results in a cascade of physiological responses [52]. The physiological level of strain varies between tissues; some examples include skin at 40%, tendons at 2-5%, and around 4% for canine TMJ discs [114, 115].

Biological tissues subjected to loading exhibit both elastic and viscous responses, and are collectively known as viscoelastic materials. The changing viscoelastic properties over time are usually related to the fluid flow within and out of the disc. Viscoelasticity is normally tested with stress-relaxation, creep, or restoration experiments which provide information on the mechanical properties as a function of time. These properties are related to how well the tissue absorbs stress and dissipates energy [52]. Stress-relaxation involves deforming the material to a set strain and recording the time required for a steady state level of stress to be achieved. The relaxation modulus is obtained by dividing the stress at steady state with the relaxation time. Creep testing involves applying an instantaneous amount of stress and maintaining it while time-dependent strain levels are recorded. Restoration tests involve applying a load then characterizing the response of the material after loading is removed. Numerous models for the behavior of TMJ discs have been proposed by assuming the material to be linearly elastic, but the time-dependent response of cartilage suggests that more complicated models are required to accurately represent mechanical behavior [64, 116-118].

The determination of the mechanical properties of TMJ discs is important for the development of finite element models and design of implantable devices. Due to the
difficulty in obtaining human tissue, and the increased chance of degeneration in cadaverous tissue, most researchers have focused on developing an applicable animal model to represent human TMJ tissues [77]. Porcine discs are currently the best representative due to their similar anatomical shape and resemblance to human discs [119-121]. Porcine discs are also readily available and very inexpensively obtained from local abattoirs. Some of the known differences between human and porcine discs include biochemical composition, chewing frequency, and intra-joint forces experienced [80, 122]. Some of the many factors that can affect the biomechanical properties measured in tissue include age, gender, weight, and animal model used for experimentation. Tissue loading history has also been observed to alter mechanical properties of condylar cartilage by inducing tissue remodeling and adaptation [123, 124].

2.6.1 Compression

Tanaka et al., (2003) found that the compressive elastic modulus was lower than the tensile modulus which suggests that the collagen fibers impart more elasticity on the disc than the proteoglycans. The disc also appeared stiffer during static loading than dynamic loading because the fluid flowed out of the disc during the first few cycles of dynamic loading [52]. Larger compressive moduli were observed for the inferior surface specifically in the posterior region [58]. Finite element models have shown that tension is prevalent in the superior side of the disc, most notably in the posterior and central regions [72]. Large shear and compressive forces were observed in FEM for these regions which correlate well with the TMJ perforation study findings in cadaverous tissues [21, 79, 125, 126]. Translational forces applied mediolaterally across the TMJ
disc may be one cause of tissue failure because traction forces are applied at the surface where the disc is relatively weak [127]. Traction forces in the TMJ are composed of friction and plowing forces, which result from movement and fluid pressurization due to loading [128]. Studies have shown that plowing forces are approximately 10 times greater than frictional forces [129, 130].

2.6.2 Anisotropy and Regional Variation

TMJ discs are often referred to as viscoelastic materials due to the time-dependent stress-strain relationships shown during mechanical testing. Discs also exhibit regional variations in composition and structure that result in the anisotropic and heterogeneous nature of the disc cartilage [9]. These have been almost universally established as the intermediate zone, anterior band, and posterior band. Compressive and tensile properties vary between regions and testing directionality due to the ECM structural and biochemical differences across the disc. Differences have also been observed between the inferior and superior surfaces of the TMJ disc during testing. Condyle cartilage has also been characterized as viscoelastic due to creep, stress relaxation, and hysteresis responses shown during tensile testing [131, 132]. The unique composition and mechanical properties of the TMJ cartilage result in significantly different physical responses when compared to other cartilages, including IVD and knee meniscus [26, 51].

Studies on the TMJ disc are primarily focused on three areas: tissue biomechanical properties, finite element models of loading, and kinematic models of TMJ motions. Biomechanical properties of large animal models have been determined in compression, tension, friction, impact, and traction loading modalities [13]. A significant
amount of variation (sometimes 100-1000 times differences) exists between results due to differences in species and testing methods. Allen et al. determined that the unconfined compression instantaneous modulus of porcine discs was ~500kPa while the relaxation modulus was approximately 30kPa [57, 58]. Kim et al. found similar results when testing porcine discs under creep indentation with the results showing an aggregate modulus of around 20kPa [59]. One group reported the elastic modulus for human discs as nearly 2MPa during creep indentation which is 10 times greater than the results for porcine discs [13]. One possible explanation for the variation is that these tissues show non-linear strain stiffening during incremental stress relaxation testing. Higher strain levels were achieved during the human creep indentation testing and the material exhibited stiffer material properties as a result [57, 58]. Since similar stress relaxation time constants were estimated from both experimental procedures, it is still safe to assume that porcine tissues can be used as an appropriate animal model [13].

2.6.3 Biomechanical Behavior Differences between Species

The compressive properties of other animal models were found to be significantly higher than those of porcine samples. Unconfined compression tests on canine samples were found to have instantaneous modulus values of around 31MPa while stress relaxation tests on bovine discs yielded an instantaneous modulus of ~15MPa [117, 133]. Specific differences in protocols such as strain rate, frequency rate, and amplitude have all been found to have a significant impact on the compressive properties of TMJ disc tissue, but it is assumed that most of the variation is due to interspecies differences in disc composition [57, 58, 113, 134]. Regional and topographical variation during
compression has been observed for TMJ discs of several species. When tested under similar conditions, bovine and porcine samples both exhibited large instantaneous moduli in the anterior region and large relaxation moduli for the medial region. However, the porcine samples had significantly lower moduli in the lateral region and large instantaneous modulus in the posterior region while these differences were not seen in the bovine model [57, 58, 133]. The general assumption is that the compressive properties vary between the mediolateral and anteroposterior directions, but the exact values and relationship are currently debated in the literature [13].

2.6.4 Dynamic Loading

Static loading of the disc represents clenching or grinding of the teeth while a dynamic loading protocol more closely models talking or chewing [52]. When the TMJ disc is cyclically loaded, the material achieves steady-state within the first 10 cycles, but a small amount of secondary creep has been noticed and is considered negligible to the overall response to loading [32]. It has been noted that the hysteresis loops of the first few cycles of loading and unloading may vary significantly, so most dynamic testing refers to this period as pre-conditioning requiring 7 to 10 cycles before steady-state can be attained [135].

Perfectly elastic materials have a phase angle ($\phi$) of 0°, where the stress and strain are perfectly in phase while perfectly viscous materials can achieve phase angles of 90°. The viscoelastic nature of disc tissue means that the stress response to cyclical strain is not completely in or out of phase, but rather somewhere in between 0 and 90°. An important parameter that can be obtained by applying a sinusoidal strain is the complex
dynamic modulus $E^*$ which consists of the real portion known as the storage modulus ($E'$) and the imaginary loss modulus ($E''$). The magnitude of the complex modulus can be determined by solving the equation $|E^*| = \delta \sigma / \delta \varepsilon$. The storage and loss moduli are determined with $E^* = E' + iE''$ where $E' = |E^*| \cos \delta$, $E'' = |E^*| \sin \delta$, $i = \sqrt{-1}$, and $\tan \delta = E''/E'$ is the loss tangent. The storage modulus represents the elastic portion of the deformation and is proportional to the energy stored during one cycle of deformation. The loss modulus represents the viscous portion and is related to the amount of energy lost or dissipated during one cycle. The tangent of the phase angle (loss tangent) is therefore the ratio of the energy lost to the energy stored during deformation, so materials with a high loss tangent exhibit a greater viscous response as a result of the internal structure and composition [52].

2.6.5 Tensile

The ultimate strength of a material is defined as the maximum stress that can be applied before fracture or continued deformation with decreasing load support. Since TMJ discs are regionally anisotropic, the testing direction has a significant impact on the tensile properties. For example, the intermediate zone tested in the anteroposterior direction the ultimate strength was 37.4MPa, but in the medio-lateral direction was only 1.6MPa [136]. For canine samples, the central zone (14.7MPa) was found to have significantly lower ultimate tensile strength than the anterior (46.7MPa) and posterior region (69.7MPa) (Table 2) [115]. Dynamic testing on the TMJ disc has been characterized at various strain and frequency levels, so direct comparison is difficult.
Many groups used 5-10% strain amplitude because studies have shown this to be the maximum strain during clenching [137]. The strain rate during normal chewing has been estimated around 0.5-1.5Hz and possibly higher during activities like gum-chewing [138].

Most studies on the tensile properties of the TMJ disc are performed in the non-linear low strain regions, referred to as the ‘toe region’, which is from 0 to 6% strain [14]. Increases to the starting level of strain serve to increase the loss and storage moduli until the linear region of the stress-strain curve is reached where these moduli remain relatively constant. Snider et al., (2008) examined the effects of dynamic tensile testing on TMJ discs and found the tissue to exhibit significant anisotropy and heterogeneity based on the direction of testing. Significant strength was found in the anteroposterior direction and is primarily transmitted through the attachments in these regions [139]. The significant difference in tensile properties between regions and directions has been attributed to the directionality of the collagen fibers [14, 115]. This is exemplified in the intermediate region where collagen fibers run anteroposteriorly, so the tensile properties in the mediolateral direction are significantly lower than in regions where fibers run in the direction of tension [139].

The storage and loss moduli generally increased as the frequency increased from 0.1 to 10 rad/sec then decreased from 10 to 100 rad/sec. Loss tangent or viscous damping occurred at low frequencies and leveled off around 10 rad/sec. Storage modulus decreased around 70 rad/sec, but this drop-off has been found to be related to the sample length between the testing grips. Several groups have found that dynamic testing creates
resonance effects which impact the mechanical properties observed. This phenomenon has been seen in other tissues including muscle, tendon, pericardium, and even synthesized gels of hyaluronic acid and collagen [139].
Table 2  Summary of the tensile modulus of the TMJ disc in various animal models [23].

<table>
<thead>
<tr>
<th>Authors</th>
<th>Location</th>
<th>Modulus (MPa)</th>
<th>Strain rate (mm/min)</th>
<th>Animal model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detamore and Athanasiou</td>
<td>Anterior band</td>
<td>9.48 ± 3.32(^a)</td>
<td>6</td>
<td>Porcine</td>
</tr>
<tr>
<td>(2003)</td>
<td>Intermediate zone</td>
<td>0.58 ± 0.39(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Posterior band</td>
<td>23.4 ± 6.5(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>14.3 ± 3.7(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>18.5 ± 4.9(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>10.6 ± 3.0(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanaka et al.,</td>
<td>Medial</td>
<td>25.9 ± 2.6</td>
<td>62.4</td>
<td>Bovine</td>
</tr>
<tr>
<td>(2003)</td>
<td>Central</td>
<td>22.9 ± 3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>24.0 ± 2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanaka et al.,</td>
<td>Central or medial</td>
<td>47.1 ± 11.2</td>
<td>—3</td>
<td>Human</td>
</tr>
<tr>
<td>(2003)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beauty et al.,</td>
<td>Center</td>
<td>3.2 ± 0.4</td>
<td>300</td>
<td>Porcine</td>
</tr>
<tr>
<td>(2001)</td>
<td></td>
<td>75.4 ± 3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.3</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Tanaka et al.,</td>
<td>Medial and central</td>
<td>60.5 ± 9.3(^a)</td>
<td>1.2</td>
<td>Human</td>
</tr>
<tr>
<td>(2006)</td>
<td></td>
<td>95.7 ± 19.4</td>
<td>~5</td>
<td></td>
</tr>
<tr>
<td>Tunne et al.,</td>
<td>Medial</td>
<td>91.9 ± 10.4</td>
<td>0.1 N/s</td>
<td>Canine</td>
</tr>
<tr>
<td>(1991)</td>
<td>Central</td>
<td>101.1 ± 22.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>83.7 ± 16.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shengyi et al.,</td>
<td>Anserior band</td>
<td>30(^a)</td>
<td>Not specified</td>
<td>Canine</td>
</tr>
<tr>
<td>(1991)</td>
<td>Intermediate zone</td>
<td>18.8(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Posterior band</td>
<td>30.1(^a)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AP, anteroposterior; ML, mediolateral.
\(^a\)All errors are standard deviations, except Beauty reports standard errors of the mean.
\(^b\)Equilibrium modulus.

2.6.6 Compression

During the first few cycles of dynamic compression, the amount of hysteresis varies significantly due to preconditioning. Steady-state properties were found to be achieved around 10 to 15 cycles with less than 5% variation between consecutive stress-strain curves. These trends support the hypothesis that the TMJ disc is crucial for shock-absorption which occurs during sudden loading due to impacts [32]. The amount of energy dissipated between cycles was sometimes reduced by 20-60% which suggests that fluid forced out of the solid matrix is slow to return in between compressions. This phenomenon becomes more obvious during higher frequencies similar to the loading
during activities like talking or chewing [53]. The amount of energy dissipation was found to be significantly dependent on strain amplitude, while frequency only affected energy dissipation during tests performed at 5% strain [53]. The overall trend is that the central region has a greater dynamic compressive modulus and more rapidly dissipates energy than other regions; this trend correlates with the prediction that the central region undergoes the greatest amount of compression during normal joint movement as shown in models (Table 3) [53, 140].

Studies have shown that high magnitude sustained stress may induce catabolic events that impact the biochemical and mechanical properties of the tissue. Intermittent loading within the physiological range was found to be required for maintaining homeostasis of the TMJ cartilage and may be dependent on the stress reduction associated with the energy dissipation in the tissue [92, 131]. Increases in strain amplitude during dynamic compression increased both the instantaneous and relaxation moduli. During small strains the hydrostatic pressure of the interstitial fluid associated with the hydrophilic components of the ECM allows the compressive force to be sustained without significant tissue deformation or fluid flow [141, 142]. High compressive strains cause fluid to exit the tissue and more of the load is carried by the solid matrix, but the exuded fluid is slow to return due to low permeability. This resulted in the instantaneous modulus being greatest during the initial cycle and only 1/3 of the initial modulus during steady state loading [113].
Table 3  Summary of regional compressive moduli for various animal models [23].

<table>
<thead>
<tr>
<th>Authors</th>
<th>Location</th>
<th>Modulus</th>
<th>Equilibrium modulus?</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim et al. (2003)</td>
<td>Anterior</td>
<td>18.8 ± 4.7 kPa</td>
<td>Yes</td>
<td>Porcine</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>18.6 ± 5.2 kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lateral</td>
<td>16.3 ± 2.1 kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medial</td>
<td>28.9 ± 12.3 kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>22.1 ± 6.5 kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beek et al. (2001)</td>
<td>Posterior</td>
<td>20 MPa</td>
<td>No</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>Intermediate zone</td>
<td>60 MPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anterior</td>
<td>30 MPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanaka et al. (1999)</td>
<td>Central</td>
<td>15.8 MPa</td>
<td>Yes</td>
<td>Canine</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>30.9 MPa</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Fontenot (1985)</td>
<td>Not specified</td>
<td>1.79 ± 0.34 MPa</td>
<td>Yes</td>
<td>Human</td>
</tr>
</tbody>
</table>

*a All reported errors are standard deviations.

2.6.7 Shear

Shear loading occurs within the TMJ disc because the compressed articulating surfaces are not parallel to one another and discs are not deformed uniformly during loading. The anisotropic and heterogeneous nature of the disc also means that local shear stresses will arise as the mechanical properties vary between regions [143, 144]. Shear stress has been found to cause irreparable damage to cartilage, but the exact relationship has not been fully characterized. Some groups have suggested that excessive shear strain may cause collagen damage and cartilage degradation that can result in development of osteoarthritis [145, 146]. The shear response of TMJ discs has been found to be dependent on the frequency and direction of the loading which may be a result of the collagen fiber alignment [147]. Other studies have found that the amount of tensile and compressive strain also has a significant impact on the shear stress [143, 148].
Tanaka et al. determined that the dynamic shear moduli \( G = \tau / \gamma \) was dependent on frequency and amplitude of shear loading as well as the compressive strain. Dynamic modulus was found to increase directly with loading frequency at every level of compressive strain [149]. Dynamic modulus at 15% was twice as high at 5% compressive strain in a similar trend between 1 and 5% strain. Loss tangent (\( \tan \delta \)) was also found to be related to compressive strain and reached the highest level at 5% then decreased with increasing compressive strain. The magnitude of loss tangent ranged from 0.2 to 0.3 and suggested that the material was primarily elastic in shear loading [149].

The effect of shear strain on shear modulus was less significant, but increasing strain increased storage and loss moduli slightly. Loss tangent increased with shear strain amplitude, but remained constant with increasing frequency. The relationship of shear with compression in the disc is consistent with results found in bovine meniscus and articular cartilage [144, 148]. This correlation may be a result of fluid pressurization during compression that decreases the size of the pores in the ECM and decreases the permeability of fluid effectively stiffening the material to shear forces [149].

### 2.6.8 Age Effect on Mechanical Properties

Changes in viscoelastic properties have been previously studied in skin and tendon, but little data has come to light about TMJ discs. A study performed by Vogel et al. investigated the impact of aging on biochemical and material properties of rat skin,
and found that tensile moduli increased with increasing age [150]. Another study by Walker et al. found that stiffness increased while viscoelasticity decreased in aging canine tendons [151]. Due to the difficulty in obtaining younger human discs, wide age ranges could only be studied in animal models. Age-related studies have primarily been done with bovine TMJ discs which have a similar shape with almost identical chewing frequencies to that of human discs [63]. Results showed that the calcium content of discs increased progressively with age, but it is unknown if this calcification is directly related to aging or with changing mechanical environments in the joint [60, 61]. Total and sulfated GAG levels have also been shown to significantly increase between mature fetuses and mature adults.

A study performed by Lai et al. demonstrated that the shear modulus of human discs increased with age and this change was caused by the increase of the collagen to water ratio [64]. With regard to the tensile properties, elastic moduli were found to be significantly higher in the medial regions than in for the central regions at all specimen ages. All regions showed increases in elastic modulus with increasing age for bovine samples [152]. Studies of the regional biomechanical properties of TMJ discs in compression have found that the intermediate zone of elderly human discs was two to three times stiffer than the anterior and posterior bands. These variations in regions were not apparent in young aged animal models, so these changes are most likely attributed to tissue remodeling during animal maturation [32]. The results of these experiments suggests that water content decreases with age as collagen and GAG levels increase in relation, so a significant stiffening of the TMJ disc cartilage occurs [63].
A study performed by Tanaka et al. on the change in compressive properties associated with age showed that the instantaneous modulus for the central region increased while it remained constant in the posterior region [63]. The compressive properties of the disc are largely correlated with the proteoglycan content, and more specifically with the presence of large chondroitin-sulfated proteoglycan molecules, such as aggrecan. These molecules are strongly hydrophilic and impede the exudation of fluid during compression thereby increasing the compressive modulus. Aggrecan was found in the highest proportion in the central region and lowest amounts in the posterior region for all ages in animal models [153]. The capacity for tissue to dissipate excessive strain energy during stress relaxation is important in preventing tissue damage [152, 154, 155]. This correlation has been observed clinically as disc fragmentation and deformation prevalence rates increase significantly with age [63].

Shear moduli in human TMJ discs were also found to increase with age [33, 64]. Tanaka et al. found that the elastic moduli of healthy TMJ discs was constant up to 50 years of age and increased soon after [156]. Another study reported that mature adult bovine discs had significantly greater elastic moduli than young adults and this correlated with smaller strains after creep. The overall elastic moduli for bovine samples were much lower than for human discs which may be due to differences in chewing motion and diet [152]. The majority of the mechanical properties are related to the collagen content and hydrophilicity of the tissue [42]. It has been found that young discs have a higher water content so fluid flow occurs more readily and the collagen fibers within the disc maintain a relaxed conformation. Mature discs contain a greater proportion of
collagen to water content, so stiffening occurs as water is more tightly held within the proteoglycans while the collagen maintains a more rigid conformation [152].

