Effects of IVD-Like Pressure and pH on Human Adipose Derived Mesenchymal Stem Cells

Alexandria Pendino
apendin@clemson.edu

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Recommended Citation
https://tigerprints.clemson.edu/all_theses/4152

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.
EFFECTS OF IVD-LIKE PRESSURE AND pH ON HUMAN ADIPOSE DERIVED MESENCHYMAL STEM CELLS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Alexandria Pendino
August 2023

Accepted by:
Dr. Jiro Nagatomi, Committee Chair
Dr. Jeremy Mercuri
Dr. William Richardson
ACKNOWLEDGMENTS

I would like to thank Dr. Nagatomi for his support throughout both my undergraduate and graduate research projects in his lab. I have learned a lot and grown as a researcher under his guidance. I would also like to thank my committee members, Dr. Mercuri and Dr. Richardson, as well as their students, for their help with providing additional insights and resources for this project.

The current and former lab members of the Cell Mechanics and Mechanobiology lab, specifically Cody Dunton PhD, Britney Hudson, Caroline Atkinson, and Collin Vogel, have been invaluable for their mentoring and assistance.

Additional thanks go to Dr. Lee Sierad, Brendyn Miller, and Ziliang Chen for their help in designing and creating the bioreactors used to complete this project, as well as the Clemson University machine shop.

Finally, thank you to everyone in the Bioengineering department who has been supportive and encouraging during this process, especially the members of the Research Troubleshooting Club.
ABSTRACT

Low back pain (LBP) affects approximately 12% of adults in the US, and of these cases, between 26 and 42% are caused by intervertebral disc degeneration (IVDD). Current treatment approaches for LBP and IVDD, such as medication and surgery, mainly focus on relieving pain and do not address current or future degeneration. As a result of reherniation, up to 15% of patients will need a second surgery. As a solution, the use of tissue engineering combining scaffolds and stem cells to regenerate the damaged structures has been proposed. There are several types of stem cells that have shown potential for regenerative treatments, primarily adipose and amnion derived mesenchymal stem cells. However, intervertebral disc (IVD) cells, specifically those in the nucleus pulposus, experience elevated pressures caused by supporting the mechanical loads applied to the spine and lower pH due to the avascular nature of the IVD, which could affect the viability of any stem cells that are placed in the IVD as part of a treatment.

The primary goals of the current research are to develop methods to create IVD conditions in vitro and to use these methods to evaluate how potential cell sources respond to these conditions. The specific aims were (1) To update a pressure-stretch bioreactor, (2) to evaluate hADMSCs response to pressure and pH conditions found in healthy and degenerated IVDs, and (3) to develop a new high-pressure bioreactor to create more realistic simulations of in vivo conditions.

Strain quantification of the new pressure stretch bioreactor done via image analysis with ImageJ showed that the new design is capable of creating up to approximately a 14% strain. Using Caspase-1 activity as a indicator, it was also determined that pressure and stretch are being applied to MYP3 cells seeded on silicone membranes. Exposure of hADMSCs to healthy and degenerated IVD conditions (0.03 or 0.275MPa and 6.5 or 7.4 pH) for six days resulted in a decreased cell density compared to the control group and an altered cell morphology. Gradually changing the pH from 7.4 to 6.5 decreased the adverse effect of the pH change on cell number. Gradually increasing the pressure to 0.275 MPa did not have the same effect and still led to a drop in cell number. Initial qPCR results for NP and chondrogenic markers at different pressures and normal pH showed mostly elevated expression levels compared to the control group.

While there is decreased cell density, hADMSCs were able to survive in IVD-like conditions, and with additional studies, may be a viable option for tissue engineering. The new pressure-stretch bioreactor is ready for use in future urothelial studies or IVD studies and the high-pressure bioreactor is in the process of further development for use in pressure-cycling based studies.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>CHAPTER ONE: INTRODUCTION AND BACKGROUND</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Intervertebral Discs</td>
<td>1</td>
</tr>
<tr>
<td>1.2.1 Mechanical Forces acting on IVDs</td>
<td>3</td>
</tr>
<tr>
<td>1.2.2 Other IVD environmental factors</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Intervertebral Disc Degeneration</td>
<td>3</td>
</tr>
<tr>
<td>1.3.1 Mechanisms of Disc Degeneration</td>
<td>4</td>
</tr>
<tr>
<td>1.3.2 Current Treatments for IVDD</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Tissue Engineering Approaches for Degenerated Discs</td>
<td>5</td>
</tr>
<tr>
<td>1.4.1 Stem cell types</td>
<td>6</td>
</tr>
<tr>
<td>1.4.2 Adipose derived mesenchymal stem cells</td>
<td>6</td>
</tr>
<tr>
<td>1.4.3 Decellularized Scaffolds</td>
<td>7</td>
</tr>
<tr>
<td>1.5 Effects of Different Stimuli on IVD Cells and MSCs In Vitro</td>
<td>8</td>
</tr>
<tr>
<td>1.5.1 Effects of Mechanical Forces on IVD Cells</td>
<td>8</td>
</tr>
<tr>
<td>1.5.2 Effects of Mechanical Forces on Stem Cells</td>
<td>8</td>
</tr>
<tr>
<td>1.5.3 Effect of pH on Stem Cells</td>
<td>9</td>
</tr>
<tr>
<td>CHAPTER 2: RESEARCH RATIONALE</td>
<td>10</td>
</tr>
<tr>
<td>2.1 Rationale</td>
<td>10</td>
</tr>
<tr>
<td>2.2 Aims</td>
<td>10</td>
</tr>
<tr>
<td>CHAPTER 3: MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>3.1 Pressure-Stretch Bioreactor</td>
<td>13</td>
</tr>
<tr>
<td>3.1.1 Application of Stretch</td>
<td>13</td>
</tr>
<tr>
<td>3.1.2 Pressure Only Configuration</td>
<td>14</td>
</tr>
<tr>
<td>3.1.3 Pressure-Stretch Configuration</td>
<td>15</td>
</tr>
<tr>
<td>3.2 Calibration of Pressure-Stretch Bioreactor</td>
<td>15</td>
</tr>
<tr>
<td>3.2.1 Strain Quantification</td>
<td>15</td>
</tr>
<tr>
<td>3.2.2 Pressure and Stretch Application to MYP3 cells</td>
<td>17</td>
</tr>
<tr>
<td>3.3 Exposure of human adipose derived mesenchymal stem cells to High Pressure / Low pH conditions</td>
<td>18</td>
</tr>
<tr>
<td>3.3.1 Cell Culture</td>
<td>18</td>
</tr>
<tr>
<td>3.3.2 High Pressure Low pH Condition</td>
<td>18</td>
</tr>
<tr>
<td>3.3.3 pH Pre-conditioning</td>
<td>19</td>
</tr>
</tbody>
</table>
Table of Figures

