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Comparing Decellularized Muscle Tissue with an Engineered Muscle Biomaterial

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Abstract

Decellularized tissues composed of extracellular matrix are commonly used for tissue transplantations but this method has many limitations. An alternative approach is to create an extracellular matrix by seeding degradable foams with cells, culturing these foams to allow extracellular matrix to be secreted from the cells, then washing away the degradable foam to collect the material that is produced. This study was interested in comparing decellularized skeletal muscle tissue to an engineered biomaterial that was seeded with skeletal muscle myoblast cells. Collagen content of decellularized tissue and engineered biomaterial were quantified using a hydroxyproline assay to compare the two samples. The engineered biomaterial collagen content value was significantly lower than the decellularized muscle tissue value. However, results did suggest that the engineered biomaterial is able to support the growth of cultured cells and excretes collagen.

1. Introduction

Tissue transplantation is severely limited by the problems of donor shortage and immune rejection. An alternative is to grow replacement tissues outside of the body using the patient's own cells. The common approach of tissue engineering has been to create tissue mimics by growing human cells on degradable polymeric foams, also known as scaffolds. These 3-D biomaterial scaffolds help provide a temporary support for the cells during growth in culture [4]. Unfortunately, studies show that once implanted these scaffolds can evoke a foreign body response by the immune system due to the synthetic polymers and other biomaterials that they are composed of. Therefore, non- synthetic alternatives are needed.

One alternative that has seen clinical success is creating implantable materials from the tissues of non-human animals. These biological materials are composed of extracellular matrix

(ECM) which is by definition, nature's ideal biological scaffold material. The ECM is specifically synthesized by the resident cells of each tissue and provides a supportive medium for blood or lymphatic vessels and nerves [3]. The multi-molecular nature of such cell derived constructs gives these naturally derived biomaterials a level of complexity and regenerative ability not observed with synthetic materials [1,2]. This harvesting process known as decellularization is useful in a number of biomedical applications such as understanding the physicochemical properties of ECM and providing a tissue specific scaffold for engineering functional tissues [11].

Yet the harvest of "fully formed" animal tissues, generally limited to skin and intestine, provides only limited opportunities to engineer the material's properties to meet a particular medical need. Fundamentally, the harvest of animal tissue is not an engineering approach and opportunities for significant innovation still exist.

Towards this end, a method to isolate the extracellular matrix in bulk from populations of cells grown in culture has been created [8.]. The strategy requires developing platforms to collect the ECM that the cells secrete. Sacrificial, open celled foams are utilized to concentrate and capture the ECM that is secreted by cells under selected growth conditions *in vitro*. Following a culture period, the underlying foam is removed by dissolution with a solvent, and the accumulated ECM is collected. The method provides a reliable means to engineer a variety of materials by selecting the cell type and the growth conditions to produce carefully engineered regenerative biomaterials with unique structures and compositions.

This study is interested in using this approach to create a material intended for the repair of damaged skeletal muscle tissue. The specific goal of the project is to use the sacrificial scaffolding approach with human muscle cells and determine the characteristics of the material

that is produced. These characteristics will be compared to decellularized muscle samples to evaluate the effectiveness of this new method.

In order to determine the characteristics of the extracellular matrix, the collagen content of the material is often found. Collagen is one of the more important structural proteins in the body being of particular importance in connective tissues by providing their durability. Specifically in skeletal muscle ECM, it accounts for 1-10% of muscle mass dry weight [12]. Knowledge of at least the amount of collagen in a particular tissue is essential for the complete understanding of the structural and mechanical properties of that tissue. The amino acid hydroxyproline is a major component of collagen and few other proteins contain this amino acid. For most tissues, it is possible to obtain a reasonable first approximation of collagen content by utilizing a biochemical assay for hydroxyproline [10].

