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Efficacy of a Novel Through-Thickness Perfusion Bioreactor to Create Scaffold-Free Tissue Engineered Cartilage

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Efficacy of a novel through-thickness perfusion bioreactor to create scaffold-free tissue engineered cartilage

By

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Articular cartilage is an avascular, aneural tissue that covers the ends of diarthroidal joints. Once damaged by disease or injury, cartilage lacks the ability to self-repair. Generating tissue engineered cartilage is an exciting field that may provide a possible solution to this problem. The purpose of this study is to determine the efficacy of a through-thickness perfusion bioreactor to generate scaffold-free tissue engineered cartilage.

The results of the study show that allowing long-term static culture to cell constructs before perfusion increases the efficacy of the bioreactor. Immediate perfusion of cell constructs in the bioreactor is shown to decrease the efficacy to produce scaffold-free constructs with desirable biomechanical and biochemical properties. The results of the study also show possible options in future works that could increase the efficacy of the bioreactor.

DEDICATION

This thesis is dedicated to Dale and Tammy Gilbert for their never ending support and encouragement as I traveled through this journey of ups and downs. Thank you for everything.

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I would like to thank Dr. Elder for all of the guidance and encouragement throughout this academic endeavor. I also would like to thank Dr. Liao, Dr. Raj Prabhu, and Dr. Jim Cooley for giving of their very valuable time and advice to help me produce the best research I could.

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CHAPTER I

INTRODUCTION

Motivation

Arthritis is the wearing out and degradation of cartilage in a joint and is a topic of particular concern. Osteoarthritis is the most common form of arthritis with the cause of degradation in the joint being from either old age or obesity. With modern medicine causing people to have longer lifespans and with 35.6% of American adults being obese [1], osteoarthritis is a problem that needs to be addressed. In 2010 it was documented that one in five adults in the United States were diagnosed with arthritis, and 50% of adults of 65 years of age or older were diagnosed with arthritis as well [1]. Also reported in 2010 was that 21.4% of overweight and 31.1% of obese Americans claimed doctor-diagnosed arthritis [2]. Another reason for looking into a solution for cartilage repair is because of the large medical costs of repair procedures. In an article published by Science Daily, there are an estimated 600,000 total knee replacements done annually in the United States. They also go to say that with each operation costing around $15,000 that the total cost for just total knee replacements alone are an estimated $9 billion [3]. Even with the prevalence of arthritis there is no cure. The typical non-surgical solutions used today for lower budget patients are methods such as stretching, pain medication, weight control, and physical therapy and are directed at alleviating the symptoms instead of repairing the affected tissue.

Articular Cartilage

Articular cartilage is a connective tissue that covers the end of long diarthoidal joints. The function of cartilage is to provide a smooth frictionless surface for joint articulation and load distribution of physical activity. Damage to cartilage tissue is problematic due to its acellular and aneural nature. This causes the cartilage to lack an appropriate self-healing response, and therefore any damage incurred to the tissue is likely to perpetuate and exacerbate thereafter. Cartilage can primarily be damaged in two ways: disease and physical injury. The disease most common in articular cartilage is osteoarthritis which is the wearing out of cartilage in the joint due to old age or obesity. Physical injury can also occur to cartilage and is commonly seen in professional athletes participating in high impact sports. Injuries to cartilage tissue be can classified into one of three categories based on the tissue depth of the injury. There is the partial thickness defect where some portion of the thickness of the cartilage has been damaged does not continue throughout the entire thickness of the cartilage. Next is the full thickness defect, and as the name describes, is an injury that affects the entire thickness of the cartilage but does not affect the underlying subchondral bone. The final classification is a subchondral defect in which the injury is through the entire thickness of the cartilage and into the underlying bone [4,5].

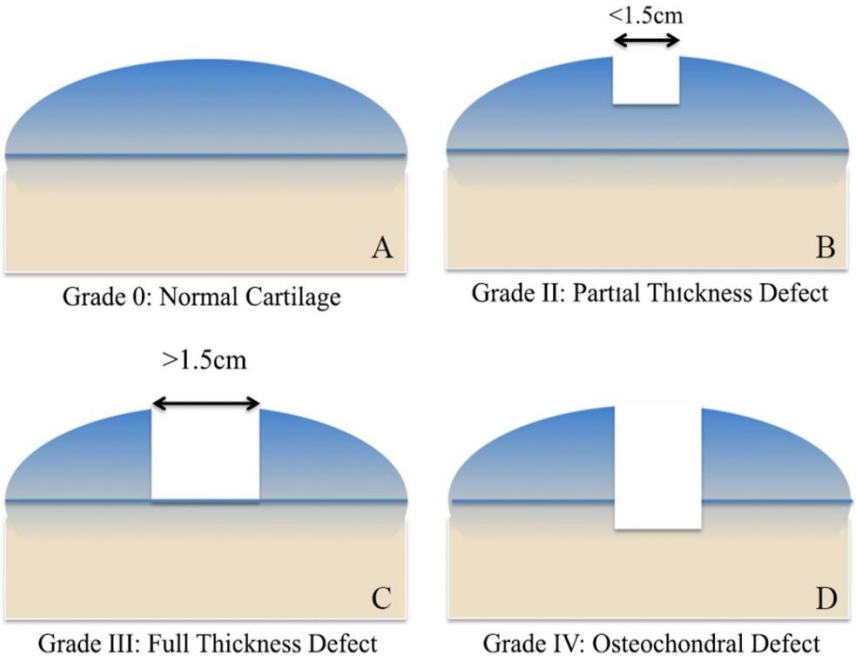

Figure 1.1 Illustration depicting the different types of cartilage defects [4].

The medical approaches that are available today to repair cartilage are not sufficient to completely repair the injured cartilage. The procedures used are directed more towards a quality of life aspect as opposed to a permanent solution to damaged cartilage. The three methods most commonly used in cartilage repair are microfracture, Chondrocyte Autologous Transfer (OAT), and Autologous Chondrocyte Implantation (ACI) [6, 7]. Microfracture is a method in which microholes are created into the subchondral bone of the defect to release mesenchymal stem cells (MSC) into the defect. The purpose is to allow the MSCs to take on the phenotype of cartilage yet the downside is that the cartilage created by the MSCs is not articular cartilage but instead fibrocartilage. Fibrocartilage has inferior mechanical properties to that of native articular cartilage and will degrade over time due to the inability of fibrocartilage to withstand the

mechanical demand of a load-bearing joint. In Chondrocyte Autologous Transfer the defect is prepared into a cylindrical hole that is to be filled. Plugs of cartilage are taken from the non-load bearing regions of the patient's joint and are used to fill the defect. The remaining space of the defect is then filled with MSCs using microfracture on the subchondral bone. While the mechanical properties of the repaired cartilage is better than that created by microfracture alone, the problem with this method is that the plugs used to fill the defect can create an uneven surface and thus poor for joint articulation. Also by taking plugs from elsewhere in the joint, even though they are non-load bearing, creates another defect that will eventually degrade and wear out over time. In Autologous Chondrocyte Implantation, cartilage is harvested from non-load bearing regions of the patient's joint. The cells are expanded in vitro for three to four weeks and then implanted into the cartilage defect underneath a periosteal flap. This method is the most promising of the three yet requires extensive physical therapy from the patient post-surgery. Also should the periosteal flap degrade or detach then another surgery will be needed. Another downside to this method is also the expensive cost of culturing and passaging cells to prepare for implantation [8].

Today tissue engineering is an exciting field that is being examined to see if it could provide a solution to both the issue of functional cartilage repair and being cost effective. But to be able to culture such tissue, one must have a fundamental understanding of the tissue.

Structure and Composition

Articular cartilage is a unique tissue because it is essentially acellular having only ~1% of the volume of tissue consisting of cartilage cells called chondrocytes. It is the

responsibility of these cells to produce and maintain the extracellular matrix that achieves the remaining volume of the tissue. The extracellular matrix (ECM) is what gives cartilage its amazing mechanical properties. The ECM has a composition of 60-85% water, 10-30% collagen, and 3-10% proteoglycans [9]. It is the collagen, proteoglycans, and chondrocytes that define cartilage and help it do its job.

Collagen

Collagen is a structural protein that composes up to 60% of the solid volume of articular cartilage. It is the collagen that is responsible for giving the tissue its tensile strength. This strength is derived from the hierarchal structure of collagen. As can be seen in Figure 1.2, the tensile strength begins on a molecular level when three polypeptide chains of collagen adhere together to form a triple helical structure which is commonly called tropocollagens. Five tropocollagens then bond to form a collagen fibril, and subsequently many collagen fibrils aggregate to bond into a collagen fiber [10]. There are many different types of collagen that exist in articular cartilage. Among them are collagen types II, III, VI, X, and XI. Of these types it is type II collagen that is most prevalent accounting for 90-95% of all collagen in the ECM of articular cartilage [11].

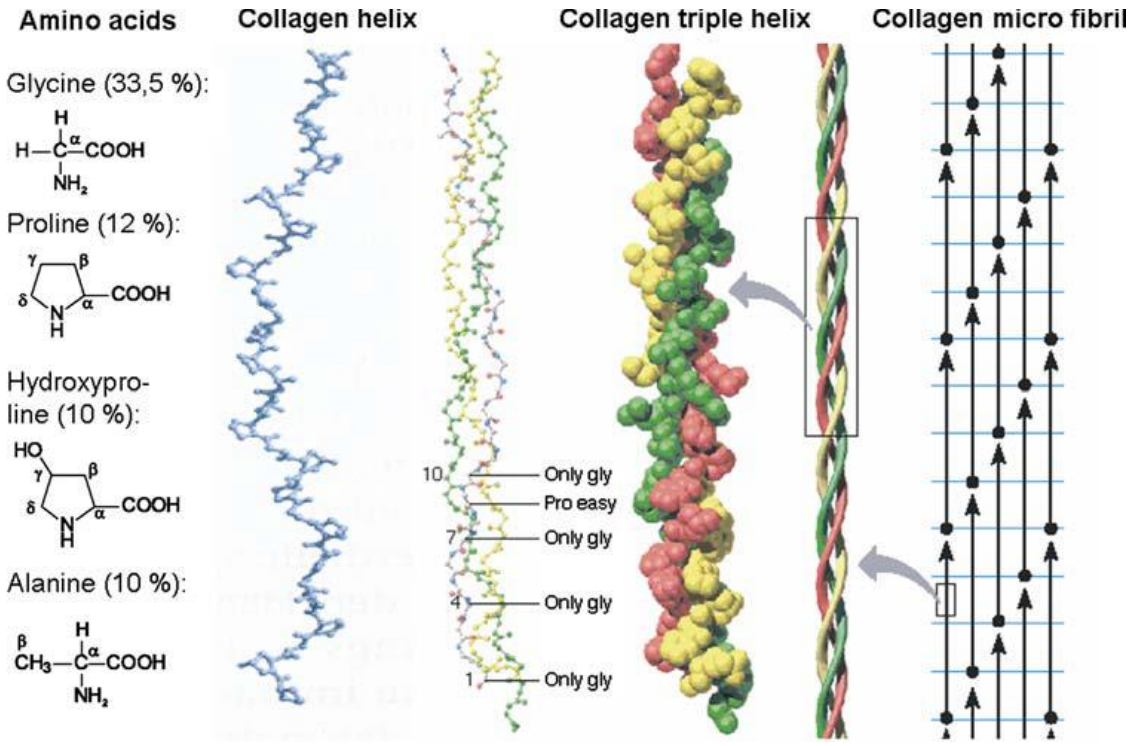

Figure 1.2 Illustration depicting the structural hierarchy of collagen [10].

