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The Effect of Cell Density and Cultivation Period on Skeletal Muscle
Extracellular Matrix Accumulation

An Undergraduate Honors College Thesis

in the

Department of Biological and Agricultural Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

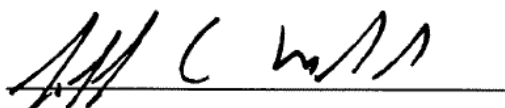
by

Ryan John Yarnall

April 26, 2013

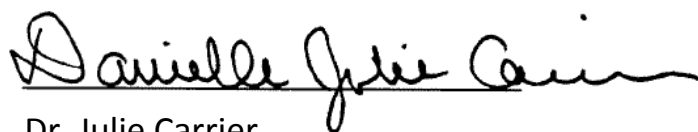
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Abstract

Traumatic skeletal muscle injuries have led to severe disability disallowing one to perform necessary daily tasks. Different methods are under current research to rebuild muscle tissue and have it function properly. One such method is the use of extracellular matrix, or ECM, retrieved from cells to create biological scaffolds providing structure for myoblast cells to grow into tissue. This research holds promise because it utilizes the body's own machinery, minimizing risk of a foreign body response. To obtain ECM scaffolding, one viable technique involves the cultivation of cells on polyurethane foams to collect and harvest ECM.

This research investigates the effect that initial cell density and cultivation period have on the accumulation of ECM material on the polyurethane scaffolds. The polyurethane foams are seeded with initial cell densities of 1×10^6 , 2×10^6 and 4×10^6 cells/foam, and the cells are cultivated for a control period of 3 weeks. In an additional experiment, the polyurethane foams are initially seeded at a control density of 2×10^6 cells/foam, and the cells are cultivated at two time periods of 2 and 4 weeks. At the end of each experiment, the polyurethane scaffolds are dissolved by dimethylacetamide solvent, and the dry weights of the resulting ECM are weighed. The results are examined to determine any trends in ECM accumulation and to suggest modifying the process of cell cultivation on polyurethane foams to increase ECM yield.

Introduction

Severe damage to skeletal muscle tissue prevents one from utilizing the damaged muscle for daily tasks. Examples of traumatic muscle injuries include crush, contusion, laceration or freezing [1]. As a method for the reformation of muscle and various other tissue types, tissue engineering has developed as a discipline that combines cell biology and engineering principles. In 1985, the term “tissue engineering” was coined by Y.C. Fung as “an interdisciplinary field that combined the technologies of biology and engineering to develop tissues to replace damaged organs” [2]. Today, extracellular matrix, or ECM, scaffolding is under investigation as an alternative to the use of synthetic biomaterials for tissue engineering purposes. Previous cell implantation was performed using non-degradable and degradable polymeric scaffolds that prompt a foreign body response (FBR) by macrophages [3]. Biologic scaffolds consisting of ECM have promise to not elicit such a reaction, because they are composed of the body’s own material. In addition, ECM biologic scaffolds contain a multi-molecular nature that supports a complex and remodelable biomaterial [3]. The biomaterial allows the body’s own “wound-healing machinery” to send biologic signals after implantation to direct proper healing [4].

The current research methods for the development of ECM scaffolding involve the cultivation of cells on polyurethane (PU) foams at a particular initial density of cells for a certain cultivation time period. The standard initial density and cultivation period are 2×10^6 cells/foam and 3 weeks, respectively [3]. At the end of the cultivation period, the PU foam is removed and the ECM is harvested to measure certain structural, biological, and mechanical properties of the material. The aim of this research is to explore methods to optimize the production of ECM using this technique. Specifically, in this project, the effect of cell seeding density and culture duration on ECM yield was explored.

Materials and Methods

Polyurethane Sacrificial Foam Synthesis

The sacrificial foams were synthesized from a medical grade polyurethane (PU) elastomer (Tecoflex SG-80, Thermedics, Woburn, MA). To fabricate a 10% solution, 2 g of PU pellets were dissolved in 20mL dimethylacetamide (DMAc) (Alfa Aesar, Wardhill, MA) in a flask set on a heat/stir pad at approximately 60 °C and low-stir. To provide a pattern for the sacrificial foams, a mixture of granulated sucrose (C&H Pure Cane Granulated White Sugar, Crockett, CA) and deionized (DI) water was placed within rubber mold cutouts (0.659 cc) on a metal plate. The ratio of sugar to DI water was 10 g to 200 μ L, and both were mixed until the sugar had the consistency of wet sand.

