Applying Biomechanical Cues to a Multilayered Cartilage Construct for Articular Cartilage Repair

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Undergraduate Group 2
580.442 Tissue Engineering Final Project

Background

The structure of articular cartilage (AC) includes three zones and is primarily composed of a collagen (90% Type II) and proteoglycan extracellular matrix (ECM) (Fox et al., 2009). The superficial zone (SZ) contains collagen parallel to the articular surface that combats shear stress. It has the highest concentration of collagen and lowest concentration of proteoglycans. The middle zone (MZ) has random collagen organization to insulate the joint from compression forces and is the thickest layer with round chondrocytes. The deep zone (DZ) has collagen perpendicular to the joint to combat tensile forces. It has the highest proteoglycan concentration and round chondrocytes are arranged in columns. A tidemark separates the true articular cartilage from deeper, calcified cartilage. These three zones are crucial to the function of AC in-vivo, especially in high-motion, weight bearing joints like the hip and knee. These joints are also the most prone to degenerative Osteoarthritis (OA) due to wear and tear. Prior engineered cartilage grafts used for repair lack this organization, which may contribute to their short longevity.

There are currently several methods being studied for use in AC products, combining inorganic and organic materials. Methods rely on use of embryonic stem cells (ESCs) (Huang et al., 2023), mesenchymal stem cells (MSCs) (Weijie et al., 2021), or induced pluripotent stem cells (iPSCs) (Khan et al., 2023). MSCs seem to be the most promising route, since culturing conditions for chondrocytes are well developed. These cells can be obtained from adipose tissue, which has become increasingly popular as a source of mature stem-cells for self-based therapies; they have minimal innervation and blood flow, and biopsies would only cause minor cosmetic changes.

Chondrocytes have different nutritional requirements from other cells (O'Hara et al., 1990). Cartilage is avascular and chondrocytes receive nutrients through diffusion of synovial fluid from the surrounding dense connective tissue, called the perichondrium. Joints with OA have reduced synovial fluid viscosity, and the avascular nature of cartilage makes it very slow to heal naturally. The daily compression exerted on cartilage aids in the diffusion of nutrients as well. For bioengineered cartilage products, it is relatively easy for media to penetrate each layer of a product compared to other tissues, due to cartilage's highly porous and hydrated structure. Providing the correct biophysical and biomechanical cues to MSCs could greatly improve the field of cartilage engineering, creating more "mature" tissues ready to withstand the rigor of motion in a joint.

Our study seeks to build off of a recent study which differentiated rabbit bone marrow MSCs into chondrocytes (Rajagopal et al., 2020). They seeded cells into a multi-layered scaffold containing three zones and tested cell viability and synthesis with the matrix. They tested in vivo models and transplanted the tissue into rabbits' knees. This research is promising, but lacks comparison to alternative scaffolds and healthy tissue. This research seeks to better understand the roles of the layers in a multi-layered scaffold, as well as the need for biomechanical cues during differentiation.

Our study plans to harvest adipocytes and culture chondrocytes. A bovine model is preferable because the thickness of bovine articular cartilage (bAC) will make it easier to visualize the structure of the native tissue; bovine joints are also large, increasing the amount of chondrocytes that can be harvested for experimental use per animal. Bovine chondrocytes and other cell-culture components are also frequently used for cartilaginous tissue engineering research, enabling this test to more closely model a single-species based construct. It has been shown that mechanical stimulation during chondrocyte culture alters expression of key chondrocyte genes in the production of a cartilage-like extracellular matrix (Petitjean et al., 2023). Therefore, we plan to add onto the Rajagopal study with experimental groups of tissues grown with mechanical stresses, specifically compression and shear stresses. We will also test if DZ cartilage is necessary to provide in a construct for adherence to bone, since we are unsure if all 3 layers are necessary for a functional cartilage implant. We propose to include an experimental group that explores this idea as well as the previously tested 3-layer construct, subjecting both scaffold types to compression, shear, and combination forces.