2.7 Nutrition and Transport

With little research published on nutrition and transport of solutes in TMJ discs, studies of intervertebral discs (IVD) can be looked at to draw comparisons between the similar cartilage discs. TMJ discs and IVD are both large avascular tissues that rely on the blood supply at the disc margins to provide influx of nutrients and removal of wastes. Therefore, these metabolites must be transported by means of diffusion from the periphery to the center of the disc to allow proper growth and development of the cellular components. As these molecules are being transported towards the center of the disc as a result of a concentration gradient, disc cells consume nutrients and produce wastes at rates that are dependent on the concentration of the substrates and pH [157]. For solutes such as oxygen and glucose, steep concentration gradients may develop that cause low concentrations in the center of the disc (below 1%) [158, 159]. No studies have directly measured these consumption rates for TMJ disc cells while several have characterized IVD cell properties in this manner. The breakdown of the nutrient transport has been hypothesized as a cause of early disc degeneration, so it becomes necessary to characterize the diffusive properties of the tissue and the consumption rates of the cells to better understand how homeostasis is maintained in a low solute environment.
2.7.1 Comparison to other Cartilage Types

The intervertebral disc is similar in many regards to the TMJ disc since both are large fibrocartilage structures situated between bone surfaces that are subjected to various loading modalities. As with all avascular tissues, the cells of both disc types rely on adjacent vasculature to provide nutrients and remove wastes via diffusion and convection [27]. The lack of blood vessels in the cartilage significantly lowers the amount of nutrients that can reach the cells and acts as a limiting factor on the proliferation and differentiation capacity of the cells in the cartilage [160]. The relatively high rate of consumption versus the slow rate of diffusion in the disc causes buildup of lactic acid as waste product of glycolysis, so the pH deep within the tissue was found to be very low [161]. Some groups have suggested that this intracellular pH is key to the tissues ability to degrade existing ECM and produce new matrix during turnover [162]. In the IVD, the nearby cartilage endplates most likely provide the pathway for nutrients to enter the disc, so when these become calcified during diseases such as scoliosis, the disc suffers degenerative processes likely a result of nutrient deficiencies [163]. Since studies on the nutrient transport and cellular consumption rates for TMJ discs are not readily found in the literature, the following sections will review these properties for IVD to underline the importance of characterizing these properties [27].

2.7.2 Nutrient Consumption Rates

Studies done by Bibby et al., (2005) measured levels of oxygen, glucose, lactic acid, and pH concurrently for IVD tissues under various starting conditions. The results
of these experiments indicated that decreasing levels of pH lowered cellular consumption of oxygen and glucose as well as decreased production of lactic acid. The effect of oxygen concentration showed similar trends of lowering metabolic processes which differed from previous studies that showed rates of glycolysis increasing at lower oxygen concentrations (positive Pasteur Effect). Glucose concentration in the range of 1-5mmol/L was found to have no effect on oxygen consumption and the consumption of glucose was 2 times greater than the production of lactic acid over a wide range of environmental conditions. This study was the first to use cultured isolated cells rather than explants to allow for greater control of the microenvironment and cell densities which may explain the significant difference in results. This experiment clearly indicates that concentrations of nutrients and metabolites and pH can significantly affect cellular activity and metabolic rates of cartilage disc cells [1]. These changes have been shown to impact matrix synthesis and degradation as well as cell viability which can be directly linked to the composition of the disc and can result in development of degenerative processes [164].

2.7.3 Disc Nutrient Environment

The nutrients required by disc cells must reach the center of the disc via simple diffusion created by gradients that result from cellular consumption. Studies have shown that imbalances between transport and cellular demand, as a result of cytokine or growth factor release or changes in osmolarity, can cause nutrient levels to fall with detrimental consequences to the cells. The balance between the biological needs of cells and physical diffusion of molecules creates a relationship between tissue thickness and cell density
that is important in understanding avascular tissues and tissue-engineered constructs [165].

Solute and waste concentrations within the tissue are balanced by the passive transport into the tissue and cell metabolic rates creating waste products that need to be removed. Static compression was shown to decrease porosity lowering the rate of diffusion through cartilage. The cells then respond to this lowered solute concentration by altering their consumption rates until a new equilibrium is attained. Therefore, concentrations of nutrients like glucose and oxygen will decrease while waste products (i.e. lactate) accumulate in the tissue. On the other hand, dynamic compression was found to have the opposite effect on transport, and these effects were strain and frequency dependent [166].

Both TMJ disc and IVD cells are subject to low pH and low oxygen environments due to the slow rate at which solutes diffuse through the tissue. The cells primarily obtain energy through glycolysis, by consuming glucose to form lactic acid and adenosine triphosphate (ATP). This energy is then used to allow cell proliferation and differentiation to build ECM components (i.e. collagen and GAG) to maintain the physiological properties of the tissue. Although studies on IVD oxygen consumption rates have shown contrasting results between the relationship of oxygen levels and glycolytic rates, all studies have shown that low pH significantly reduces glycolysis, the rate of O₂ uptake, and production of ATP [1, 97]. Synthetic processes were found to be greatest around 5% oxygen concentration with sulfate incorporation (indicator of GAG synthesis) being hindered as oxygen concentrations decreased towards 1% oxygen. Low
levels of pH are assumed to inhibit synthesis of ECM components without slowing degradation, so a net loss of matrix occurs. Since disc cells obtain most of their energy from glycolysis, glucose concentrations below 0.2mM have been shown to cause cell death within twenty-four hours [97].

2.8 Modeling

Since it is difficult to observe the TMJ disc in vivo, finite element models (FEM) have been developed to fill this gap in information by predicting how the tissue responds to various loading conditions. In vivo testing of discs would require invasive equipment to measure stress distribution, such as strain gages that would alter the biomechanical properties and affect the results of experiments [20, 21]. The complex nature of the cartilage is simplified using mathematical models that attempt to predict the response found in native tissues. The TMJ disc has been previously modeled with the viscoelastic model, biphasic theory, and most recently multiphasic theory. Validation of FEMs must be done by combining experimental testing with computational results to determine the accuracy of predictions [127]. Extensive characterization of the biomechanical, anatomical, and cellular characteristics of the disc must be well researched before a FEM can be created that will accurately predict behavior under changing conditions [20].

Accurate FEMs that are proven with validation can serve as predictive models that help researchers characterize the differences between normal and abnormal disc behavior. This understanding may help researchers to understand TMD etiology and develop treatments based on results found during modeling. FEMs may also provide
physicians with another method of diagnosing TMD in the early stages, before irreparable tissue damage occurs. Models would also enable surgeons to examine the joint environment and functionality of a patient’s joint before and after invasive surgery is performed [167]. Models could be used to track the growth and development of the TMJ over time, so that tissue remodeling resulting from property changes could be better understood [168]. Various implants could also be modeled and tested computationally without all of the costs of designing and developing a prototype [20].

**2.8.1 Biphasic Theory**

The TMJ disc was found to have time-dependent viscoelastic properties during creep and stress relaxation tests which can be modeled with the biphasic theory initially proposed by Mow et. al. [169]. This theory has already been applied to tissues that display anisotropy, nonlinearity, and heterogeneity [170, 171]. The biphasic theory is most accurate when used to model small strains that are less than 25% in which the linearity assumption remains valid [127]. The biphasic theory works on the simplification of the tissue into two phases: the solid phase which includes the ECM components and cells, and the fluid phase which is mainly comprised of the interstitial fluid within the solid matrix. Spilker et al., (2009) performed plowing tests that found that ~96% of the load was held by the fluid pressure within the disc. The significant impact of the interaction between solid and fluid components emphasizes the importance of modeling the tissue using the biphasic theory and more advanced approaches [127].
As the tissue is loaded, the volume changes and creates a pressure gradient that generates interstitial fluid flow. The fluid can only move through the pores of the solid matrix causing friction and resulting in the viscoelastic behavior found in cartilage. The biphasic theory developed by Mow et al., (1980) assumes that tissue is a composite material consisting of two principle phases: a solid phase and a fluid phase. The solid phase is assumed to be elastic and permeable to the flow of the fluid phase. This theory also suggests that loaded tissue is subjected to three modes of internal forces: 1) the stress generated from the deformation of the solid matrix components; 2) the fluid pressure due to the incompressibility of water; and 3) the friction created from the two phases moving against one another [169]. The permeability of cartilage tissue is very low (~10^{-16} \text{m}^4 \cdot \text{N}^{-1} \cdot \text{s}) which suggests that under compression the fluid pressure sustains a significant portion of the loading. One study found that 95% of the loading was supported by interstitial fluid pressurization. During confined compression this fluid load support lasted longer than 500 seconds and is presumed to be even longer in vivo [142].

Under the linear biphasic theory of cartilage, a simplified version of the biphasic theory, the solid phase is assumed to be isotropic and linearly elastic. The friction associated with fluid flow can also be simplified by using a linear equivalent of Darcy’s law [169]. Darcy’s law shows that fluid flow rate is linearly related with tissue permeability, tissue thickness, and pressure drop [172]. In this simplified model, only the Young’s modulus, Poisson’s ratio, and hydraulic permeability are required to fully characterize the biphasic properties of the material [172]. The Young’s modulus can be obtained with unconfined compression tests and the Poisson’s ratio can be determined by
measuring the lateral expansion during unconfined compression [173, 174]. Confined compression testing is sometimes done because the aggregate modulus can be directly calculated by dividing the load by the strain at equilibrium. The hydraulic permeability can then be obtained by curve-fitting the creep curve during a confined compression test [169, 172, 173].

2.8.2 Triphasic Theory

The charged sulfate (SO$_3^-$) and carboxyl (COO$^-$) groups on the GAGs in the ECM create a high negative charge concentration known as the fixed charge density (FCD) (0.04 to 0.2 mEq·mL$^{-1}$ in normal cartilage) [171, 175, 176]. The negative charge associated with the tissue must be balanced by mobile positive charges (e.g., Na$^+$) to maintain the electroneutrality principle [176]. As a large amount of mobile ions build up in the tissue, a gradient between the surrounding solution and cartilage is created which is known as Donnan osmotic pressure [171, 175]. Donnan osmotic pressure has been found to significantly influence tissue hydration, ion transport, mechanical behavior, and even the biochemical composition [177].

The triphasic theory originally developed by Lai et al. in 1991 incorporates the effects of osmotic pressure and ion transport by building on the principles of the biphasic model [171]. Studies showed that the osmotic pressure can contribute up to 30-50% of the stiffness found in cartilage [178]. The addition of the ion phase to the biphasic theory incorporates the effect of dissolved ionic species of both positive and negative charges [171, 175, 176, 179]. The interaction of the solid, fluid, and ionic phases grants the tissue significant strength in resisting compressive and shear stresses [169, 171, 180].
Triphasic theory has been used to model viscoelasticity, swelling, and electrokinetic behavior found in various charged, hydrated soft tissues [114].

Triphasic modeling can also incorporate the transport of solutes and waste as a result of tissue permeability and deformation. Using a new formulation of the triphasic theory to model the consumption and transport of nutrients in the IVD, Huang et al., (2008) determined that dynamic compression increased local oxygen concentrations and reduced lactate accumulation. Dynamic loading also increased the consumption rate of oxygen and production of lactate in the simulated cartilage. Static loading had the opposite effect, with lactate build-up and oxygen levels decreasing. These results are in agreement with trends and predictions found in the literature [166].

2.8.3 Finite Element Models

Development of an accurate predictive finite element model requires input of accurate geometric and anatomical properties of the material in question. Specifically, a TMJ model would require the mechanical properties of the cartilage under various loading conditions, the biochemical composition (collagen, PG, and water content of each region), and the correlation between structure and function (Figure 5) [172]. Finite element models in 2D have severe limitations in predicting the complex 3D behavior of the TMJ, but many properties can be estimated from the simplistic representation [11]. Beek et al. used a 3D model to predict mechanical responses of the TMJ during clenching with various initial restraint conditions preset [21]. Another model developed by Takaka et al. examined the stress distribution of the jaw during opening and analyzed the variation in properties between joints with and without derangement [79, 181]. Tanaka et
al. also developed a FEM of magnetic resonance images that suggests that increases in friction between articulating surfaces may be one of the causes of disc displacement [80].

Palomar et al. developed a 3D model that included both joints, but only considered the movements to be symmetric during clenching which is a major simplification [182]. Another FEM developed by Koolstra and van Eijden, combined the rigid-body model with models of the discs, and incorporated the articulating surfaces during jaw movement [183]. This FEM showed that the structure of the TMJ allowed regulation of the mechanical properties by controlling fluid movement and redistribution. The fluid was also found to be replenished as the joint moved during normal function. This study once again did not include the impact of the surrounding connective tissues and asymmetric movement of the TMJ [184].

A more recent FEM developed by Palomar et al. added the most important connective ligaments, three body contact between all parts of the joints, and biomechanical behavior of soft tissues during nonsymmetrical movement [185]. During right lateral movement, maximum stresses were reported in the posterior region of the right disc and anterior region of the left disc. Higher shear stresses were found in the left disc, specifically in the lateral part of the posterior band, and tensile stress was significantly higher in the left disc. The results of this asymmetric bruxism FEM suggest that the lateral regions of both discs are more prone to higher loads and more likely to suffer perforations [79, 149, 181].
Figure 6  Finite element mesh of TMJ disc, surrounding bone surfaces, and articulating cartilage [55].
Chapter 3 Compression Properties

3.1 Introduction

The primary function of the TMJ disc is to provide mechanical support and prevent bone to bone contacts that can result in significant damage and loss of joint function. Sustained mechanical loading can cause decreases in fluid load support and increased friction that may result in excessive tissue deformation and wear [186]. The mechanism through which mechanical loading initiates pathological events within the TMJ disc is poorly understood and therefore the focus of Aim 1. One requirement for studying TMJ pathology and development of tissue engineered treatments is the characterization of disc mechanical properties and the resulting biological responses. These properties will be crucial in determining a suitable animal model as well as for building a predictive model of TMJ disorders.

As discussed in Chapter 2, many studies have characterized the compressive, tensile, and shear mechanical properties of a variety of animal species. Several groups concluded that the pig is the best experimental model of the TMJ after comparison to sheep, cows, dogs, cats, rabbits, rats, and goats [9, 33]. In particular, selection of the pig was attributed to the similar size of TMJ structures, shape of the disc, and omnivorous diet. In addition, the pig and human TMJs have been shown to have similar gross morphology and structure, including the disc and its attachments. Moreover, both pig and human TMJs have similar range of motion.
During jaw motion, the fossa remains stationary while the condyle bone articulates. As a result, the sandwiched TMJ disc is subjected to a variety of compressive, tensile, and shear forces. Due to the complexity of the joint and loading modalities, several mathematical models were developed to closely approximate the physiological responses of tissues during loading. In many published studies, the TMJ disc was treated as a single-phase viscoelastic solid, and to our knowledge only one study on the porcine TMJ disc has used a biphasic model to fit the indentation data [59]. Finite element models based on the well-known biphasic (poroelastic) theory have been developed to examine the viscoelastic mechanical behavior and loading support mechanism of the TMJ discs [187]. In these biphasic models, the TMJ disc tissue was considered as a mixture of water and solid phases.

In the following chapter, we sought to measure the biphasic mechanical properties of porcine TMJ discs before applying the same techniques to human cartilage allowing for direct comparisons. Very few studies have characterized the viscoelastic properties of the human TMJ disc which may be due to difficulty in obtaining samples [32, 116]. These reasons served as the rationale for measuring the biphasic mechanical properties of porcine and human TMJ discs. Studies were also done to determine the dynamic compressive properties during loading in order to simulate jaw motion. These studies focused on the impact of regional structure and frequency of loading in order to characterize the viscoelastic and anisotropic nature of the tissue.
3.2 Materials and Methods

3.2.1 Porcine Specimen preparation

Twelve TMJ discs from the left joint were harvested from pig heads (6-8 month old, Yorkshire, male) obtained from a local slaughterhouse within two hours of sacrifice. The discs were immediately photographed, morphologically examined, and wrapped in gauze soaked in a normal saline solution with protease inhibitors and stored at −80 °C until mechanical testing. It has been reported that mechanical properties of porcine discs were retained over five freeze–thaw cycles [58]. Discs exhibiting any abnormalities (i.e. fissures or bruising) were discarded.

Cylindrical tissue plugs were obtained from the anterior, intermediate, lateral, medial, and posterior regions of the TMJ disc (Figure 7), using a 5 mm corneal trephine (Biomedical Research Instruments Inc., Silver Spring, MD). Thin layers from the superior and inferior surfaces were removed via sledge microtome (Model SM2400, Leica Instruments, Nussloch, Germany) with freezing stage (Model BFS-30, Physitemp Instruments Inc., Clifton, NJ) to eliminate the natural concave shape of the disc and allow for a flat surface during mechanical testing. Confined compression samples had an average height of 1.4mm and a diameter of 5mm.
Figure 7 Schematic of specimen preparation. The region and size of test specimens are shown.

3.2.2 Human Sample preparation

Human TMJ discs from the left joint of fresh cadavers were extracted in conjunction with the Medical University of South Carolina’s Gross Anatomy Laboratory under institutional approval. Discs exhibiting physical signs of degeneration including calcification or tears were discarded. In total, twelve morphologically healthy TMJ discs from twelve male human heads (mean age=78 years) were sectioned and used for mechanical testing. To limit the heterogeneity of the experiment, only samples from male cadavers were used. The same sample preparation methods as described for porcine samples were used to obtain cylindrical discs from 5 regions with an average height of 1.4 mm and a diameter of 5 mm.
3.2.3 Porosity (Water Volume Fraction) Measurement

The volume fraction of water for each specimen was determined gravimetrically in PBS (pH 7.4) using a density determination kit and an analytical balance (Sartorius YDK01, German). Samples were thawed in room temperature PBS and due to the low GAG content did not swell significantly. This measurement technique was developed by Gu et al. [188] and is based on Archimedes’ principle where the volume \( V \) of a completely submerged object in a fluid with density \( \rho \) is given as:

\[
V = \frac{F_b}{\rho g}
\]

Equation 1

where \( F_b \) is the buoyancy force and \( g \) is acceleration due to gravity. The buoyancy force is calculated from the difference in the weight of the specimen in air, \( W_{\text{wet}} \), and the weight of the specimen in PBS, \( W_{\text{PBS}} \). Therefore, the volume of the specimen weighed in PBS can be determined by:

\[
V = \frac{W_{\text{wet}} - W_{\text{PBS}}}{\rho_{\text{PBS}} g}
\]

Equation 2

where \( \rho_{\text{PBS}} \) is the density of PBS solution (~1.005g/cm³). The volume of water in the sample \( V^w \) can then be calculated with:

\[
V^w = \frac{W_{\text{wet}} - W_{\text{dry}}}{\rho_{\text{w}} g}
\]

Equation 3

where \( W_{\text{dry}} \) is the dry weight (after lyophilization) of the specimen and \( \rho_{\text{w}} \) is the density of water. Therefore, the initial volume fraction of water in the tissue, \( \phi^w_0 \), can be calculated from the ratio of water volume to wet tissue volume as:
\[
\phi^w_0 = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{wet}} - W_{\text{PBS}}} \frac{\rho_{\text{PBS}}}{\rho_w}
\]
Equation 4

\(W_{\text{wet}}\) and \(W_{\text{PBS}}\) are measured prior to mechanical testing while \(W_{\text{dry}}\) is measured after lyophilizing the sample. The water volume fraction of the specimen at different compression levels (\(\phi^w\)) can be calculated by:

\[
\phi^w = \frac{\phi^w_0 + e}{1 + e}
\]
Equation 5

where \(e\) is the tissue dilatation [171]. Considering one-dimensional confined compression in this study, the tissue dilatation is equal to the compressive strain. The volume fraction of solid (\(\phi^s\)) is related to the volume fraction of water by \(\phi^s = 1 - \phi^w\).

### 3.2.4 Collagen and GAG content

Biochemical analysis was performed on the lyophilized specimens. The total collagen content was determined with a modified chloramine-T hydroxyproline assay (Sigma-Aldrich Co., St. Louis, USA) [189]. Dry tissue (2.0 mg ± 0.5 mg) was solubilized with 0.5 mL 500 \(\mu\)g/mL papain in 50 mM phosphate buffer (pH 6.5) containing 2 mM N-acetyl cysteine and 10 mM EDTA at 65°C for 48 hours. One hundred microliters of this solution was hydrolyzed by 0.5 mL 2.5 N NaOH for 2 hours at 110°C, then 0.5 ml 6 N HCl was added to each microcentrifuge tube. Twenty \(\mu\)L of each sample was assayed for total collagen content by a modified chloramine-T hydroxyproline assay. Instead of using hydroxyproline standards, collagen standards (Accurate Chemical and Scientific Corporation, Westbury, NY) were chosen for a more direct comparison [190, 191].
The total glycosaminoglycan (GAG) content was quantified using a Blyscan Glycosaminoglycan Assay kit. (Biocolor, Newtonabbey, Northern Ireland). Lyophilized samples (2.0 mg ±0.5 mg) were treated with 1 mL of 4 M guanidinium chloride, 50 mM sodium acetate, (pH 5.8), 5 mM benzamidine/HCl, 10 mM N-ethylmaleimide 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C for 48 hours. After extraction, the samples were centrifuged at 3,000g for 10 min to separate the extracted collagen-rich cartilage pellet from the supernatant containing the intact proteoglycans and fragments. The supernatant was removed and stored at 70°C. Total sulfated GAG was quantified according to the kit based on 1, 9-dimethylmethylene blue binding using standards provided by the manufacturer.