Proteoglycans

Proteoglycans make up another 30% of the solid weight of articular cartilage. There are two different types of proteoglycans in the ECM of cartilage and are classified as either nonaggregating or aggregating. The nonaggregating class of proteoglycans consist of a core protein to which sulfated glycosaminoglycans attach as can be seen in Figure 1.3. GAG being hydrophilic in nature attracts and binds water molecules which can then act as a hydrostatic pressure when the tissue is rapidly compressed. This is how cartilage is able to absorb and withstand such large compressive loads exerted by the body [9].

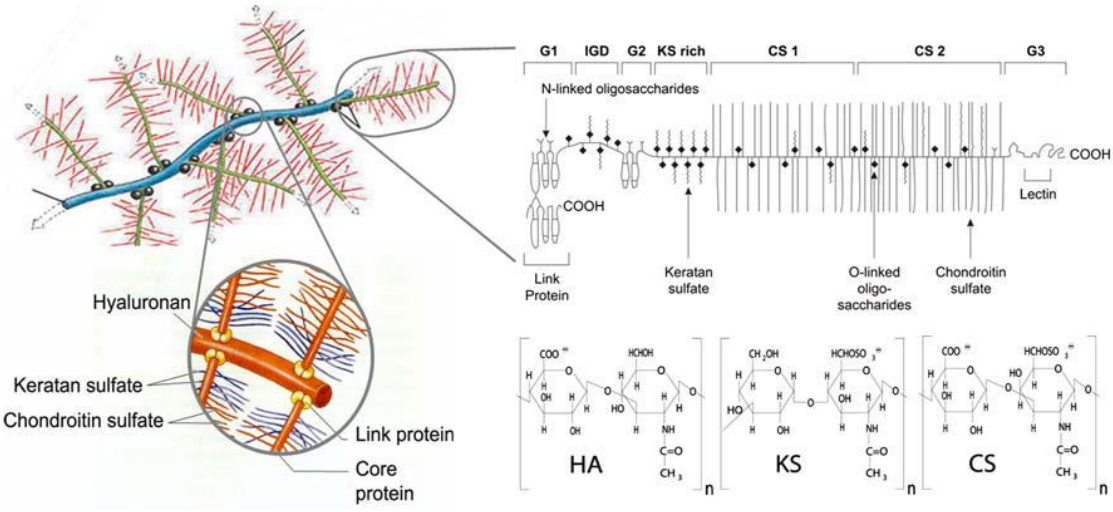

Figure 1.3 Illustration depicting the structure of GAG [9].

Chondrocytes

Chondrocytes are the only cells found in articular cartilage. While only making up ~1% of the total volume of the tissue, they are responsible for the delicate upkeep of the correct composition of the ECM to keep the tissue healthy and functioning. Each chondrocyte is typically isolated from other cells in the tissue in chambers called cartilage lacunae [12]. Since articular cartilage lacks a nervous system, the chondrocytes rely on mechanical stimuli from the surrounding ECM to be able to respond to the external environment. Also with the tissue lacking a vascular system the cells must rely on diffusion aided by the passive motion of the joint to both receive nutrients and remove waste.

Methods of Cell Culture

Chondrocytes can be cultured multiple ways based on the structural or compositional needs and shape requirements of the tissue. Articular cartilage is often

cultured in monolayer in common cell culture flasks yet when large numbers of cells are needed in 3-D culture such as on a scaffold or in a hydrogel, these methods are insufficient. This is where bioreactors can be used to culture large amounts of chondrocytes in 3-D culture. The function of the bioreactor is to regulate the environment cells are grown in by controlling factors such as pH through waste removal, gas exchange of O2, temperature, nutrient and growth factor transfer. While there are many different models of perfusion bioreactors and each tailor to the growth and development of a specific tissue there are no clinically used bioreactors that create scaffold-free tissue engineered cartilage constructs for the repair of partial-thickness defects. This objective of this study is to create a through-thickness perfusion bioreactor with a high efficacy for producing scaffold-free tissue engineered cartilage with superior properties to constructs cultured under static conditions [13, 14].

CHAPTER II

BIOREACTOR CHARACTERIZATION

Introduction

While some bioreactors are used clinically, it is not uncommon for custom bioreactors to be built by labs and private companies to better meet the needs of the required tissue. The needs of this study was to have a bioreactor that can generate large scaffold-free tissue engineered cartilage with properties better than that of static culture. Therefore a custom bioreactor chamber was constructed with this goal in mind. The bioreactor chamber was designed as a cylindrical vertical flow chamber with an inlet and outlet at top and bottom for media flow. The bioreactor was constructed from polysulfone to allow autoclaving of the entire chamber. The center was hollowed out and made so that with a rubber gasket, a 6-well plate transwell insert would fit tightly in the middle of the bioreactor which ensures that there is no flow that bypasses the cell culture insert as can be seen in Figure 2.2. This restricts and forces all cell culture media to pass through the membrane of the transwell insert and thus the cell culture. An 8.5mm hole was also drilled in the lid of the bioreactor so that a sterile syringe filter could be fitted to the lid to ensure sterile gas exchange. To transport media throughout the bioreactor system, 2.79mm diameter Manostat® silicon tubing was used to connect the reservoir media bottle to the inlet and outlet of the bioreactor. A Manostat® Carter® 4/8 Cassette Pump (Thermo Fischer Scientific, Waltham, MA) was used to pump media through the system.

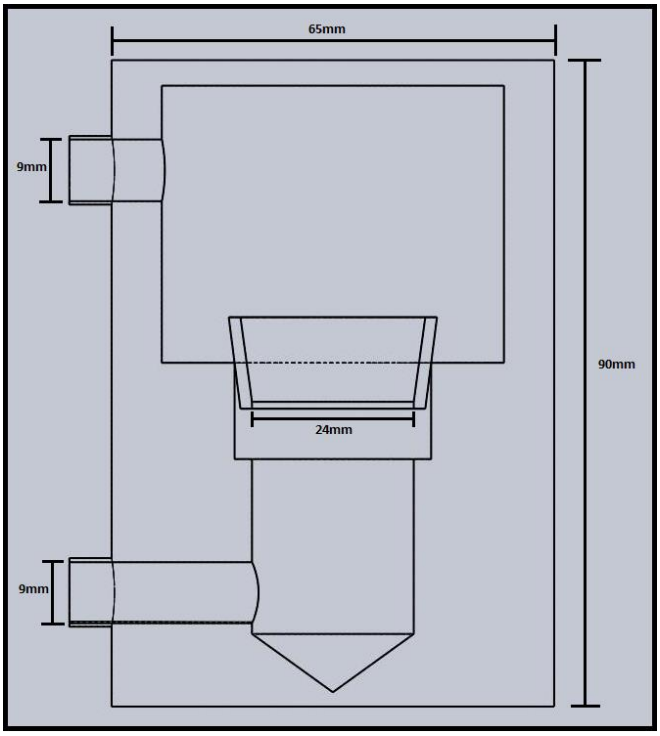

Figure 2.1 Illustration depicting the shape and specifications of the bioreactor

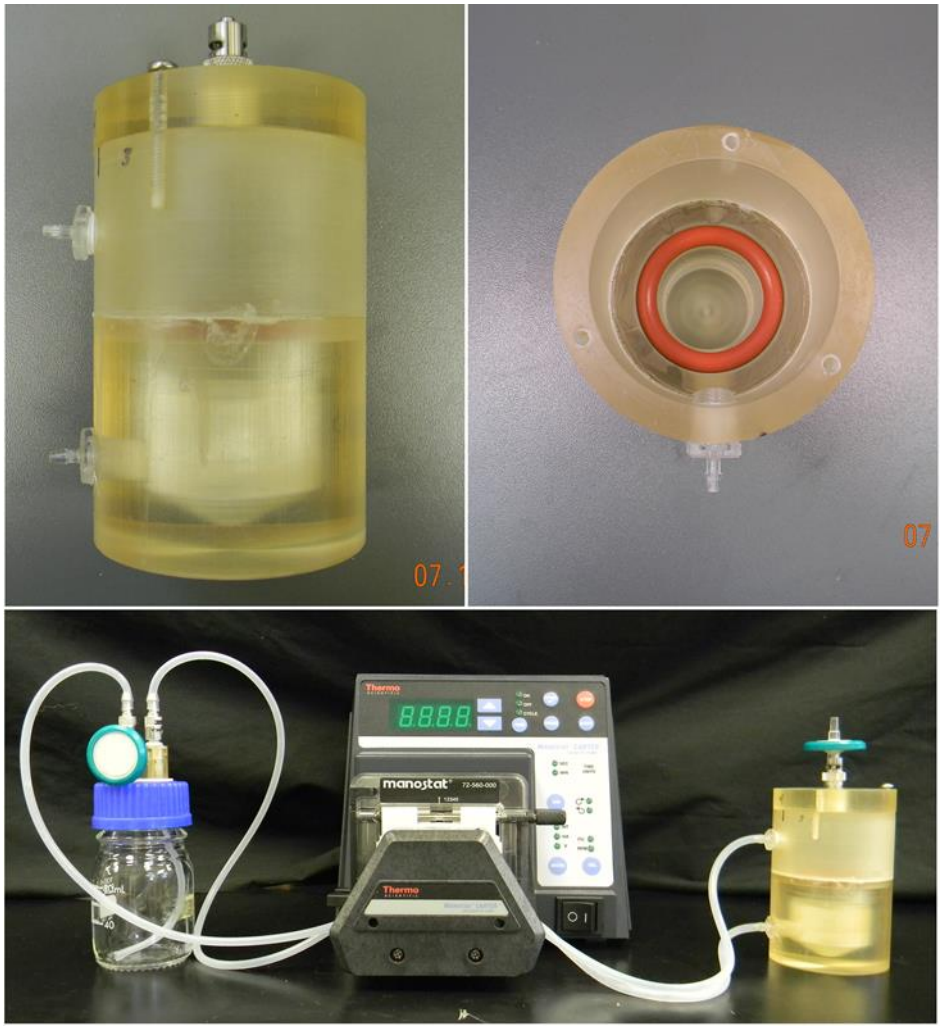

Figure 2.2 Photographs illustrating the setup of the bioreactor for cell culture

While the use of this custom-built through thickness perfusion bioreactor will theoretically be beneficial for cell culture, the bioreactor chamber has to be characterized to determine if it is viable for cell culture. Temperature and pH were two characteristics examined due to the long assembly time of the bioreactor after autoclaving and implementation of cells into the bioreactor and the detrimental effects these two characteristics might have on cell culture from the bioreactor being out of the incubator

prior to cell culture. Another point of interest was the ability to repetitively autoclave the entire bioreactor system sans pump. In a clinical setting, the ability to easily sterilize the entire system would be of great benefit and add to the ease of use of the bioreactor.

Methods

Temperature and pH

To measure temperature and pH, the bioreactor was put in a 37°C cell culture incubator and continuously perfused with media and empty of cell culture. Temperature was taken at increasing intervals using a voltmeter with a thermal couple. For the first temperature test, temperature was taken through the front of the incubator and measuring temperature and taking a media sample for pH. The concern with this method was that, especially early on with quick time points, the temperature in the incubator would be inhibited from reaching its desired temperature of 37 degrees Celsius. Therefore, a second temperature test was run in which only temperature was taken. The voltmeter was placed outside the incubator and the thermal couple threaded through to the inside of the incubator and into the bioreactor. This way the internal temperature of the bioreactor could be taken without compromising the temperature of the incubator and therefore more representative of the conditions of cell culture during perfusion.