Figure 1: Pressure-Stretch Bioreactor ................................................................. 14
Figure 2: Pressure-only bioreactor ................................................................. 15
Figure 3: Stretch-Quantification Set Up ......................................................... 16
Figure 4: Measurement Markings for Strain Quantification ......................... 17
Figure 5: High Pressure Bioreactor Design ..................................................... 22
Figure 6: %Strain vs Negative HP ................................................................. 24
Figure 7: Radial Max Strain Quantification ....................................................... 25
Figure 8: Caspase-1 Activity Fold Change ...................................................... 26
Figure 9: Coomassie Blue Staining of hADMSCs under IVD-like Conditions ... 27
Figure 10: DAPI Staining of hADMSCs under IVD-like Conditions .............. 28
Figure 11: Change in Average Cell Density Under IVD-like Conditions ......... 29
Figure 12: Change in Average Cell Density Compared to Seeding Density Under IVD-like Conditions ................................................................. 30
Figure 13: hADMSC Gene Expression via qPCR After Exposure to IVD-like Conditions ................................................................. 31
Figure 14: Change in Average Cell Density in Response to Applied Pressure .... 32
Figure 15: Change in Average Cell Density Compared to Seeding Density in Response to Applied Pressure ................................................................. 33
Figure 16: DAPI and Coomassie Blue Staining of hADMSCs After Exposure to Pressure Conditions ................................................................. 34
Figure 17: Change in Average Cell Density in Response to pH Change .......... 35
Figure 18: Change in Average Cell Density Compared to Seeding Density in Response to pH Change ................................................................. 36
Figure 19: DAPI and Coomassie Blue Staining of hADMSCs After Exposure to pH Changes .. 37

Tables

Table 1: IVD-like conditions used for Exposure of hADMSCs ......................... 19
Table 2: PCR Primer Sequences .................................................................. 21
CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1 Introduction

Back pain is a common ailment that can range from occasional pain to chronic pain. Low back pain (LBP) is a specific type of back problem that affects approximately 12% of the US adult population, and 9.4% of the global population although this percentage can vary based on different demographic factors, such as age and sex. LBP is also one of the biggest contributors to disability across the global population. There are several different causes of low back pain, but a very common one is intervertebral disc degeneration (IVDD) or degenerative disc disease (DDD). Treatments for LBP result in an annual health care expenditure of over 100 billion USD per year in the United States, and the amount that individuals with LBP spend on treatments is increasing.

1.2 Intervertebral Discs

Intervertebral discs (IVD) are structures in between the vertebrae of the spine that help to provide flexibility and stability to the spine. The discs are composed of three components: the nucleus pulposus, the annular fibrosis and two endplates. Each of these components has a different composition and function within the disc.

The nucleus pulposus (NP) is the gel-like center of the IVD. The cells within the NP are similar to chondrocytes, and the extracellular matrix is composed mostly of type II collagen and aggrecan. The abundance of aggrecan attracts and retains water that allows the NP to swell slightly, and as a result, resist the compressive forces experienced by the spine. In children, the cells in the NP resemble notochordal cells, but as they age, the cells more closely resemble chondrocytes. The density of cells in the NP is about 5000/mm³ within the NP. In addition to physical characteristics, NP cells can also be identified by certain phenotypic markers. Some common ones
include Paired box 1 (PAX1), Forkhead box F1 (FOXF1), Aggrecan (ACAN), and Type II collagen α1 (COL2A1). For aggrecan and collagen, the marker specific to NP cells is actually the ratio of type II collagen to aggrecan, since both markers are common in several cell types, including chondrocytes⁸,⁹. These markers have been shown to be elevated in NP cells compared to cartilage tissue, which can experience similar mechanical forces and can also be degraded in the case of osteoarthritic tissue.

The annulus fibrosus (AF) is the outer fibrocartilaginous portion of the disc that surround the NP. The exact composition varies throughout the AF; closer to the center of the disc, it is more like the NP with higher levels of type II collagen. Towards the outside of the disc, there is more type I collagen. The AF is made up of concentric rings containing parallel collagen fibers and cells that tend to resemble fibroblasts⁷. The function of the AF is to help contain the NP in the center of the disc, and also provide more resistance to tensile forces that IVDs experience⁵.

The endplate portion of the IVD is a thin layer of hyaline cartilage. The function of the endplate is primarily to provide an interface between the disc and vertebra, and it also helps with movement of molecules and water into and out of the disc⁵,⁷.

The IVD is avascular, making nutrient transport to the cells, especially those in the NP, difficult. The blood supply at the edges of the discs feeds into small capillaries that end at the endplates⁷. The lack of blood flow to the inner portions of the IVD means that there is a possibility of cell death or damage to the NP if something changes with the endplates, such as calcification, that prevents nutrients from getting through to the inner portion³. There are also nerves in the disk, which are mostly present in the outer layers of the AF⁷. Similar to blood vessels, these nerves do not protrude all the way to the NP in normal IVDs¹⁰.
1.2.1 Mechanical Forces acting on IVDs

The primary function of the IVD is to help absorb and distribute the loads that are placed on the spine. In a normal, healthy spine, the pressure within the disc can range from 0.1MPa to over 2MPa, depending on position and activity\(^\text{11}\). The lowest pressures occur when lying down, and pressure increases as activity increases from sitting, to standing, to walking, with the highest pressures occurring when lifting a heavy object with incorrect technique\(^\text{11}\). The loading experienced by IVDs increases in different postures as well, from prone, lateral, upright standing, and upright sitting; these loads also increase when bending forward or backward in either standing or sitting positions\(^\text{12}\). It has also been found that the intradiscal pressure decreases as degeneration increases when measured in vivo. In a prone body position, the intradiscal pressure is around 90kPa in a normal disc, while the intradiscal pressure in a severely degenerated IVD is around 10kPa\(^\text{12}\).

1.2.2 Other IVD environmental factors

In addition to mechanical forces, the IVD has other factors that make it a unique environment. The avascular nature of the NP region results in the tissue being hypoxic with a lowered pH\(^\text{8,13}\). The pH of healthy discs is normally between 7.0 and 7.2\(^\text{14}\). The acidic environment is created by less oxygen and other nutrients being delivered to the center of the disc. This also results in hypoxia-inducible factor 1 (HIF-1\(\alpha\)) expression more consistently than in non-hypoxic tissues\(^\text{8}\).

1.3 Intervertebral Disc Degeneration

Intervertebral disc degeneration (IVDD) refers to a breakdown of the structures of the IVD that provide support for the spine. There are different mechanisms that can cause degeneration, but as the discs age, there are some natural changes that occur that result in degeneration. One common marker of disc degeneration is the presence of inflammatory cytokines, specifically IL-1.
and TNF-α, which are both thought to contribute to degeneration\textsuperscript{15}. Additionally, in degenerated IVDs the pH range decreases to under 7.0 to as low as 5.5 in very severe cases\textsuperscript{14}.

### 1.3.1 Mechanisms of Disc Degeneration

There are several mechanisms through which disc degeneration can occur. One problem that can lead to degeneration is a lack of nutrients or decreased transport of nutrients to the disc. A decreased nutrient supply can lead to changes in the ECM composition, leading to disk degeneration since a lack of proper nutrients can prevent the IVD from properly responding to loads and injury\textsuperscript{13,16}. Genetic mutations that affect genes associated with IVDs and their composition, such as ACAN, different collagen genes, and others can also lead to degeneration due to their effect on the structure of the disc. Gene mutations that affect inflammatory responses can also increase disc degeneration via disruption of the anabolic/catabolic balance needed to maintain disc integrity\textsuperscript{16}.