### 3.2.5 Confined Creep Compression

Compression tests were performed in PBS (pH 7.4) with a TA Instruments Dynamic Mechanical Analyzer (DMA) Q800 (TA Instruments Inc., New Castle, DE). The testing chamber with the sample was maintained at 37±0.5 °C by a temperature controlled furnace. The precision of the DMA for force was 0.01 mN while the precision for strain measurements was 1 nm. A uniaxial, confined compression test chamber was custom designed and built to enable 1-dimensional compression during testing (Figure 8A). The chamber allowed for the sample to be immersed in PBS to prevent dehydration during experimentation. The specimen was confined laterally by a stainless steel ring and compressed axially between a titanium porous platen (20 μm average pore size) on the bottom and the test probe (5 mm nominal diameter) on top [192, 193]. The porous platen
allowed for fluid flow through the sample during compression ensuring that forces were not maintained by fluid pressure within the tissue.

Figure 8B shows schematically the mechanical testing protocol which will be described in further detail. The testing protocol of confined compression was similar to previous studies on IVD tissues and hydrogels [193, 194]. First, the specimen was subjected to a minute compressive tare load (5 mN) to measure specimen height prior to the addition of PBS and this height served as the initial height for all mechanical tests. The specimen was then compressed to the height corresponding to 10% compressive strain (relative to the initial height). This offset strain was to ensure interdigitation between the porous platen and the specimen and to fully confine the specimen at its periphery. The specimen was allowed to reach equilibrium at this strain (~60 min) and the stress was then recorded. Then a 2 hour creep test was performed by applying a stress equal to 1.2 times the equilibrium stress at 10% strain. Steady state was achieved after 2 hours of compression with the resulting creep strain at the end of the experiment on average 2–3% of the initial specimen height. This small creep strain was chosen to satisfy the linear biphasic theory.

The equilibrium compressive aggregate modulus ($H_A$) and hydraulic permeability coefficient ($k$) were then determined by curve-fitting the creep data to the biphasic theory developed by Mow et al. [169]:

$$
\frac{u(0,t)}{h} = \frac{F}{H_A} \left[ 1 - \frac{2}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(n+\frac{1}{2})^2} \exp \left( -\frac{H_A k(n+\frac{1}{2})^2 \pi^2}{h^2} t \right) \right]
$$

Equation 6
Where \( u(t) \) is the displacement of the tissue under constant stress, \( h \) is the tissue thickness, \( t \) is the time of loading, and \( F_0 \) is the applied stress. The aggregate modulus is a material property related to the stiffness at equilibrium (zero fluid flow) and is related to the Young’s modulus \( (E) \) and Poisson ratio \( (\nu) \) with this equation:

\[
H_A = \frac{E(1 - \nu)}{(1 + \nu)(1 - 2\nu)}
\]

Equation 7

Hydraulic permeability is related to the ease at which fluid can move through the pores of the solid material. Both of these material properties are essential in developing a constitutive model of the complex mechanical environment in the TMJ disc.

**Figure 8** (A) Schematic of confined testing chamber. (B) Confined compression protocol.
3.2.6 Dynamic Compression

After creep testing, the specimen was subjected to a dynamic frequency scan test over the range of 0.01 to 3 Hz. The amplitude of the sinusoidal dynamic stress was equal to 50% of the static offset stress (i.e. creep stress). The criterion for choosing the amplitude of the dynamic loading was based on the dynamic displacement amplitude of the specimen at 3 Hz ($\geq$ 5 microns). Using this requirement, our study showed that the maximum dynamic strain, occurring at the lowest testing frequency (0.01 Hz), was less than 2.0%. Therefore, the dynamic response of the specimen could still be considered within the linear range [195]. The dynamic complex modulus and phase angle were automatically recorded by the DMA over the frequency range. As discussed in Chapter 2, the magnitude of the complex modulus can be calculated by solving the equation $|E^*| = \frac{\delta}{\delta_0}$. The phase angle is calculated as the inverse tangent of the ratio of viscous modulus to elastic modulus or $\delta = \tan^{-1} E''/E'$. A perfectly elastic material would have a phase angle 0° while a perfectly viscous material would achieve 90° phase angles. Therefore, viscoelastic materials such as biological tissues would fall between these extremes. Characterizing the viscoelastic dynamic properties is essential for better understanding the behavior of cartilage and developing accurate models of joint mechanics.

3.2.7 Statistical analysis

The mechanical and biochemical properties were examined for significant differences between disc regions using SPSS statistics software (SPSS 16.0, IBM, NY).
One-way ANOVA and Tukey’s post hoc tests were performed to determine if significant differences existed. Linear regression was performed to correlate biphasic properties with tissue composition. Statistical differences were reported at $p$-values <0.05.

### 3.3 Porcine Confined Compression Results

#### 3.3.1 Creep Compression Behavior

The creep data were well fitted to the biphasic theory to determine the equilibrium compressive aggregate modulus ($H_A$) and hydraulic permeability ($k$) (Figure 9). The aggregate moduli of the peripheral bands of the disc (anterior: 61.33±10.37 kPa and posterior: 71.20±11.94 kPa) were approximately 20% lower than the regions running mediolaterally (medial: 97.43±13.53 kPa, intermediate: 79.27±11.73 kPa, and lateral: 73.65±9.12 kPa) (Figure 10A). The intermediate, lateral and posterior regions showed significant differences for hydraulic permeability (Figure 10B). The anterior and medial regions (anterior: $42.03\pm2.36\times10^{-15}$ m$^4$/Ns and medial: $40.26\pm7.16\times10^{-15}$ m$^4$/Ns) were ~36% less permeable than the other regions of the disc (intermediate: $60.51\pm9.06\times10^{-15}$ m$^4$/Ns, lateral: $62.81\pm6.24\times10^{-15}$ m$^4$/Ns, and posterior: $61.04\pm6.84\times10^{-15}$ m$^4$/Ns).
Figure 9 Typical biphasic creep behavior of a TMJ disc specimen. A good agreement is shown between the theoretical prediction and the experimental result.
Figure 10  (a) Mean and standard deviation values for aggregate modulus of the five regions of interest. Significant regional variations were detected. *P<0.05 compared to anterior, lateral, and posterior regions. (b) Mean and standard deviations for permeability of the five regions of interest. Significant regional variations were detected. *P<0.05 compared to anterior and medial regions.
3.3.2 Dynamic compression behavior

The dynamic compression behavior of the porcine TMJ disc was highly frequency-dependent. The complex modulus, a ratio of dynamic stress to dynamic strain, increased with increasing frequency (0.01–5.0 Hz) in all five disc regions (Figure 11A). Standard deviations values were withheld from the figure for clarity. The average standard deviation was 50% of the complex modulus magnitude at that frequency. The lateral region displayed significantly lower complex moduli over all frequencies (average complex moduli over 0.01–5 Hz for peripheral regions: 2,955–32,300 kPa and central regions: 1,960–22,560 kPa). The three central regions had complex moduli approximately 30% less in magnitude over all frequencies tested. The phase angle shift, a measure of energy lost in one cycle of oscillation in a viscous deformation, decreased with increasing frequency in all five disc regions (Figure 11B). Phase angle standard deviation values were approximately 25% of the average phase angle value for that frequency. All regions demonstrated very similar material responses to increasing frequency.
Figure 11 (A) Mean complex modulus of each region over the frequency range of 0.01–5 Hz. (B) Mean phase angle over five regions of interest.
3.3.3 Biochemical composition

Mean and standard deviation values of the water content (% wet weight), volume fraction of water, total collagen content (% dry weight), and total GAG content (% dry weight) of porcine TMJ discs were listed in Table 4 for each disc region. The average tissue water volume fraction across the human TMJ disc was 67.3%. Significant differences were only found for the lateral and posterior regions for collagen content. The average total collagen and GAG content by dry weight were 68.1% and 4.7% by dry weight, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Water Content (% Total Weight)</th>
<th>Collagen Content (% Dry Weight)</th>
<th>GAG Content (% Dry Weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>67.8 ± 4.5</td>
<td>69.7 ± 11.8</td>
<td>5.0 ± 1.9</td>
</tr>
<tr>
<td>Intermediate</td>
<td>67.1 ± 3.4</td>
<td>75.2 ± 12.5</td>
<td>4.6 ± 1.3</td>
</tr>
<tr>
<td>Lateral</td>
<td>67.86 ± 4.0</td>
<td>65.7 ± 15.6*</td>
<td>5.1 ± 2.0</td>
</tr>
<tr>
<td>Medial</td>
<td>67.1 ± 3.6</td>
<td>69.9 ± 10.8</td>
<td>5.1 ± 2.7</td>
</tr>
<tr>
<td>Posterior</td>
<td>66.9 ± 3.5</td>
<td>61.2 ± 14.8*</td>
<td>3.8 ± 1.16</td>
</tr>
<tr>
<td>Total</td>
<td><strong>67.3 ± 3.8</strong></td>
<td><strong>68.1 ± 13.9</strong></td>
<td><strong>4.7 ± 1.9</strong></td>
</tr>
</tbody>
</table>
3.4 Porcine Confined Compression Discussion

As discussed in Chapter 2, many studies have measured TMJ disc mechanical properties in a variety of animal species. Due to similarities in anatomy and diet the porcine disc serves as the best model for comparisons with human discs. In this study, the biphasic compressive properties of porcine discs were measured to validate the model and to develop protocols for human disc testing. In addition, the biochemical composition was analyzed to correlate with the biomechanical properties measured. Previous studies have found that structure-function relationships exist and result from the anisotropic extracellular matrix distribution [13].

It has been hypothesized that the compressive mechanical load on the TMJ disc is mainly supported by fluid through the fluid pressurization effect [52]. A biphasic finite element model of the porcine TMJ disc showed that more than 90% of the load during plowing experiments was supported by the fluid phase [127]. However, it is not clear how the biphasic mechanical properties affect the dynamic behavior of TMJ disc tissue during compression. Therefore, the objective of this study was to determine the regional biphasic viscoelastic properties, as well as dynamic properties, of porcine TMJ discs in confined compression. Confined compression allows for well controlled 1-D strain state experiments, and compared to indentation and unconfined compression, fewer parameters are needed to be determined by the curve fit in order to obtain more reliable results [169]. The biochemical composition of the disc was also determined to correlate with the biphasic mechanical properties. The effect of the aggregate modulus and permeability on
the dynamic properties was examined to further delineate the load supporting mechanisms in the TMJ disc under compression.

The confined creep data of porcine TMJ discs in this study were well fitted to the biphasic model with an average $R^2$ value of 0.987. This result indicates that confined compression is a reliable testing method to characterize biphasic properties of TMJ disc tissues. In this study, the average aggregate modulus was 76.6kPa which is approximately 3 times greater than the value measured in an indentation compression study by Kim et al. The average hydraulic permeability was $53.33 \times 10^{-15} \text{ m}^4/\text{Ns}$ which is 2 times higher than the value determined in the indentation study [59]. These variations in mechanical properties may be related to the sample differences including age and gender as well as loading configuration.

Differences in biochemical composition and structure distinguish the disc into three regions: the anterior band, intermediate zone, and posterior band [196]. Detamore et al., (2006) measured the average water content of porcine TMJ discs as 71±2% of the total weight compared to the 67±4% of our discs. Their studies also measured ~80-90% collagen by dry weight while we only measured 68.1±13.9%. The measured GAG content was approximately 5% by dry weight for both studies [10, 38-40]. Overall the results of our biochemical analysis were not significantly different from previously published results.

Studies have shown a strong correlation between equilibrium aggregate modulus and permeability with water and proteoglycan content. Aggregate modulus was found to vary inversely with water content and directly with proteoglycan content [197]. In this
study, no significant differences were found for either water or proteoglycan content. As for collagen content, the regions with the lowest amount of collagen also demonstrated the lowest aggregate moduli. Collagen is thought to mediate tensile properties with orientation of fibers being more important than overall content. Studies have shown that hydraulic permeability was proportional to water content and inversely proportional with proteoglycan content, but these trends were not observed in this study [194, 198].

Significant regional variation was detected for the medial region for aggregate modulus. The hydraulic permeability in the intermediate, lateral, and posterior regions was significantly higher than in the anterior and medial regions. There was no direct correlation between aggregate modulus and permeability which may be a result of the relative young age of pigs tested. Eight to ten months is considered a young adult stage, and the anisotropy and inhomogeneity described in the literature may not be fully developed. The dynamic modulus was significantly lower for the lateral region and was 1/2 to 1/3 of the other 4 regions over the frequency range. The dynamic modulus was 30-400 times (over 0.01-5Hz) greater than the aggregate or equilibrium modulus under static compression. When hydrated soft tissues are subjected to dynamic compression of loading frequencies higher than the characteristic frequency of the tissue, the interstitial fluid pressure will increase significantly and the tissue becomes stiffer due to the fluid pressurization effect [142]. Theoretical and experimental studies in articular cartilage and IVD have shown that the fluid pressurization effect is related to the biphasic mechanical properties of the tissue, such as hydraulic permeability and equilibrium compressive modulus [192, 193]. This viscoelastic phenomenon is further demonstrated
by the decreasing phase angle during low frequency compression that stabilizes around 1-2Hz which is within the physiological rate associated with chewing [199].

In summary, regional biomechanical and biochemical characterization of the porcine TMJ disc was conducted to validate the confined compression loading modality and to develop protocols for future testing of human discs. Due to the relative biochemical uniformity of the disc, few correlations between mechanical properties and ECM distribution could be drawn. Overall, the magnitude of the aggregate modulus and hydraulic permeability were similar to other previous porcine mechanical studies. The dynamic modulus was found to be several hundred times higher than static modulus which is potentially related to the instantaneous fluid load support resulting from pressurization within the tissue.

3.5 Human Confined Compression Results

3.5.1 Creep compression behavior

The aggregate moduli of the peripheral bands of the disc (anterior: 18.61±3.02 kPa and posterior: 25.44±7.27 kPa) were approximately 1/3 that of the regions running mediolaterally (medial: 59.81±11.2 kPa, intermediate: 74.93±10.99 kPa, and lateral: 74.51±12.23 kPa) (Figure 12A). Significant differences were also observed for hydraulic permeability (Figure 12B) The anterior and posterior regions (anterior: 8.95±1.17×10⁻¹⁵ m⁴/Ns and posterior: 8.07±1.55×10⁻¹⁵ m⁴/Ns) were ~40% more
permeable than the central regions of the disc (intermediate: $3.64 \pm 0.67 \times 10^{-15} \text{ m}^4/\text{Ns}$, lateral: $3.74 \pm 0.78 \times 10^{-15} \text{ m}^4/\text{Ns}$, and medial: $3.86 \pm 0.70 \times 10^{-15} \text{ m}^4/\text{Ns}$). Therefore, a strong inverse relationship between aggregate modulus and permeability was found with significant regional variation.
**Figure 12** (A) Mean and standard deviation values for aggregate modulus of the five regions of interest. Significant regional variations were detected. *P<0.05 compared to anterior and posterior regions. (B) Mean and standard deviations for permeability of the five regions of interest. Significant regional variations were detected. *P<0.05 compared to anterior and posterior regions.
3.5.2 Dynamic compression behavior

The dynamic compression behavior of the human TMJ disc was highly frequency-dependent. The complex modulus increased with increasing frequency (0.01–3.0 Hz) in all five disc regions (Figure 13A). The anterior and posterior regions displayed lower complex moduli over all frequencies (average complex moduli over 0.01–3 Hz for peripheral regions: 171.8–609.3 kPa and central regions: 454.6–1613.0 kPa). The three central regions had complex moduli approximately 2.5–3 times greater in magnitude over all frequencies tested. The phase angle shift decreased with increasing frequency in all five disc regions (Figure 13B). The anterior region demonstrated a greater phase angle over all tested frequencies indicating a greater viscous response during dynamic compression.
Figure 13 (A) Mean complex modulus of each region over the frequency range of 0.01–3 Hz. (B) Mean phase angle over five regions of interest.

3.5.3 Biochemical composition

Mean and standard deviation values of the water content (% wet weight), volume fraction of water, total collagen content (% dry weight), and total GAG content (% dry
weight) of human TMJ discs were listed in Table 5 for each disc region. The average tissue water volume fraction across the human TMJ disc was 80%. Significant differences were found between the central three regions and the posterior region which contained ~3% more water by total weight. Although it was not significantly different, anterior region samples on average contained slightly more water. The average total collagen and GAG content were 62% and 3.2% by dry weight, respectively. There were no significant differences of total collagen and GAG content in disc regions for human TMJ discs.

Table 5 Results (mean±SD) of biochemical assays measuring water, total collagen, and total GAG content for each region of human TMJ discs. Significant differences were only found for water content, showing the posterior region contained a greater percentage of water than all other regions.

<table>
<thead>
<tr>
<th></th>
<th>Water Content (% Total Weight)</th>
<th>Water volume faction (%)</th>
<th>Collagen Content (% Dry Weight)</th>
<th>GAG Content (% Dry Weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>74.7±5.6</td>
<td>80.9±4.8</td>
<td>61.9±10.2</td>
<td>3.2±1.1</td>
</tr>
<tr>
<td>Intermediate</td>
<td>73.5±2.7</td>
<td>79.0±3.0</td>
<td>60.2±13.0</td>
<td>3.1±1.0</td>
</tr>
<tr>
<td>Lateral</td>
<td>73.0±2.5</td>
<td>78.7±2.5</td>
<td>61.6±12.0</td>
<td>3.2±1.2</td>
</tr>
<tr>
<td>Medial</td>
<td>73.5±3.1</td>
<td>79.4±1.8</td>
<td>63.1±8.9</td>
<td>3.2±1.4</td>
</tr>
<tr>
<td>Posterior</td>
<td>78.5±5.2</td>
<td>82.8±4.5</td>
<td>62.5±14.7</td>
<td>3.3±1.5</td>
</tr>
<tr>
<td>Total</td>
<td><strong>74.5±4.2</strong></td>
<td><strong>80.0±3.6</strong></td>
<td><strong>62.0±11.4</strong></td>
<td><strong>3.2±1.4</strong></td>
</tr>
</tbody>
</table>
3.5.4 Correlation between biphasic properties and tissue composition

The correlation between the aggregate modulus and water content, as well as the correlation between the permeability and water content, was found statistically significant, as shown in Figure 14. The aggregate modulus was negatively correlated with the water content ($R=-0.587, P<0.0001$), while the hydraulic permeability positively correlated with the water content ($R=0.522, P<0.0003$).
Figure 14 (A) Correlation between the aggregate modulus and the water content. Significant negative correlation was detected ($R = -0.578, P<0.0001$). (B) Correlation between the hydraulic permeability and the water content. Significant positive correlation was detected ($R = 0.522, P<0.0003$).

3.6 Human Confined Compression Discussion

The equilibrium aggregate modulus and permeability of cartilaginous tissues are highly correlated with the water and proteoglycan content [197]. In this study, we
confirmed that the aggregate modulus of human TMJ discs negatively correlates with the water content, while the hydraulic permeability positively correlates with the water content (Figure 14B). The correlation between the GAG content and biphasic properties, however, was not statistically significant due to extremely low GAG content in human TMJ disc tissues. The average water volume fraction of the human TMJ disc was 80% which is higher than the typical value for adult human articular cartilage (75%). Moreover, the human TMJ disc has 3.2% (dry weight) total GAG content which is significantly lower than that of human articular cartilage (15–25%) [200].

Regional variations were detected for both aggregate modulus and permeability for human samples with the anterior and posterior regions being significantly softer and more permeable than the other regions of the disc. These regional differences are likely due to the higher water content in the anterior and posterior regions compared to the central regions. Beek et al. examined seven human discs under indentation compression on the anterior band, intermediate zone, and posterior band [32]. They reported a similar observation that the modulus of the intermediate zone was approximately 2 and 3 times the modulus of the anterior and posterior bands, respectively.

The dynamic modulus of the TMJ disc is much greater than the equilibrium modulus even at a low frequency of 0.01 Hz. This is due to the interstitial fluid pressurization effect occurring within the tissue under dynamic loading conditions [142]. Based on the biphasic theory, the value of the dynamic modulus of hydrated soft tissue in the confined compression is proportional to $H_A \alpha$ (for $\alpha \gg 1$, which is true for this study). $\alpha$ is an amplification factor, reflecting the effect of fluid pressurization on
dynamic modulus, and is given by: 
\[ \alpha = \sqrt{\frac{2\pi h^2 H_A}{k}} \]
(where \( h \) is the thickness of the sample) [192, 195]. This relationship indicates that the sample with higher aggregate modulus and lower permeability correlates to a higher dynamic modulus. Using the biphasic mechanical properties obtained through creep experiments in this study, the ratio of the value of \( \sqrt{H_A/k} \) for the central regions to that of the anterior and posterior regions was approximately 2.68, which agrees well with the averaged modulus ratio (2.75) of the central regions to the anterior and posterior regions over all loading frequencies.