Autoclavability

In order to determine if the bioreactor could be easily and repetitively sterilized, each portion of the perfusion system sans pump was individually placed in steam autoclavable pouches. All components of the bioreactor were steam autoclaved at 140°C for 45 minutes. After autoclaving, the bioreactor system was assembled and media cycled

to ensure structural integrity as well as overall sterility. This process was repeated five times to ensure consistency.

Fluid flow model

To determine how cell culture media reacts inside of the bioreactor as well as at the surface of the membrane, a simplistic fluid flow profile was created using the FLOEXPRESS simulation from SolidWorks® 2013 Student Edition (Dassault Systèmes SolidWorks Corporation, Waltham, MA). The first model was created using only the cell culture chamber with a transwell insert but omitted cells to get a base value and idea as to what pressure and flow velocities were like within the bioreactor. The model was to be used to help determine if the flow rate across the membrane of the transwell was uniform or if there was a discrepancy in the flow rates between the centroid and perimeter of the membrane. The flow rate of the peristaltic pump was determined both theoretically and experimentally to ensure that the boundary conditions set for the inlet were accurate, and the flow rate of the pump was found to be 1 mL/min. Thus the inlet boundary condition was set to have a volumetric flow rate of 1 mL/min and the outlet boundary condition was set as an atmospheric pressure outlet. The model was run using water as an analog for cell culture medium, assuming laminar flow, accounting for gravity, using 442 iterations leading to a convergent solution.

The second model was created to simulate flow within the bioreactor accounting for a cell suspension included in the transwell insert. Therefore, all assumptions and boundary conditions from the first model were maintained. The difference in this model being that a lid was created of 1mm thickness on the membrane of the transwell to simulate the presence of a cell suspension. Using the permeability coefficient for

multipotent mesenchymal tissue as determined by Loboa et al. [15] as a basis for the permeability of the neonatal chondrocyte cell suspension, the pressure differential (ΔP) across the cell suspension was estimated using the equation following Darcy's law:

$$v = k(\frac{\Delta P}{\mu \times \Delta x}) \tag{2.1}$$

where v is the volumetric flowrate set at 1 mL/min, k is the permeability constant obtained from Loboa et al. to be 1×10-13 m4/N·s for multipotent mesenchymal tissue, μ is the dynamic viscosity of water at 37°C which is 686.5 Pa·s, and Δx is the thickness of the cell suspension which is 1mm. Using this information the material properties were set in the engineering database of SolidWorks® for the lid that represented the cell suspension. The porosity of the cell suspension was set at 75% which is the median value for the porosity of articular cartilage as reported by Jin et al. [16]. The model was run under the same conditions and assumptions as the first model using water as a culture medium analog, assuming the effects of gravity and laminar flow. The calculations repeated for 400 iterations leading to a convergent solution.

The third model was developed simulating cartilage that would have been perfused in the bioreactor for a long duration. Following the same methods as the second model, the physical properties of the 1mm thick lid representing the cells were set in the engineering database following Equation 1 but changing the value of the permeability coefficient. The value for the articular cartilage permeability coefficient is 4.8×10-15 m4/N·s from Loboa et al. [15] and was used in conjunction with Darcy's Law for the calculations in the same fashion as in the second model. The model was run with all of

the same assumptions and boundary conditions as the other models, and the calculations repeated for 400 iterations to a converging solution.

Results

Temperature and pH

The first temperature test revealed a rapidly tapering increase of the internal temperature of the bioreactor taking six hours for the internal temperature of the bioreactor to reach that of the incubator. The pH test showed that there was no large increase in the pH for the duration of the temperature experiment. The second temperature test showed a more rapid initial increase in temperature taking less than three hours to reach equilibrium temperature with that of the incubator.

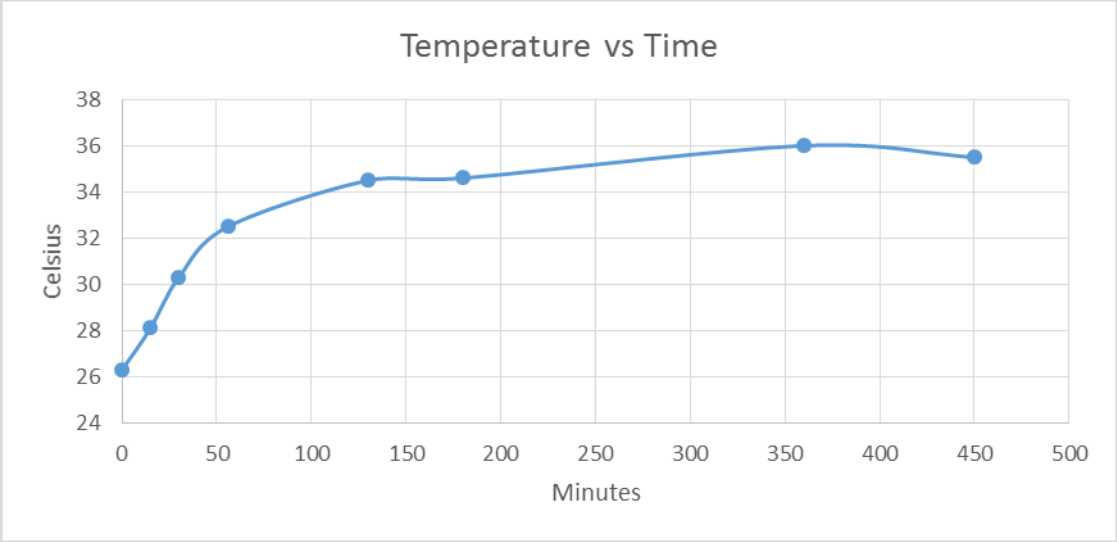

Figure 2.3 A scatter plot showing Temperature vs Time.

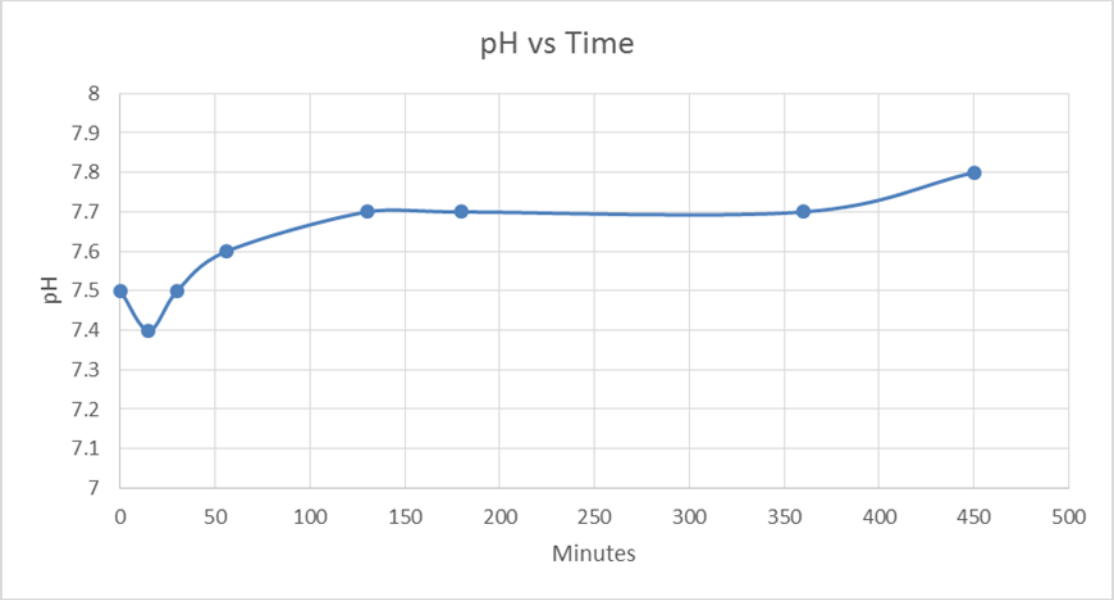

Figure 2.4 A scatter plot showing pH vs Time.

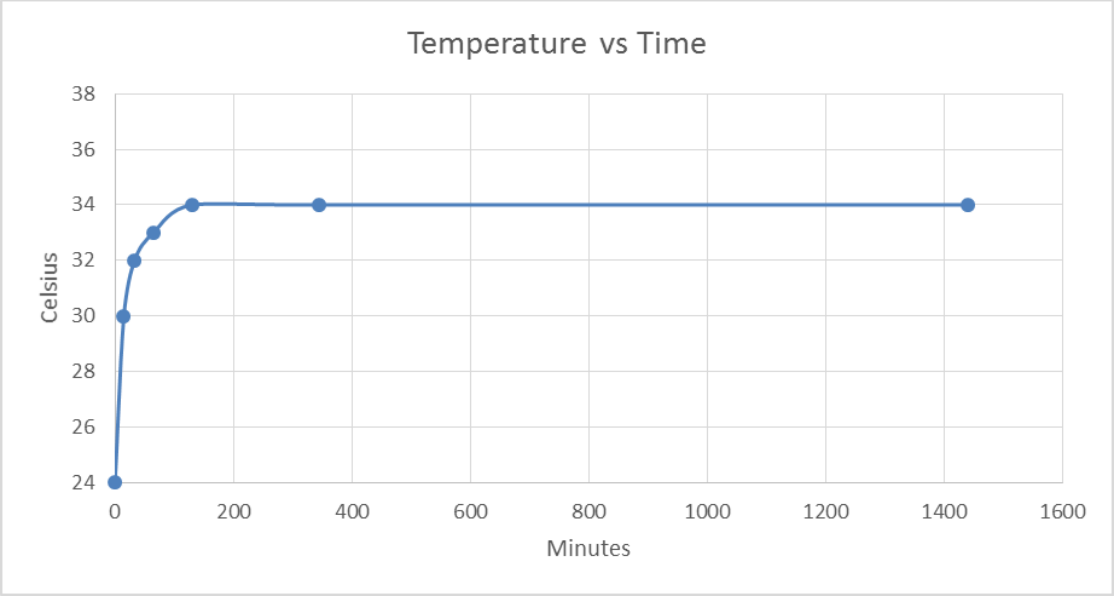

Figure 2.5 A scatter plot showing Temperature vs Time.

Fluid Model

The initial flow model shows relatively uniform flow velocity (≥5.592×10-5 m/s) throughout the bioreactor chamber as well as at the membrane with the exception of the inlet (5.033×10-4 m/s) and outlet (3.355×10-4 m/s) where the flow volume is restricted. The pressure of the fluid within the bioreactor shows that there is increased pressure in the bottom of the chamber (101620.55 Pa) and decreases as the flow approaches the outlet (101065.87 Pa) and at the surface of the membrane the pressure was uniform (101313.21 Pa).