Environmental and general health factors can also contribute to IVDD. General obesity, and specifically obesity at a younger age, has been found to contribute to an increased risk of development of IVDD\textsuperscript{16,17}.

Additionally, cell senescence can contribute to degeneration. The senescent cells lose the ability to divide, which can affect the IVD’s ability to maintain its catabolic/anabolic balance and overall structural integrity. Degenerated IVDs have higher populations of senescent cells and increased levels of reactive oxygen species that can contribute to further degeneration and cell death\textsuperscript{16,18}.

### 1.3.2 Current Treatments for IVDD

Many of the current treatments for IVDD are primarily focused on relieving the symptoms of low back pain. One of the primary treatments is pharmacological, normally using nonsteroidal
anti-inflammatory drugs to reduce inflammation induced pain. One concern with treating IVDD and the associated LBP with medication is the risk of misuse of or addiction to stronger pain medications\textsuperscript{19}.

Alternatives to medication include rehabilitation and physical therapy to help prevent or treat IVDD. Both medication and physical therapy can help to treat the pain symptoms associated with IVDD, but the degeneration is not fixed and can continue to worsen. If conservative treatments fail, then the next treatment option is surgery. Options for surgery include decompression or spinal fusion in more severe cases\textsuperscript{3}.

IVDD can result in a disc herniation, where the material of the IVD is displaced beyond what is considered normal. Pain and inflammation from disc herniation are often caused by the protrusion of the NP pressing against a nerve, but herniation is not always painful. Surgical treatment for this complication is typically a discectomy, where the protruding part of the disc is removed\textsuperscript{20}. Other surgical options include laminectomy and spinal fusion, but all surgical options involve a risk for surgical complications. These complications include recurrent herniation and damage or degeneration of the surrounding discs, which can lead to the need for more surgeries\textsuperscript{21}. The percentage of patients who need additional surgeries varies based on several factors, but it is between 1\% and 15\% over a five-year period\textsuperscript{22}.

1.4 Tissue Engineering Approaches for Degenerated Discs

As medicine is advancing, new treatments for IVDD are emerging. Like many other fields, tissue engineering is a promising field with the potential to treat and repair damage from disc degeneration. Generally, tissue engineering involves the use of stem cells with or without a scaffold to help regenerate or replace damaged tissue\textsuperscript{23}. Within tissue engineering, there are
various sources for stem cells that can be used and scaffolds that can be made to aid in delivering
the stem cells.

1.4.1 Stem cell types

There are several different types of cells that can be used for tissue engineering. Some
studied cell types are pre-differentiated cells from the NP or other regions of the IVD or
chondrocytes, adult mesenchymal stem cells, or induced pluripotent stem cells (iPSC)
19. The
common types of mesenchymal stem cells (MSC) are adipose-derived (ADMSC) and bone
marrow-derived (BMSC) and a less commonly used type is amnion-derived (AMMSC). This
refers to where the MSC is sourced from, although all MSCs share common properties, such as
multi-potency and what cell types they can differentiate into. Common differential pathways
involve MSCs differentiating into bone, fat, chondrocyte, muscle, neuron, islet, and liver cells, all
of which are influenced by genetic and environmental factors24. Once they are harvested, MSCs
can be cultured in vitro and influenced to differentiate into the desired cell type, before being used
for transplants.

1.4.2 Adipose derived mesenchymal stem cells

ADMSCs are derived from adipose or fat tissue, but they are similar to other MSCs in their
differentiation potential. One advantage of ADMSCs over bone-marrow MSCs is that the
procedure to harvest them is less invasive than harvesting bone marrow, but the culturing process
is similar. ADMSCs are able to differentiate into adipocytes, osteocytes, chondrocytes, and
myocytes, with varying efficiency in vitro; of these, adipocytes are the most easily differentiated
while myocytes are the least efficient. Differentiating ADMSCs requires specific conditions in
vitro, such as added supplements or other factors25. Some work has already been done on the
potential of using ADMSCs in tissue engineering solutions, specifically related to IVDD. Under
inflammatory conditions, ADMSCs show increased levels of pro-inflammatory cytokines and proliferation and decreased levels of GAG production, along with decreased levels of GAG-containing ECM production\textsuperscript{26}. Their ability to proliferate under inflammatory conditions is promising for use in tissue engineering, but a healthy IVD contains high levels of GAG molecules that would need to be replenished after degeneration.

1.4.3 Decellularized Scaffolds

Another important aspect of a tissue engineering solution for IVDD is the scaffold used to deliver cells to the IVD. The scaffolds used need to be biocompatible, porous, and biodegradable, depending on the purpose. Scaffolds can be made from tissue that has been treated to have the appropriate properties, or they can be fabricated from biomaterials. Common methods include 3D printing, stereolithography, selective laser sintering, and fused deposition modeling\textsuperscript{27}.

A method that has already been studied for IVDD is the use of a decellularized NP scaffold. Using a bovine IVD, the NP can be harvested and used to create a decellularized scaffold. The process of creating this scaffold involves removing the cells from a NP sample, while leaving the tissue-specific extra-cellular matrix behind. The decellularization protocol also needs to be optimized for maximum DNA content removal and maximum GAG and ECM content preservation\textsuperscript{28}. This is important to make sure that the scaffold does not illicit an immune response when implanted and can withstand the forces within the IVD. Mechanical testing on scaffolds made using this method showed that the scaffolds have similar viscoelastic properties to IVDs. While not exactly the same, they behave similarly enough to make them a candidate for tissue engineering solutions to IVD. It was also found that ethanol-mediated compaction and cross-linking of the scaffold results in improved mechanical properties with similar biocompatibility for seeding cells into the scaffold\textsuperscript{29}. It was also shown that these methods increased resistance to
enzyme degradation and while the study was limited, these results are promising for how a scaffold could be used in vivo for IVDD repair.

1.5 Effects of Different Stimuli on IVD Cells and MSCs In Vitro

1.5.1 Effects of Mechanical Forces on IVD Cells

Several studies have been done to determine the effect that mechanical forces have on both NP and AF cells. One finding from these studies is that applying high mechanical strain to healthy IVD cells results in upregulation of pro-inflammatory genes including TLR2, TLR4, and TNF and increased cytokine levels, also indicating inflammation. The conditions of strain used for this study do not fully represent what IVDs experience under normal conditions, but the spine can be exposed to excessive strain under certain conditions\textsuperscript{30}. It has also been found that cyclic tensile strain applied to AF cells is frequency dependent. As the frequency of the strain increases, there is more change in matrix regulation that can lead to a breakdown of the structures. Degenerated and non-degenerated cells were also compared, and it was found that the response to strain and its frequency were more dramatic in the nondegenerated samples\textsuperscript{31}. The effects of pressure or compression have also been studied. When comparing the effects of no loading to a physiological load or overloading on NP cells, it was found that a normal load increased cell viability and collagen-II production. Additionally, matrix-degrading enzymes were increased in response to overloading, showing that degeneration may be related to the forces that act on the spine\textsuperscript{32}.