2. Methods and Materials

2.1 Sacrificial Foam Preparation

The sacrificial foam scaffolds were fabricated from medical grade polyurethane (Tecoflex SG-80, Thermedics) using a phase inversion process [7.] Briefly, SG80-A polyurethane (PU) pellets were dissolved in the solvent dimethylacedamide (DMAC) (10% w/v) overnight at 60°C. Next, approximately 10 g of sugar and 200 uL DI were mixed and added to molds to create a sugar-filled mold (Figure 1a). Excess sugar/water mixture was carefully scraped off of molds. (Figure 1b). Sugar-filled molds were heated at 75°C until dry (at least 20 minutes). Once dry, molds were removed and transferred to fume hood. 2 ml SG80-A/DMAC solution was added to each mold. The main objective of this part is to apply as much solution to the mold without applying a greater volume than the mold's volume [Figure 1c]. The sugar mold should appear saturated. The

molds were then carefully placed in a container filled with DI until completely submerged for 24 hours, which both precipitated the PU solution and dissolved the sugar scaffold[Figure 1d]. The polymer scaffolds were removed from the molds and placed in a separate DI bath and were stirred for 24 hours. The polyurethane foams contained a network of pores consistent with the prior location of the sugar scaffold.
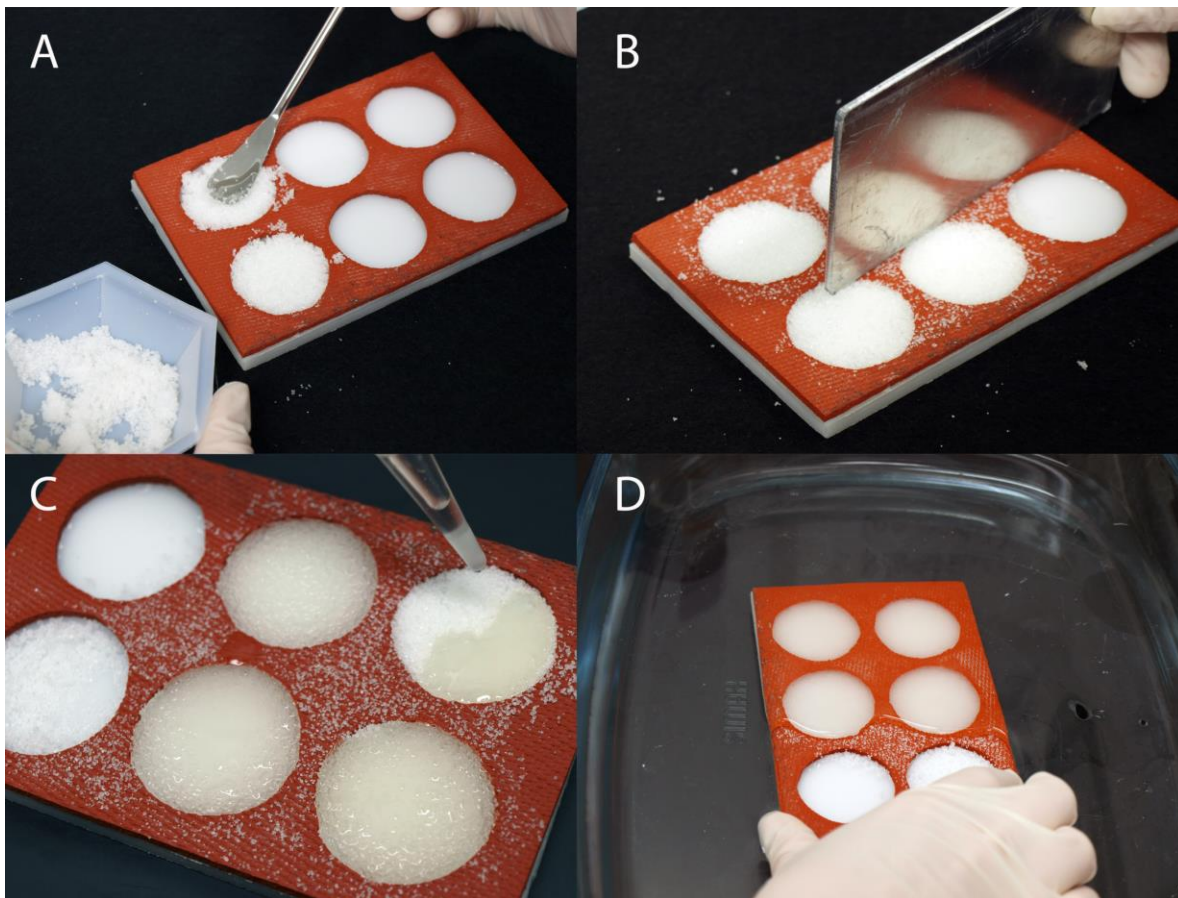