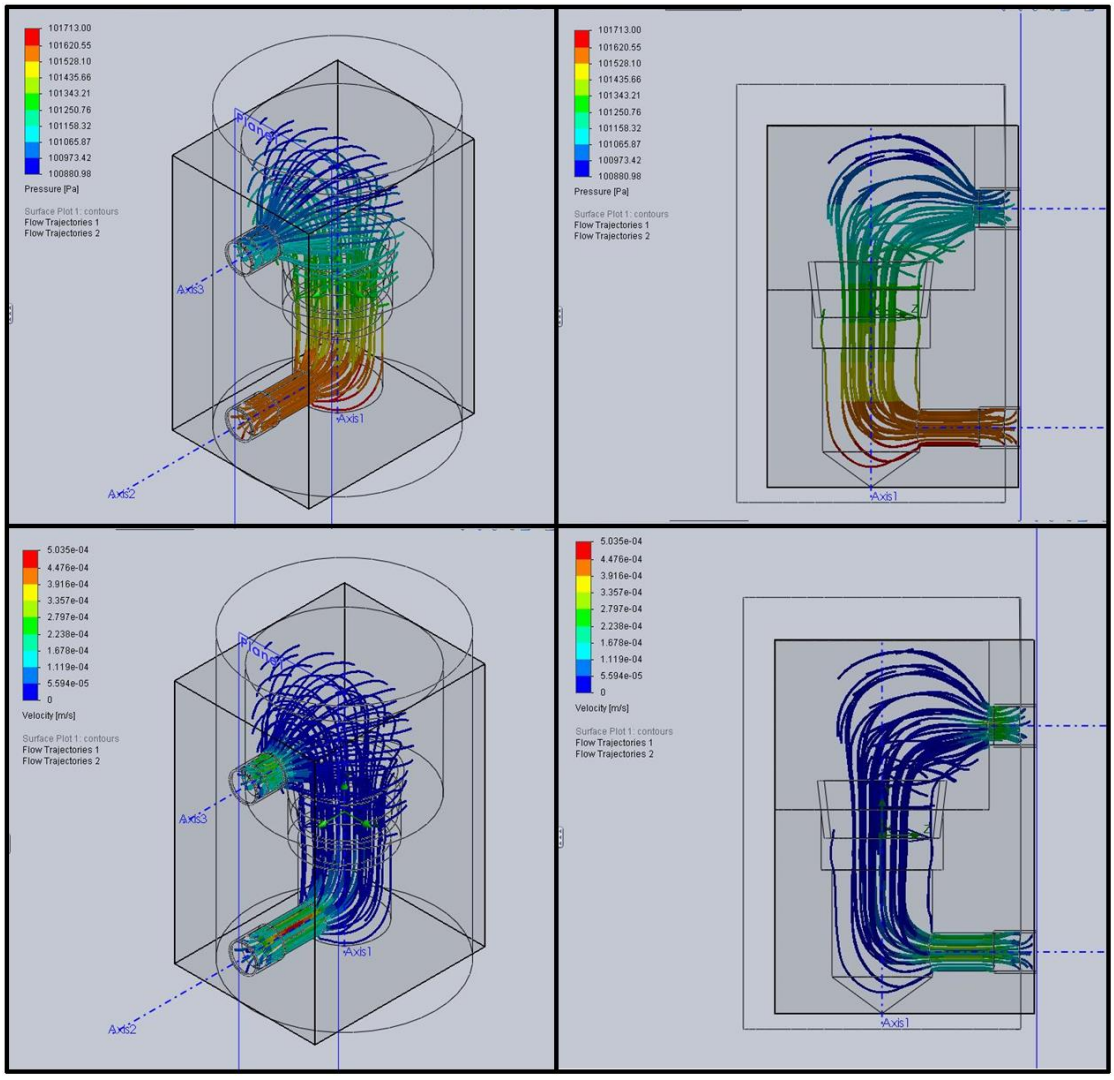

Figure 2.6 Visual representation of the first bioreactor flow model without cell culture

The second flow model created with a cell suspension in the transwell insert displays a pressure differential from either side of the membrane as can be seen in Figure 2.7. The pressure across the cell suspension observed in the figure appears to be uniform across the width of the membrane (between 129851.82 and 123478.79 Pa). The velocity of the fluid across the membrane in Model 2 (≥ 9.488×10-5 m/s) is higher than in Model

1 (≥5.592×10-5 m/s) which is to be expected with the lower permeability of the cell suspension relative to the membrane of the empty transwell insert.

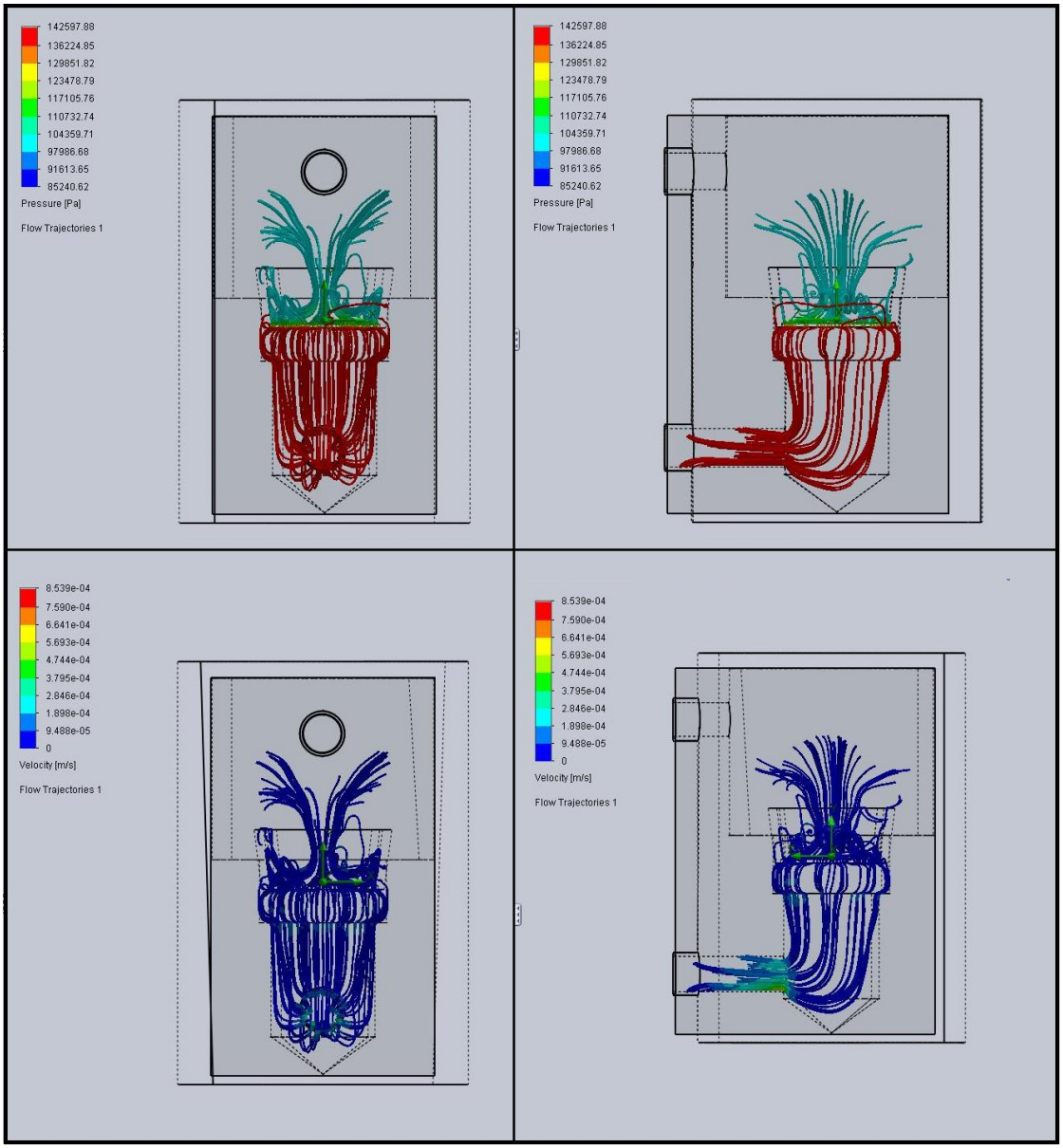

Figure 2.7 Visual representation of the second bioreactor flow model with cell suspension

The third flow model shows a pressure profile that has uniform pressure across the membrane of the transwell being between 101349.75 Pa and 101256.00 Pa. The flow velocity profile shows a uniform velocity of media across the membrane at 2.507×10-4 m/s. The velocity also increases to 5.014×10-4 m/s once the fluid has passed the lower permeability of the cell construct but still seems to be uniform across the width of the membrane.

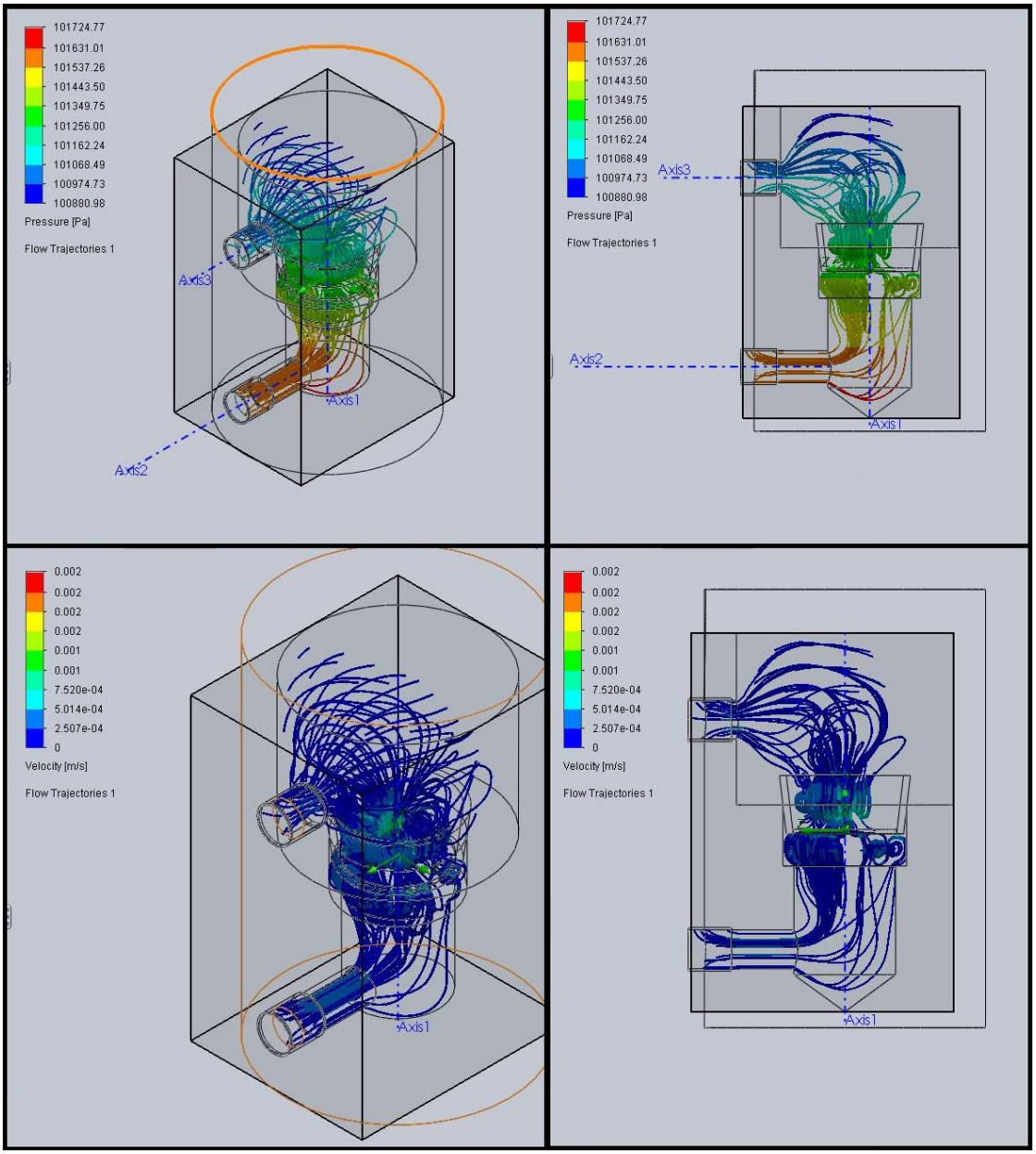

Figure 2.8 Visual representation of the third bioreactor flow model with articular cartilage