Clinical Impact:

OA affects over 32.5 million adults in the United States and approximately 7% of the world's population. OA is caused by AC degeneration in high-motion joints. Cartilage degeneration causes inflammation of the joint and damage to bone due to loss of crucial protection. Most commonly affected joints are knees, hands, feet, and spine. Cartilage can also become injured through sports or other accidents. Cartilage injuries are typically graded from 0 (healthy, smooth cartilage surface) to IV (full-thickness defect with exposure of the bone), where cases of grades III and IV require surgical intervention, and cannot heal on their own (Wasyleczko et al., 2020).

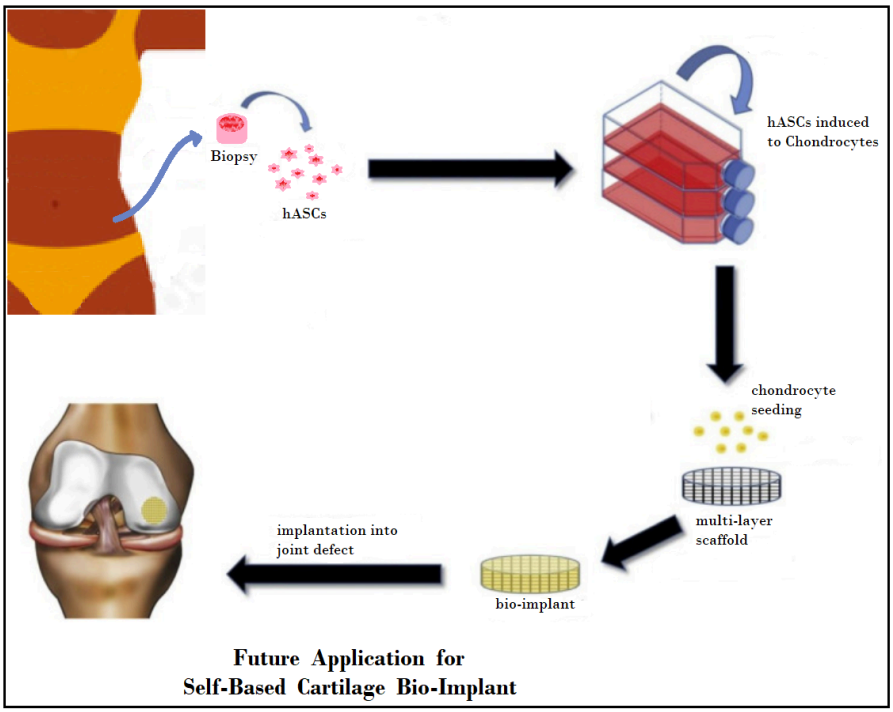

Figure 1: Proposed Future Application
Adipose tissue collected from the patient's abdomen will be induced into chondrocytes in-vitro, and seeded into the multi-layered construct described in this proposal. After maturation in the combination bioreactor, the self-based cartilage implant can be placed into the defect in a single surgery. Figure was adapted from (Wasyleczko, Sikorska, and Chwojnowski, 2020).

The current clinical gold standard for AC injuries that require surgery are microfracture, autograft (own cartilage), allograft (cadaverous/donor tissue), autologous chondrocyte implantation (ACI), and matrix autologous cartilage implantation (MACI). These methods tend to have short effective lifespans, and autologous cartilage samples require taking cartilage from other regions in the joint, which requires more invasion into the joint as well as creates a potential site for future damage. Allografts risk immune compromization and require a donor, and acellular artificial cartilages are not adaptable or durable. By culturing a cartilage tissue from adipose-derived mesenchymal stem cells (ADMSCs), the construct can be created with a minimally invasive biopsy, and will reduce the pain and healing time for the patient (Figure 1).

Since OA develops slowly over many years, the weeks needed to produce this engineered construct will not likely cause an large increase in harm to the patient, making an engineered tissue highly suitable if it can be made to withstand physiological conditions in the joint. The use of ADMSCs will also avoid the risk of culturing an implant from already stressed or damaged cells from an arthritic joint.