1.5.2 Effects of Mechanical Forces on Stem Cells

Mesenchymal stem cells (MSCs) do not normally experience high amounts of mechanical forces in vivo; however, studies have been done on how these forces might affect MSCs and their differentiation. Depending on the culture conditions, application of pressure or strain can influence MSC differentiation. Specifically, hydrostatic pressure and compression lead to a chondrogenic
cell type. Tensile strain, along with fluid shear, can influence MSCs into osteogenic differentiation. For in vitro testing, the type of cell culture also influences how forces affect MSCs. Using a hydrogel-based culture method, it was found that mechanical loading influenced cells to chondrogenic differentiation without use of any pre-differentiation methods.

1.5.3 Effect of pH on Stem Cells

In addition to mechanical loading, MSCs are not normally exposed to the acidic environment that is found inside either healthy or degenerated IVDs. Culture media pH levels similar to those found in IVDs (7.4, 7.1, 6.8, 6.5) have been shown to decrease cell viability and proliferation of ADMSCs and increase the percentage of apoptotic or necrotic cells. Additionally, the lowered pH caused a decrease in gene and protein expression of aggrecan and collagen-II, which are major elements of the ECM in IVDs. These effects were found in both young and mature cell sources. Additionally, a lower pH also alters the cell morphology to one with less cytoskeleton and more rounded cells. Different stem cell sources may have different abilities to adapt to a low pH, but they still experience the negative effects of low pH.
CHAPTER 2: RESEARCH RATIONALE

2.1 Rationale

Most of the current treatments for IVDD focus primarily on symptom treatment, which can result in further degeneration and can be expensive for continuous treatment. Similar to other branches of medicine, tissue engineering is a promising next step for IVDD treatment. One of the primary challenges for the use of tissue engineering as a treatment is survival of the implanted cells in the environment of the IVD. The NP and AF have different structures and functions within the disc, but both can be affected by IVDD. The NP experiences high amounts of elevated pressure as it supports motion and stability in the spine and the AF experiences high levels of strain. The forces acting on both healthy and degenerated IVDs need to be accounted for in creating tissue engineered solutions for IVDD. Additionally, the low pH environment created within the IVD also creates challenges for using other cell types in repair solutions for IVDD. The primary goals of the current research are to develop methods to create IVD conditions in vitro and to use these methods to evaluate how potential cell sources respond to these conditions.

2.2 Aims

- **Aim 1:** To update a pressure-stretch bioreactor
  - Rationale: Previous versions of a pressure-stretch bioreactor have been used for urothelial cell studies, but aspects of the design could be improved. Additional changes were also needed in order to use the bioreactor for IVD studies as well. Testing of the bioreactor was needed to ensure that pressure and stretch were being applied correctly to cells and to quantify the amount of strain that was applied.
  - Approach: Previous experiments completed with the old bioreactor design were repeated to compare results to ensure that cells experience pressure and stretch with
the new design. Specifically, MYP3 cells were exposed to pressure and strain, both separately and simultaneously. Cell lysates were then used for determination of Caspase-1 activity, which can be a marker of inflammation caused by mechanical stimulation. Strain quantification was accomplished with images analyzed with ImageJ software.

- **Aim 2:** To evaluate hADMSCs response to pressure and pH conditions found in healthy and degenerated IVDs
  
  - Rationale: When hADMSCs are implanted they would be exposed to high pressure and low pH conditions, so their ability to survive in these conditions needs to be evaluated. Determining the effects of pressure and low pH on different types of stem cells can help to find the best option for tissue-engineering solutions for IVDD.
  
  - Approach: hADMSCs were exposed to elevated pressure (0.03MPa or 0.275MPa) and low pH (pH 6.5 instead of physiological pH7.4) then analyzed visually for proliferation and cell morphology. Cells were also analyzed for gene expression to see if pressure can trigger differentiation into NP-like cells.

- **Aim 3:** To develop a new high-pressure bioreactor to create more realistic simulations of in vivo conditions
  
  - Rationale: IVDs specifically experience high levels of pressure that are difficult to replicate in vitro. The goals of the new bioreactor are to achieve higher pressures more similar to those seen in IVDs, to connect it to a pressure cycling program that can more accurately simulate what IVDs experience during the day, and to expose more samples at one time.
o Approach: Different variations were designed and then 3D printed to test the design before the final version was machined. Initial work on testing the components, including calibration of a pressure transducer, was also completed.
CHAPTER 3: MATERIALS AND METHODS

3.1 Pressure-Stretch Bioreactor

The current design of the pressure-stretch bioreactor was created to improve on the previous version\textsuperscript{39}. The bioreactor consists of an upper and lower chamber that are separated by a silicone membrane construct. The chambers are sealed around the membrane with the use of bolts and wingnuts to hold the upper and lower chambers together. There are two different configurations of the device that allow for either pressure and stretch to be applied independently or concurrently, or for only higher pressures to be applied. The silicone membrane construct is a circular piece of 40A silicone that is sandwiched between two nylon washers and held in place by superglue. The glue was allowed to cure for 24 hours before the constructs were sterilized via sonication in 70% ethanol for 60 minutes followed by UV sterilization for 15 minutes if cells were going to be seeded on the membranes.

3.1.1 Application of Stretch

Stretch was applied to cells seeded on gelatin coated silicone membranes by allowing the upper chamber to remain open, while negative pressure is applied to the bottom chamber via a syringe or syringe pump set to pull a set volume over an amount of time. The post in the lower chamber was lightly coated with vacuum grease to aid in the membrane’s ability to stretch over the post. The lower chamber and connected tubes were filled with water or PBS.
3.1.2 Pressure Only Configuration

The application of pressure to cells seeded on the silicone membrane was achieved by sealing the lower chamber filled with water or PBS and then applying positive pressure to the upper chamber via a syringe. The other end of the upper chamber was attached to a pressure gauge to ensure that the correct amount of pressure was applied. To achieve elevated pressures for longer periods of time, a different bottom chamber was used that did not have the same openings as the one used to apply stretch or the center post.
3.1.3 Pressure-Stretch Configuration

To apply pressure and stretch simultaneously but independently to the cells, both the top and bottom chambers were closed on one end and connected to pressure/vacuum source on the other. The conditions for stretch only and pressure only need to be met, so the bottom chamber was filled with water or PBS. Stretch was applied first, followed by pressure, using the same methods as applying each individually.