In summary, regional biomechanical and biochemical characterization of the human TMJ disc was conducted to address the lack of data on human TMJ disc tissue currently available in the literature. The higher water content and lower GAG content result in the human TMJ disc being much softer and permeable than human knee articular cartilage. The central regions of the disc have higher aggregate modulus and lower permeability when compared to the anterior and posterior regions, which correlated to the region-dependent water content. The dynamic modulus of the specimens in confined compression is related to the aggregate modulus and the hydraulic permeability of the tissue as a result of the biphasic nature of the tissue. The region of the TMJ disc with higher aggregate modulus and lower permeability had a higher dynamic modulus. The fluid pressurization plays a significant role in the load support of the TMJ disc under dynamic loading conditions.
Table 6 Comparison of the biphasic mechanical properties of human TMJ discs, porcine TMJ discs, and articular cartilage.

<table>
<thead>
<tr>
<th></th>
<th>$H_A$ (kPa)</th>
<th>$k \times 10^{15}$ (m$^4$/Ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human knee femoral head [201]</td>
<td>1207 ± 606</td>
<td>0.895 ± 0.54</td>
</tr>
<tr>
<td>Human knee meniscus [201]</td>
<td>604 ± 154</td>
<td>1.45 ± 0.61</td>
</tr>
<tr>
<td>Porcine confined disc</td>
<td>76.6 ± 11.34</td>
<td>67.7 ± 30.7</td>
</tr>
<tr>
<td>Human confined disc</td>
<td>50.66 ± 8.94</td>
<td>5.65 ± 0.97</td>
</tr>
<tr>
<td>Porcine confined condyle</td>
<td>17.24 ± 10.1</td>
<td>266.3 ± 96.3</td>
</tr>
</tbody>
</table>

3.7 Conclusions

In this study, the biphasic mechanical properties of the human TMJ disc were measured in order to compare the results with porcine TMJ discs as well as with other diarthrodial joints (Table 6). This study found the average aggregate modulus of the human TMJ disc to be 50.66 kPa which is 1/25 of human hip joint cartilage and 1/12 of human knee joint cartilage. The average permeability of the human TMJ disc was $5.652 \times 10^{-15}$ m$^4$/Ns which is 6 and 4 times higher than in human hip and knee cartilages, respectively [201]. A study by Kim et al. using indentation tests found similar results with the porcine TMJ disc being softer and more permeable than human articular cartilage with the average aggregate modulus of the porcine TMJ disc as 21 kPa and an average permeability of $24.1 \times 10^{-15}$ m$^4$/Ns [59]. Porcine condyle cartilage was found to have average aggregate moduli 20% that of porcine TMJ discs and permeability values 4 times greater. These findings indicate that the TMJ disc has significantly different mechanical properties than other diarthrodial joints and is likely due to the difference in tissue composition and structure.
The average aggregate modulus of porcine TMJ discs was 76.6kPa compared to 50.66kPa in human samples (33% higher). The average hydraulic permeability of porcine samples was 53.33 $\times 10^{-15}$ m$^4$/Ns compared to 5.652$\times 10^{-15}$ m$^4$/Ns (~10x higher). Average water content in porcine samples was 7% lower while collagen and GAG content were 6% and 1% higher, respectively. These significant differences in ECM content and distribution may cause the variation in mechanical properties.

Over the range of 0.01-3Hz, porcine TMJ discs exhibited an average complex moduli 10x higher than moduli found in human samples. This result could largely be related to the higher average aggregate modulus found in porcine samples. The average phase angle for porcine samples at 1Hz was 7° compared to 16° for human samples indicating a more significant elastic response. This is likely due to the higher water content of human discs which contributes greatly to the viscous properties of the material.

In summary, the biphasic mechanical properties of porcine and human TMJ discs were measured to validate the use of the porcine model and to correlate mechanical function with biochemical structure. Due to the lack of biochemical regional variation in porcine discs, no correlation for biphasic properties could be drawn. On the other hand, significant correlation between aggregate modulus and permeability with water content was found in human confined compression studies. The results of dynamic testing indicated the necessity for characterizing the viscoelastic properties of the TMJ disc. Although significant species variation for mechanical and biochemical properties was found, the porcine model remains the most accessible animal species to compare with human samples.
Chapter 4 Shear Properties

4.1 Introduction

As part of Aim 1, the biomechanical properties of the TMJ disc were characterized to better understand the complex function and environment in the cartilage tissue. Due to the incongruities between bone and cartilage surfaces, non-uniform deformation of the disc will result in the development of local shear stress. Another potential cause of shear stresses is the variation of ECM structure and distribution across the disc. Collagen fibers have a ring-like alignment along the disc periphery and run anteroposteriorly through the central region of the disc [13]. Due to the low GAG content of TMJ discs, shear properties are largely believed to be associated with collagen content and orientation. Previous studies have shown that excessive shear stress can result in fatigue and permanent damage of the TMJ disc. The static and dynamic shear properties have not been fully examined in relation to varying frequency, strain, and region.

As discussed in Chapter 2, the shear modulus was largely dependent on frequency and direction of loading which may be a result of biochemical anisotropy [147]. It is generally believed that tensile loads occurring within the cartilage are a result of shear and friction produced during joint motion [20]. Studies have found that the viscoelasticity of the disc during tension and shear, unlike compression, is largely flow-independent and primarily supported by the solid phase [202]. These findings highlighted the importance of testing within a reasonable frequency (~0.5-2 Hz during
chewing) and under sufficient compressive loading (10% strain during clenching) to maintain a physiological basis.

Using rotational shear experiments, the region-dependent equilibrium modulus and dynamic viscoelastic properties of porcine TMJ discs were examined in the following chapter. Due to the anisotropic and viscoelastic properties of the disc, the shear properties were examined in relation to frequency, shear strain, and regional impact. The results of Chapter 3 showed us that the porcine animal model was an acceptable analog for human samples. Therefore, the goal of this study was to measure the viscoelastic shear properties of the porcine model and to determine how these related to disc structure and function.

4.2 Materials and Methods

4.2.1 Sample Preparation

Twelve TMJ discs from the left joint were harvested from pig heads (6-8 month old, Yorkshire, male) and prepared according to the protocols described in Chapter 3. Shear samples from the five disc regions had an average height of 1.0mm and a diameter of 5mm.

4.2.2 Testing Configuration

Cylindrical tissue samples were placed in custom designed shear chamber that allowed for complete immersion in PBS to prevent dehydration (Figure 15). Prior to
testing, 200 grit sandpaper was affixed to both surfaces of the stainless steel 8mm parallel plate geometry with cyancrylate glue. This was necessary to prevent sample slipping during high frequency rotations. The sandpaper was allowed to soak in PBS prior to zeroing of the gap to prevent errors in the measurement of sample height.

Rotational shear experiments were performed with a TA Instruments AR G2 (New Castle, DE) at 37°C maintained by a water cooled Peltier plate. The instrument has a displacement resolution of 25nrad and torque resolution of 0.1nN/m. Initial height was measured by lowering the probe onto the sample and recording the height occurring at 5mN of force. Samples were then compressed 10% of the initial measured height to ensure full contact of the surface and prevent slipping during rotation. Studies have suggested that this amount of strain corresponds with jaw clenching [122].

4.2.3 Loading Protocol

Three testing protocols were used to measure the static and dynamic viscoelastic properties of the disc. These included a frequency sweep, strain sweep, and a stress relaxation test (Figure 16). The frequency sweep was over a range of 0.1-10 radians/sec (0.016-1.59Hz) at a constant angular strain of 0.05 radians (~2.86°). The strain sweep was over a strain range of 0.005 to 0.15 radians (~0.29°-8.6°) at an angular frequency of 10rad/sec. The stress relaxation occurred in strain steps of 0.05 radian increases from 0.005-0.15 radians with each level maintained for 900 seconds to measure the equilibrium shear modulus. Small shear strains were used to measure the flow-independent material properties. Complex modulus and phase angle were recorded for
dynamic experiments. Shear modulus was calculated from the slope of the stress versus strain curve generated from each stress relaxation step.

**Figure 15** Schematic of rotational shear chamber

**Figure 16** Schematic of frequency sweep, strain sweep, and stress relaxation experiments
4.3 Results

4.3.1 Frequency Sweep

In the shear frequency experiments, the magnitude of the complex modulus $|G^*|$ and phase angle were significantly frequency dependent. Complex modulus values for the posterior region were higher than other regions over the entire frequency range. Average standard deviations for complex moduli were approximately 45% of average moduli values. At a frequency of 6.28 rad/sec (~1Hz), the average $|G^*|$ of the central regions was 11.22±5.2 kPa, 10.20±4.9 kPa for the anterior, and 15.38±6.07 kPa for the posterior region. Phase angle decreased with increasing frequency until 1 rad/sec was reached then began to increase with increasing frequency. Phase angle values only varied between 11°-15° and showed no regional variation. Average phase angle standard deviations were approximately 15% of measured phase angles.
Figure 17 Shear frequency sweep of porcine TMJ discs. A) Relationship of complex modulus with frequency. B) Relationship of phase angle with frequency
4.3.2 Strain Sweep

In the strain sweep experiments, complex modulus significantly decreased with increasing rotational strains. The posterior region once again was significantly higher for $|G^*|$ over all frequencies tested. Average standard deviations were 40% of the magnitude of complex modulus values. Phase angle increased with increasing strain for all regions with the posterior and intermediate regions significantly higher. Phase angles only varied between $11^\circ$-15$^\circ$ and showed no significant regional variation. Average phase angle standard deviations were 15% of measured values.
Figure 18 Strain sweep of porcine TMJ discs. (A) Complex modulus (B) Phase angle
4.3.3 Equilibrium Shear Modulus

The rotational shear equilibrium modulus was measured by performing stepwise stress relaxations at increasing strain steps. The slope of the resulting stress versus strain curve at equilibrium was used to calculate the modulus. No significant regional variation was detected, but the posterior region had the highest average modulus value. The overall average equilibrium modulus was 3.53±1.61 kPa.

![Bar chart showing average ± standard deviation of regional shear modulus.](image)

**Figure 19** Average ± standard deviation of regional shear modulus.

4.4 Discussion

Due to the structure and motion of the TMJ joint, shear forces occur as a result of non-uniform loading on the tissue. These viscoelastic shear properties are dependent on direction, region, and loading frequency. Some groups have suggested that synovial fluid
may reduce friction and drop shear forces to negligible levels, but abnormal loading as a result of clenching or grinding can reduce the fluid boundary resulting in direct cartilage contacts [203]. Other studies have found that dynamic shear stress or excessive shear strain can result in fatigue failure of the TMJ disc [204]. Pathological loading may cause permanent collagen damage and cartilage degradation that can result in development of osteoarthritis [145, 146].

Lai et al., (2008) investigated the static shear properties of human TMJ discs and found the shear moduli of the peripheral regions to be significantly higher than the central portions. In our study, the posterior region exhibited significantly higher complex moduli for frequency and strain sweeps as well as a higher equilibrium modulus. The anterior region did not show increased moduli for any of the experiments. Lai’s study also found significant increases in shear moduli with increasing age suggesting changes in composition over time. The average shear modulus measured in the Lai study ranged between 1-1.75MPa which was several hundred times greater than the values measured in this study (~3kPa). These significant differences are likely a result of testing configuration differences. In the Lai study, tissue samples were attached to the testing apparatus with adhesive which could infiltrate the tissue and alter the viscoelastic properties measured. In previous studies, samples were also tested to failure which resulted in forces much higher than those experienced in vivo [64].

Zhu et al., (2004) found that the magnitude of the dynamic shear modulus increased with increasing compressive strain. Dynamic modulus was found to increase directly with loading frequency at every level of compressive strain [149]. A possible
explanation is that compression might lead to stretching of the collagen fibers running anteroposteriorly thereby strengthening the tissue to shear loading in certain directions [148]. The relationship of shear with compression in the disc is consistent with results found in bovine meniscus and articular cartilage [144, 148]. This correlation may also be a result of fluid pressurization during compression that decreases the size of the pores in the ECM which lowers the permeability of fluid, effectively stiffening the material to shear forces [149].

Previous studies found the tensile and shear properties of TMJ discs to be largely fluid flow independent suggesting much greater load support from the solid phase of the tissue [202]. These findings were further supported with the results of our dynamic frequency and strain sweeps. Phase angles only varied between 11°-15° which was a significantly smaller range than observed during dynamic compression experiments in Chapter 3 (~15°-30°). These findings suggest that during small rotational strain experiments, the solid phase of the tissue behaves elastically and only minor viscous behavior as a result of fluid flow can be measured. Shear modulus values were also significantly lower than confined compression dynamic modulus values. These results are likely due to the small strains achieved during rotational shear.

4.5 Conclusions

In Aim 1 (Chapters 3 and 4), the viscoelastic confined compression and shear properties of TMJ discs were measured to correlate mechanical function with structure.
As hypothesized, the biochemical composition of the tissue significantly affected the regional mechanical properties. For human discs, significant correlation between mechanical properties and water content were observed. Dynamic experiments on the TMJ discs were measured to correlate with frequency, strain, and region. Significant stiffening during dynamic loading was observed and is likely a result of fluid pressurization within the tissue.

Shear experiments focused on the fluid-flow independent material properties of the disc occurring at small shear strains. Due to the lack of biochemical regional variation in porcine discs, no correlation for properties could be drawn. In our studies, complex modulus was found to significantly increase with increasing frequency, but decrease with increasing rotational strain. Due to differences in testing configuration, overall magnitude of results varied from findings in the literature, but similar trends were observed. These studies were undertaken to characterize the TMJ material properties in order to understand the complex biomechanical environment as a result of loading.
5.1 Introduction

The TMJ disc is a large avascular structure and the nutrients required by disc cells are supplied by blood vessels and synovial fluid at the margins of the disc [196, 205]. 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 disc. These gradients can profoundly affect disc cell viability, matrix synthesis, and response to inflammatory factors [206, 207]. Disc cells require sufficient supply of nutrients and removal of waste products to maintain normal cellular functions. Imbalances in solute transport can result in pathological changes that may result in cell death or tissue degeneration.

The transport of small solutes (e.g., ions, oxygen, and glucose) within avascular cartilaginous tissues mainly depends on diffusion [208]. Solute movement as a result of convective flow has been found to be minor for small solutes such as glucose or oxygen [209, 210]. The rate of solute diffusion in tissue is governed by solute diffusivities which are affected by the composition and structure of the matrix, as well as mechanical strains on the tissue. Mechanical loading has been found to affect nutrient supply by altering the diffusion distance, tissue water content, and cell behavior [211]. Solute diffusivities in articular cartilage and IVD have been measured using a variety of techniques, including magnetic resonance imaging (MRI), tracking the net movement of radiolabeled solutes, and fluorescence or radiotracer desorption [212-215]. However, very few data of
solute diffusivities are available for the TMJ disc. Compared to articular cartilage and IVD, the TMJ disc has unique matrix composition and structure with higher collagen content (mainly type I) and much lower GAG and water content [9]. Therefore, the data from articular cartilage and IVD cannot be extrapolated to explain TMJ disc transport properties.

As discussed in previous chapters, significant differences in biochemical composition and structure are reasons for classifying the disc into several regions. In Aim 1, these regional variations were found to have significant impact on the viscoelastic mechanical properties of the tissue. This finding leads to the hypothesis that solute diffusion may also be inhomogeneous and anistropic. Chapter 3 also demonstrated that the porcine TMJ disc could be an acceptable analog for human discs during the development of testing protocols. Therefore, the first study of this Aim 2 will characterize the strain-dependent electrical conductivity of the tissue in porcine and human TMJ discs.

Electrical conductivity is a material property of biological tissues and is related to ion diffusivities within the tissue. Using an electrical conductivity method, the effect of mechanical strains on ion transport has previously been studied in hydrogels and IVD [216, 217]. The relationship between ion diffusivities and tissue porosity were further determined in those studies. In this study, we adopted this method to study the impact of mechanical loading on the solute transport in TMJ discs. We hypothesized that the electrical conductivity of the porcine TMJ disc is mechanical strain-dependent due to changes in tissue porosity caused by tissue compression. We also hypothesized that the
electrical conductivity is region-dependent within the TMJ disc due to its unique composition and structure. The objective of this aim will be to determine the impact of mechanical strain on solute transport in TMJ disc tissues in order to characterize nutritional supply.

5.2 Materials and Methods

5.2.1 Specimen Preparation

Twelve porcine and twenty-four human (twelve male and twelve female) TMJ discs were obtained and prepared as previously described in Chapter 3. Porcine TMJ discs came from pigs aged 8-10 months. The mean cadaver age was 76.0 for males and 79.6 for females. The porcine discs (n = 60) had an average height of 1.69±0.28mm and the human discs (n=120) had an average height of 1.49±0.46mm after flattening the surfaces via microtome. Water content was measured for all samples as described in Chapter 3. Three conductivity measurements, corresponding to three levels of compressive strain (0%, 10%, and 20%) were performed on each specimen.

5.2.2 Height Measurement

Sample heights were measured with a custom-built current sensing micrometer. The device consisted of a micrometer with an accuracy of ±3µm (Mitutoyo Corp., Kanagawa, Japan) connected to a current sensing circuit to indicate when the measuring
tip was in contact with the sample surface. The current sensing circuit consisted of a voltage comparator, relay, and an LED that lit up when the circuit was completed. This allowed for more accurate readings without significant compression of the sample during thickness measurements.

5.2.3 Electrical Conductivity Measurement

The method and apparatus for measuring electrical conductivity of tissues were previously developed and reported by Gu et al [218]. The conductivity apparatus consists of two stainless steel current electrodes coaxial to two Teflon-coated Ag/AgCl voltage electrodes placed on the top and bottom of a cylindrical nonconductive plexiglass chamber with a diameter of 5mm (Figure 20). Ag/AgCl wires were chosen due to resistance to corrosion and stability during measurements.

To ensure consistent measurements, the chamber was calibrated prior to experiments using a conductivity standard solution of 12.9mS/cm at 23°C (0.1M KCl Orion Research Inc., Boston MA). Calibration curves of chamber height (gap between measurement electrodes) versus measured resistance were created for the standard solution and PBS (bathing solution during testing). Calibration heights of 0.9, 1.6, and 2.9mm were maintained using Plexiglas spacers. Prior to sample insertion, the chamber was filled with each standard and the voltage electrodes were shifted until resistance measurements fit calibration curves. All calibrations and measurements were taken at room temperature (22.5° C).
Disc samples were thawed at room temperature in PBS and placed inside the test chamber with special care to remove air bubbles. The upper electrode was then placed on top and the micrometer lowered to maintain the initial sample height. Measurements were taken with a Source Meter (Model 2400, Keithley Instruments, Inc., Cleveland, OH), providing 3µA of direct current with offset compensation. This setting automatically subtracted the resistance measurement taken at 3µA from one taken at 0µA current to decrease errors associated with thermal noise. Applying the four-wire method the resistance (Ω) values across the specimens were measured at a very low, constant current density of 0.015 mA/cm². This current density allowed for stable measurements to be taken without damaging the tissue or electrodes. Due to the degradative effects of direct current on Ag/AgCl wires conductivity was measured again with the direction of current reversed. Stability of the electrodes was also checked by recalibrating between sample changes.

The electrical conductivity (χ) values of the specimens were calculated by:

\[ \chi = \frac{h}{\Omega A} \],

Equation 8

where \( h \) and \( A \) are the height and cross-sectional areas of the specimens respectively. The electrical conductivity of the specimen was measured at 0%, 10%, and 20% compression levels. The confined compression of the tissue specimen was achieved by lowering the micrometer to the desired height. The specimen was allowed to reach equilibrium (i.e., no fluid flow) following compression by waiting 15 minutes.
Figure 20 Schematic of apparatus for measuring electrical conductivity, consisting of two stainless steel current electrodes, two Ag/AgCl voltage-sensing electrodes, a nonconductive plexiglass chamber, a current sensing micrometer and a sourcemeter.