Discussion

Temperature and pH

Given the temperature data obtained it is apparent that the bioreactor should never be taken out of the incubator until the end of the perfused cell culture duration. The second temperature test indicates that with the incubator undisturbed it takes three hours for the internal temperature of the bioreactor to equilibrate to that of the incubator. This could be detrimental to the proliferation and culture of the neonatal chondrocytes upon introduction to the bioreactor system and thus caused an alteration to the procedure to preparing the bioreactor to receive cell culture. The results of the second temperature tests caused from that point forward for the bioreactor to be assembled and placed in the incubator and then perfused with media until the chamber was full. Once the perfusion system was primed, the perfusion was stopped and the bioreactor was allowed to set for three hours to allow the internal temperature of the bioreactor to equilibrate to that of the incubator. Only after temperature equilibration was the cell suspension allowed to be introduced into the bioreactor to ensure the minimum amount of heat loss from the bioreactor was possible. Should the cell culture media need changing, then it should be done inside the incubator by replacing the reservoir media bottle. With the pH data staying relatively stable, the perfusion of the bioreactor does work in stabilizing the pH of the system and proves the autoclaving of the entire perfusion system works in successfully sterilizing the system for cell culture.

Flow Model

The first model showed exactly what would be expected from the system with the volumetric flow rate being uniform throughout the entire system. This is expected

because of the peristaltic pump having the tubing of both the inlet and the outlet being pumped at the same rate and thus the volumetric flow rate of the inlet should match the outlet in theory. The pressure of the bioreactor shown from the first model gave a reasonable result lending a good basis for comparison against the other two models. The third model also has a uniform profile across the membrane of the transwell insert with both velocity and pressure profiles. While the velocity in the third model does increase slightly once it has passed the cell construct, the flow still appears uniform across the width of the transwell. The difference between the third model and the first model is the backflow that appears below the membrane and slightly above the membrane of the transwell in the third model. This is expected with the lower permeability cartilage construct inserted in the transwell. Should further models be created in the future, the bioreactor should include the 8.5mm hole in the lid of the bioreactor that accommodates the sterile syringe filter for gas exchange. This would allow for three boundary conditions to be created with the hole in the lid being for an environmental pressure outlet and the inlet and outlet being a volumetric flow inlet and volumetric flow outlet each set to 1 mL/min. While the current model is still an accurate representation of what is occurring within the bioreactor, the suggested changes would give even greater accuracy to the model. Regardless of possible future models, the results of the current models indicate that there is uniform flow across the surface of the transwell membrane at the given speed of the pump. This indicates that there is no cause for concern that the selected speed of 1 mL/min of the pump nor the geometry of the bioreactor chamber causes any inhomogeneity laterally across the cell construct.

CHAPTER III

CELL CULTURE

Introduction

Of the tissue culture methods available today, perfusion bioreactors are the method of choice in clinical settings for producing large tissue engineered constructs. Bioreactors are used to help control the environment in which the cells are cultured. Another beneficial characteristic is that bioreactors help speed up the rate of cell culture on a large scale and are a means to standardize the result of the cell culture which makes it appealing to the clinical market [17]. Yet there are many different models of perfusion bioreactors and each tailor to the growth and specific development of a tissue. When discussing tissue engineered cartilage there are two major types of bioreactors: spinner flasks and rotating wall vessel bioreactors.

Spinner flasks produce cartilage tissue with better mechanical properties than constructs cultured under static conditions. Even with generating better properties than statically grown cartilage, the spinner flask does have its draw backs. The speed of the spinner bar in the flask creates high shear stress which can alter the biophysical properties of the developing tissue. The turbulent flow often leads to the creation of fibrocartilage in the tissue engineered construct which is an unwanted characteristic if the cartilage is to be implanted into a patient [18]. This generated fibrocartilage has inferior mechanical

properties than healthy articular cartilage and will degrade over time if implanted into a defect of a patient.

The rotating wall vessel bioreactor is the most readily used bioreactor in clinical settings. Its design consists of two independently rotating concentric cylindrical chambers. The inner cylinder spins and is what causes the media to flow circularly around the chamber with the inner cylinder as the centroid. The outer cylinder also spins and functions to house the inner cylinder and hold both the cell constructs and cell culture media. By having both the inner and outer cylinder rotating in the same direction, the cell constructs in between the two cylinders are left in a state of perpetual free fall. It has been shown that rotating wall vessel bioreactors generate tissue engineered cartilage with superior mechanical properties than that of both statically grown and spinner flask cultured cartilage. Yet there is a downside to rotating wall vessel bioreactors and that is tissue constructs grown in the bioreactor are not usually uniform. This can be undesirable in tissue engineered cartilage constructs that rely on uniform thickness to provide a smooth articulating surface. Another factor about the rotating vessel wall, as with all of the bioreactor types previously stated, is the reliance of the tissue engineered cartilage on having a scaffold to grow on. In the rotating wall vessel the constructs can often times come in contact with the wall of the bioreactor and thus needs the scaffold to help give added mechanical support to refrain from deformation and cell construct damage [19].

While both of these bioreactors have pros and cons to culturing different tissues, neither of the aforementioned bioreactors can create scaffold-free tissue engineered cartilage for the repair of partial-thickness defects.

Experiment One

Cell Source

For Experiment One, neonatal porcine chondrocytes were used as the cell source. Stillborn neonatal piglets were obtained from Prestige Farms (Crawford, MS) and cartilage was harvested from the stifle joints under sterile conditions. The harvested cartilage was minced by hand and placed in a beaker with a magnetic stir bar and Dulbecco Modified Eagle's Medium (DMEM)(Sigma-Aldrich, St. Louis, MO) containing 1% Antibiotic-Antimycotic (ABAM), 10% Fetal Bovine Serum (FBS), and 1mg/mL Type II Collagenase. The cartilage was allowed to dissolve in this solution with constant stirring in the incubator at 37°C overnight. After 24 hours, the resulting cell suspension was centrifuged at 300rcf for 5 minutes, media with collagenase removed, and resuspended in DMEM with 10% FBS and 1% ABAM. A cell count of the cell suspension was conducted using a BioRad TC10™ Automated Cell Counter. By adding trypan blue to the cell count suspension, the automated cell counter gave an accurate cell count out of the percentage of cells alive to ensure the correct amount of neonatal chondrocytes were used for each experiment.

Methods

In the first experiment two 6-well plate transwell inserts (EMD Millipore, Billerica, MA) were individually seeded with 5.5x107 neonatal porcine chondrocytes and allowed to grow in static culture for four weeks using DMEM containing 1% ABAM and 10% FBS with media changed every fifth day. After the four week period, one transwell was transferred to the perfusion bioreactor and allowed to culture under constant perfusion of 1 mL/min for one week. Defined chondrogenic medium (DCM) with TGF-

β3 was perfused through the bioreactor at a speed of 1mL/min using a Manostat® Carter® Peristaltic Pump. The other transwell insert remained in static culture in DCM with TGF- β3 to serve as a control. Defined chondrogenic medium was made using DMEM containing 1% v/v ITS + Premix, 1mM sodium pyruvate, 0.1μM dexamethasone 50μg/mL ascorbate-2 phosphate, 40μg/mL L-proline, and 1% v/v antibiotic-antimycotic solution. After one week of culture, the transwell inserts were removed from the bioreactor and the cell construct was tested biomechanically, and then sectioned for testing biochemically and histologically.

Biomechanical Evaluation

For biomechanical data at the end of Experiment One, each construct was immediately tested for thickness and Young's elastic modulus by unconfined indentation testing with a Mach-1 Micromechanical Testing System (Biomomentum, Laval, QC, Canada). Stress relaxation tests were conducted using 25% strain for multiple points around the construct. The Young's elastic modulus was calculated using the following equation:

$$E = \frac{F(1-v)}{2ka\omega_0} \tag{3.1}$$

where F is the applied load, v is the Poisson's ratio, k is a scaling factor (a function of the aspect ratio (a/h) and v), ω0 is the indenter displacement, and h is the thickness of the sample [20]. The scaling factor, k, is obtained from a previous study done by Haynes et al [21].

Total Hydroxyproline Measurement

Total hydroxyproline content was used as an indicator of collagen content and determined by the Chloramine T assay established by Reddy and Enwemeka [22]. For Experiment One, all hydroxyproline values were standardized to DNA content of the same sample. DNA content was gathered by adding 100µL of lysis buffer (0.1% Triton X-100, 5 mM Tris-HCl, pH 8.0, 20 mM EDTA) to each sample, vortexed for 30 seconds, and then spun at 15000 rcf for 3 minutes. This was then used to quantify DNA content by Hoescht. Afterward, NaOH was added to the remaining pellet and the Chloramine-T assay was started. All samples were homogenized using liquid nitrogen to freeze the cells and a bio-pulverizer to lyse the cells. Each sample was placed in a microcentrifuge tube with 200 µL of NaOH and placed on the vortex shaker for 5 minutes. Aliquots of sample were taken from each sample and brought to 50 µL with 4N NaOH and then autoclaved at 120°C for 20 minutes to hydrolyze each sample. Then 450µL of chloramine-T reagent was added and then set at room temperature for 25 minutes. Next 500µL of Ehrlich's reagent was added to each sample and placed in a hot water bath at 65°C for 20 minutes before reading absorbance at 550nm using a BioTek µQuant Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Total hydroxyproline content was calculated from the standard curve by using stock solution of 1mg/mL of hydroxyproline.

GAG and DNA Measurement

For the measurement of GAG and DNA each sample was digested in a 1% papain solution with 50mM sodium acetate overnight at 60°C. The samples were spun at 10000 rcf for three minutes and DNA content was measured using the Hoechst method. Fifty

microliters of sample were added to 2mL of 1×TNE buffer containing 0.2µg/mL Hoechst dye and raw fluorescent intensity was measured using a GloMax®-Multi Jr Single Tube Multimode Reader (Promega, Corporation, Madison, WI). A standard curve for DNA was created using calf thymus DNA.

The same sample digestion that was used for DNA quantification was also used to obtain GAG content. For GAG quantification, each sample was added to 250µL of DMB solution and absorbance was read at 530nm and 590nm using the BioTek µQuant Microplate Spectrophotometer. GAG content was calculated from the standard curve obtained by using chondroitin sulfate from a Blyscan Glycosaminoglycan Assay kit (Biocolor, Newtonabby, Northern Ireland).