Hypothesis:

Over time, the 2-layer cartilage construct will become more histologically similar to the 3-layer construct when cultured on top of a bone-like matrix for 28 days, indicating that 2-layers could be sufficient for future in vivo use. When cultured in biomechanical reactors for 28 days, the constructs will have Compressive and Shear Moduli closest to healthy AC from the combination shear-compression reactor, as well as show the three expected layers found in healthy AC.

Specific Aims

Aim 1: Evaluate formation of multi-layered cartilage in 2 or 3 layered scaffold.

Bovine ADMSCs (bADMSCs) will be seeded and on 3-layer scaffolds described by Rajagopal et al. as well as novel 2-layer scaffolds we have developed to exclude the DZ AC layer. Scaffolds will then be placed on a calcium rich, rigid bone-like layer and cultured. Histological evaluation of the tissues will be performed over 28 days, with samples taken every 7 days. In order to evaluate the engineered cartilage tissue, both organizational and functional properties will be tested. First, the percentage of surviving cells in the tissue and distribution within the scaffold will be assessed using the Invitrogen Live/Dead Cell Viability kit. This will also

allow us to evaluate longevity of cells in different layers across conditions. Testing of the organization of the tissue will be done through histological analysis. Immunohistochemistry stainings for type 2 collagen and type 10 collagen will be performed at each timepoint. Functional testing of compressive and shear behavior consists of a stress-relaxation where step strain (e.g. 20%) is applied and held over a period of time. Results between 2 and 3-layer groups will be compared as well as evaluated against published data detailing the behavior of native human cartilage under compressive, shear, and tensile conditions (Smith et al., 2000).

Aim 2: Evaluate effects of shear, compression, and combination forces on development of tissue.

Four different experimental groups will be established that test the effect of compressive, shear, combination, and control conditions (see Figure 3) on organization and functionality of 2 and 3-layer AC tissue products described in Aim 1. After the 28 day seeding period, each condition will be tested for compression and shear strengths. To test compression behavior, each construct will be exposed to unconfined metal platens. First, the samples will be exposed to a ramp of stress until linear strain response ceases to occur. Then, constant strain in a confined chamber will be applied to observe behavior through creep indentation. From the compressive test, the Compressive Modulus is obtained, and Shear Modulus is obtained through analysis of the creep indentation results.

Experimental Methods

Summary of Sample Number, Controls, and Statistical Analysis

20 independent cartilage constructs of each type (2-layer, 3-layer) will be cultured in each of the 4 bioreactors in Figure 2. Histological samples will be obtained from each condition at 7, 14, 21, and 28 days, taking cross-sectional slices through 3 of the constructs from each bioreactor at each time, using a total of 12 constructs per bioreactor. 6 of the remaining 8 will be used to determine the Compressive and Shear Moduli of the constructs at 28 days (3 per modulus). The remaining 2 constructs are left in reserve in case of failure or damage to one of the testing cartilage constructs before analysis. Overall, this project will need a total of 160 cartilage constructs, and may require several bioreactors or successive testing.

By using 3 constructs per test for both the histological tests (Aim 1) and the mechanical tests (Aim 2), some degree of statistical significance can be determined, and a range of success considered. Statistical analysis will be done using a 2-tailed T-test, assuming uneven sample variance; this method will indicate if variation between experimental conditions is likely to be due to random error.

Native bAC will be used as a control for all experimental conditions, as a representation of what an ideally in-vitro cartilage construct should resemble. It will undergo histological analysis as well as the compression and shear testing, to establish a qualitative baseline for Aim 1 and a quantitative baseline for Aim 2.

Cell Sourcing

Bovine adipose tissue will be collected from a healthy adult cow. Flow cytometry will be used to separate out bADMSCs, which will be confirmed through immunocytochemistry analysis of CD105+, CD90+, CD73+, CD271+, CD45-, CD34-, and CD31- surface markers (Calabrese et al., 2017). These confirmed and isolated MSCs can then be artificially induced to produce chondrocytes.