5.2.5 Ion Diffusivity Calculation

The TMJ disc was considered as uncharged in this study, since biochemical studies have shown that the GAG contents of both human and porcine TMJ disc are very low (< 5% dry weight) compared to hyaline cartilage and IVD [48, 219]. For uncharged tissue in NaCl, the relative diffusivity \( (D/D_0) \) of NaCl is simply related to the relative conductivity by [216]:
\[
\frac{D}{D_0} = \frac{\phi w \chi}{\chi_0},
\]
Equation 9

where \( D = (D^+ + D^-)/2 \) is the mean ion diffusivity of \( \text{Na}^+ \) and \( \text{Cl}^- \) in tissue, \( D_0 \) is the mean ion diffusivity in the bathing solution, \( \phi w \) is the water content, \( \chi \) is the electrical conductivity of the tissue, and \( \chi_0 \) is the conductivity of the bathing solution. In our analysis, the ions in PBS were assumed to be \( \text{Na}^+ \) and \( \text{Cl}^- \), since the electrical current was essentially carried by \( \text{Na}^+ \) and \( \text{Cl}^- \) ions which are dominant ionic components in PBS.

5.2.6 Statistical Analysis

Twelve porcine and 24 human discs were tested for each 5 disc regions (total 60 specimens), and the porosity, electrical conductivity, and relative ion diffusivity of each specimen were determined under 3 compressive strains. Two-way ANOVA and Tukey’s post hoc tests were performed using SPSS statistic software to determine if significant differences existed between regions and compressive strains. Linear regression was performed to correlate ion diffusivity with tissue porosity. Statistical differences were reported at p-values < 0.05.

5.3 Porcine Conductivity Results

5.3.1 Electrical Conductivity

The effects of compressive strain on electrical conductivity in the five disc regions of porcine TMJ discs are shown in Figure 21A. There was a significant decrease
in electrical conductivity with increases of compressive strain in all five disc regions (P<0.00002). The average disc electrical conductivity (mean ± SD) at 0% strain was 2.97±0.90 mS/cm, decreased to 2.65±0.74 mS/cm (-10.8%) at 10% strain, and 2.29±0.67 mS/cm (-22.3%) at 20% compressive strain. Significant regional variation of electrical conductivities was also detected at all strain levels (P<0.022). The electrical conductivity in the anterior region was significantly higher than the value in posterior region. No significant interaction was found between the level of strain and the region of the disc.

5.3.2 Porosity

The effect of compressive strain on tissue porosity of TMJ discs in the five disc regions is shown in Figure 21B. There was a significant decrease in water content with increases of compressive strain in all five disc regions (P<0.00001). The average disc porosity (mean ± SD) at 0% strain was 0.726±0.038, decreased to 0.696±0.042 (-4.1%) at 10% strain, and 0.658±0.047 (-9.4%) at 20% compressive strain. Significant regional variation of porosity was also detected at all strain levels (P<0.0004). The porosity in the anterior region was significantly higher than the value in posterior region. No significant interaction was found between the level of strain and the region of the disc.

5.3.3 Ion Diffusivity

Electrical conductivities of a PBS at 22°C were measured using an Orion conductivity meter (Model 150Aplus, Beverly, MA). The value of 15.5 mS/cm for
solution conductivity was used to normalize the measured conductivity data of tissues in Equation 9. The effect of compressive strain on ion diffusivity in the five disc regions of the TMJ disc is shown in Figure 21C. There was a significant decrease in ion diffusivity with increases of compressive strain in all five disc regions (P<0.006). The average disc relative ion diffusivity (mean ± SD) at 0% strain was 0.263±0.073, decreased to 0.245±0.062 (-6.8%) at 10% strain, and 0.225±0.061 (-16.9%) at 20% compressive strain. Significant regional variation of the ion diffusivity was also detected at all strain levels (P<0.05). The ion diffusivity in the anterior region was significantly higher than the value in the posterior region. No significant interaction was found between the level of strain and the region of the disc.
Figure 21 Effect of compressive strains on regional distribution of (A) electrical conductivity, (B) porosity (water volume fraction), (C) and relative ion diffusivity of porcine TMJ disc. The symbol (*) indicates significance compared to anterior/posterior regions for the post hoc test ($p<0.05$).
5.4 Discussion

The objective of this study was to investigate the effect of mechanical strain on solute transport in porcine TMJ discs using electrical conductivity methods. The measured electrical conductivity and calculated ion diffusivity from this study indicated that compressive mechanical strain impeded solute transport through the disc. Our results also showed that the solute transport properties of the TMJ disc were region dependent.
The decrease in electrical conductivity with increasing mechanical strain in the TMJ disc is mainly due to the porosity (water content) reduction caused by increasing compression. Previous studies in hydrogel and IVD have shown that electrical conductivity decreases with decreasing tissue hydration, which is attributed to a decrease in ion diffusivities with decreasing tissue hydration [216, 217]. In this study, we confirmed that the ion diffusivity of the TMJ disc positively correlated with the water content (Figure 22). Tissue compression caused fluid exudation and a corresponding decrease in tissue porosity (water content). In this study, the water volume fraction of the TMJ disc was decreased by 4.1% for 10% compression, and 9.4% for 20% compression. The reduction of water content resulted in decreased ion diffusivity in the tissue and subsequent decreased electrical conductivity of TMJ disc. In this study, the averaged ion diffusivity of Na\(^+\) and Cl\(^-\) in TMJ discs was decreased by 6.8% for 10% compression, and 16.9% for 20% compression. This is in agreement with previous studies reporting decreasing diffusivity of solutes with increasing static compressive strain in articular cartilage [214, 220], as well as IVD tissues [221, 222]. For example, Jackson et al. reported that the glucose diffusivity in bovine IVD is decreased by 27.5% for 10% compression, and 51% for 20% compression [221]. The strain-dependent electrical conductivity and ion diffusivity indicated that mechanical strain impeded solute transport in TMJ discs.

Our results showed that the transport properties of the TMJ disc are region-dependent. The electrical conductivity and ion diffusivity in the anterior region are significantly higher than in the posterior region. This finding is consistent with the
region-dependant solute diffusion of 4KDa FITC-Dextran in porcine TMJ disc determined by FRAP techniques [223]. This regional difference is likely due to the significant differences of tissue hydration between these two regions. Our study confirmed that the ion diffusivity in TMJ disc is positively correlated with the tissue water content. The results of this study showed that the water volume fraction in the anterior region (0.749±0.045) is significantly higher than that in the posterior region (0.707±0.017). The correlation between the diffusivity and tissue water content suggests that the diffusive transport in porcine TMJ discs is dependent on tissue composition. The inhomogeneous distribution of tissue components results in region-dependent diffusivity in TMJ disc tissues.

5.5 Human Conductivity Results

5.5.1 Electrical Conductivity

The effects of compressive strain on electrical conductivity are shown in Figure 23A for all five human TMJ disc regions using combined gender data. A significant decrease in electrical conductivity was found with increases of compressive strain in all five disc regions (P<0.05). Average conductivity at 0% strain was 5.45mS/cm, this decreased to 4.72mS/cm (-13.3%) at 10% strain and to 4.10mS/cm (-24.8%) at 20% compressive strain. Significant differences were observed between the three compressive
strains (P<0.05). However, there was no correlation between regions and electrical conductivity.

The effect of gender on electrical conductivity was examined by separating the data by gender and compressive strain in Figure 24. The female group conductivity at 0% strain was 5.79 mS/cm, decreased to 4.97 mS/cm (-14.2) at 10% strain, and 4.29 mS/cm (-25.7%) for 20% strain. The male group electrical conductivity was 5.14 mS/cm at 0% strain, decreased to 4.50 mS/cm (-12.3%) at 10% strain, and 3.93 mS/cm (-23.5%) for 20% strain. The average female group conductivity was higher than the average male group conductivity for all strain levels (P<0.05). The conductivity for the female group also decreased more significantly with each increasing level of strain (P<0.05).

5.5.2 Porosity

The effect of compressive strain on porosity or water volume fraction of TMJ discs in the five disc regions is shown in Figure 23B. There was a significant decrease in water content with increases in compressive strains in all five disc regions (P<0.05). The average disc porosity (mean ± SD) at 0% strain was 0.766 ± 0.022, at 10% strain decreased to 0.738 ± 0.025 (-3.4%) and for 20% strain decreased to 0.700 ± 0.028 (-7.6%). No regional variation of porosity was detected through all strain levels (P<0.05).
5.5.3 Ion Diffusivity

Figure 23C shows the effect of compressive strain on ion diffusivity in the five disc regions on the human TMJ disc. A significant decrease in ion diffusivity was found with an increase in all levels of compressive strain in all five disc regions (P<0.05). The average disc relative ion diffusivity (mean ± SD) at 0% strain was 0.269 ± 0.069, at 10% strain decreased to 0.225 ± 0.060 (-16.4%) and for 20% compressive strain to 0.187 ± 0.053 (-30.4%). Diffusivity was significantly related with compressive strain (P<0.05), but no regional variation was detected.

The correlation between the relative ion diffusivity and the water volume fraction was compared in Figure 25. Increasing porosity was found to correlate with increased relative diffusivity for both genders (P<0.0001). The female group data fit the linear curve-fit closer as shown with the smaller R value (0.622 compared to 0.423).
Electrical Conductivity (mS/cm)

Disc Average: 5.45±1.32 0% Strain
4.72±1.20 10% Strain
4.10±1.07 20% Strain

Water volume fraction

Disc Average: 0.766±0.021 0% Strain
0.738±0.025 10% Strain
0.700±0.028 20% Strain
Figure 23  Effect of compressive strains on regional distribution of (A) electrical conductivity, (B) porosity (water volume fraction), (C) and relative ion diffusivity of human TMJ disc. The symbol (*) indicates significance compared to anterior/posterior regions for the post hoc test (P<0.05).

Figure 24  Effect of gender and compressive strain on electrical conductivity.
Figure 25 Correlation between the relative ion diffusivity and the water volume fraction (porosity). Significant positive correlation was detected (R= 0.423 for male and R=0.622 for female).

5.6 Discussion

The objective of this study was to investigate the effect of mechanical strain, gender, and disc region on solute transport in human TMJ discs using electrical conductivity methods. Electrical conductivity and ion diffusivity were significantly impeded with increasing levels of compressive strain. The results also indicated that solute transport properties for human TMJ discs were significantly gender dependent but not region-dependent.

In this study, we confirmed that the ion diffusivity of the TMJ disc positively correlated with the water content. The water volume fraction of the TMJ disc was
decreased by 3.4% for 10% compression, and 7.6% for 20% compression. Tissue compression resulted in porosity decreases which lowered the diffusivity in the tissue. In this study, the averaged ion diffusivity of Na\(^+\) and Cl\(^-\) in TMJ discs was decreased by 14.9% for 10% compression, and 31.2% for 20% compression. These trends were in good agreement with those observed in porcine disc conductivity experiments validating the testing procedures and further confirming the pig as a suitable animal model.

The lack of regional variation for transport properties in human discs is likely due to the relative uniformity of measured water content. Ion diffusivity was found to be well correlated with water content as observed in Figure 25. The female group showed higher conductivity which may be related to the slightly higher water content. Strain affected the female group more significantly as each 10% increase in strain caused a 2% larger drop in diffusivity for female samples. The slope of the female group linear curve-fit was steeper, indicating that female group diffusivity increased more with porosity increases. This suggests that compressive strain has a greater impact on the transport of solutes in female discs than male. As discussed in Chapter 2, gender discrepancies in TMJ disorder prevalence have been found, but have yet to be explained. Some potential hypotheses include hormonal developmental differences and varying responses to pain sensations.

5.7 Conclusions

The average porcine conductivity was approximately 45.5% of the average human TMJ disc conductivity and the average water content was 6% lower. A strong correlation
between water content and electrical conductivity was found for both studies. The differences in conductivity are likely associated with disc structural differences, water content variation, and relative age of the samples tested. The electrical conductivity and tissue porosity of cartilaginous tissues were compared and listed in Table 7. The electrical conductivity in TMJ discs from both species had the lowest values compared with human articular cartilage, porcine annulus fibrosis, and human annulus fibrosis. Consequently, the mean relative diffusivity of Na\(^+\) and Cl\(^-\) in TMJ discs (porcine: 0.263±0.073, human: 0.269±0.069) was lower than that in human articular cartilage (0.35-0.45) [176] and human annulus fibrosis (0.35-0.45) [224]. The slower solute diffusion in the TMJ disc is likely due to its lower tissue water content compared to other cartilaginous tissues (Table 4). The TMJ disc is a large avascular structure [196, 205], so the balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells establishes a concentration gradient inside the disc. This study showed that solute diffusivities in the TMJ disc are much lower than the values in other cartilaginous tissues. Compressive mechanical strains were also found to further impede solute diffusion in the TMJ. Therefore, it is likely that a steep nutrient gradient may exist in TMJ discs. Such a steep gradient will be very vulnerable to any pathological event which impedes nutrient supply, such as sustained joint loading due to jaw clenching. These studies provided important insight into the electrical and solute transport behaviors in TMJ discs under mechanical loading and aid in the understanding of TMJ pathophysiology related to tissue nutrition.
Table 7  Comparison of electrical conductivity and tissue porosity between the TMJ disc and other cartilaginous tissue.

<table>
<thead>
<tr>
<th></th>
<th>Electrical conductivity (mS/cm)</th>
<th>Water volume fraction (porosity)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine TMJ disc</td>
<td>2.97±0.90</td>
<td>0.726±0.038</td>
<td>Present study</td>
</tr>
<tr>
<td>Human TMJ disc</td>
<td>5.45±1.32</td>
<td>0.766±0.021</td>
<td>Present Study</td>
</tr>
<tr>
<td>Human articular cartilage</td>
<td>6-10</td>
<td>0.8</td>
<td>[225]</td>
</tr>
<tr>
<td>Porcine annulus fibrosis</td>
<td>5.60±0.89</td>
<td>0.74±0.03</td>
<td>[218]</td>
</tr>
<tr>
<td>Human annulus fibrosis</td>
<td>7.5±0.8</td>
<td>0.80±0.02</td>
<td>[217]</td>
</tr>
</tbody>
</table>
Chapter 6 Glucose and Lactate Diffusion

6.1 Introduction

As discussed in the previous chapter, the delivery of nutrients is limited by the rate of diffusion through the tissue (particularly for small solutes). Nutrients such as glucose and oxygen are important for energy metabolism and ECM maintenance. Cells consume oxygen and glucose to generate adenosine triphosphate (ATP) for energy and produce lactic acid as a waste product during glycolysis. The build-up of lactic acid can result in the lowering of pH which can affect cell viability and metabolism [211]. Studies on the IVD have found that matrix turnover and cell viability can be significantly affected by lack oxygen and glucose [226]. Therefore it is necessary to measure the rates at which nutrients can be delivered and wastes can be removed to better understand the local cellular environment.

Chapter 5 demonstrated that even transport of small ions such as Na⁺ and Cl⁻ could be impeded via mechanical loading. The effect of mechanical strains on small nutrient diffusivities has not been investigated in TMJ discs and is important for assessing the nutrient concentrations available for disc cells. As the jaw undergoes normal function, the TMJ disc is subjected to a variety of loading modalities which alter the physical properties and capacity for solutes to diffuse through the tissue. This suggests that deviation from physiological nutrient levels in the TMJ disc due to insufficient solute transport may initiate tissue remodeling and matrix degradation. Compression has significant impact on diffusion distance and water content of the tissue.
in turn affecting solute transport. Determining the diffusion of solutes is therefore important in understanding transport mechanisms and the resulting biological responses of disc cells. Therefore, this study characterized the unsteady-state strain-dependent diffusion properties of glucose and lactate through TMJ discs using diffusion chambers. We hypothesized that regional variation existed due to anisotropic ECM distribution as well as significant strain dependence resulting from fluid exudation during compression.

6.2 Materials and Methods

6.2.1 Specimen Preparation

Twelve TMJ discs from the right joint of pigs aged 8-10 months were removed and prepared as previously described in Chapter 3. The prepared cylindrical disks from the 5 regions (n=60) had a height of 1.69±0.29mm and a diameter of 6mm. Sample heights were measured with the current sensing micrometer described in Chapter 5.

6.2.2 Diffusion Measurements

A custom diffusion chamber was built for the purpose of measuring glucose and lactate diffusivity through TMJ disc tissues. The device consisted of two non-conductive acrylic solution chambers with a channel separated by the specimen holder (Figure 26). Samples were confined between two rigid hydrophilic porous polyethylene (PE) plates to maintain uniform compressive strains and prevent sample swelling. The PE plates had thickness of 0.5mm and pores sized 50-90 µm which allowed for unimpeded solute diffusion due to the nanoscale size of glucose and lactate molecules. A nitrile o-ring was
placed in a groove around the periphery of the sample to prevent swelling and leaking. Sample height was maintained with spacers cut to the appropriate sample thickness and were placed between the chambers to prevent tissue overcompression.

After thawing the sample at room temperature, the tissue was confined in the acrylic chamber at the appropriate initial height. Both the upstream and downstream wells were filled with a 3x concentrated PBS solution to wash out any residual glucose or lactate in the tissue. This concentrated PBS solution was also used to fill the downstream well during experiments to balance the osmolarity of the glucose/lactate solution of the upstream well. This was important in preventing any electrical potential gradients from developing as a result of osmolarity differences between the wells. The height of the solution in each well was also kept equal to prevent hydrostatic pressure resulting in convective flow.

To begin the experiment, 500 µL of 20mg/mL glucose with 10mg/mL lactate mixed into 1x concentration PBS was pipetted into the upstream chamber while 200 µL of 3x concentrated PBS solution was pipetted into the downstream chamber. The entire diffusion chamber was placed on a stir plate in an incubator to maintain 37°C and utilize stir bars to prevent stagnant boundary layer formation. Solution volumes were chosen to allow concentration measurements to be measurable over reasonable periods of diffusion (15 minutes) estimated from the literature [221].

At the end of each 15 minute time interval, the downstream solution was removed and collected in micro-centrifuge tubes for measurement of glucose and lactate concentrations. Chambers were flushed with PBS and wiped dry before adding fresh
solutions to each well as described above. This was repeated every 15 minutes until the same concentration (within 5%) was measured 3 times concurrently, suggesting that steady state had been reached (~after 2 hours).

At the end of the final 15 minute interval, the tissue was allowed to re-equilibrate in PBS solution for at least 1 hour. This was done to wash out a significant portion of the residual glucose and lactate in the tissue. Following re-equilibration, the spacer was changed to allow measurements to be taken at 10% and 20%.

![Figure 26 Schematic of glucose/lactate diffusion chamber.](image)

**6.2.3 Measurements**

Glucose and lactate concentrations were measured with a YSI 2700 Select Biochemistry Analyzer (YSI Inc., OH). The instrument works by removing 15μL of the downstream solution and mixing it with a buffer solution in a test chamber. A probe on either side holds a membrane with immobilized oxidase enzymes specific to the substrate of interest which reacts with the solution in the chamber. The substrate diffuses through membrane, reacts with the enzymes and produces hydrogen peroxide. The hydrogen
peroxide is oxidized at a platinum electrode to produce electrons which is measured as current. The electron flow is proportional to the hydrogen peroxide concentration and the initial substrate concentration. The instrument is capable of measuring glucose and lactate with the ranges of 0-9g/L and 0-2.67g/L, respectively.

### 6.2.4 Diffusion Process

This chamber allows for the one-dimensional steady-state glucose and lactate diffusivity to be measured. This technique has been previously used for measuring diffusivities through IVD tissues [221]. A sample of known thickness and with zero initial concentration of solutes (assumed after washing with PBS) is clamped between the wells of the diffusion chamber. The glucose/lactate solution is introduced into the upstream chamber and allowed to gradually diffuse across the tissue into the downstream chamber.