Fig. 1. Sugar/water mixture being added to molds (A). Excess sugar/water mixture carefully being removed (B) After drying, adding SG280-A/DMAC solution to each mold (C) then carefully submerging molds into DI (D).

2.2 Cell Seeding and Culturing

PU foams were coated overnight in a fibronectin solution (20ug/ml) to encourage attachment of cells. Skeletal cells (L6,ATTCC, Manasas VA) were expanded to the necessary number in culture and seeded into the sacrificial foams at a density of 2 million cell/cm3 (approximately 2

million cells/ sample). All samples were cultured for four weeks and maintained in a growth medium consisting of DMEM/F12 supplemented with 10% fetal bovine serum (FBS), gentamicin, and 1mM ascorbic acid (Sigma, St. Louis, MO). Growth media was changed every 2-3 days. Samples were stimulated with TF-Beta1(4ng/ml) to stimulate ECM production.

2.3 Material Extraction

At the end of the culture period, the sacrificial polyurethane foams were removed. Samples were rinsed in phosphate buffered saline to remove residual media, and incubated in DMAC for 72 hours at 37 degree Celsius. The solvent was exchanged four times during the seventy-two hour incubation period, twice on the first day and then daily thereafter. Following DMAC incubation, the remaining material was rinsed three times in DI water, frozen, and lyophilized.

2.4 Animals and decellularization of skeletal muscle

Rat quadriceps muscles were dissected from rats to obtain native muscle tissue for comparison of data. Following dissection, right quadriceps muscles were decellularized using a previously published protocol [3]. Samples were submersed in 1% sodium dodecyl sulphate (SDS) in distilled water and solution was exchanged every 48 h for one week. At least 4ml of SDS solution was used for each sample. At the end of the decellularization procedure, the muscles were thoroughly washed by means of three incubations lasting 30 min in PBS. Decellularized muscles underwent a rinsing period in distilled water for one week. (Solution was exchanged every 24 h.)

2.5 Material Testing

Samples were prepared for scanning electron microscopy (SEM) to analyze the topography and physical characteristics of material collected. Samples were imaged with assistance from the University of Arkansas Nanotechnology Imaging Facility.

Collagen was quantified in engineered tissue and native rat muscle using a hydroxyproline assay. The method described is a modification of an assay first introduced by Stegemann and Sterr [9]. Sample hydrolysis was performed by combining hydrochloric acid with engineered tissue in a 1mL: 2mg ratio and rat muscle in 1mL:1mg ratio and hydrolyzing for 16-20 hours at -20°C. Samples were diluted with dH20 (40uL sample in 160uL H20). Hydroxyproline buffer was made by combining 25 g citric acid monohydrate in 600mL dH20, 6 mL glacial acetic acid, 36 g sodium acetate, 17 g sodium hydroxide, 150 1-propanol, and 750 uL toluene. Hydroxyproline standard was created by dissolving 10.1 mg hydroxyproline in 100mL hydroxyproline buffer (101mg/uL). Standard was diluted 3:2 ratio across a 96 well plate. 125uL of each sample was added into triplicate on the 96 well plate. A buffer was created to oxidize the hydroxyproline using a solution made of 122.5 mg Chloramine–T dissolved in 3.5 mL 1-proponal and 28mL hydroxyproline buffer. 100ul of buffer was added to samples. Plate was covered and incubated for 20 minutes at room temperature. Next a buffer containing 1.05g 40dimethylamniobenzaldehyde in 5mL proponal and 1.85 mL perchloric acid was created for staining hydroxyproline. 100ul of this buffer was added to each sample and standard dilution then plate was covered and incubated at 65°C for 25 minutes. Plates were read at 570nm absorbency. All samples were corrected for volume of sample loaded and multiplied by volume of HCL used in hydrolysis to determine ug hydroxyproline per mg of tissue. Statistical analysis (t-test) was performed to compare collagen content values of two groups. A 0.05 level of significance was used.

