Chondrogenesis of Mesenchymal Stem Cells with a Hydrostatic Pressure Bioreactor

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Abstract

Arthritis is a disease that will affect 50% of people by the age of 85. Because there is no current treatment methods to directly correct the problem, there is a need to understand and generate a method to directly treat the cartilage. Through a tissue engineered approach, the use of a bioreactor, scaffold and cells, a matrix in which cartilage should flourish will be generated. Bioreactors are devices that simulate a biologically active environment for cell culture. The bioreactor design for this study will have the following qualifications: mechanical stimulation, inexpensive, small enough to fit into an incubator. The mechanical stimulation that this bioreactor will be applying is hydrostatic pressure. Mesenchymal Stem Cells (MSC) will be used for their capability to differentiate into chondrocytes. Along with the bioreactor and cells, the last component is a scaffold composed of hyaluronic acid and collagen. Together these items will create a pseudo-physiological environment for cartilage.

Introduction

One of the most prominent forms of cartilage damage is arthritis. Arthritis is the painful inflammation and stiffness of the joints. Arthritis affects 22.7% of adults in the United States and is the leading cause of disability (8.6%). There are two main forms of arthritis are osteoarthritis and rheumatoid arthritis. Osteoarthritis is the most common type of arthritis that affects about 51% of people that have arthritis. Osteoarthritis is caused by wear and tear of the joint over time. However, there are a few factors that can increase the chance of getting osteoarthritis such as genetics, weight, injury, and overuse. The second most common form of arthritis is rheumatoid arthritis. This form of arthritis affects 2.4% of people in the United States. Unlike osteoarthritis, rheumatoid arthritis is not caused by the wear and tear on the joint, but instead is an autoimmune disease; the immune system of a person with rheumatoid arthritis is actually attacking their cartilage because it recognizes it as a foreign body causing deterioration within the cartilage.

Current treatments for arthritis can be broken up into two main categories. The first being medications. Medications that are used for the treatment of arthritis are pain relievers and anti-inflammatories. Both of these medicines are used to only relieve pain for the person and not actually cure the disease. Another form of medicine that is only
used for rheumatoid arthritis are disease modifying drugs which only slow the progression of rheumatoid arthritis and does not treat the symptoms or pain caused by the disease.

The second category is surgery. One type of surgery used for treatment of arthritis is arthroscopy. Arthroscopy is used to remove debris that is in the joint, but does not actually treat arthritis. Second type of surgery is arthroplasty where they remove part or the whole joint depending on what is being affected by the arthritis. This form is very invasive and expensive way to treat the arthritis problem. A third form of surgery is joint fusion. Joint fusion is when the joint is essentially removed and two bones are fused together. This form of surgery can be helpful because it can remove the movement of the two bones against each other, but can cause future problems because it takes away the natural movement of the body. A fourth type of surgery is osteotomy. Osteotomy is when a portion of bone is removed to regain the natural movement of the joint. This form of surgery is invasive and also does not solve the the deterioration of the cartilage caused by arthritis. The treatment of arthritis is very costly and if the deterioration is not accounted for then the pain of arthritis will continue and cost even more.

According to the CDC the total cost of arthritis in 2003, the most recent data available, was $127.8 billion. $80.8 billion of this was directly related to medical expenditures. This was up by 24% from 1997. Assuming the increase remained constant for the next 12 years, there would be a total cost in 2015 would be approximately $177.3 billion. There is also an expected increase in the number of people that will have arthritis. From 2012 to 2030 of 14.5 million more people will be affected by this disease. Arthritis is also the highest cause of disability among adults in the United States. The focus of this project effects elderly, obese, overweight, healthy, and youth. However, healthy people and youth are less likely to have arthritis by a large percentage than elderly, obese, and overweight.

Additionally we believe that our influential stakeholders would be clinical researchers, medical doctors, patients, hospitals, nurses, and insurance companies with the decision makers being the insurance companies, medical doctors, and hospitals. Based off of the large population that is affected by arthritis these stakeholders are greatly affected by this disease.