3.2 Calibration of Pressure-Stretch Bioreactor

3.2.1 Strain Quantification

To correlate the applied negative pressure to the resulting membrane strain, an open top viewing chamber was designed and fabricated by 3D printing (Figure). The membrane was stamped with a circular pattern (Figure 3) via a 3D printed stamp and inkpad. The membrane construct was placed in the bioreactor with the open top chamber and a camera (iPhone7/13) was positioned so that the edges of the membrane were at the edge of the view of the camera. Images
were taken before and after negative pressure was applied to the bottom chamber. The images were analyzed using FIJI (ImageJ) to determine the change in radial distance between the stamped rings that could be seen over the post at 16 points (Figure 4). These measurements were used to calculate radial and circumferential strain. The strain values were compared to recorded negative pressure values to find the relationship between the two. The maximum strain was also found using a similar method. Instead of applying a set amount of negative pressure, it was applied until the membrane would not stretch anymore. The pressure value was recorded and the strain was calculated again to determine how much strain the system was capable of applying to the membranes.

Figure 3: Stretch-Quantification Set Up – 3D printed stamp and ink pad used (left) and open top chamber used for imaging (right)
3.2.2 Pressure and Stretch Application to MYP3 cells

An immortalized rat urothelium cell line (MYP3 cells) was cultured in F12-K media with 10% FBS under standard conditions (37°C and 5% CO₂/95% air environment) until confluency and then seeded onto sterile gelatin coated-silicone membranes at 8.4x10⁵ cells/mL. Cell seeding was restricted to only the center of the membrane equal in diameter to the post in the lower chamber of the bioreactor. The cells were left for 24 hours before being exposed to any of the experimental conditions.

Using the bioreactor described above, the cells were exposed to pressure (40 cmH₂O for 1 minute), stretch (gradual strain to 15% over 15 minutes), or pressure and stretch (pressure applied at the end of the stretch condition). A control group was also included that was placed in the bioreactor without pressure or stretch applied for 15 minutes.

At the end of exposure, the cells were immediately lysed with a lysis buffer designed for use with a Caspase-1 assay. The cell lysates were then stored with a Caspase storage buffer and frozen at -80°C. The cell lysates were then thawed and used in an assay to measure Caspase-1.
activity using an established protocol\textsuperscript{40}. The results of this were then analyzed compared to the control group.

3.3 Exposure of human adipose derived mesenchymal stem cells to High Pressure / Low pH conditions

3.3.1 Cell Culture

Human adipose derived mesenchymal stem cells (hADMSCs) were cultured to 80% confluency in low glucose DMEM media (Sigma) supplemented with 10% FBS and 1% Ab/Am or 1% Pen-Strep antibiotic. The media was adjusted to the desired pH (7.4 for normal cell culture) using an amount of sodium bicarbonate based on a curve of pH vs concentration of sodium bicarbonate. This curve was created by making a solution of media powder and DI water at 1g/L. The concentration of sodium bicarbonate was gradually increased and the pH was recorded at each concentration. Cells were cultured in T-75 flasks under standard cell culture conditions (37°C and 5% CO\textsubscript{2}/95% air environment), with media changes every 3 days, and then seeded onto the appropriate material for testing.

3.3.2 High Pressure Low pH Condition

After passaging, hADMSCs were seeded onto 15mm Thermanox plastic treated coverslips at a density of 50,000 cells/coverslip. The cells were left in minimal media for 2 hours, and then 1.5mL of media was added to the wells containing the coverslips. After 24 hours, the IVD condition exposure was started. Cells were exposed to one of six conditions (Table 1) for six days, starting with a change to the media pH. The pH was kept constant for the whole trial and the cells were exposed to a pressure condition for 1 hour per day. The low pH condition was achieved by placing the cells in cell culture media that was made with a set concentration of sodium bicarbonate to achieve a pH of 6.5. The coverslips were transferred to the bottom well of high-pressure
bioreactor chamber with 5mL of the same pH media. The chamber was connected to an air compressor, which was adjusted to a set PSI value to achieve the desired pressure. This pressure was validated via a separate pressure gauge before the experiments were conducted. The cells were then left for 24 hours after the last pressure exposure before any analysis was performed.

<table>
<thead>
<tr>
<th></th>
<th>0MPa</th>
<th>0.03MPa</th>
<th>0.275MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal pH (7.4)</td>
<td>Control</td>
<td>Low pressure, normal pH</td>
<td>Healthy IVD</td>
</tr>
<tr>
<td>Low pH (6.5)</td>
<td>Low pH control</td>
<td>Degenerated IVD</td>
<td>High pressure, low pH</td>
</tr>
</tbody>
</table>

Table 1: IVD-like conditions used for Exposure of hADMSCs

3.3.3 pH Pre-conditioning

In response to results from the exposure to IVD-like conditions, the effects of a sudden change in pH on the cells was examined. In order to accomplish this, three groups were established: a normal pH control, a low pH control with a sudden change in pH, and a gradual pH change. hADMSCs were seeded into a six-well plate at a density of 2.5x10^5 cells/well. The cells were allowed to stay in normal pH (7.4) media for 24 hours (day 0). Media was changed for all groups every 24 hour. On day 1, the gradual change group was changed to a 7.1pH media, followed by 6.8pH on day 2, and 6.5pH on day 3. After day 3, the cells were allowed to stay in culture for three more days.

3.3.4 Pressure Pre-conditioning

Similar to the pH condition, pressure pre-conditioning was also examined. Similar groups were established: a control group with no pressure, a high-pressure control group, and a gradual pressure change group. hADMSCs were seeded onto Thermanox coverslips at 50,000 cells/coverslip and all groups experienced no pressure for the first 24 hours (day 0). The high-pressure control group was exposed to 0.275MPa for 1 hour/day for 6 days and the control group
received no pressure exposure. The gradual change group was exposed to 0.05MPa on day 1 for 1 hour and the pressure was increased by 0.05MPa for days 2-5 (0.1MPa, 0.15MPa, 0.2MPa, 0.25MPa). On day 6, the gradual change group was exposed to the same pressure as the high pressure control group (0.275MPa).

3.3.5 Cell Proliferation Analysis via DAPI staining

At the conclusion of a trial, the cells were removed from the incubator and fixed using 10% neutral buffered formalin (NBF). For staining with DAPI to see only the nuclei of the cells, the samples were permeabilized with 0.2% Triton X-100 in PBS before staining with a XM DAPI solution. After staining, the samples were imaged within 24 hours (Nikon Eclipse E2000-2 microscope, EXFO X-City 120, and Ham camera). The images were then opened with FIJI. The images were converted to binary using the adjust threshold feature and the cells were counted with the analyze particles tool.

3.3.6 Cell Morphology Analysis via Coomassie Blue Staining

At the conclusion of a trial, the cells were removed from the incubator and fixed using 10% NBF. After rinsing with PBS, the samples were stained using an adapted protocol41. Samples were stained with 0.2% w/v Coomassie blue solution in 50% Methanol, 40% ddH_{2}O, and 10% acetic acid for 15 minutes. The samples were then rinsed with ddH_{2}O and imaged with brightfield microscopy (Nikon Eclipse E2000-2 microscope and Hama camera). The images were opened in FIJI and viewed for cell morphology or counted using the cell counter feature.

3.3.6 PCR analysis

Following pressure and pH exposure, cells were lifted and stored in RNALater at 4°C. RNA was extracted via the RNAEasy kit (Qiagen) following the kit instructions. RNA purity was assed using a Take3 plate reader. The samples were then frozen at -80°C and thawed. All samples
were then diluted and samples that did not meet the required purity underwent DNA digestion and were read again.