Induction of bADMSCs into Chondrocytes

Ideal chondrocyte growth media have already been developed and outlined (Isyar et al., 2016). Materials for a chondrocyte media include ITS, RPMI-1640 DMEM, FBS, PS, Amphotericin B, and L-glutamine. As described, insulin, transferrin, and selenious acid containing premix solution (ITS) will be used to stimulate proliferation of chondrocytes. RPMI-1640 DMEM is also a necessary component because it contains glucose, amino acids, and vitamins at the right pH. These components provide the necessary nutrition and contribute to the right functionality of the cells. Inactivated fetal bovine serum (FBS) provides key extracellular matrix proteins and growth factors. Penicillin-streptomycin (PS) and Amphotericin B are included because they prevent contamination of the chondrocytes. EDTA, PBS, and DMSO are also necessary to have on hand for detachment of cells and washing steps.

Scaffold and Scaffold Attachment Construction

Two versions of a chitosan-gelatin (CG) scaffold were developed to mimic the structure of articular cartilage. CG was selected for its ability to mimic the orientation of collagen fibers in different AC zones, as well as behave similarly to the extracellular matrix found in AC (Rajagopal et al., 2020). 4 mm diameter and 3 mm thick scaffolds with either the SZ and MZ regions (2-layer) or SZ, MZ, and DZ layers (3-layer) will be created through sequential unidirectional freezing to create the aligned fibers of the SZ and DZ (Rajagopal et al., 2020). The 2-layer method is a modification, allowing for better analysis of the attachment method of chondrocytes to a bony surface, a crucial aspect of cartilage grafting.

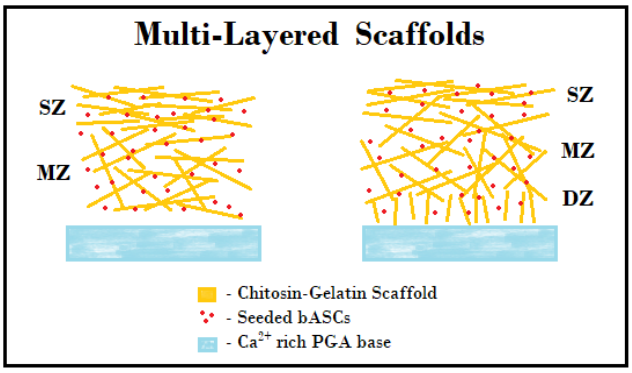

Figure 2: Multi-Layered Scaffold Structures. (Left) 2-layer chitosan-gelatin (CG) scaffold (superficial and middle zones). (Right) 3-layer CG scaffold (superficial, middle, and deep zones). Scaffolds are seeded with bASCs and placed on a Ca^{2+} rich PGA base.

These scaffolds will then be placed over a Ca^{2+} rich PGA base that represents the stiffness of bone. PGA is a linear polyester with a high crystallinity, estimated to have a strength of around 7 GPa; it is biodegradable over a period of 6-12 months (Shetye et al., 2017), providing ample time for graft attachment before the bottom platform under the scaffolds disappears. Calcium phosphate will be incorporated into the woven ester structure (Okazaki et al., 1989), to recreate the bone-like calcified layer below the tidemark on AC. This layer will simulate the surface the multi-layered scaffold would be expected to anchor to in-vivo.