Histological Evaluation

For histological evaluation, a sample from static and perfused culture was fixed in 10% buffered formalin at the end of Experiment One. The samples were taken to Mississippi State University Clay Lyle Entomology Lab and examined under an SEM microscope to examine the surface geometry of the tissues. Preparation of the samples for the SEM microscope included dehydrating the samples in an ever increasing v/v percentage of ethanol to water before mounting occurred.

Statistical Analysis

Statistical analysis was conducted to determine statistical significance between static and perfused groups of each experiment. This was done using Microsoft Excel 2013 with a two-tailed t-test assuming unequal variance (α=0.05).

Rationale

The design of the first experiment was to follow most of what is published in literature. This was to give tissue engineered cartilage six weeks to grow as most tissue engineered cartilage experiments use either a six to eight week time frame for maturation of cartilage constructs. Thus our experiment was set up to give the cartilage four weeks to consolidate and form a self-cohesive construct and then perfuse it for seven to ten days to observe the effect of perfusion on the scaffold-free cartilage construct.

Results

Gross Morphology

The gross morphology of the constructs from Experiment One is shown in Figure 3.1. The morphology indicates that the perfused construct had a far more homogeneous surface than that of its statically cultured counterpart. The perfused construct was more pliable and easily handled when compared to the static construct. With regard to the perfused construct, the membrane from the transwell insert was completely removed allowing for a relative homogeneous scaffold-free perfused construct that could be easily handed with forceps. The morphology of the perfused construct was more desirable compared to the static construct which had the transwell insert membrane intact on the construct to ensure reliable handling of the cartilage construct without damage to the tissue because of its inhomogeneity.

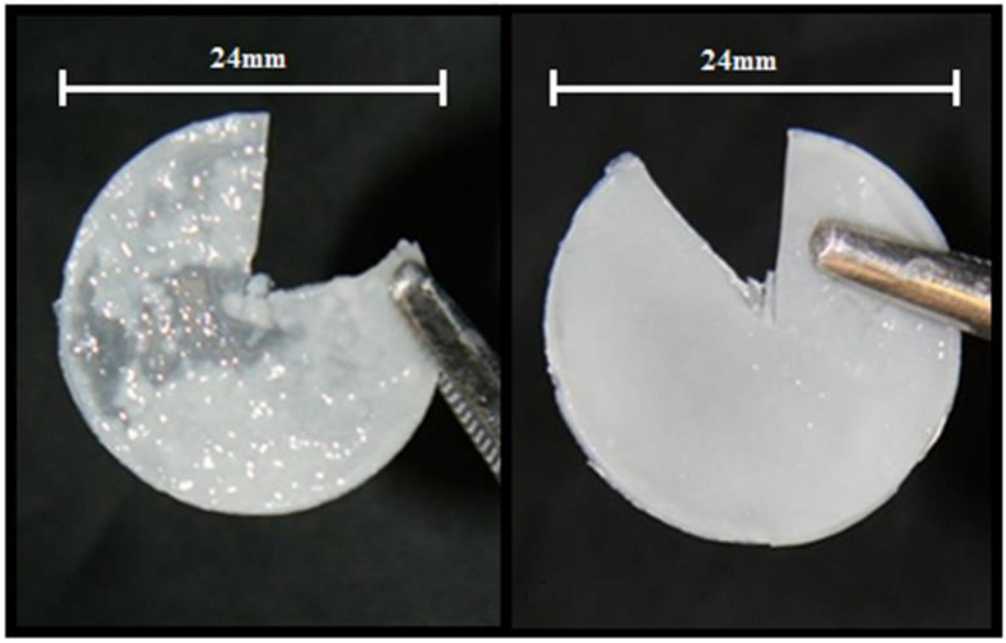

Figure 3.1 Photographs showing the gross morphology of the statically cultured cartilage construct (left) and perfused cartilage construct (right)

Biomechanical and Biochemical Analysis

Young's elastic modulus for Experiment One static and perfused constructs are shown in Table 3.1. The modulus for Experiment One, static (37.6±12.7 kPa) and perfused (86.6±23.9 kPa) were each averaged across 6 different test locations on the same construct. There is a statistically significant difference in the means of the Young's Modulus between static and perfused constructs in Experiment One.

For total hydroxyproline content each construct, static and perfused, had three samples used to determine hydroxyproline content. Experiment one used each sample obtained for hydroxyproline content and also obtained total DNA content to use to normalize the hydroxyproline values. All three values from each construct were averaged as hydroxyproline/DNA. There is no significant difference between the mean

hydroxyproline/DNA values of static (.301±.04) and perfused (.354±.08) constructs in Experiment One.

Total GAG content, shown in Table3.1, was normalized against total DNA content obtained from the same samples with three samples taken from each construct of all experiments. There is was no significant difference between the average GAG/DNA values of static (100.10±24.6) and perfused (75.17±5.0) of Experiment One.

Table 3.1 Biomechanical and biochemical data of cartilage constructs form Experiment One

		Young's Elastic Modulus (kPa)	Hydroxyproline /DNA	GAG/DNA
Experiment One	Static	37.6(±12.7)	.301(±.04)	100.10(±24.6)
	Perfused	86.6(±23.9)*	.354(±.08)	75.17(±5.0)

(*) Indicates statistical significance

Histological Evaluation

A scanning electron microscopy was used to further evaluate the homogeneity between the surfaces of the perfused and static constructs of Experiment One. At lower magnification (46X for static and 53X for perfused) it is clear that while neither sample is perfectly homogeneous across the surface, the perfused sample of Experiment One is more homogeneous than the statically cultured sample. This is reinforced by the SEM pictures taken at a higher magnification of 299X showing more closely the homogeneity disparity between the two samples.

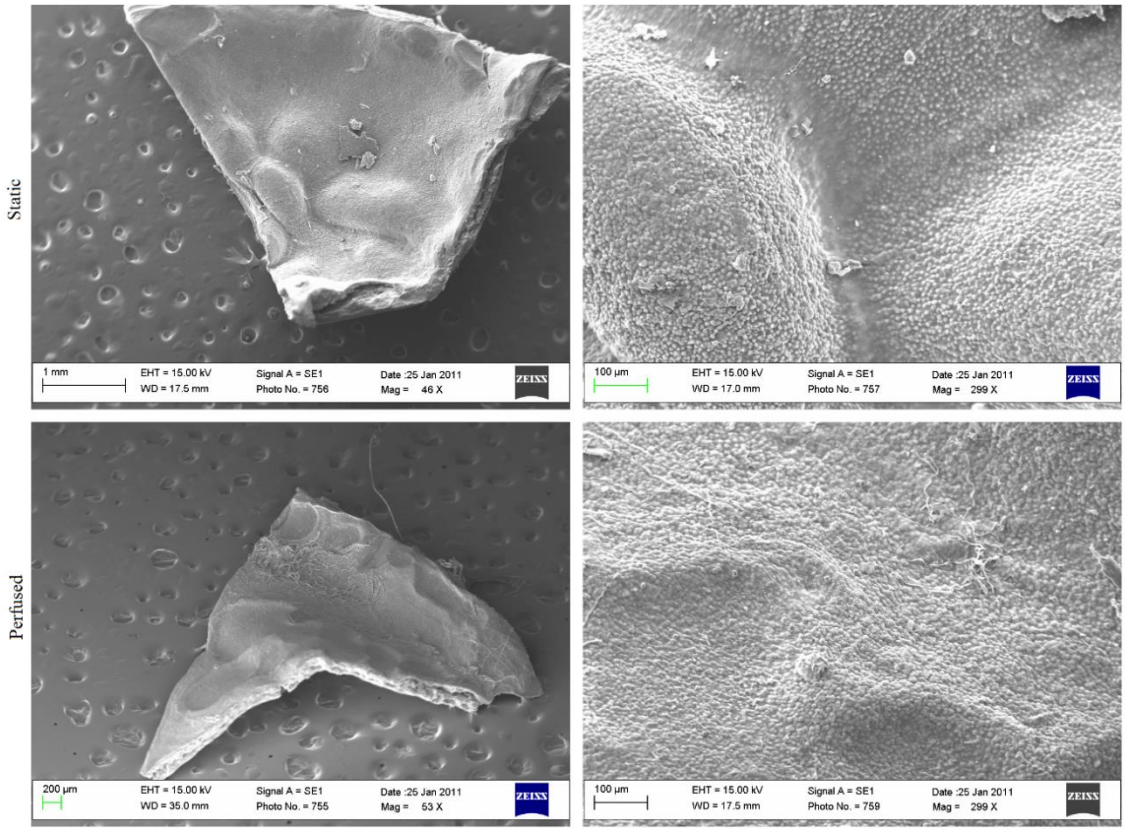

Figure 3.2 High resolution SEM images of Experiment One constructs Statically cultured (top row) and perfused (bottom row)

Experiment Two

Methods

The cell source for Experiment Two was the same as in Experiment One using the same cell count per cell culture insert. The second method involved immediate implementation and perfusion of the cell suspension in the transwell of the bioreactor, without the four week static culture period. The transwell inserts were coated in collagen using a 5% v/v collagen solution using rat tail collagen in phosphate buffered saline. The inserts were allowed to sit in the solution in a 6-well plate overnight and then air dried inside the tissue culture fume hood. After air drying, the transwell inserts were sterilized

under ultraviolet light for 25 minutes. The cell suspension retrieved from the overnight collagenase solution was cell counted and pipetted into the transwell insert so that each would have approximately 5.5×107 cells/insert. Both bioreactor chambers were filled with DCM containing TGF-β3 and allowed to sit 24 hours after pipetting the cell suspension into the transwell to let the cells to settle and allow cell attachment. After 24 hours, the pump to one bioreactor was turned on with a speed of ~1mL per minute while the other bioreactor chamber maintained static media as a control. The flow of the media was from top to bottom of the perfused bioreactor chamber and was allowed to flow continuously for one week. After the one week period, the transwell inserts were removed and the cell construct was tested biomechanically, and then sectioned for testing biochemically and histologically.

Histological and biochemical evaluation of the constructs were conducted the same way as in Experiment One. The only difference between the evaluation methods of Experiment One and Two is that in Experiment Two the constructs had hydroxyproline content normalized to dry weight instead of DNA content as in Experiment One.

Rationale

The second method used a much shorter time frame due to the inability to keep the cultured cartilage sterile for four continuous weeks with Experiment One's experimental setup. The transwell insert needed a large amount of media for static culture because of the 55 million cells in the transwell. Placing the transwell in a 6-well plate would not suffice because the media would be depleted and would have to be changed every twelve hours for four weeks. To remedy the problem of contamination from Experiment One, this experiment was conducted with the cell suspension added

immediately into the bioreactor. This was so that the possibility of contamination from long term culture would not be a possibility and would still be able to determine if the bioreactor was beneficial to the culturing of scaffold-free tissue engineered cartilage. In the second experiment, the flow of the bioreactor was from the top down. The rationale behind this was that if the flow was from the bottom to the top, then the cell suspension would never have a chance to consolidate. So with the flow reversed, the cell suspension would be forced to the bottom of the transwell and given the chance to consolidate. We also decided to coat the membrane of the insert with collagen to help with the aggregation of the cell suspension into a construct.

Results

Gross Morphology

The morphology of the constructs created by the methods of Experiment two are grossly different between perfused and static culture as seen in Figure 3.3. Both constructs underwent contraction upon cell culture. Yet as Figure 3.3 indicates, the statically cultured construct contracted by a far greater degree than that of the perfused construct. This is due to the compressive forces exhibited on the construct by the perfused media from top to bottom in the bioreactor chamber.