This project leads to an alternative to all current forms of treatments. This tissue engineered approach would be to use stem cells, a scaffold, and a bioreactor to differentiate the stem cells into chondrocytes, the building block of cartilage. Although this treatment is still in the early stages of research, it could be very effective in the future. The stages of this research that have already been performed are chondrogenesis, stem cell differentiation into chondrocytes, they have all been ound to use growth factors. Growth factors are cellular receptors that aid with the proliferation
and differentiation process. Growth factors are also very expensive, which causes the current research to not be a viable option for treatment. This project however is trying to negate the use of growth factors. Therefore it could be used to prove that by inject stem cells or a stem cell scaffold construct into the joints of patients and cause the regeneration of cartilage. In the future this process could become cheaper and faster which would eliminate the need for invasive surgeries and treatments that does not resolve the issues of arthritis.

**Details of Design Concept**

*Type of Cell:*

For our project we are going to use Rat Bone Marrow Mesenchymal Stem Cells (MSCs). The purpose for using these type of cells are that they can be easily harvested, are low maintenance, and are capable of differentiating into chondrocytes. Stem cells are also much better at proliferating and renewal; therefore a larger cell culture can be obtained with stem cells rather than adult cells.

*Scaffold Design:*

![Figure 1: Structure of the cross linking of Collagen and Hyaluronic Acid](image)

The design of our scaffold will consist of two materials, collagen and hyaluronic acid (Figure 1). This design will give us good biocompatibility because both of these materials are found within the extracellular matrix of collagen. Through research we have also found that this combination has resulted in good viability. In order to fabricate this scaffold we will use the process of freeze drying. Freeze drying is a simple process that we can succeed at creating the appropriate pore size that is needed for our cells to differentiate in.
Bioreactor Design:

The design (Figure 2) of the bioreactor that we plan to create differentiation of our Mesenchymal Stem Cells is a hydrostatic bioreactor. The main design of our bioreactor contains four parts. The first part that will contain the scaffold and media is the cup. The cup will be created by a ¼” thick piece of cylindrical polycarbonate (Lexan). The cylindrical chamber will be have an inner diameter of 3”. It will also be approximately 4” in height. The second main part of the main design is the lid of the bioreactor. The lid will also be created out of 1/4” thick cylindrical polycarbonate. However, the inner diameter will need to be 3.5” in order to fit over the cup. In the top of the lid there will be an adaptor for the hose to attach to. The adaptor will vary in size
based on the size of the hose. The hose will attach from the adaptor to a reservoir that will be used to pump more media into the chamber and create the hydrostatic pressure. The reservoir chamber will also be made of polycarbonate but will have a diameter 3/4” because of the diameter of the pneumatic piston being used to apply the pressure to the fluid. The purpose of the reservoir is to make as close to a one to one ratio between the psi applied and the psi of the fluid in the bioreactor. If the ¾” piston were to be attached to a 3” head and applied 100 psi to the fluid then there would be less pressure than 100 psi. This design will allow for adequate pressure to be applied to the fluid, a sealed chamber to maintain the pressure, and a friendly environment for the cells to replicate in.

Methods
In order to ensure our results have control to compare to, there will be both a positive and negative control. The Positive control will be that the stem cells can differentiate and furthermore have the potential to differentiate on the seeded scaffold design created with the use of growth factors. Growth factors are used for these two steps to ensure the differentiation of the stem cells and are representative of the standard methods of differentiation. They are preventing the differentiation from failing. The negative control will be a seeded scaffold that does not have growth factors and does not have the hydrostatic pressure. Our hypothesis is that cells will not differentiate on the seeded scaffold without growth factors and does not endure the hydrostatic pressure, however differentiation will be able to be seen on seeded scaffold that also do not have growth factors but do experience hydrostatic pressure. To test the controls as well as our hypothesis, there will be numerous phases tested.

Our first step is to follow a protocol of differentiating MSCs into chondrocytes using a pellet approach (Appendix 1). This differentiation will be using Transforming Growth Factor- Beta 1 (TGF-B1). Using histology and microscopes, we can then determine is chondrocytes are present. In parallel to this, the differentiation of MSCs to chondrocytes will be tested on the scaffold construct. Once again this test will be using TGF-B1. These test are ran in parallel to allow for time to correct the scaffold construct if necessary and to eliminate any doubt as to whether or not our scaffold will be able to produce chondrocytes.

The next step would be to then do a study without growth factors. In this step we will have three different pressures we will be testing. The first trial would be to allow growth on the scaffold in a free swelling environment and no pressure; this will be the sample that has MSCs seeded onto the scaffold but does not undergo hydrostatic pressure. The second trial would be to allow growth on the scaffold while applying ~50 psi of pressure in our hydrostatic bioreactor for one hour a day and five days a week.
The third trial would be to allow growth on the scaffold while applying ~100 psi of pressure in our hydrostatic bioreactor for one hour a day and five days a week.