Biochemical and Biomechanical Induced Differentiation

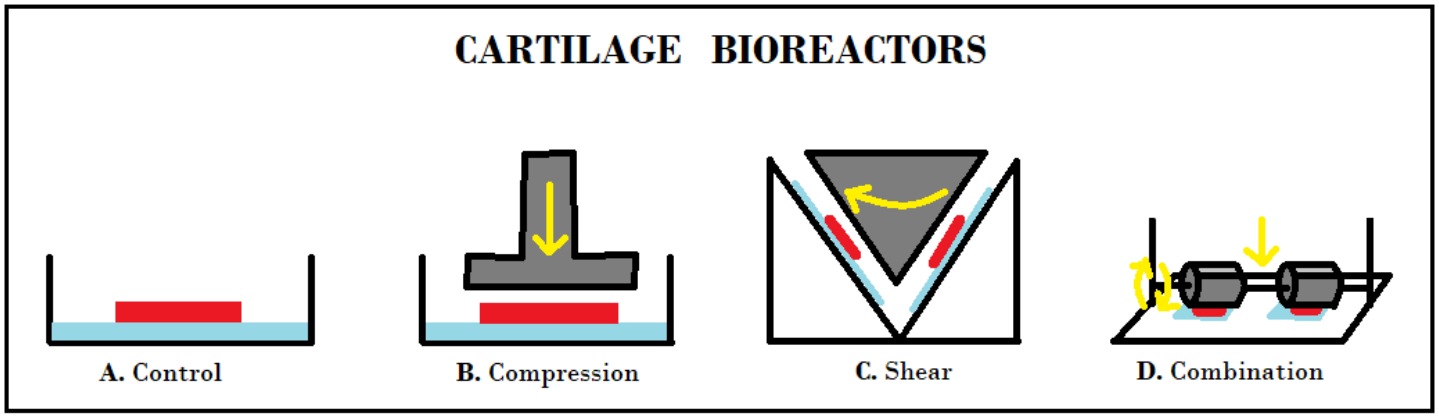

Figure 3: Cartilage Bioreactors for Mechanical Stimulation. *(A) Control culture conditions in a simple growth chamber. (B) Compression will be exerted at a constant rate and pressure in a compression chamber that permits lateral expansion. (C) Shear forces will be exerted continuously via a cone viscometer. (D) The combination bioreactor is designed to exert both compressive and shear forces during cell culture, by suspending spinning wheels over wells containing the tissue construct; the wheels can be raised and lowered to exert pressure while also spinning to exert shear force (Shahin and Doran, 2015).*

Cell-seeded scaffolds will be differentiated into the chondrogenic lineage using Dulbecco's Modified Eagle Medium (DMEM)-high glucose supplemented with dexamethasone (100 µM), ascorbate-2-phosphate (40 µg/ml), 1 × insulin-transferrin-sodium selenite + 1 media supplement, L-proline (40 µg/ml), and sodium pyruvate (1 mM) (Isyar et al., 2016). To maintain sterility, the cells will then be placed in four distinct bioreactors under the same general conditions of 37°C and 5% CO_2 (See figure 3).

Although shear and compression are loading components of the in vivo environment of healthy AC, research on mechanical testing for shear on cartilage will be low relative to compression. The DZ AC will resist compression, while the SZ AC will resist shear stress that occurs as a result of joint movement and is about 20% of the thickness of AC (Schulz & Bader, 2007). Collagen production, GAG production, and chondrocyte

proliferation will be influenced positively by shear and compressive forces. Human articular cartilage (hAC) has an Equilibrium Shear Modulus of 2.6 MPa (Schulz & Bader, 2007).

- An unconfined loading chamber that applies 10% strain at a frequency of 1 Hz will be used for reactor B (Figure 3), to apply compression force in the z-direction only.
- A cone viscometer system in reactor C will apply a constant and even shear force of 1.6 Pa through continual rotation of the internal cone.
- A combination of shear and compression forces will be applied using a unique combination reactor, where recessed wells hold the tissue constructs so that a rotating wheel can be raised and lowered onto the constructs (Shahin & Doran, 2015).
- A control bioreactor with no mechanical forces will also be used to culture the constructs.

Schematics of the bioreactors are present in Figure 3. Constructs will be cultured for a total of 28 days, based on the tri-layer strategy previously discussed (Rajagopal et al., 2020). After this period, it will be expected that the chondrocytes have created a dense ECM that resembles native AC. Several of the constructs will be tested to determine Compression and Shear Moduli of the mature cartilage implant, to evaluate how the cartilage might perform upon implantation.

Immunostaining

At each designated time point, three samples from each experimental group will be sectioned on a plane to capture all layers of the cartilage and subsequently processed for paraffin embedding. Subsequent to paraffin embedding, tissues will be sliced to achieve a thickness ranging between 5-7 micrometers. These sections will then be affixed onto glass slides and then deparaffinized and rehydrated.