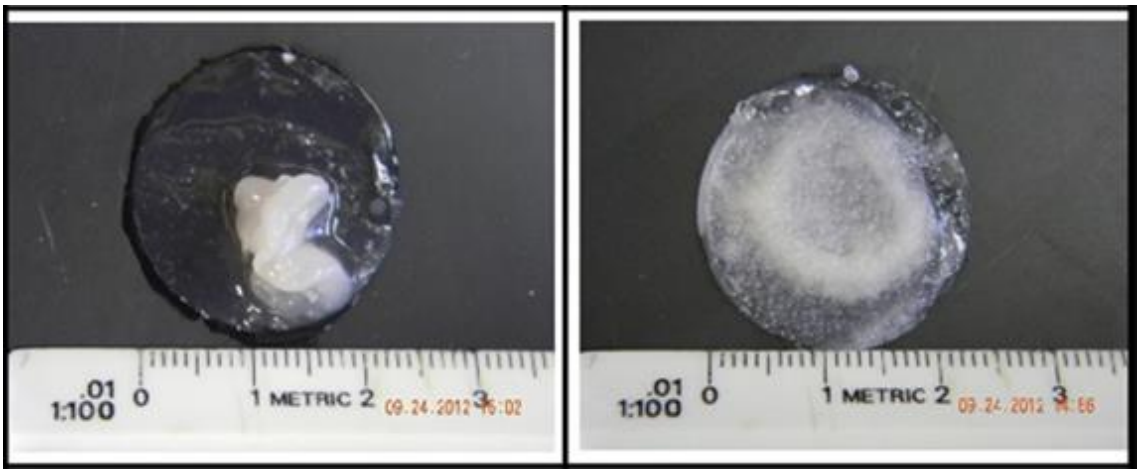

Figure 3.3 Images of gross morphology of statically cultured cartilage constructs (left) and perfused cartilage construct (right) of Experiment Two

Biomechanical and Biochemical Evaluation

The Young's Elastic modulus for the constructs of Experiment Two are shown in Table 3.2. The modulus for the static construct of Experiment Two (20.97±5.7 kPa) was not statically different from the modulus of the perfused construct (15.83±12.1 kPa). Total hydroxyproline content normalized to wet weight was not statistically different between statically cultured construct (6.2×104±2.21×10-4) and perfused culture constructs (1.8×10-4±9.69×10-5). Total GAG content normalized to DNA content was also not statically different between the static construct (.03978±.00782) and perfused construct (.04452±.00454) of Experiment Two.

Table 3.2 Biomechanical and biochemical data of cartilage constructs from Experiment Two

		Young's Elastic Modulus (kPa)	Hydroxyproline/Wet Weight	GAG/DNA
Experiment Two	Static	20.97(±5.7)	6.2×10^{-4} ($\pm2.21\times10^{-4}$)	.03978(±.00782)
	Perfused	15.83(±12.1)	1.8×10^{-4} ($\pm9.69\times10^{-5}$)	.04452(±.00454)

(*) Indicates statistical significance

Histological Evaluation

The unstained recut slides for Experiment Two were toluidine blue stained to determine proteoglycan content and separately stained for Type II collagen. The static histological sample for Experiment Two showed a more intense staining in both Type II collagen and Toluidine blue than all other slides of the same stain respectively. This indicates that in the static construct of Experiment two there is a large amount of proteoglycan content and a decent amount of Type II collagen. The perfused construct in Experiment Two stained moderately for Type II collagen and stained with weak intensity for Toluidine blue. The staining on the perfused construct of Experiment two indicates a lower amount of Type II collagen relative to its static counterpart and contains an ECM with low proteoglycan content. Staining for Type II collagen is shown in Figure # and Toluidine blue staining is shown in Figure 3.4.

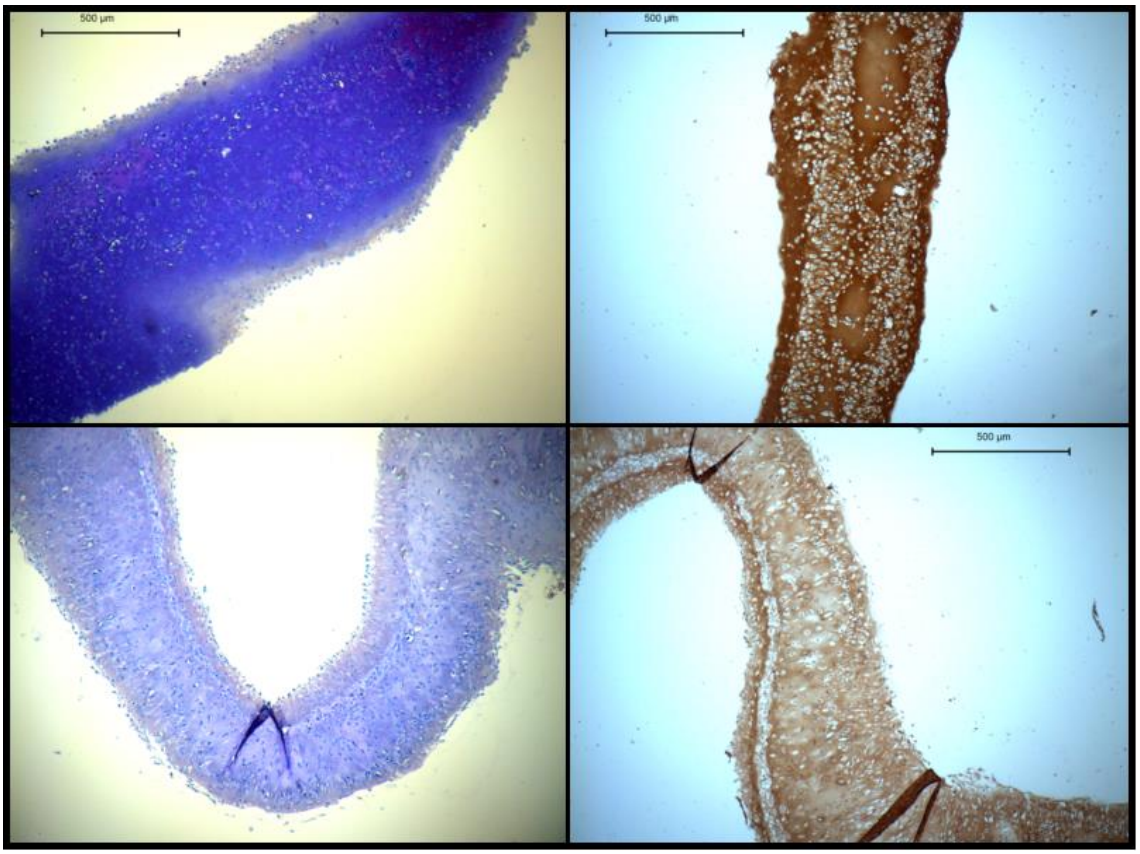

Figure 3.4 High resolution images of Experiment Two constructs stained for Toluidine Blue and Type II Collagen

Statically cultured cartilage construct (top row), Perfused cartilage construct (bottom row), Toluidine Blue stain (left column), Type II Collagen stain (right column)

Experiment Three

Methods

The cell source used in the third experiment is the same as in the previous two experiments using the same cell count per insert. The methods of Experiment Three follow the same methods as that in Experiment Two using the same cell count, culture media, time frame, etc. The difference between the Experiment Two and Three is that in Experiment Three, the direction of perfusion in the bioreactor is from the bottom of the chamber to the top. The cell suspension was allowed to statically culture inside the

bioreactor for 24 hours in the collagen coated insert and then perfused continuously for ten days. At the end of the experiment, the constructs were tested biomechanically and then sections for biochemical and histological evaluation.

Results

Gross Morphology

The gross morphology of the constructs from Experiment Three displayed a high variance in the colors between the static and perfused constructs. Also the thickness is far greater in the perfused construct than in the statically culture construct as can be seen in Figure 3.5.

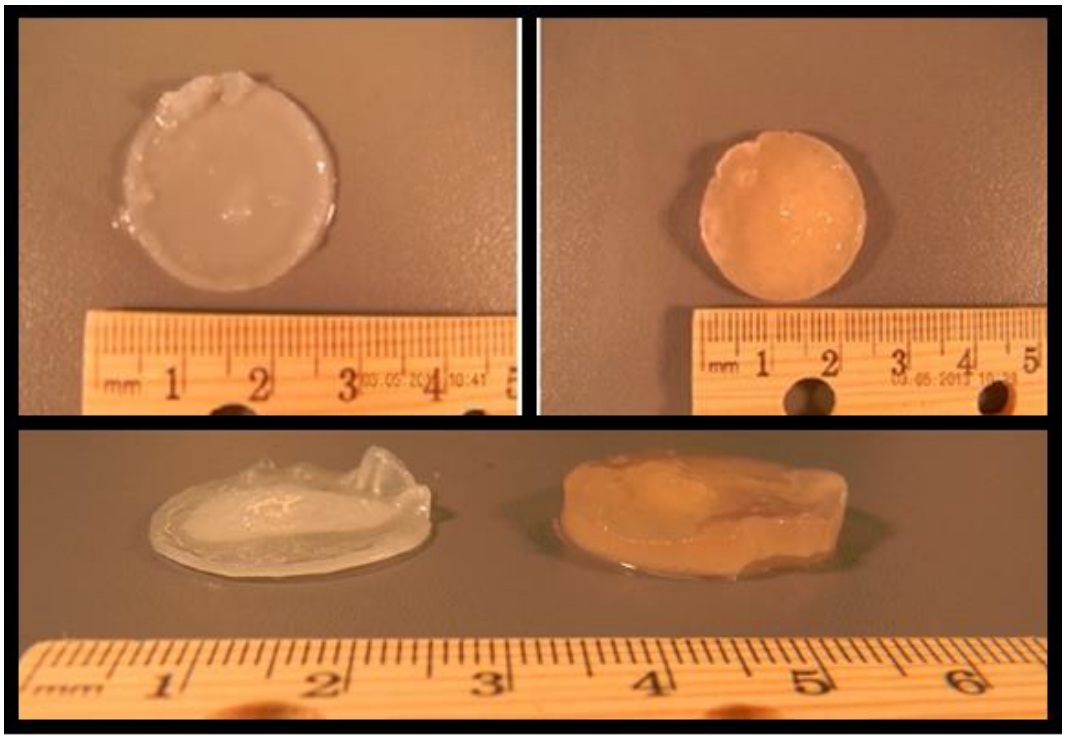

Figure 3.5 Images of the gross morphology of cartilage constructs of Experiment Three

Statically cultured cartilage construct (top left), Perfused cartilage construct (top right), comparison of thickness between the two constructs (bottom)

Biomechanical and Biochemical Evaluation

The Young's Modulus for Experiment Three was obtained from the stress relaxation data gathered from both perfused and statically cultured constructs and as seen in Table 3.3 there is a significant difference between the modulus of the static construct (46.55±2.4 kPa) and perfused construct (2.41±1.0kPa). The hydroxyproline content of both constructs was gathered and normalized to wet weight as was done in Experiment Two. There was no statistically significant difference between the Hydroxyproline content of the statically cultured construct ($5.1\times10^{4}\pm2.43\times10^{-4}$) and the perfused construct ($2.9\times10^{-4}\pm2.78\times10^{-4}$). The total GAG content of both constructs was determined and normalized to DNA content as was done in Experiment Two. With Experiment Three, there was no significant difference between the total GAG/DNA content of the statically cultured construct (.11987±.02715) and the perfused construct (.14409±.08356).