PCR primers were designed using the Primer Design tool from IDT and checked using NCBI Primer BLAST software (Table 2). PCR was performed on the samples using the SuperScript™ III Platinum™ SYBR™ Green One-Step qRT-PCR kit (Thermo-Fisher) for the genes FOXF1, PAX1, SOX9, and GAPDH as a housekeeping gene. Gene expression was analyzed using the ΔΔCt method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXF1</td>
<td>5’-CCTTCCAGACTCCCAAGAGATAG-3’</td>
<td>5’-TCCTCTTGTCGCCAGCAGTTAC -3’</td>
</tr>
<tr>
<td>PAX1</td>
<td>5’-GGGAGAAGAGCTGGGAAATG-3’</td>
<td>5’-CACAGGATACAGGGAACAAGAG-3’</td>
</tr>
<tr>
<td>SOX9</td>
<td>5’-CTCTACTCCACCTTCACCTACA-3’</td>
<td>5’-CTGGTTGGTCCTCTCTTTTCTTC-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CAAGAGCACAAGAGGAAGAGAG-3’</td>
<td>5’-GGTTGAGCACAGGGTACTTTAT-3’</td>
</tr>
</tbody>
</table>

Table 2: PCR Primer Sequences - Forward and reverse primer sequences used in PCR analysis of hADMSC cells

3.4 Design and Fabrication of High Pressure Bioreactor

A new high-pressure bioreactor was developed with the intended function of being able to hold multiple samples at one time and expose them evenly to elevated hydrostatic pressure. Ideally, this design will allow for more accurate IVD-like pressures to be applied to the cells, specifically around 1MPa of pressure. Prototypes and the final design were made in SolidWorks and initial prototypes were 3D printed with Biomed Amber resin (FormLabs). The final design was machined from aluminum completely for the upper chamber and the lower chamber was aluminum with well inserts 3D printed with Biomed Amber resin.
3.4.1 Pressure Cycling Function

One of the goals for the new design is to more accurately simulate IVD-like conditions, including the various pressures that the discs experience throughout the day. This can be accomplished via a pressure cycling program. The physical part of this program consists of a 3-way solenoid valve, a proportional solenoid valve, and a pressure transducer attached to a pressure source and the chamber. The components are also connected to a NI-DAQ usb 6000 in order to be
controlled by a LabVIEW program. For testing purposes, each piece of the system was controlled individually with a LabVIEW code before the final code was assembled.

3.5 Statistical Analysis

Statistical analysis was performed using Prism Graphpad software. For analyzing the effects of combined pressure and pH exposure, multi-variable ANOVA was used. For all other trials, one-way ANOVA was used to compare experimental groups to the control group. T-tests were also performed. P-values of less than 0.05 were considered to be significant.
CHAPTER 4: RESULTS

4.1 Pressure-Stretch Bioreactor Testing

4.1.1 Pressure Stretch Bioreactor Testing

Testing was done to quantify the amount of strain correlated to specific negative pressures, as well as what the maximum achievable strain is with the current set-up. When measuring %strain vs negative pressure, as the pressure increased, the strain increased as well. Using this method, the maximum strain was found to be around 22.5% (Figure 6). During the testing for the maximum strain, it was found to be around 14% (Figure 7). The measurements for both tests were taken between the inner four rings of the stamped membranes in four directions, designated by 0°, 90°, 180°, and -90° (Figure 4) and then averaged together.

![Strain vs Negative Pressure](image)

Figure 6: %Strain vs Negative HP – Recorded negative pressure values compared to calculated % strain. Data are +/- SEM
4.1.2 MYP3 Caspase Activity

Using the updated pressure-stretch bioreactor, MYP3 cells were exposed to pressure and stretch conditions. Caspase-1 activity was measured using a previously described assay to determine if the cells were experiencing the intended forces. The results of this assay showed a trend towards elevated activity levels in the pressure, stretch, and pressure+stretch groups when compared to the control group. The pressure group was only slightly elevated above the control group, while the stretch and stretch+pressure groups showed more of a change.
**Figure 8: Caspase-1 Activity Fold Change** – Caspase-1 activity levels compared to a control group with no pressure or strain application. N=8, data are +/- SEM

### 4.2 hADMSC exposure to elevated pressure and low pH

#### 4.2.1 Coomassie Blue Staining and Cell Morphology

Coomassie blue staining was used to look at the changes in cell morphology. When compared to the normal pH groups at the same pressure, there was more room in between the cells in the low pH groups. At high pressure, the difference was less noticeable. There was a similar trend across pressure groups at the same pH. As the pressure increased, there was more space between the cells in the images. The change was less noticeable between the control and low pressure groups than the change between the control and high pressure group.

As the cell density decreased, the cell shape also changed. When the cells were further apart, they were also more rounded. In the higher density samples, the cells were more elongated and generally extended in one direction.
4.2.2 DAPI Staining and Cell Counts

DAPI staining was used to count the number of cells present on the coverslips after pressure and low pH exposure. The average number of cells per 0.2mm² was calculated and values were compared to the control group that was kept at a normal pH with no additional pressure. For the no pressure and low-pressure groups, the lower pH resulted in fewer cells present compared to the normal pH conditions. Additionally, the normal pH groups showed fewer cells when low and high pressure were applied compared to the no pressure group. In the high pressure group, there was less change between the normal and low pH groups.

The average cell density was also compared to the starting density that was seeded on the coverslips. Both the normal and low pH controls were elevated above the starting density, but the low pH was less elevated than the normal pH. The low pressure, normal pH group was also elevated above the starting density and was below the normal pH control and above the low pH
control. The low pressure, low pH group was decreased from the starting density and lower than the normal pH group at the same pressure. The high-pressure groups were decreased from the starting density and control groups at both pH values but were similar to each other.

**Figure 10: DAPI Staining of hADMSCs under IVD-like Conditions** – DAPI stained images showing nuclei of hADMSCs after exposure to healthy and degenerated IVD-like conditions.
Figure 11: Change in Average Cell Density Under IVD-like Conditions – Average fold change in the number of cells per 0.2mm$^2$ on treated cover slips after exposure to healthy and degenerated IVD conditions. Cells counted with ImageJ particle analyzer then compared to no pressure, normal pH control group. N=3 (n=2 low pH control), data are +/- SEM
Normalized Change in Cell Density (Compared to Starting Density)

![Graph showing normalized change in cell density](image)

**Normal pH**

**Low pH**

**Figure 12: Change in Average Cell Density Compared to Seeding Density Under IVD-like Conditions** – Average fold change in the number of cells per 0.2mm² on treated cover slips compared to initial seeding density after exposure to healthy and degenerated IVD conditions. Cells counted with ImageJ particle analyzer then compared to starting density. N=2 (n=1 low pH control), data are +/- SEM

4.2.3 PCR Results

qPCR was done on one sample of hADMSC cells that were exposed to low and high pressure at normal pH. PAX1 and FOXF1 were used as NP markers and SOX9 was used as a chondrogenic marker. The goal was to evaluate if any potential differentiation is occurring when pressure is applied. PAX1 levels were more elevated from the low pressure exposure than the high pressure. FOXF1 expression was elevated in the low pressure group but decreased at the higher pressure. SOX9 expression was elevated in both pressure groups although the high pressure group was very elevated above the control.
Figure 13: hADMSC Gene Expression via qPCR After Exposure to IVD-like Conditions – Relative gene expression for hADMSCs exposed to normal pH and high or low IVD-like pressures compared to a no pressure control group. GAPDH was used as housekeeping gene. N=1, data are +/- SEM between duplicates.