The movement of a solute from a region of higher concentration to lower concentration due to random motion is known as diffusion and was first modeled by Fick’s Law:

$$ J = -D \frac{\partial C}{\partial z} $$

Equation 10

where $J$ is the diffusive flux, $D$ is the diffusion coefficient, $C$ is the concentration of the solute, and $z$ is the distance for solute transport. In this equation we are solving for the apparent diffusivity, $D_{app}$, which incorporates the effect of the partition coefficient, $K$ ($D_{app}=K D_{eff}$ where $D_{eff}$ is the effective diffusion).
The concentration gradient can be estimated as the concentration difference, \( \Delta C \), across the thickness of the tissue, \( h \). The diffusive flux, \( J \), can be defined as the mass flow rate, \( \Delta Q \), of the solute per unit area, \( A \):

\[
J = -D_{app} \frac{dC}{dx} = -D \frac{\Delta C}{h}
\]

Equation 11

where \( V \) is the volume of the downstream solution, and \( t \) is the time of diffusion. This relationship is therefore equal to:

\[
\frac{V_{down} \frac{dC_{down}}{dt}}{Adt} = -D_{app} \frac{dC}{dx}
\]

Equation 13

where \( V_{down} \) is the volume in the downstream chamber, and \( C_{down} \) is the solute concentration in the downstream chamber. Assuming that steady state is achieved, where the distribution of solutes across the tissue is linear (then \( -\frac{dC}{dx} = \frac{(C_{up} - C_{down})}{h} \)) and by cross multiplying we obtain:

\[
\frac{dC_{down}}{dt} = \frac{AD_{app} (C_{up} - C_{down})}{V_{down} h}
\]

Equation 14

\[
\frac{dC_{down}}{(C_{up} - C_{down})} = \frac{AD_{app}}{V_{down} h} dt
\]

Equation 15

Let \( C' = C_{up} - C_{down} \). Therefore, \( dC' = -dC_{down} \) due to the assumption that the upstream concentration is constant. The equation can be rewritten as:
\[- \frac{dC}{C} = \frac{AD_{\text{app}}}{V_{\text{down}} h} dt \]

Equation 16

Integrating this,

\[ \int_{C_{\text{down}(t_0)}}^{C_{\text{down}(t)}} \frac{dC}{C} = \int_{t_0}^{t} \frac{AD_{\text{app}}}{V_{\text{down}} h} dt \]

Equation 17

We eventually obtain

\[ \ln \frac{C_{\text{up}} - C_{\text{down}(t_0)}}{C_{\text{up}} - C_{\text{down}(t)}} = \frac{AD_{\text{app}}}{V_{\text{down}} h} (t - t_0) \]

Equation 18

Where \( C_{\text{down}(t_0)} \) is the concentration in the downstream well at \( t_0 \) and \( C_{\text{down}(t)} \) is at time \( t \).

Solving for \( D_{\text{app}} \) yields:

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

Equation 19

The apparent diffusion coefficient can therefore be calculated with this equation once steady state is reached.

6.2.5 Statistics

Twelve porcine TMJ discs were tested for each 5 disc regions (total 60 specimens), and the glucose and lactate diffusivity of each specimen were determined under 3 compressive strains. Two-way ANOVA and Tukey’s post hoc tests were performed using SPSS statistic software to determine if significant differences existed between regions and compressive strains.
6.3 Results

The apparent glucose and lactate diffusivities were measured for porcine TMJ discs at 0%, 10%, and 20% compressive strains (Figure 27 and 28). Diffusivities were measured for 5 disc regions at 37° C. Average glucose diffusivity at 0% strain was $5.22 \pm 0.99 \times 10^{-11}$ m$^2$/sec, decreased to $4.77 \pm 1.24 \times 10^{-11}$ m$^2$/sec (-8.5%) at 10%, and $3.65 \pm 0.84 \times 10^{-11}$ m$^2$/sec (-30%) for 20%. The average lactate diffusivity was $8.24 \pm 1.18 \times 10^{-11}$ m$^2$/sec, $7.80 \pm 1.38 \times 10^{-11}$ m$^2$/sec (-5.3%) and $6.28 \pm 1.02 \times 10^{-11}$ m$^2$/sec (-24%) for 0, 10, and 20% strains, respectively. Diffusivity for both solutes was found to be higher in the lateral region, but these results were not significant. The ratio of glucose to lactate diffusivity was an average 0.60. Tests were conducted at 37° C with stir bars to prevent stagnant boundary formation. Increases in compressive strain significantly decreased average diffusivity for both glucose and lactate (P<0.05).
Figure 27 Effect of compressive strain on glucose diffusion by region.

Figure 28 Effect of compressive strain on lactate diffusion by region.
6.4 Discussion

One dimensional steady state diffusion measurements to determine apparent glucose diffusivity have been used in other cartilaginous tissue [221, 227]. At the present time, no studies have measured the glucose and lactate diffusivities of TMJ disc tissues. Other studies have reported significant strain dependence for diffusion in IVD and knee articular cartilage. Diffusivity of glucose and lactate decreased with increasing compressive strain, with significance occurring at 20% compression. This trend is likely a result of fluid being forced out of the tissue during loading. The relationship between compressive strain and decreased diffusivity appeared to be more significant in the IVD and may be a result of the higher overall water content. Nutrient transport has been found to be highly correlated with tissue porosity for the transport of solutes including ions and dextran [220].

Diffusivity studies on other cartilage tissues found significant anisotropy as a result of structure and direction of ECM components. In our studies, no significant anisotropy was observed in diffusion measurements and may be a result of the lack of regional water and ECM content variation (Table 4). The lateral region was found to have higher overall diffusivity, but these results were not found to be significant.

Compression of 10% decreased diffusivity by approximately 8.5% while 20% decreased diffusivity 30% compared to the diffusivity at initial height.

Gu et al. determined the axial apparent glucose diffusivity of the IVD to be $1.38 \times 10^{-10}$ m$^2$/sec at 0% strain, $1.00 \times 10^{-10}$ m$^2$/sec at 10% strain (-27.5%), and $7.65 \times 10^{-11}$ m$^2$/sec (-44.5%) for 20% compressive strain [221]. The average glucose diffusivity for
IVD tissues was ~2-2.5 times greater than the values measured in TMJ disc tissues. As with conductivity, these results may be associated with the overall lower water content of TMJ tissues compared to IVD and articular cartilage.

The results of this study indicated a significant interaction between compressive strain and lowered nutrient transport. Compression of the sample significantly impeded solute diffusion at 20% strain suggesting potential difficulty for nutrient transport and waste removal during sustained or pathological loading. The minor regional variation is potentially related to inhomogeneous distribution of collagen fibers and glycosaminoglycan molecules. This study further confirmed that compressive strain induced by mechanical loading may impact disc nutritional supply and serve as a possible cause of TMJ pathologies.

Glucose diffusion occurred at 60% the rate of lactate diffusion and is related to the molecular weight or atomic size of the solute. Glucose has a molecular weight of 180g/mol and an estimated radius of 0.365nm while lactate has a molecular weight of 90g/mol and radius of 0.23nm. While glucose is an uncharged ring-shaped structure, lactate forms a charged anion in solution which may interact with the charged components of the tissue ECM. All of these factors contribute to the ability of the solutes to diffuse through the pores of the TMJ disc. Glucose is important for energy metabolism and ECM production while lactate removal is necessary to prevent acidic buildup. These studies have shown that mechanical strain significantly impacts the rate of solute transport in the disc tissue which can have significant biological consequences.
6.5 Conclusions

The experiments of Aim 2 were crucial in understanding the impact of mechanical loading on nutrient transport in TMJ disc tissues. Chapter 5 focused on the diffusion of small charged ions which are important for disc cells to maintaining osmotic balance. The solutes discussed in Chapter 6 are important in cellular energy metabolism and regulation of pH. The relatively low diffusivity of solutes in the TMJ discs suggests that steep nutrient gradients can develop and are affected by mechanical loading. Significantly lower diffusivities were found in TMJ discs than other cartilaginous tissues which may result from lower overall water content. Compressive loading was found to significantly decrease the delivery of nutrients and is likely associated with fluid exudation and decreased tissue porosity. These findings support our hypothesis that pathological mechanical loading can result in decreased nutrient levels deep within the TMJ discs.
Chapter 7 Oxygen Consumption Rates

7.1 Introduction

As previously discussed, the balance between the rate of nutrient diffusion through the matrix and the rate of consumption by disc cells potentially establishes a concentration gradient within the disc. The nutrients are supplied from blood vessels connected to the disc or synovial fluid filling the joint cavity [45]. While nutrients diffuse through the tissue, cells utilize these solutes for energy metabolism and matrix synthesis, and produce waste products that must be removed. The balance between nutrient delivery and usage results in a gradient that must be maintained in a healthy joint. Recent studies have shown that hypoxia with inflammation modulates gene expressions of tenascin-C and matrix metalloproteinases in TMJ disc cells [206, 207].

Understanding these gradients is therefore important for studying TMJ disc physiology and pathophysiology. Due to the difficulty of measuring these gradients in vivo, mathematical models have been used to predict the nutrient environment inside cartilaginous tissues. Most of these models have primarily studied oxygen as it is regarded as an important factor directly affecting cell biological activity [228]. Oxygen gradients have been modeled for growth cartilage [229], articular cartilage [230], engineered cartilage [231, 232] and the intervertebral disc (IVD) [157, 166]. It has also been shown that the oxygen consumption rates of IVD cells and articular cartilage cells are dependent on the culture conditions. In order to obtain a realistic prediction of in vivo nutrient distribution, metabolic rates of cells have to be taken into account in the
theoretical model. Therefore, measuring the oxygen consumption rate of TMJ disc cells is crucial for precise theoretical analyses of nutrient transport in the TMJ disc. In this study, we will determine cellular consumption rates of oxygen in cultured cell suspensions, 3D agarose constructs, and fresh tissue explants of porcine TMJ disc. The obtained functional relationships between nutrient consumption rates, oxygen tension, glucose concentration, and pH value will be important in understanding the mechanism of the energy metabolism of TMJ disc cells.

7.2 Materials and Methods

7.2.1 Specimen Preparation

A total of twenty pig heads (American Yorkshire, male, aged ~ 8-10 months) were collected from a local abattoir within 2 hours of slaughter. The entire TMJ with capsule intact was removed en bloc. Joints were opened under a sterile dissection hood; TMJ discs were then removed and washed with 5-6 changes of phosphate buffered saline. Five TMJ discs of the left joints were used to determine cell density distribution via confocal laser scanning microscopy. Five TMJ discs of the corresponding right joints were used to determine DNA content to validate cell density measurements. Ten discs from both left and right joints were then used to measure the oxygen consumption rate of tissue explants.
7.2.2 Cell Suspensions

In order to determine the optimum protocol conditions, TMJ disc cells were tested as suspensions, 3D agarose gels, and as explants for comparison. The washed TMJ discs were digested with Trypsin-EDTA and Collagenase Type-II. The resulting digestions were filtered and re-suspended in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (Invitrogen Corp., NY). Cells were cultured for ~10 days before second passage cells could be experimentally tested at densities of ½, 1, 2, and 4 million cells/mL. Cell densities were checked via hemocytometer prior to re-suspension. To test the effect of nutrient environment, suspensions were incubated for 24-hours at various glucose levels (1, 2.5, 5, and 25mM) to allow cells to adapt. Media was treated with HEPES to alter the pH to 6.2, 6.8, and 7.4 to determine the effect of pH on consumption rate.

7.2.3 3D Agarose Gels

Second passage digested cell suspensions were mixed with equal volumes of 4% low-melting temperature agarose gel solutions (made with PBS) to produce 2% gels. Gel solutions were poured into 5mm diameter acrylic molds to produce cylinders with 1mm height. Before tests, these disc shape constructs were incubated for one day in DMEM containing 10% FBS, and 1% antibiotic-antimycotic at 37° C. Discs were diced (pieces smaller than 0.1mm) to maximize the surface area for diffusion and to prevent development of diffusion gradients.
7.2.4 Explants

Fresh TMJ disc explants (~0.075g wet weight/explant) were harvested from 5 regions and the tissue volume of each explant was determined in PBS based on the Archimedes’ principle using the method described in Chapter 3. Each region will be normalized by cell density values determined from confocal measurements and validated with DNA content assays. Explants were immediately diced into small pieces (<0.1mm) to minimize the concentration gradient of oxygen within the explant and then placed into a metabolism chamber containing DMEM with 5mM glucose at pH 7.4. This glucose concentration is considered physiological in synovial fluid [233] and was selected for measuring basal oxygen consumption rate of the TMJ disc in this study. At the end of experiments, the explant pieces were fully digested and the cell viability was examined via trypan blue exclusion (greater than 90% viability).

7.2.5 Oxygen Chamber

The chamber for measuring oxygen concentration consists of two titanium 500uL chambers with acrylic sealing plug that creates a seal via fluid column and hydrostatic pressure. The medium used in the metabolism chamber had been preheated to 37°C and stirred in air for 10 minutes to establish constant initial dissolved oxygen concentration. Micro stir bars were set to a low speed setting to prevent stagnant fluid layer development and evenly distribute solutes in the chamber. Chambers were calibrated before experiments with 0% O₂ standard solution (Oakton Instruments, IL) and fully oxygenated media.
The concentration of dissolved oxygen in the culture medium at 37°C and atmospheric pressure was 200 μmol/L (or 6.4 mg/L). The oxygen consumption rates of TMJ disc cells were measured in a stirred, water-jacketed chamber maintained at 37°C (Instech Laboratories, Plymouth Meeting, PA) (Figure 29). After the chamber was sealed, real time dissolved oxygen concentration in the medium was recorded every 30 seconds by a fiber optic oxygen sensor (Ocean Optics, Dunedin, FL) until the oxygen concentrations fell to 0.95 μmol/L (0.1% oxygen). O₂ tension is measured with a fiber optic needle probe coated with a captured fluorescent dye. A reading is generated from the intensity spectrum by calculating the area under the curve (centered around 600nm). At the end of the experiments, the glucose concentration and pH of the culture medium were measured. The decrease in glucose concentration and the change of pH were found to be minimal.

**Figure 29** Schematic of oxygen consumption rate chamber.
7.2.6 Cell Density Measurement

A confocal microscopy based technique was developed to determine the \textit{in situ} surface-regional cell distribution of the TMJ disc. The volume-based cell density measurements were accomplished by counting cell numbers in specific volumes from reconstructed three-dimensional (3D) images. Each porcine TMJ disc was divided into five regions: anterior, intermediate, posterior, lateral and medial as previously described. These specimens were then sectioned into three layers (100µm each) along the superior-inferior axis using a microtome (SM2400, Leica Microsystems GmbH, Wetzlar, Germany). The nuclei of samples were stained with DRAQ5™ (Biostatus Limited, Leicestershire, UK) and all samples were scanned with a Leica TCS SP5 Confocal Microscope System (Leica Microsystems, Inc., Exton, PA). 2D image series were acquired by Z-stack scanning with a 1µm step in the Z-direction and a Field of View in the X-Y plane of 387.5µm×387.5µm. 3D images of stained cells were reconstructed in the image processing software based on the Z-stack image series, and cell density measurements were obtained from these by counting cell numbers in observed tissue volumes (Figure 30)
7.2.7 DNA Content Measurement

The above technique was validated by measuring the DNA content of corresponding disc tissues from the same animals. Tissue plugs were punched from five regions of the right side discs, and the volume of each specimen was determined in PBS using a density determination kit (Sartorius YDK01, Germany) and an analytical balance based on the Archimedes’ principle [193]. The specimens were lyophilized for 2 days and then digested in 1 mL of papain solution (125 µg/ml papain; Worthington Biomedical Corporation), 100 mM phosphate buffer, 10 mM cysteine and 10 mM EDTA, at pH 6.3 and 60°C for 36 hours. The DNA content was determined using a DNA Quantitation Kit, Fluorescence Assay (Sigma, St. Louis, MO, USA). A conversion factor of 7.7 pg DNA per TMJ disc cell was used [48]. The cell density of the specimen was determined by taking the ratio of cell number to tissue volume.

Figure 30 Schematic of confocal slices used for 3D cell counting.
7.2.8 OCR Measurement

A typical plot of dissolved oxygen concentration over time is shown in Figure 31A. The rate of oxygen consumption in the TMJ disc cells enclosed in the metabolism chamber can be calculated from the recorded decrease in oxygen concentration versus time. Based on our pilot study, the relationship between the oxygen consumption rate and oxygen concentration can be expressed using the Michaelis-Menten equation:

\[ R = \frac{V_{\text{max}} \times C}{K_m + C} \]  
Equation 20

where \( R \) is oxygen consumption rate (\( \mu \text{mol/mL tissue/hr} \)), \( V_{\text{max}} \) is the maximum oxygen consumption rate (\( \mu \text{mol/mL tissue/hr} \)), \( K_m \) is the Michaelis-Menten constant (\( \mu \text{mol/L} \)), and \( C \) is the oxygen concentration in the chamber (\( \mu \text{mol/L} \)). Based on the conservation of mass, the time rate of oxygen concentration change (\( dC/dt \)) in the sealed chamber is given by:

\[ \frac{dC}{dt} = -\frac{1000V_{\text{max}}C}{K_m + C} \cdot \frac{\text{Vol}_{\text{tissue}}}{\text{Vol}_{\text{chamber}}} \]  
Equation 21

where \( \text{Vol}_{\text{tissue}} \) is the tissue volume of the explant (mL), \( \text{Vol}_{\text{chamber}} \) is the volume of the metabolism chamber (mL). In this study, the chamber volume is 0.5mL. Integrating Equation 21, we can determine the oxygen concentration in the chamber over the time:

\[ t = \frac{K_m}{1000V_{\text{max}} \text{Vol}_{\text{tissue}}/\text{Vol}_{\text{chamber}}} \ln\left(\frac{C_0}{C}\right) + \frac{C_0 - C}{1000V_{\text{max}} \text{Vol}_{\text{tissue}}/\text{Vol}_{\text{chamber}}} \]  
Equation 22

where \( C_0 \) is the initial (t=0) oxygen concentration in the chamber. Curve-fitting the recorded oxygen concentration data to Equation 22, we determined the kinetic coefficients \( V_{\text{max}} \) and \( K_m \) to establish the functional relationship between the oxygen
consumption rate $R$ and the oxygen concentration $C$. Each measured volume base $V_{max}$ was then normalized by the cell density either measured via hemocytometer or confocal microscopy.
Figure 31 (A) Typical record of dissolved oxygen concentration in the chamber over time. The experimental data were curve fit to Equation 22. (B) The oxygen consumption rate was plotted based on the Michaelis–Menten equation with determined parameters $V_{\text{max}}$ and $K_m$. 
7.3 Results

7.3.1 Effect of Cell Seeding and Environmental Conditions

Based on the studies on cell density, 1 million cells per mL was the most appropriate in terms of experimental time and approximation with the Michaelis-Menten curvefit. Suspensions and 3D gels had similar $V_{\text{max}}$ and $K_m$ values while explants had significantly lower consumption rates (Figure 32A). Cell suspension results show that low pH increases the consumption rate of disc cells significantly (Figure 32B). Glucose concentration did not appear to have significant effect on consumption rates. Normalized consumption rates for suspensions and 3D cultured cells had similar consumption rates while explants were lower.
Figure 32 (A) O₂ consumption rates comparison with other cartilaginous tissues[25] [158]. (B) Effect of pH on oxygen consumption rate.
7.3.2 Distribution of Cell Density

The surface-regional distribution of the volume based cell density in porcine TMJ discs was determined in situ using confocal microscopy techniques. Confocal assessment yielded an overall cell density (mean, 95% CI) of 51.3(21.3-81.3)×10^6 cells/mL in wet tissue. Surface-regional variations in cell density along the superoinferior, anteroposterior, and mediolateral axes are depicted in Table 8. Along the 3 axes, statistically significant differences in total cell numbers were observed only along the anteroposterior and mediolateral axes, with no statistical difference between layers along the superoinferior axis. Although there was no statistically significant difference along the superoinferior axis, the cell density in the middle layer was lower than in the superior and inferior layers. Along the anteroposterior axis, the anterior band had 25.5% higher cell density than the intermediate zone (p<0.02) and 29.1% higher than the posterior band (p<0.008), with no significant difference between the intermediate and posterior band. Along the mediolateral axes, the medial region had 26.2% higher cell density than the intermediate zone (p<0.04) and 25.4% higher than the lateral region (p<0.045), although there was no significant difference between the intermediate zone and lateral region.
Table 8 Surface-regional distribution of cell density (mean, 95% CI): superioinferior direction ($P=0.415$), anteroposterior direction ($P<0.01$) and mediolateral direction ($P<0.04$).

<table>
<thead>
<tr>
<th>Surface/region</th>
<th>Cell density ($\times 10^6$ cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoinferior</td>
<td>Superior 53.6 (20.1–87.1)</td>
</tr>
<tr>
<td></td>
<td>Middle 48.1 (22.8–73.4)</td>
</tr>
<tr>
<td></td>
<td>Inferior 52.6 (21.6–83.6)</td>
</tr>
<tr>
<td>Anteroposterior</td>
<td>Anterior 58.5 (31.8–85.2)*</td>
</tr>
<tr>
<td></td>
<td>Intermediate 46.6 (23.9–69.3)</td>
</tr>
<tr>
<td></td>
<td>Posterior 45.3 (22.8–67.8)</td>
</tr>
<tr>
<td>Mediolateral</td>
<td>Intermediate 58.8 (24.5–93.1)*</td>
</tr>
<tr>
<td></td>
<td>Medial 46.6 (23.9–69.3)</td>
</tr>
<tr>
<td></td>
<td>Lateral 46.9 (14.2–79.6)</td>
</tr>
</tbody>
</table>

7.3.3 Cell Density Validation using DNA Assays

The total DNA content in the TMJ disc was $0.42(0.32-0.518)$ mg/mL in wet tissue. Correspondingly, the cell density across the disc was calculated as $54.6(42.4-66.7)\times10^6$ cells/mL wet tissue. The comparison of cell density between the confocal microscopy technique and DNA assay is shown in Figure 33. The cell densities measured by the DNA assay was comparable to the measurements of the confocal technique. There were no significant differences between the two methods for cell density measurements in any of the five regions.
Figure 33  Comparison of cell density between the confocal microscopy technique \((n = 5)\) and DNA assay \((n = 5)\). The error bar is a 95% CI. No significant differences were detected in each disc region.