In order to visualize the effectiveness of our methods we would use different imaging modalities. To image the pore sizes of the scaffolds we would use the Environmental Scanning Electron Microscope (ESEM). To image the cells and their viability we would use the Confocal Fluorescence Microscope along with staining to count live and dead cells. We would also use the Inverted Phase Microscope to image the cells to determine differentiation into chondrocytes. Finally, we would use histology to determine the cell type.

**Timeline**

Through a division of tasks we are able to separate the work equally that allows us to remain on schedule with our constructed timeline. Although we have designated expertise on each task of this project, it is not to say we are divided front. Together we will be accomplishing tasks with one member being the frontrunner for each. The divisions of this project are laid out in Figure 3.

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**Figure 3: Diagram of the division of tasks**
Nick Denney is the leader in the bioreactor. He is in charge of researching different bioreactor techniques for chondrogenesis, different materials to construct it with, different designs that currently exist. He will also be in charge of knowing cartilage properties to ensure we are applying suitable pressures and the correct types of loads.

Alex Eddington will be in charge of the cell cultures, differentiation processes, and scaffolding. The cell culture consists of seeding, splitting, counting, taking care of, and freezing of cells. The differentiation processes will consist of those with growth factors and those without. The delegation of finding protocol and materials for these processes falls underneath Alex. Lastly she is responsible for the scaffolding. She is to research scaffold materials that are suitable and scaffolding techniques.

With the efforts combined as well as factoring in the time in which each step will take, a timeline was constructed. This timeline allows for replication, testing and adjustments of devices and procedures. Figure 4 shows the intended timeline for this project.

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<tr>
<th>Task Name</th>
<th>Start</th>
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<th>December</th>
<th>January</th>
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Figure 4: Timeline of project

Budget

The budget of this project was created with collaboration between the group members and the advisors. The group members began the discussion of the budget with the advisors in mid October. To gain a better understanding of vendors and costs of specific items the discussion and faculty support was beneficial. In Table 1, the breakdown of the cost can be found. The bioreactor was budgeted out to be $700 to allow for the need to redesign and rework the bioreactor if necessary. The bioreactor will be using pneumatics which needs the pistons and the system for controlling the pneumatics, which is considered in the $700. The material used for the container itself is polycarbonate from Lexan, which is factored into the budget as well. Lastly for the bioreactor we will need tubing. The stem cells will be provided from Therese Bou-akl and collected from rat bone marrow. This is estimated to cost $500. For the growth factors and disposal equipment, vendors that will be used are Sigma-Aldrich and Thermo Fisher Science. Growth factors are expensive, so that they are budgeted out to
be $1000. While disposal equipment is common and relatively cheap, it has been budgeted out to be $400. Examples of this equipment consists of pipette tips, test tubes, petri dishes. Last thing budgeted out for is materials for the cells, including but not limited media, hyaluronic acid, and collagen. This was estimated out to $400. The total budget for this is $3000. Half of it will be funded for by LESA Foundation. The remaining costs will be covered by collaborators.

<table>
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<th>Item</th>
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<td>Growth Factors</td>
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<td>Disposable equipment (Pipette Tips, Test Tubes, etc.)</td>
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<td>Materials for Cells (Media, Hyaluronic Acid, Collagen, etc.)</td>
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<td>Funding from LESA</td>
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Table 1: Budget for project
Appendix

1. MSC Differentiation Protocol
   1. 2x10^5 BM MSC cells in 0.5 ml of medium were centrifuged at 300 g for 10 mins in a 15-ml polypropylene tube to form a pellet.
   2. Without disturbing the pellet, cells were cultured for 21 days in media:
      a. medium consisted of DMEM-HG supplemented with 1% FCS and 10 ng/ml TGF-b1, 0.5 lg/ml of insulin, 50 IM ascorbic acid
   3. Media is changed every 3 days.
   4. On day 21, cell aggregates are fixed in 10% formaldehyde for 1 hr at room temperature, dehydrated in serial ethanol dilutions, and embedded in paraffin blocks. Paraffin sections (4-Im thick) were stained for histologic analysis with HE, Mallory (Biotec)

Acknowledgements

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References