4.3 hADMSC pre-conditioning to elevated pressure and low pH

4.3.1 Pressure Pre-conditioning

hADMSCs that were exposed to either gradually increasing pressure or a sudden high pressure were stained with both DAPI for cell counting and Coomassie Blue for cell morphology. The cell counting shows that, when compared to the no pressure control group, the gradual and sudden groups had less cell density. The sudden group was also elevated above the gradual group. The final density was also compared to the starting seeding density of the cells. All three groups were elevated above the starting density, and followed the same trends, with control having the highest change, followed by the sudden group and then gradual group.

Coomassie blue staining images show that the cells in the control group are closer together with a thinner shape than the cells in either of the pressure groups. The cell shape is similar between the groups, but there were some more rounded cells in the gradual change group compared to the no pressure control and high pressure groups.
Figure 14: Change in Average Cell Density in Response to Applied Pressure - Average fold change in the number of cells per 0.2mm² on treated cover slips after exposure to sudden or gradual increased pressure compared to a no pressure control group. N=4, data are +/- SEM, **** p<0.0001 (One-way ANOVA and T-test)
Figure 15: Change in Average Cell Density Compared to Seeding Density in Response to Applied Pressure - Average fold change in the number of cells per 0.2mm² compared to seeding density on treated cover slips after exposure to sudden or gradual increased pressure compared to a no pressure control group. N=4, data are +/- SEM
Figure 16: DAPI and Coomassie Blue Staining of hADMSCs After Exposure to Pressure Conditions – No pressure control (left), gradual change (middle), and sudden change (right) in pressure. DAPI staining (top) shows the nuclei of cells and Coomassie Blue staining (bottom) shows whole cell morphology. Representative images selected from all images taken.

4.3.2 pH Pre-conditioning

hADMSCs exposed to either a gradual or sudden change in pH were also stained with DAPI for cell counting and Coomassie Blue for cell morphology. When the average cell density was compared to the control group, the gradual change group density was slightly lower and the sudden change group density was lower than both the control and gradual groups. The final cell densities were also compared to the initial seeding density. All three groups were elevated above the starting density. The control and gradual groups were similar and more elevated above the starting density than the sudden change group.

Coomassie blue staining also showed that in the control and gradual pH change groups, cells were closer together and more elongated. In the sudden change low pH group, there is more room in between the cells and their shape is slightly rounder.
Figure 17: Change in Average Cell Density in Response to pH Change – Average change in the number of cells per 0.2mm² compared to control (pH 7.4) group after a gradual or sudden change to low pH media. N=3, data are +/- SEM, **p<0.01 (One-way ANOVA and T-test)
Figure 18: Change in Average Cell Density Compared to Seeding Density in Response to pH Change - Average change in the number of cells per 0.2mm² compared to initial seeding density after a gradual or sudden change to low pH media. N=3, data are +/- SEM
Figure 19: DAPI and Coomassie Blue Staining of hADMSCs After Exposure to pH Changes – Normal ph 7.4 (left), gradual change (pH 7.4 to 6.5) (middle), and sudden change (pH 6.5) (right) in pressure. DAPI staining (top) shows the nuclei of cells and Coomassie Blue staining (bottom) shows whole cell morphology. Representative images selected from all images taken.

4.4 High-Pressure Bioreactor Testing

One of the initial tests done for the high-pressure bioreactor was calibrating the pressure transducer that will be used as part of the control system. It was found to be a linear relationship with pressure value corresponding linearly to output voltages. This was tested with both a simple LabView code and a voltmeter with similar results.
CHAPTER 5: DISCUSSION

5.1 Pressure-Stretch Bioreactor

One of the main improvements made to this version of the bioreactor was the addition of the post in the center of the lower chamber. In the previous version, when negative pressure was applied, the silicone membrane the cells are seeded on would deform down into the bottom chamber and the result was a nonuniform distribution of strain. When cells are only seeded onto the portion of the membrane that sits on top of the post in the new design, this nonuniform strain should be eliminated. Additionally, the previous design was rectangular which sometimes led to a wrinkling of the membrane when the chambers were tightened. In the new design, the area around the well was square shaped and silicone membranes were glued between nylon washers to better hold it in place. The new bioreactor was also 3D printed with BioMed Amber resin and the bolts and washers used to hold the upper and lower chambers together were changed to stainless steel. Threaded parts were removed from the design to increase longevity.

Previous experiments completed using the old design of the pressure-stretch bioreactor used a total strain of 15% to simulate specific bladder conditions, so the goal with the new design was to achieve the same amount of strain or more. Initial testing to determine the maximum amount of strain that can be applied to the membranes showed that the average maximum strain was around 14%. Later testing to determine the correlation between negative pressure and strain showed that a negative pressure of 0.09MPa resulted in a strain of 22.5%. This difference most likely arose from a slight change in the set up to attach a pressure gauge or glare from overhead lights causing distortions on the stamped lines for measurements. These results do show that the bioreactor is capable of achieving strains that are physiologically relevant for use in urothelial studies.
5.1.1 MYP3 Caspase-1 Activity

Previous studies used Caspase-1 activity to determine if pressure, stretch, or pressure+stretch was causing an inflammasome activation\textsuperscript{40}. The results from these previous studies showed that all three conditions caused an increase in Caspase-1 when compared to a control group, although they were not all elevated by the same amount. The results of the current study using the new bioreactor show similar results. Applying pressure and stretch independently or simultaneously resulted in elevated Caspase-1 activity levels compared to the control. Comparing these results indicates that the redesigned bioreactor is transferring the applied pressure and stretch to cells seeded on the silicone membrane. The levels were not the same as the results from the previous study, but the differences could be due to variation in protocols that accompanied the changes made to the bioreactor.