7.3.4 Oxygen Consumption Rate

A typical curve-fit of the recorded oxygen concentration data with Equation 22 is shown in Figure 34A. Good agreement was found between the experimental data and theoretical curve-fitting with \(R^2=0.989\pm0.007\) \((n=40)\), indicating that the relationship between the oxygen consumption rate and oxygen concentration can be well expressed using the Michaelis-Menten equation with the two parameters \(V_{max}\) and \(K_m\). The \(V_{max}\) is the maximum oxygen consumption rate at high oxygen tension, and the \(K_m\) is the oxygen tension at which the oxygen consumption rate decreases to 50% of the \(V_{max}\) (Figure 34B). The overall tissue volume based \(V_{max}\) was 1.44(0.44-2.44) \(\mu\text{mol/mL}\) wet tissue/hr. One-way ANOVA showed that the volume based \(V_{max}\) was significantly region-dependent
The medial region had the highest, while the anterior had the lowest regional consumption rate (Table 9). There was no significant difference between the medial, intermediate, and lateral regions, as well as between the anterior and posterior bands. However, the averaged volume based $V_{max}$ of central regions ($1.69(0.55-2.83)\mu$mol/mL wet tissue/hr), including intermediate, lateral, and medial, was 76% higher than the averaged value of the anterior and posterior bands ($0.96(0.39-1.53)\mu$mol/mL wet tissue/hr). The overall $K_m$ was $19.1(13.6-24.6)\mu$mol/L with no significant regional differences found.

The cell based $V_{max}$ was calculated by normalizing each volume based $V_{max}$ with the corresponding regional mean value for cell density obtained from confocal microscopy. The overall cell based $V_{max}$ was $28.7(12.2-45.2)\text{ nmol/10}^6 \text{ cells/hr}$. Compared to the volume based $V_{max}$, region-dependency was further enhanced in the cell based $V_{max}$ ($p<0.005$) with similar trends of regional distribution (Table 9). The average cell based $V_{max}$ of central regions ($34.4(13.6-55.2)\text{ nmol/10}^6 \text{ cells/hr}$), including intermediate, lateral, and medial, was 72% higher than the averaged value of anterior and posterior bands ($20.0(7.8-32.2)\text{ nmol/10}^6 \text{ cells/hr}$).

The relationships between the oxygen consumption rate and oxygen concentration in the five disc regions are plotted in Figure 34A using the Michaelis-Menten equation with averaged $V_{max}$ and $K_m$. The oxygen consumption rate was relatively constant and fairly independent of oxygen tension until the oxygen tension fell below 5%. Below 5% oxygen, the rate fell in a highly concentration-dependent manner. Based on the Michaelis-Menten equation, the sensitivity of the oxygen consumption rate to the oxygen
tension is solely controlled by the parameter $K_m$. Using the averaged $K_m$ (19.1 $\mu$mol/L) of this study, the oxygen consumption rate relative to that at 21% oxygen tension (1% oxygen = 9.5 $\mu$mol/L) was shown in Figure 34B. The relative oxygen consumption rate was 0.91 at 10% oxygen, 0.78 at 5% oxygen, 0.55 at 2% oxygen, and 0.36 at 1% oxygen.
Figure 34 (A) Relationship between the oxygen consumption rate and the oxygen tension for five regions. (B) Predicted relationship between the relative oxygen consumption rate and the oxygen tension.
Table 9 Regional distribution of oxygen consumption rate (n = 4): volume based $V_{\text{max}}$ (significantly region-dependent, $P < 0.02$), cell based $V_{\text{max}}$ (significantly region-dependent, $P < 0.005$), and kinetic constant $K_m$ (not significantly region-dependent, $P = 0.965$)

<table>
<thead>
<tr>
<th>Region</th>
<th>Volume based $V_{\text{max}}$ ($\mu$mol/mL tissue/h)</th>
<th>Cell based $V_{\text{max}}$ (nmol/10^6 cells/h)</th>
<th>$K_m$ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>0.95 (0.28–1.62)</td>
<td>18.3 (5.4–31.2)</td>
<td>17.2 (5.6–28.8)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>1.47 (0.19–2.75)</td>
<td>31.6 (0.6–62.6)</td>
<td>17.3 (4.6–30.0)</td>
</tr>
<tr>
<td>Lateral</td>
<td>1.67 (0.42–2.92)</td>
<td>35.6 (8.4–62.8)</td>
<td>20.9 (4.4–37.4)</td>
</tr>
<tr>
<td>Medial</td>
<td>2.16 (0.38–3.94)*</td>
<td>36.7 (6.5–66.9)*</td>
<td>23.6 (13.2–34.0)</td>
</tr>
<tr>
<td>Posterior</td>
<td>0.98 (0.45–1.51)</td>
<td>21.5 (9.9–33.1)</td>
<td>17.8 (10.4–25.2)</td>
</tr>
</tbody>
</table>

The symbol (*) indicates significance compared to anterior/posterior bands for the post hoc test ($P < 0.05$).

Table 10 Comparison of tissue thickness and cell density between knee articular cartilage and the TMJ disc

<table>
<thead>
<tr>
<th>Type of joint and species</th>
<th>Tissue thickness (mm)</th>
<th>Cell density ($\times 10^6$ cells/mL)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit knee</td>
<td>0.21</td>
<td>188</td>
<td>[232]</td>
</tr>
<tr>
<td>Dog knee</td>
<td>0.67</td>
<td>44.4</td>
<td>[232]</td>
</tr>
<tr>
<td>Bovine knee</td>
<td>1.7</td>
<td>19.8</td>
<td>[232]</td>
</tr>
<tr>
<td>Human knee</td>
<td>2.3</td>
<td>14.1</td>
<td>[232]</td>
</tr>
<tr>
<td>Porcine TMJ disc</td>
<td>2–4</td>
<td>51.3</td>
<td>Present study</td>
</tr>
</tbody>
</table>
Table 11 Comparison of cell based oxygen consumption rate between the TMJ disc and other cartilaginous tissues

<table>
<thead>
<tr>
<th>Type of joint and species</th>
<th>Subpopulation</th>
<th>$V_{\text{max}}$ consumption rate (nmol/10^6 cells/h)</th>
<th>$K_m$(μmol/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine articular chondrocytes</td>
<td>Superficial</td>
<td>3.2</td>
<td>68</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Deep</td>
<td>6.6</td>
<td>63</td>
<td>[25]</td>
</tr>
<tr>
<td>Porcine IVD</td>
<td>AF</td>
<td>6.0</td>
<td>35.7</td>
<td>[158]</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>11.5</td>
<td>6.8</td>
<td>[158]</td>
</tr>
<tr>
<td>Porcine TMJ disc</td>
<td>Anterior</td>
<td>18.3</td>
<td>17.2</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>Central</td>
<td>34.4</td>
<td>20.6</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>Posterior</td>
<td>21.5</td>
<td>17.8</td>
<td>Present study</td>
</tr>
</tbody>
</table>

7.4 Discussion

Since the TMJ disc is avascular [23, 196, 205], the consumption rate 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 basal oxygen consumption rate in porcine TMJ discs and further examine the effect of oxygen tension on those rates. Preliminary studies were done to determine the optimum cell seeding technique and environmental conditions. Cells were tested as suspensions, 3D agarose gel constructs, and fresh disc explants. Cell suspensions were tested in a variety of pH and glucose conditions.

Recent studies have shown that the oxygen consumption of isolated articular chondrocytes increases with in vitro culture duration [234]. Oxygen consumption increased exponentially in that previous study within the first week and had doubled.
within the first 24 hours. The increase in oxygen consumption capacity could not be negated by culturing the cells under reduced oxygen atmospheres (2% and 5% O₂), thought to fall within the physiological range of oxygen tensions [235]. These same trends were observed when comparing cultured suspensions and 3D gels with fresh explants (Figure 32A). Due to this finding, we decided to focus on explant studies to prevent loss of cell phenotype and to more accurately predict in vivo consumption rates. Therefore, in this study, fresh TMJ disc explants were used to determine baseline oxygen consumption rates at the physiological glucose concentration of 5mm.

In using tissue explants, it became necessary to measure the distribution of volume based cell density to determine the oxygen consumption rate on a per-cell basis. Conventional histology slices can only provide cell numbers per unit area [24]. Although enzymatic cell isolation can determine cell numbers per tissue volume, the sequential enzymatic digestion may lose a significant amount of cells [236]. Therefore in this study, a confocal microscopy based technique was developed to determine the in situ surface-regional cell distribution of the TMJ disc. The confocal measurements were performed in three layers (i.e., superior, middle, and inferior) for each disc region. Multiple cell layers were found in each 3D confocal imaging data set. Therefore, the confocal measurement did provide real volume based cell number for each disc region. The confocal assessment yielded an overall cell density of 51.3(21.3-81.3)×10⁶cells/mL wet tissue in porcine TMJ disc. Our validation assessment using DNA content yielded the overall cell density of 54.6(42.4-66.7)×10⁶cells/mL. Those values are comparable to the cell density of 50×10⁶cells/mL for porcine TMJ discs using a DNA assay in the
literature [48]. However, complete enzymatic digestion of the bovine TMJ disc yielded a cell density of $20 \times 10^6$ cells/mL wet tissue (assuming wet tissue density=$1.08$ g/mL) [18].

While there is no layer dependency, our results showed that porcine TMJ disc has higher cell density in the anterior and medial regions. This is consistent with the DNA distribution of the porcine TMJ disc in the literature [48]. Previous qualitative studies have also demonstrated that cells were more numerous in the anterior band compared with the intermediate zone in rabbit and primate TMJ discs [36, 42]. One possible explanation is that the peripheral regions have higher cellularity due to a better nutrient supply from surrounding tissues. It is also likely due to an inhomogeneous mechanical strain distribution within the TMJ disc during jaw function. In contrast, a recent study quantitatively indicated that the anterior band has fewer cells than the intermediate zone and posterior band [24]. Note that the cell density in that study was measured by counting cells on histological slides which cannot be translated for 3D tissue volume based cell density.

On a per-cell basis, the oxygen consumption rate of articular cartilage and IVD are remarkably lower than vascularized tissues (~ 2-5% of liver or kidney tissue rates) [25] since articular chondrocytes [228, 233] and IVD cells [158] obtain their energy primarily through glycolysis. The rates of oxygen consumption in articular cartilage [230, 237] and IVD [1, 238] depend on the local oxygen tension. The consumption of oxygen decreases as oxygen tension decreases and is regionally dependent. The deep zone articular chondrocytes had higher oxygen consumption rates than superficial zone cells [237]. In IVD, the nucleus pulposus cells have a higher rate than annulus fibrosus.
cells [226, 238, 239]. Compared to articular cartilage and other fibrocartilaginous tissues (e.g., IVD or knee meniscus), the TMJ disc has a unique matrix composition and cell phenotype [9, 18, 240]. Differences in biochemical composition and structure distinguish three regions of the TMJ disc: anterior band, intermediate zone, and posterior band. Based on the cell morphological studies it appears that the TMJ disc contains an inhomogeneous distribution of a mixed cell population of fibroblast-like cells and chondrocyte-like cells, which are distinct from chondrocytes from hyaline cartilage [24]. These differences imply that the nutrient consumption rate in the TMJ disc may be region-dependent and different from the rates of articular cartilage.

The tissue thickness and cell density of the TMJ discs from this study were compared to those of knee joint cartilage in Table 9. Stockwell et al. showed that, in general, thinner cartilage has higher cell density than thicker cartilage due to the limitation of nutrient diffusion [25]. The TMJ disc has a bi-concave shape and the thickness of the disc in the superior and inferior direction varies across the surface between 2-4mm. Considering the larger thickness of the TMJ disc, the cell density of TMJ discs is higher than expected. This implies that the TMJ disc might have greater demand of nutrients than previously estimated.

The oxygen consumption rate of the TMJ disc was measured in a sealed metabolism chamber. This approach has been used to investigate the effect of oxygen tension on the oxygen consumption rate of isolated articular chondrocytes [230] and IVD cells [238]. Those studies have shown that the relationship between the oxygen consumption rate and oxygen tension can be modeled by the Michaelis-Menten equation
with the two parameters $V_{\text{max}}$ and $K_m$. Our results revealed that the kinetics of the oxygen consumption rate of TMJ disc explant can also be well expressed by this equation. Due to small $K_m$, the oxygen consumption rate of the TMJ disc was relatively constant until the oxygen tension fell below 5%. Below 5% oxygen, the rate fell in a concentration-dependent manner. This finding is similar to the association between the oxygen consumption rate and oxygen concentration for articular cartilage [230, 237] and IVD [238].

The oxygen consumption rates of tissue explants are usually determined at 21% O$_2$. It is apparent that the tissue volume based oxygen consumption rate of the TMJ disc is the highest among the cartilaginous tissues listed in Table 10. Although the TMJ disc has a high cell density compared to other cartilage, the maximum cell based oxygen consumption rate ($V_{\text{max}}$) of TMJ disc cells is still about 3 times higher than for articular chondrocytes and IVD cells (Table 11). Both chondrocytes and IVD cells obtain their energy primarily through Embden-Meyerhof-Parnas (EMP) pathway glycolysis, even in the presence of high oxygen tension [158, 228, 233]. Therefore, the oxygen consumption rate of those cells is exceptionally low. It has even been reported that the mitochondria of chondrocytes in situ lack certain cytochromes that are required for a fully functional electron transport chain [241]. Cell morphological studies using electron microscopy 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 [24]. The chondrocyte-like cells in the TMJ disc do not appear to exhibit the distinct pericellular capsule typical of articular chondrocytes [240].
Moreover, there are significant differences in organelle content between articular chondrocytes and chondrocyte-like cells in TMJ disc, which likely suggest differences in cellular behavior. The chondrocyte-like cells in TMJ discs have a greater number of mitochondria, suggesting a higher metabolic activity than articular chondrocytes [24]. The overall higher oxygen consumption rate determined in this study might be related to some extent of oxidative phosphorylation in TMJ disc cells. Detamore et al. reported that the distributions of cell subpopulations in TMJ disc are significantly region-dependent [24]. This might lead to the region-dependent oxygen consumption rate determined in this study. For example, the intermediate zone of TMJ disc had a higher oxygen consumption rate possibly due to the relatively higher number of chondrocyte-like cells in this region.

Due to the difficulty of measuring nutrient concentrations in vivo, mathematical models have been used to estimate them in cartilaginous tissues. The results of these calculations indicate that there is a steep gradient of oxygen in normal cartilage and the concentration can be as low as 1% [230]. The normal adult human TMJ disc is a large avascular structure [23, 196, 205], although some research has shown vasculature in young animal [24] and human discs [242], as well as degenerated human discs [243]. Considering the higher cell density and oxygen consumption rate of TMJ disc cells, a steeper oxygen gradient potentially exists in the normal TMJ disc. Such a steep oxygen gradient would make this tissue uniquely vulnerable to any pathological event which impedes nutrient supply, such as sustained joint loading due to jaw clenching [244]. To precisely predict the nutrient environment using a mathematical model, it is crucial to
determine quantitative relationships between the nutrient consumption rates and the local nutrient concentrations. In this study, the relationship between the oxygen consumption rate and oxygen tension was established for the TMJ disc. Studies on articular cartilage and IVD have shown that nutrient consumption rates are dependent not only on a single substrate but also on other nutrients. For instance, stimulation of oxygen uptake at low glucose concentrations (the Crabtree effect) was observed in articular cartilage [233, 245-247].

7.5 Conclusions

In summary, the distributions of cell density and basal oxygen consumption rate in five TMJ disc regions were determined using porcine tissue explants. The impact of the oxygen tension on the oxygen consumption rate was investigated and a quantitative relationship between them was established. The TMJ disc had a higher cellularity compared to articular cartilage. The cell density of the TMJ disc was region-dependent, and the anterior and medial regions had higher values compared to intermediate, lateral, and posterior regions. Compared to articular cartilage and IVD, the TMJ disc had a higher oxygen consumption rate on a tissue volume basis, as well as on a per-cell basis. The central regions, including intermediate, lateral, and medial, had a higher average oxygen consumption rate than anterior and posterior bands. The oxygen consumption rate was also dependent on the oxygen tension. At high oxygen tension, the oxygen consumption rate remained constant, and only dropped significantly as oxygen tension
fell below 5%. This relationship can be well expressed by the Michaelis-Menten equation. Considering the higher cell density and oxygen consumption rate of TMJ disc cells, a steeper oxygen gradient potentially exists in the normal TMJ disc. Such an oxygen gradient will likely be very vulnerable to any pathological event that can impede nutrient supply, and ultimately result in tissue degeneration.
Chapter 8 Effect of Nutrient Environment on Energy

Metabolism, Proliferation, and Differentiation

8.1 Introduction

In order to better understand the TMJ disc environment, further research into the nutrient utilization and energy metabolism is required. The balance between cellular maintenance, differentiation, and proliferation is coupled with nutrient delivery and utilization. The interaction between disc transport properties and metabolic demand affects the local environment of the disc tissue, in turn, altering the cell response. As mentioned in the previous chapter, most studies have been focused on the oxygen consumption rates in cartilaginous tissues. Therefore, few have studied the coupled relationship of glucose and lactate in the TMJ disc. Glucose is an essential nutrient for energy production and serves as a building block for cellular growth. Lactate is a waste product of energy metabolism during glycolysis. The build-up of lactic acid can result in the lowering of pH which can affect cell viability and metabolism [211]. Further characterization of the metabolic requirements is necessary for developing predictive models of in vivo nutrient distribution.

As discussed in previous chapters, mechanical loading significantly impedes the nutrient pathway resulting in development of a steep solute gradient. As a result, TMJ disc cells deep within the tissue are likely subjected to a low nutrient, high pH environment. Studies on the IVD have found that matrix turnover and cell viability can
be significantly affected by the lack of oxygen and glucose [226]. Therefore it is important to study the nutrient environment effect on cell proliferation and differentiation behavior of disc cells. The avascular nature of the TMJ disc suggests that cells are capable of producing ATP and ECM components at low nutrient levels which may be further compounded by compressive strains. In these studies, TMJ disc cells were cultured in a variety of conditions to determine the protein synthesis, ATP production, and cell proliferation. These results were coupled with a study to determine the in vitro glucose consumption rates of disc cells in various nutrient concentrations. This chapter will therefore discuss the glucose consumption and lactate production rates of TMJ disc explants and provide insight into energy metabolism and nutrient utilization.

8.2 Methods

8.2.1 Glucose Consumption Rate

A total of six pig 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 en bloc. Joints were opened under a sterile dissection hood; TMJ discs were then removed and washed with 5-6 changes of phosphate buffered saline. Fresh TMJ disc explants (~0.1 g wet weight/explant) were harvested from 5 regions and the tissue volume of each explant was determined in PBS based on the Archimedes’ principle using the previously described method. Each region was normalized by cell density values determined from confocal measurements described in the previous
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 FBS free DMEM glucose solutions at 0.5, 1, 5, 10, and 25mM. The incubator had been preset at 2.5, 5, and 21% O₂ prior to plating of explants. Glucose and lactate concentrations were measured with the instrument described in Chapter 6. These measurements were taken at 4 and 18 hours time. Glucose consumption (GCR) and lactate production (LPR) rates were calculated from the resulting concentration difference, cell densities, and time. At the end of experiments, the explant pieces were fully digested and the cell viability was examined via trypan blue exclusion (greater than 90% viability).

8.2.2 TMJ Disc Cell Isolation and Culture

Porcine discs were harvested under sterile conditions within 2 hours of animal death. Samples were washed 3 times in 1% antibiotic/mycotic PBS, resuspended in 0.25% Trypsin-1mM EDTA for an hour, and then digested overnight at 37°C with 0.1% (weight/vol) collagenase II (Worthington Biochemical Corp., Lakewood, NJ) in DMEM containing 10% fetal bovine serum. Digestions were strained through a 70µm filter, washed, and re-suspended in DMEM supplemented with 10% FBS (HyClone Laboratories, Inc), 1% penicillin/streptomycin (Gibco Brl) and 25 ug/ mL ascorbic acid.