3.0 Results

3.1 Visual Observations

Thin sectioning of engineered material revealed a porous network similar to ECM material and residual cellular fragments as shown in Figure 2. These results suggest the collection of ECM from muscle fibroblast is viable.
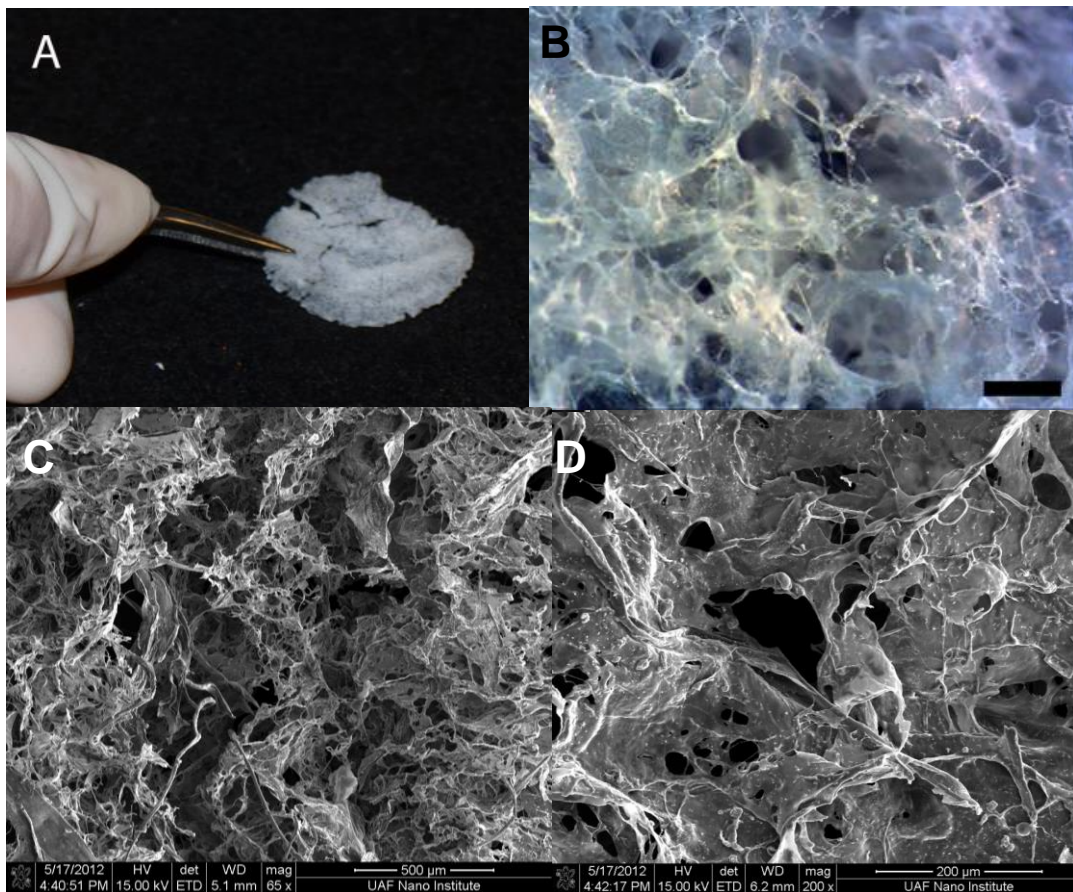

Fig. 2. (A) ECM biomaterial prepared using muscle myoblast cells (L6 cells) shown in bulk form. (B, C, D) Magnified view to show surface and texture.