Table 3.3 Biomechanical and biochemical data for static and perfused cartilage constructs of Experiment Three

		Young's Elastic Modulus (kPa)	Hydroxyproline /Wet Weight	GAG/DNA
Experiment Three	Static	46.55(±2.4)	5.1×104 (±2.43×10-4)	.11987 (±.02715)
	Perfused	2.41(±1.0)	2.9×10-4 (±2.78×10-4)	.14409 (±.08356)

(*) Indicates statistically significant

Histological Evaluation

For Experiment Three, both the static and perfused constructs stained with similarly moderate intensities for both Type II collagen and Toluidine blue. This indicates that there is not much difference in the moderate amount of proteoglycan content between the two constructs and the same result indicative in the amount of Type II collagen.

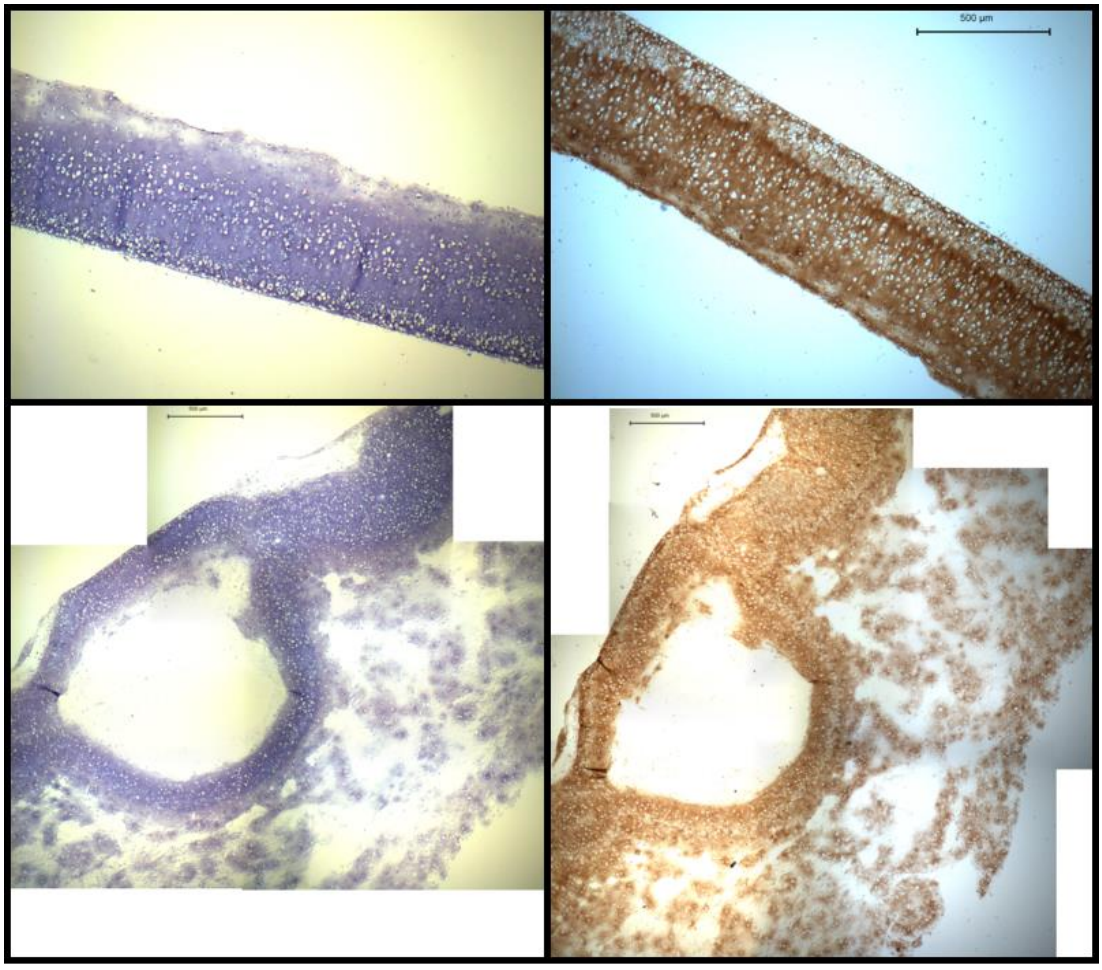

Figure 3.6 Histology of Experiment Three cartilage constructs stained for Toluidine Blue and Type II Collagen

Statically cultured cartilage constructs (top row), Perfused cartilage constructs (bottom row), Toluidine Blue stain (left column), Type II Collagen stain (right column).

Discussion

The first experiment provided the most promising results by creating a perfused tissue engineered construct with superior mechanical qualities than the statically cultured construct. By having the chondrocytes culture statically for four weeks before being put into the bioreactor, the cells were allowed to self-aggregate into a scaffold-less construct and thus helped the tissue to withstand the shear forces exhibited from the continuous flow. In the first experiment, the scaffold-free tissue engineered construct created by the perfusion bioreactor had a more uniform thickness than the construct created by static culture as was observed in the SEM pictures in Figure 3.2. This is a very desirable characteristic for smooth articulation of the joint if used for experimentation in vivo. Also the Young's elastic modulus of the perfused construct was over twice as large as the Young's modulus in the static control. But with the inability to replicate the results of Experiment one due to the difficulty of maintaining sterility when culturing $5.5x10^7$ cells/insert for four weeks, it became apparent that a different approach to testing the bioreactor should be explored. By perfusing the bioreactor from the top of the chamber to the bottom theoretically would let the cell suspension to be perfused immediately instead of waiting for four weeks of static culture to implement perfusion. While the transwell did prevent the cell suspension from being dislodged or disrupted, especially with the use of the collagen coated transwell insert, the mechanical data shows that the results of the first experiment were not duplicated by the methods used in the second experiment. The draw back from this method of experiment two was that the bioreactor continued to overflow. On multiple occasions the bioreactor had to have the perfusion stopped so that the bioreactor could be opened and have the transwell resituated so that there was no

media by-flow. The resistance of the cell suspension caused the flow above the transwell to back up and cause DMEM to overflow from the top of the bioreactor. Also with the use of the peristaltic pump the outlet volumetric flow rate was equal to the inlet, and therefore the area of the bioreactor below the transwell became void of DMEM creating an air pocket. The pressure from this air pocket caused the transwell to dislodge from the rubber O-ring holding the transwell in place creating the need to reset the transwell. Despite this repetitive disturbance to the cell culture, the amount of collagen and GAG produced relative to the amount of cells present was many times greater than experiment one. While the method employed by Experiment Two did not yield better mechanical results, the far better biochemical results are a testament to the metabolic activity of the neonatal chondrocytes. With the third experiment the method of adding cells instantly but flowing from the bottom to the top of the bioreactor chamber was to avoid the constant overflow from experiment two. As both the histology and mechanical data indicate the immediate addition of perfusion to the cell suspension, even with the collagen coated transwell, was detrimental to the development of the cartilage construct. The introduction of the shear stresses caused by perfusion so early on did not allow the cell suspension to self-assemble into a solidified construct as would have happened with 4 weeks of static culture. The air capsule displayed in both the Toluidine blue and Type II collagen staining of the perfused histology in experiment three is the result of shear stresses on the cells [23].

CHAPTER IV

CONCLUSION

The goal of the study was to determine the efficacy of the bioreactor to produce tissue engineered cartilage. While there is evidence that long term culture can result in production of scaffold-free tissue engineered cartilage, there is no way to ensure duplicity without having a reliable way to maintain sterility while culturing the cells under static conditions for a prolonged period before perfusion. While the bioreactor does indeed function to adequately maintain a stable temperature for cell culture and allow for more regulated pH through perfusion, the issue of being able to produce a scaffold-free tissue engineered construct able to be used to resurface a joint or fill a defect is of interest. While it is very interesting to note that the bioreactor was able to produce a cartilage construct that could be easily handled after only ten days of perfusion, the construct had properties, both biochemically and mechanically, inferior to what would be desired in a construct to be used for implantation in vivo. The results clearly indicates that the bioreactor has a very low efficacy to produce cartilage by using immediate perfusion. Moving forward, the solution to these problems will depend on the intent in which the bioreactor will be used: for either rapid development of tissue engineered cartilage or for the long term growth of a tissue engineered construct.

If the goal of the bioreactor is to produce tissue engineered cartilage as quickly as possible then the bioreactor should be modified to allow media perfusion from the top of

the chamber to the bottom without a chance of overflow. This can be accomplished by remaking the lid without the attachment adapter for a syringe filter for gas exchange. Also putting a rubber gasket between the lid and the chamber will keep media from leaking out. This solution is simple and pragmatic as the results indicate that flow using the bottom port as the inlet creates too much shear on the cells and does not allow them to aggregate. While the same amount of shear is experienced by the cells with the flow in the opposite direction, the membrane of the transwell acts as a barrier to the cells placing them under compression as well. This also keeps the construct from developing air pockets as the cells proliferate as seen in the histology of Experiment Three. Another thing that would have to be used if rapidly developing scaffold-free cartilage using is the collagen coated transwells. While the cell suspension does not have time to aggregate into a self-adhering construct as was the case with the constructs of Experiment One, the collagen coated transwells are benefical to the attachment of some of the cells in the cell suspension to the membrane of the transwell. Yet with the results of the Young's modulus of Experiment One versus the other two experiments, the clear indicator is that the long duration of static culture before implementation into the bioreactor yields constructs with better biochemical and biomechanical properties. Still there are problems that should be addressed before further experimentation with prolonged culture before perfusion.

Should the intended use of the bioreactor be to create scaffold-free tissue engineered constructs using static culture over a long time period, of four to eight weeks, before implantation into a defect then the solution is greatly different than the aforementioned solution. Experiment One shows that the bioreactor has the capacity to

generate relatively homogeneous constructs comparative to its statically cultured equivalent. While the bioreactor has the capacity to generate cartilage constructs of a superior quality, it lacks the ability to reliably duplicate the results. The key to this is to be able to consistently culture a construct with a large amount of cells without the risk of contamination. The solution to this is to limit the human interaction with the cell construct while the cells are in static culture before perfusion is implemented. Another factor to consider is limiting the amount of unfiltered gas exchange to the cell constructs which was a particular problem with trying to repeat the methods of Experiment One. The proper method is to simply start culturing the cells in the bioreactor from the beginning. A slight modification to the bioreactor can be used to allow for the change of media without the use of perfusion. A valve could be used on the bottom inlet could allow for the draining of cell culture media when the media needed to be changed without risk of contamination. A clamp on the tubing leading into the bottom inlet would serve to hold media in the chamber while culturing the cells. Media could then be replaced in the bioreactor through the hole in the lid for the syringe filter. This will negate the problem from experiment one with culturing the cells in a nested beaker which allowed for too much unfiltered air exposure causing contamination over a four week period. By culturing the cell suspension in the bioreactor from the beginning limits the amount of human interaction and places the cells in an already sterile environment. This approach could give the cell suspension the time it needs to coalesce into a scaffold-free cartilage construct that could better handle the shear stress generated by media perfusion. This could yield better constructs with improved biomechanical and biochemical properties,

thus increasing the efficacy of the through-thickness bioreactor to produce scaffold-free tissue engineered cartilage.

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