Isolated TMJ disc cells were seeded in 75 cm² tissue culture flasks at 1 × 10⁴ cells/ cm² (100cm² dish) at 21% oxygen and 5% carbon dioxide at 37°C in DMEM (cell counted with hemocytometer). The media was changed every 2 days, and upon reaching
confluence (within 2 weeks), first-passage (P1) cells were detached with trypsin-EDTA (Invitrogen, Paisley, Renfrewshire, UK). Cells were re-plated at a 1:2 ratio and cultured to produce a second passage (P2) monolayer for use in experiments. The cells of an aliquot sample were counted and viability was quantified by trypan blue exclusion (0.4% in buffered saline solution).

8.2.3 WST-1 Assay for Examining Cell Proliferation

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 proliferation with extremely low cytotoxicity. Ten percent working solution was made by mixing one part volume of cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) 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).

8.2.4 ATP Measurement

At the end of the incubation period in oxygenated or hypoxic media, the cell suspension was mixed with a cell lysis solution (alkaline to inactivate endogenous ATPases and to stabilize released ATP) in a 96-well plate in the ratio of 2:1 and shaken for 5 minutes. One-hundred and fifty µL of the mixture was then combined with 50 µL of substrate (luciferase/luciferin) and shaken for a further 5 minutes. The plate was allowed to dark-adapt for 10 minutes in the luminometer before luminescence counting was initiated.
8.2.5 Lactate Assay

Lactate dehydrogenase converts lactate and NAD\(^+\) into pyruvate and NADH (Eton Bioscience Inc). The lactate Assay kit is based on the reduction of the terazolium salt in a NADH-coupled enzyme reaction to formazan, which exhibits an absorbance maximum at 490 nm. The intensity of the absorbance is proportional to the lactate concentration in samples. Fresh culture media was used as the background reading.

8.2.6 Collagen Synthesis Assay

The TMJ disc cells were exposed to 20 \(\mu\)Ci/mL (2,3-3H) proline incorporation into TMJ disc cells in 2 mL of medium for the final 24 hours of incubation. The cell layer was washed three times with PBS and homogenized with a polyton in 0.2% Triton-X 100 and 50 mM Tris/HCl. The cell homogenate was digested with 0.02% collagenase and incubated for 4h at 37°C. After incubation, the each sample was added the 10% TCA /0.5% tannic acid, centrifuged at 4,000 rpm at 4°C for 10 min 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.

8.2.7 Proteoglycan Synthesis Assay

The matrix-forming TMJ disc cells were exposed to 5.0 \(\mu\)Ci/mL (\(^{35}\)S) sulfate incorporation into TMJ disc cells in 2 mL of Medium for the final 4 hours of incubation. The cell layer was washed three times with PBS and solubilized with 2 mg/mL of Pronase E in 5 mM CaCl\(_2\) and 0.2 M Tris/HCl at 56°C for 3 hours. The precipitates incorporating (\(^{35}\)S) sulfate were collected on glass-fiber filter papers and washed three
times with cetyl pyrinium chloride (CPC). The radioactivities of the cells precipitated with CPC were measured in a scintillation counter.

8.2.8 Statistical Analysis

Comparisons were performed by one-way ANOVA analysis of variance with LSD as a post hoc test. Probabilities of less than 0.05 were considered to be significant.

8.3 Results

8.3.1 Glucose Consumption Rate Results

Glucose concentrations were measured at 4 and 18 hour time points and glucose consumption rates (GCR) were determined from the resulting concentration difference, cell densities, and time (Figure 35). The results showed significant substrate dependence as increasing glucose concentration increased the average GCR. The concentration of oxygen also had a significant effect on the GCR as the average GCR was 16.7±4.94 for 2.5%, 15.02±4.90 for 5%, and 6.80±3.06 nmol/million cells/hour for 21% oxygen concentrations. This trend of increasing GCR with decreasing oxygen concentration is indicative of a positive Pasteur Effect.

8.3.2 Lactate Production Rate Results

Lactate production rates (LPR) were measured in a similar manner as the GCR. Increasing glucose concentration resulted in increasing LPR with significant variation between the oxygen levels (Figure 36). Lactate production remained around 5 nmol/million cells/hour for 21% O₂ for all glucose concentrations. Increasing oxygen
concentrations significantly decreased LPR with 26.21±6.52 for 2.5%, 12.38±4.58 for 5%, and 5.28±1.81 nmol/million cells/hour for 21% oxygen concentration. The ratio of glucose consumption to lactate production rate increased with increasing glucose concentrations (Figure 37). The ratio also increased with increasing oxygen tension and was most significant for 21% oxygen.

![Figure 35 Average glucose consumption rate (GCR) varied by glucose and oxygen concentration](image)

**Figure 35** Average glucose consumption rate (GCR) varied by glucose and oxygen concentration
Figure 36  Average lactate production rate (LPR) varied by glucose and oxygen concentration.

Figure 37  The ratio of glucose consumption to lactate production rates.
8.3.3 Differentiation and Proliferation Results

In this study, we set a wide range of oxygen and glucose concentrations during cell culture to determine the influence of nutrient levels on TMJ disc cell behavior (Figure 38). Increasing glucose concentrations during culture increased cell proliferation, collagen synthesis, and GAG synthesis. ATP production on average increased with increasing oxygen concentration, but did not show significant correlation with glucose concentration. The absence of glucose significantly reduced cell proliferation. Increased oxygen tension resulted in decreased cell proliferation and increased collagen and GAG synthesis.

**Figure 38** Effect of glucose and oxygen concentrations on (A) cell proliferation and (B) ATP production (C) collagen synthesis, and (D) GAG synthesis. Glucose was found to be the limiting nutrient for the survival of cells.
8.4 Discussion

Studying TMJ disc metabolic behavior and energy requirements in a variety of nutrient environments is an important step in understanding disc pathology and degeneration. As shown in previous chapters, mechanical loading can cause significant decreases in solute transport directly limiting the nutrient supply to disc cells. The local nutrient environment impacts the consumption rates and availability of nutrients for energy metabolism. Cells respond to the changing environment by regulating differentiation and proliferation which in turn affects the nutrient demand. Therefore, this study sought to determine the effect of glucose and oxygen environment on cell differentiation and proliferation as a result of cellular consumption rates.

Aerobic respiration is a more efficient means of energy production, but requires a greater availability of oxygen. Glycolysis can occur under anaerobic conditions and results in the production of lactate. As discussed in previous chapters, TMJ discs have lower glucose diffusivity but significantly higher oxygen consumption rates compared to the IVD. Glycolysis has been found to be the dominant means of energy production in IVD and articular cartilage, while the behavior has not been studied in the TMJ. The increased ATP production coupled with relatively flat glucose consumption rate at high oxygen concentrations suggests that ATP production via oxidative phosphorylation is promoted by increased exposure to oxygen. This relationship suggests that TMJ disc cells can readily shift between glycolysis and oxidative phosphorylation as a result of
nutrient availability. Fernando et al. also discovered that ATP production was significantly affected by static and dynamic compressive loading [248]. These results support our hypothesis that mechanical forces can alter the local nutrient environment which affects cellular metabolic activities.

Glucose and oxygen concentrations had significant impact on cellular proliferation and differentiation. In the absence of glucose, cell proliferation was essentially negligible for all oxygen tensions suggesting that glucose is the limiting factor for the survival of disc cells. In the presence of glucose, increased oxygen tension decreased the amount of cell proliferation suggesting that behavioral changes were occurring in response to environmental shifts. Collagen and GAG synthesis increased with increasing oxygen tension, suggesting that cells begin to differentiate more in the presence of oxygen as long as sufficient glucose was supplied. Coupled with the results of the glucose consumption rate studies, it appears that glycolysis is preferred at low oxygen concentrations. High GCR and low LPR at high oxygen concentrations suggests a shift towards oxidative phosphorlation. Cell proliferation occurs at lower oxygen levels while ECM production is greatly increased with higher oxygen concentrations. These results suggest that TMJ disc cells are highly adaptable based on the nutrient concentrations supplied.

GCR was substrate dependent suggesting that nutrient utilization was highly regulated and affected metabolic activities. The positive Pasteur Effect shows that at low oxygen concentrations TMJ disc cells prefer glycolysis for energy needs and switch at higher oxygen. The transition point likely occurs between 2.5% and 5% O2 tension (the
1:2 ratio of glucose consumption to lactate production associated with glycolysis disappears). Both oxygen and glucose consumption rate data suggest that the TMJ disc cells have a higher metabolic rate compared to other cartilaginous tissues. However, the basic metabolism of TMJ disc cells is virtually unknown. The low glucose consumption at high O₂ found in TMJ discs is significantly different from trends in other cartilage types and may be related to cell type. As discussed in Chapter 2, several cell phenotypes have been found in TMJ disc tissues and at the present time no studies have investigated the energy metabolism of each cell population. The significant difference in energy metabolism between cartilage types may be due to the higher proportion of fibroblasts in TMJ discs.

The 0.5 GCR/LPR ratio at low oxygen hints that the glycolytic pathway is preferred (during glycolysis, 1 glucose molecule results in 2 lactate molecules produced). High glucose consumption at high glucose (without lactate production) could be associated with glycogen storage or ECM production. Lactate production rate was also found to increase with glucose concentration for 2.5 and 5% oxygen, but remained flat for 21% O₂. This suggests that at low oxygen glycolytic activity is limited by the level of glucose readily available, but at high oxygen glucose is being used for purposes other than energy metabolism. No changes in lactate production at high oxygen content also suggests greater utilization of oxidative phosphorylation for energy needs.

Explants allow for the closest approximation to in vivo cell behavior, but issues with cell density, cell death, and contamination are risks. Studies have found that cells in vitro can lose their phenotypes rapidly and not provide any real information on true cell
metabolism. As mentioned in Chapter 2, the TMJ disc is composed of a variety of cell types, and each of these may require varying amounts of nutritional supply so future work may involve sorting cells based on phenotype. At this present time, cell markers distinguishing between chondrocytes, fibroblasts, and fibrochondrocytes of the TMJ have yet to be distinguished. The cell types vary on shape, organelle structure, and ECM production capabilities. Another possible future study involves the inhibition of mitochondrial metabolic activity in order to determine the full effect of environmental changes in glucose and oxygen concentrations. These studies would allow full distinction of glucose and oxygen utilization for energy, differentiation, or proliferation. Further work into coupling diffusion models with cell consumption data to better create an environmental model of the TMJ is also required.

8.5 Conclusions

It appears that glucose may be the limiting nutrient for the survival of disc cells, rather than oxygen. At 5mmol glucose and 21% oxygen (conditions tested in Chapter 7), oxygen consumption rate was approximately 3 times higher than the glucose consumption rate and 6 times higher than the lactate production rate. Oxygen consumption rates were significantly higher while glucose consumption rates were lower compared to articular cartilage and IVD. Oxygen consumption rates of TMJ disc cells are highly substrate dependent as rates only begin to drop when below 5% oxygen tension. High glucose concentration was found to suppresses oxygen consumption
(Crabtree effect). Average consumption rates of TMJ disc cells were 3-5 times greater than in other cartilaginous tissues. At low levels of oxygen the energy metabolism of TMJ disc cells is most likely anaerobic, but at high levels of oxygen both aerobic and anaerobic pathways might be present.

The behavior and metabolism of TMJ disc cells is highly dictated by nutrient concentrations. Rates of production of matrix proteins are severely inhibited at low oxygen and/or glucose. The decrease in cell proliferation and increases in GAG and collagen synthesis associated with increased O$_2$ tension indicate increased cell differentiation in response to the nutrients available. ATP production trends appear to be more complex and may be related to the transition from cell proliferation to differentiation related to nutrient environment. It becomes apparent that TMJ disc cell behavior is dictated by nutrient concentrations, so pathological changes in the nutrient transport pathway can have significant impact on cell health and potentially result in degeneration. Therefore, this study determined the impact of nutrient levels on cell energy metabolism, differentiation, and proliferation.
Chapter 9 Overall Conclusions and Future Directions

9.1 Conclusions

The major impact of this work is the characterization of the biomechanical, nutrient transport, and consumption rate properties of porcine and human TMJ discs. This information will be useful in understanding the root cause of TMJ disorders and developing models for predicting \textit{in vivo} changes as a result of pathological loading or trauma. These studies underlined the impact of mechanical loading on altering material properties that resulted in changes to the local nutrient environment of the disc. Furthermore, these studies showed that nutrient concentrations had significant effect on the biological responses in regards to proliferation and differentiation. These biological responses therefore affected the intrinsic properties of the tissue resulting in a cascade of changes potentially resulting in degenerative processes. These studies supported our general hypothesis that sustained mechanical loading can alter solute transport and nutrient concentrations in the TMJ disc, resulting in changes to the cellular metabolism, tissue composition, and mechanical function, ultimately leading to disc pathologies.

Chapter 3: In this study, the biphasic mechanical properties of porcine and human TMJ discs were measured to validate the use of the porcine model and to correlate mechanical function with biochemical structure. As hypothesized, the biochemical composition of the tissue significantly affected the regional mechanical properties. The results of dynamic testing indicated the necessity for characterizing the viscoelastic properties of
the TMJ disc. Although significant species variation for mechanical and biochemical properties was found, the porcine model remains the most accessible animal species to compare with human samples. The most notable finding was how significantly different the TMJ disc tissues were from other cartilage surfaces in porcine and human systems. The results of these experiments were essential in describing the tissue mechanical responses to loading and describing the material properties of the disc.

**Chapter 4:** In this study, the viscoelastic shear properties of porcine TMJ discs were measured to correlate with frequency, strain, and region. These experiments focused on the fluid-flow independent material properties of the disc occurring at small shear strains. In our studies, complex modulus was found to significantly increase with increasing frequency, but decrease with increasing rotational shear strain. Studies on the dynamic shear properties of the TMJ disc have found a non-linear dependence on frequency which is potentially due to the viscoelastic properties of the ECM components. Due to differences in testing configuration, overall magnitude of results varied from findings in the literature, but similar trends were observed. These studies were undertaken to characterize the TMJ material properties in order to understand the complex biomechanical environment as a result of loading.

**Chapter 5:** This experiment measured the regional porcine and human TMJ disc small ion transport properties. This study showed that solute diffusivities in the TMJ disc are much lower than the values in other cartilaginous tissues and that compressive
mechanical strain can further impede solute diffusion in the TMJ. Therefore, it is likely that a steeper nutrient gradient may exist in TMJ discs. Such a steep gradient will be very vulnerable to any pathological event which impedes nutrient supply, such as sustained joint loading due to jaw clenching. These studies provided important insight into the electrical and solute transport behaviors in TMJ discs under mechanical loading and aid in the understanding of TMJ pathophysiology related to tissue nutrition.

**Chapter 6:** This experiment measured the one dimensional regional glucose and lactate diffusivities under compressive strains. As with conductivity, compressive strain significantly impeded solute transport further suggesting that sustained pathological loading can affect the TMJ disc nutrient environment. The transport of these solutes is particularly important in relation to the energy metabolism and behavior of disc cells. The glucose diffusivities were also significantly lower than the values found in intervertebral discs and articular cartilage further emphasizing the difficulty of transport in the TMJ disc. Compressive loading was found to significantly decrease the delivery of nutrients and is likely associated with fluid exudation and decreased tissue porosity. These findings support our hypothesis that pathological mechanical loading can result in decreased nutrient levels deep within the TMJ discs.

**Chapter 7:** This experiment measured the oxygen consumption rates of TMJ disc cells in a variety of environmental and cell seeding conditions to determine the optimum testing protocol. The results of these experiments indicated that culturing had a significant effect
on altering the phenotype and resulting cell behavior. Therefore, explant experiments would yield results most similar to \textit{in vivo} consumption rates as long as accurate measurements for cell density could be obtained. These studies also showed that oxygen consumption rates were significantly substrate and nutrient environment dependent with high consumption at high concentrations. TMJ disc consumption rates were also significantly different from rates found in other cartilage types.

**Chapter 8:** This chapter investigated the glucose consumption rates of porcine TMJ discs explants in order to couple the nutrient supply with energy metabolism. TMJ disc glucose consumption rates were highly substrate and nutrient environment dependent. It appears that glucose may be the limiting nutrient for the survival of disc cells, rather than oxygen. Increased levels of oxygen decreased cell proliferation and increased collagen and GAG synthesis suggesting that oxygen significantly modulated cell differentiation. The significant increase in glucose consumption without lactate production at high oxygen suggests that TMJ disc cells are capable of shifting from glycolysis to oxidative phosphorylation in response to nutrient availability. These findings indicate that the TMJ disc is unique in nutrient requirements and energy metabolism which may be a result of cell phenotype differences. This further supports our hypothesis that nutrient environment heavily dictates cellular behavior and insufficient nutrient supply may be associated with degenerative cell processes.
9.2 Challenges

Chapter 3 and 4: In our sample preparation, the superficial layers from the top and bottom surface were removed by a sledge microtome to create flat surfaces for the confined compression test. For the porcine TMJ disc, it has been reported that there is significant surface variation between mechanical properties. Therefore, it is necessary in the future to examine the surface differences of the biphasic mechanical properties in human discs.

Chapter 5: Due to the low measured GAG content, the effects of tissue swelling and hydration are assumed to be negligible. This assumption also leads to the simplification of low fixed charge density in the tissue. In the future, it will be necessary to measure the effect of these assumptions on conductivity of the tissue.

Chapter 6: A major limitation of this experiment is the difficulty in measuring partition coefficient. Without fully washing out the sample, residual solutes would shift the diffusion curve and skew partition coefficient calculations. Without the partition coefficient it is therefore impossible to accurately determine the effective or intrinsic diffusion properties of the tissue. The use of porous platen was necessary to maintain compressive strains on samples may have resulted in stagnant boundary formation. Another issue in the use of PE porous platen is that samples may bow or flex while
confined and may not be exactly the strain desired. Reusable metallic platen would result in difficulty for cleaning and potential damage to the chamber wells. These issues may contribute to the lack of significant diffusivity variation at 10% compressive strain. In the future, these effects need to be further explored to determine the amount of error introduced by testing configuration.

Chapter 7 and 8: Consumption rates were measured for TMJ disc explants and normalized with previously obtained cell density values. These experiments were done under the assumption that viability was not significantly affected during testing. In order to minimize gradient development, tissues were diced making viability testing difficult. Therefore, studies need to be undertaken to validate these assumptions.

9.3 Future Goals

The goal of this project was to characterize the unique biomechanical, transport, and cellular properties of the TMJ disc in order to develop a model of the TMJ disc. By incorporating realistic tissue properties, a predictive 3D finite element can be developed to investigate the effect of sustained mechanical loading on nutrient transport and cell metabolic behavior. This model can be further built into a TMD diagnostic tool based on patient specific MRI and jaw tracking data. This work will help to build new strategies for TMD treatment and can be applied to tissue engineering approaches in other cartilaginous tissues.
**Chapter 3 and 4:** We hope to further study the anisotropic mechanical and transport properties of the TMJ disc with emphasis on comparing porcine and human samples. Future work can be done to investigate the effect of age and gender on mechanical properties. Histological studies to further correlate tissue structure and composition with material properties would also provide insight into TMJ disc function. These studies would be crucial in characterizing the biomechanical environment in the tissue under loading conditions to better understand TMJ degenerative processes. Other loading modalities including tension, plowing, and indentation experiments have all been undertaken for TMJ discs, therefore development of a comprehensive model utilizing all of this data could also be a future study.

**Chapter 5 and 6:** We hope to further study the effects of compressive strains on nutrient transport by investigating the diffusivity of other important solutes in TMJ discs. These include oxygen, growth factors, and cytokines which play significant roles in cell behavior and tissue remodeling. These transport properties will be useful in developing functional relationships between tissue water content and nutrient availability.

**Chapter 7 and 8:** Chapter 7 only determined the oxygen consumption rate of TMJ disc at 5mM glucose. Therefore, it is necessary to investigate the effect of glucose on the oxygen consumption rate in a future study. It will also be valuable to study the coupling of oxygen consumption, glucose consumption, and lactate production to fully understand
the energy metabolism in the TMJ disc. One method for testing this would be to block mitochondrial activity to limit energy metabolism entirely to glycolysis. Future studies could also determine methods for sorting the cell types in order to isolate and determine the metabolic contributions of each.

Future work for this research involves incorporating the TMJ disc properties into a predictive 3D finite element model of the *in vivo* TMJ environment. This model can be further developed into a TMD diagnostic tool based on patient specific magnetic resonance images (MRI) and jaw tracking data. The work outlined in this thesis represents progress towards understanding the mechanisms of TMJ pathobiology in order to develop new strategies for diagnosis and treatment from a bioengineering perspective. The techniques and approaches outlined in this project will therefore be useful in developing new strategies for TMJ disc tissue regeneration and can be translated into tissue engineering applications in other cartilaginous tissues.
References