3.2 Collagen content

After performing hydroxyproline assay, the engineered biomaterial (n=4) was determined to have a 1.55±.44% collagen content and the decellularized muscle (n=4) had a 8.39±3.51% collagen content. Collagen content was calculated as function of hydroxyproline, a major amino

acid in collagen, and the results are shown in Figure 3. The difference between engineered muscle matrix and decellularized muscle collagen concentration was statistically significant.

4.0 Discussion

The biomaterial produced displayed encouraging results with a porous network common to an extracellular matrix. However, the physical structure of the material produced didn't reveal the same structure of skeletal muscle ECM [12]. Due to the components of skeletal muscle tissue, its ECM tends to have a structure revealing a honeycomb when a cross section is analyzed. The engineered biomaterial tended to have a structure similar to the sacrificial foam it was originally seeded on. It would be beneficial to create foams similar to skeletal muscle tissues because the material grown according to the
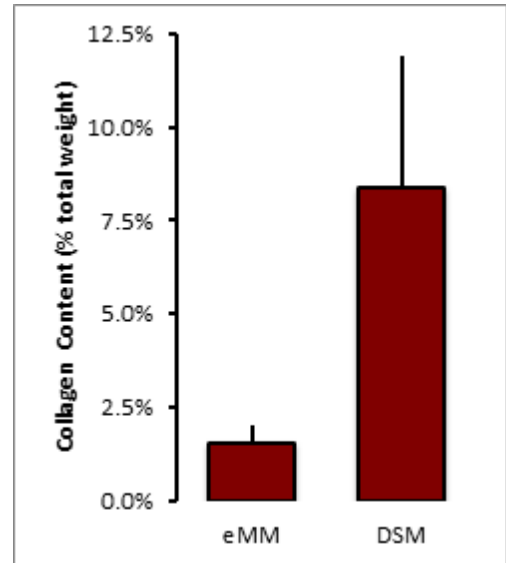

Figure 3. Hydroxyproline assay was performed on engineered muscle matrix (EMM) and decellularized muscle (DSM). Results reveal collagen content as a percent of total weight (mean±SD, n=4)

process in this study tends to favor the foam it is seeded on.

Because the collagen content of the engineered tissue samples is significantly lower than the decellularized tissue samples, studies can be performed in the future to encourage growth of collagen in the engineered samples. Specifically, transforming growth factor-β (TGF-β) is a hormonally active polypeptide that controls cell growth and differentiation. TGF-β has been shown to induce fibroblasts to produce increased amount of collagen and fibronectin [13]. Therefore, it would be beneficial to conduct a study manipulating the amount of TGF-β seeded on cell foams and comparing the corresponding collagen content.

Another means of promoting collagen growth would be to seed the foams with more cells. This study seeded the cells at a density of 2 million cells/cm3 so it would be advantageous to increase this amount to see if growth is encouraged or if there appears to be a density limit for foams. Correspondingly, growth might increase with increased cell culturing time.

Not addressed in this study is the foreign body response to this cell-derived ECM. The host response to biologic scaffold materials composed of ECM involves both the innate and acquired immune system, and the response is affected by device specific variables including the intended clinical application, the source of the raw material/tissue from which the ECM is harvested, and the processing steps involved in manufacturing [14]. However, a cell derived ECM is the ideal scaffold because it is specifically synthesized by the resident cells of each tissue and is likely biocompatible since host cells produce their own matrix [3]. The cytotoxicity of the material still needs to be addressed before it can be fully reproduced in the future.

5.0 Conclusion

The results of this study have revealed that there is an approach to creating a cell derived extracellular matrix that with further research can be compared to decellularized muscle. Because traditional extracellular matrix scaffolds induce the foreign body response and are limited by donor organs, the ability to create such a material would be beneficial for a variety of medical treatments requiring muscle growth.

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