Histological assessments will be performed using Hematoxylin and Eosin (H/E) and Masson's Trichrome stainings. These techniques will aim to qualitatively assess chondrocyte proliferation, cartilage layer formation, and the orientation of the cartilage matrix. Safranin O staining will be utilized to assess proteoglycan content, with staining intensity quantified using ImageJ. Additionally, Picrosirius Red staining will be employed to visualize collagen distribution within the cartilage matrix (Sun et al., 2012).

Immunohistochemistry will involve the use of primary antibodies, specifically mouse anti-collagen type 2 (II-II6B3, dilution 1:5) and rabbit anti-collagen type 10 (234196, dilution 1:250), to stain for type 2 collagen and type 10 collagen, respectively. The density and distribution of type 2 collagen staining in the regenerated cartilage will be quantified using ImageJ v1.52a software (Rajagopal et al., 2020).

A sample of native bAC will be obtained from a healthy adult cow. The bAC will be histologically analyzed using the same methods as above, working quickly to avoid tissue necrosis. This sample will be used for qualitative comparison with the experimental groups, to observe trends in layer formation, cell health, and cell-density between the experiments compared to a “Grade 0” cartilage tissue.

Live/Dead analysis

In order to view the viability of cells within the different scaffold regions a Live/Dead Viability/Cytotoxicity kit will be used. 100–200 μm slices will be cut out using a surgical blade such that all scaffold layers were included in the slice. After a 30 min incubation in Live/Dead assay reagents, fluorescence microscopy was used to visualize the live/dead cell distributions. A fluorescein filter (485±10 nm) for calcein AM was used to view the live cells and a rhodamine filter (530±12.5 nm) for ethidium homodimer-1 was used to view the dead cells (Kim et al. 2003).

Compressive Modulus testing of final constructs

The first mechanical test for all the experimental groups will be to measure the Compressive Modulus through unconfined compression using the ElectroForce Biodynamic 5500 instrument. A 25 mm circular metal platen will be attached to the instrument in order to provide a compressing surface. Another 25 mm platen will be used as a base to support the petri dish the sample is placed on. The platen will increase its force on the cartilage from a 0% to a 20% strain at 0.01 mm/s. As is common practice, the gradient of the stress-strain curve between 10% and 15% strain will be analyzed and used to calculate the Compressive Modulus (Kabir et al., 2020).

$$\text{Young's Modulus: } E = \frac{\sigma}{\epsilon}$$

σ = compression stress, ϵ = axial strain

Compressive testing is crucial for understanding the material's ability to withstand and respond to compressive forces, mirroring the physiological conditions experienced by cartilage in vivo. Successful testing will result in tissue with a Compressive Modulus that matches that of bAC at 0.38 ± 0.12 MPa (Schinagl et al., 1997), mimicking MZ and DZ properties of bAC.

Creep/Shear Modulus testing of final constructs

Creep indentation will be performed in a confined setting. Each sample was loaded into impermeable metal platens, then compressed with flat, porous metal under a constant load of 0.7 g for one hour. Finite element analysis with a linear biphasic model via COMSOL Multiphysics software will be used on the experimental data to obtain the Shear Modulus (Donahue et al., 2022).

$$\text{Shear Modulus: } G = \frac{\tau}{\gamma} = \frac{F/A}{\Delta x/l},$$

F = force, A = top-plane surface area, Δx = deformation laterally, l = height of construct

This method was chosen to determine Shear Modulus and explore creep curve behavior corresponding to the stress that bAC is under. Low loading is necessary to avoid tissue damage; strain during creep testing in confinement can occasionally exceed 50%, significantly changing the local composition of a sample (Patel et al., 2019). 0.7 g has been a standard load used for creep indentation previously (Brown et al., 2018). However, there is a risk that this load affected the cartilage microstructure in unpredicted ways or the software modeling was inaccurate. If we engineer the tissue successfully, the Shear Moduli of our constructs will be in range of the moduli of bAC, which is about 0.1-1.5 MPa (Jahn et al., 2016). A corresponding shear modulus indicates that the SZ of our engineered cartilage is able to withstand shear like bAC.

Citations

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