5.2 hADMSC exposure to elevated pressure and low pH

Elevated pressure values were chosen for this study based on both literature value and the capabilities of the set-up of the bioreactor system. The air compressor being used for the pressure-only version of the bioreactor was only rated for 60psi/0.41MPa and above values of 40psi/0.275MPa, the bioreactor experienced more leaking from the chambers and the connections to the air compressor. For this reason, 0.275MPa was chosen for the high pressure “healthy” IVD group, since it is within the physiological range as well\textsuperscript{11,12}. The low pressure “degenerated” IVD group was chosen to be at 0.03MPa since that is within the range of pressures experienced by a degenerated IVD (Sato). pH levels were chosen similarly; physiological pH is generally considered to be 7.4 and degenerated IVDs experience a pH level that is lowered to under 6.8 due to structural changes and lack of oxygen.
5.2.1 Cell Morphology and Density

The cell morphology and cell density of the samples that were exposed to a combination of elevated pressure and low pH conditions to mimic IVD conditions showed changes in response to both conditions. When the pressure was increased for the normal and low pH groups there was a decrease in cell density when compared to the respective normal control group. The same was true when comparing normal and low pH groups at the same pressure. Similar trends were also seen when comparing the final cell density to the initial seeding density. In the samples with decreased cell density, there was also altered cell morphology. When the cell density was increased, the cells had a more linear, elongated shape. The lower cell density samples had cells with a more rounded shape, which is consistent with previous studies examining the effects of low pH on MSCs\(^\text{37}\). This relationship between cell density and morphology is similar to cells under standard culture conditions. As they reach confluency, they become more elongated.

The decreases in cell density between both the increased pressure and low pH groups show that both conditions can have an effect on cell proliferation or survival. A previous study has shown that under inflammatory conditions, hADMSC growth was increased; however, inflammatory conditions created via pro-inflammatory cytokines may different than any inflammation that was caused by the increased pressure and lowered pH in this study\(^\text{26}\).

5.2.2 hADMSC Gene Expression

Markers for both NP cells and chondrogenic cells were chosen to evaluate if differentiation was occurring. A chondrogenic marker (SOX9) was chosen as well as the NP markers since there are some similarities between the NP and cartilage and hADMSCs have previously been shown to differentiate into chondrogenic cells\(^\text{6,25}\). The NP markers chosen were PAX1 and FOXF1 which have been shown to be elevated in NP cells when compared to chondrocytes and AF cells\(^\text{42}\). PAX1
and FOXF1 are both transcription factors that are likely involved in embryonic development. SOX9 has previously been shown to have increased expression levels in hADMSCs under applied hydrostatic pressure, which is consistent with these results as well33.

The results of the qPCR on samples exposed to elevated pressures are only preliminary, but they do show some increases. PAX1 was increased for both the low and high pressure groups while FOXF1 was only elevated in the low pressure group. SOX9 showed a very large increase in the high pressure group. With a small sample size, these results are not significant enough to determine if differentiation is occurring, but they do serve as a starting point and confirmation that the genes are being expressed to some level in hADMSCs.

5.2.3 Pressure and pH Preconditioning

Pressure and pH preconditioning tests were both completed to further examine the effects of pressure and pH individually on hADMSCs, as well as how a gradual change in conditions compared to a sudden change. The results of the pressure pre-conditioning showed that a sudden change to 0.275MPa resulted in a smaller change in cell density than a gradual change. The change between the control and sudden change groups was different than the similar groups in the IVD-like condition experiments, even though the conditions were similar. While there are more conditions to consider, these results show that slowly acclimating hADMSCs to the elevated pressures found in IVDs may not increase their ability to survive within an IVD.

A gradual change in pH did reduce the negative effects that a low pH has on hADMSC culture. The group with a gradual pH change to 6.5 from 7.4 was much closer in final cell density than the group that was immediately changed to 6.5. These results indicate that slowly changing the pH of the cell culture media may help to prepare hADMSCs for use in IVDs. Other studies
have shown that in MSCs derived from other sources, acidic conditions increase the stem cell phenotype, which may have implications for how readily they differentiate⁴³.

The results of both the pressure and pH preconditioning experiments have other factors that need to be taken into account when determining if preconditioning will actually help stem cells survive in the IVD environment. In both studies, the sudden change group was exposed to the more extreme condition for the full six days. The gradual change group in the pH experiment was only in 6.5 pH media for three days, and the gradual pressure group was only exposed to the maximum pressure for one day of the trial. This difference in exposure time could account for some of the decrease in cell density, especially in the pH trials.

5.3 High Pressure Bioreactor

The primary goal of this bioreactor was to apply more accurate physiological conditions to cells in vitro. For use with IVD cells specifically, the goal is to reach 1MPa of applied pressure, which would cover a larger range of pressures that the IVD experiences daily. Additionally, creating a pressure cycling program allows for a range of pressure to be applied during a set time period. In relation to IVD conditions, this would more accurately model the pressure experienced by the disc in prone, sitting, and standing positions that a person would be in throughout the day. The design also features 9 individual wells so that more samples can be exposed at once.

The initial prototypes were 3D printed with BioMed Amber resin, but the final design was machined out of aluminum and then anodized to ensure that it would be resistant to corrosion and easier to sterilize while being able to hold up to higher pressures. The initial prototype was also too large for the 3D printer to handle easily and the final product ended up with warping that would prevent it from being properly pressurized. The well-inserts are still 3D printed to ensure that they are biocompatible since they are what the cells will be in contact with.
A previous pressure cycling protocol was developed previously for use with urothelial pressure and this was used as a starting point for developing the physical and LabView components of a new protocol\textsuperscript{40}. The LabView code controls different solenoid valves and receives data from a pressure transducer. Depending on the data or timing, valves can be opened or closed to change the pressure inside the chamber. The only physical testing that has been completed so far is calibrating the pressure transducer to convert the voltage readings to pressure values.
CHAPTER 6: CONCLUSIONS AND FUTURE WORK

6.1 Pressure-Stretch Bioreactor

The redesign of the pressure-stretch bioreactor was successful in still applying pressure and stretch to cells seeded on silicone membranes. For improvements over the previous design, the silicone membranes are no longer being pulled down when negative pressure is applied, so the cells are experiencing a more even distribution of strain. Additionally, the new design allowing for the option of applying higher pressure expands its use beyond the original applications for urothelial cell culture and testing.

6.2 Effects of Elevated Pressure and Low pH on hADMSCS

The overall results from exposure to healthy and degenerated IVD like conditions show that hADMSCs could be a viable cell source for tissue engineered IVD repair. While the conditions did decrease cell density, the cells were not wiped out by the changes in pH and pressure. Additionally, pre-conditioning cells to a lower pH may help to decrease the negative effects of low pH.

6.2.1 Future Work for IVD conditions

Further work is needed to fully determine if hADMSCs could be used to repair damaged IVDs. One major component of tissue engineering is the scaffold used to implant the stem cells, so it would be necessary to perform tests combining the hADMSCs and decellularized scaffolds. Testing would include similar testing to 2D cell culture tests to determine if the cells are capable of surviving IVD-like conditions while in the scaffold. For both 2D and 3D cell culture, more tests are needed to determine if differentiation into the desired cell type, or an undesired cell type, is occurring.
6.3 High Pressure Bioreactor

The work done on the high pressure bioreactor was mostly focused on the physical portion of it. Compared to previous prototypes and designs, it should be able to withstand higher pressures, as well as expose more samples to higher pressure at one time. Now that the physical design is finalized and has been made, the next steps involve testing the chamber to ensure that it will pressurize all wells evenly and creating the physical and software components for pressure cycling. The work for this will involve creating LabView codes to control a series of valves and a pressure transducer that will ultimately function to better model the conditions that IVDs experience in vivo.
REFERENCES