The sucrose-filled molds were then placed in a gravity convection (VWR International, Radnor, PA) oven at 75 °C for 20 minutes for drying. After the molds became dry,

approximately 2 mL of the 10% DMAc solution was added to each individual mold in a fume hood. The solution saturated each sugar-filled mold without having a greater volume than the mold itself. The molds were then submerged completely into a DI water bath at 21 °C and allowed to soak for 24 hours to remove the sugar template. After 24 hours, the molds were removed from the DI water bath. The individual polymer scaffolds were removed from the mold cutouts and inspected for equally porous sides to ensure they would not impede cell proliferation. The polymer scaffolds were then soaked in a different DI water bath with minor agitation for 24 hours. After the 24 hour period, the DI water in the container was discarded and replaced. The scaffolds were then soaked for an additional 24 hours. The polyurethane scaffolds were removed from the DI water bath and placed into a 50 mL tube (VWR International, Radnor, PA) and freeze dried (Cat No: 7382020, Labconco, Kansas City, MO).

Polyurethane Foam Sterilization

In a chemical hood, the foams were placed into 50 mL sterilized tubes and soaked in 70% isopropyl alcohol (IPA). The tubes were then positioned in a vacuum chamber (Thermo Fisher Scientific, Waltham, MA) at room temperature for 20 minutes. After removing the 50 mL tubes from the vacuum chamber, the 70% IPA was discarded from the tubes and sterile phosphate buffered saline (PBS) (Gibco Life Technologies, Carlsbad, CA) was added. The foams were soaked in PBS for 3 minutes. Then, PBS was removed and rinsed with sterile PBS repeated twice. After the last sterile PBS rinse and removal, a fibronectin solution (Sigma-Aldrich, St. Louis, MO) was added to the tube to promote cellular attachment to the polyurethane scaffolds. The 50 mL tubes were stored at 4 °C and incubated overnight.

Cell Seeding

The cells used in this research were rat muscle myoblasts (ATCC, Manassas, VA). Frozen cells were thawed and plated in a sterile T-75 flask (Greiner Bio-One, Monroe, NC). They were expanded in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture (DMEM)/F12 (Gibco Life Technologies, Carlsbad, CA) with 10 % fetal bovine serum (FBS) (Gibco Life Technologies, Carlsbad, CA) and 25 µg/mL gentamicin (Gibco Life Technologies, Carlsbad, CA).

After 2 days of cellular growth in the T-75 flask, the cells were transferred to a T-175 flask. Under sterile conditions, the DMEM/F12 media was removed from the T-75 flask. The flask was then treated with 5 mL of a 0.25% trypsin/1mM ethylenediaminetetraacetic acid (EDTA) (Amresco, Solon, OH) solution and gently agitated to promote cellular detachment. Five mL of DMEM/F12 media were added to the flask to neutralize the Trypsin/EDTA solution. The cellular mixture was transferred from the flask to a 15 mL sterilized tube. The 15 mL tube was centrifuged at a rate of 300g for 5 minutes to pelletize. After centrifugation, the media and Trypsin/EDTA solution were carefully removed from the tube. Approximately 7 mL of DMEM/F12 media was added to the tube. To break up the cellular pellet, the media was agitated in the tube using a pipette. The cells in the DMEM/F12 media were transferred from the 15 mL tube to the T-175 flask, and 10 mL of additional DMEM/F12 media were added to the flask for a total of 17 mL of media. The T-175 flask was incubated until a sufficient number of cells were available for cell-seeding onto the polyurethane foams.

For seeding the cells on the polyurethane foams, 6 mL of trypsin was added to the T-175 flask to prevent cell adhesion. Six mL of DMEM/F12 media was subsequently added to

neutralize the acidic trypsin. The mixture was transferred from the flask to a 50 mL tube and centrifuged at 300 g for 5 minutes. The liquid from the tube was removed, leaving the cell pellet at the bottom. The cell pellet was broken up and mixed with 5 mL of DMEM/F12 media. Fifty μL of the mixture was added to a vial along with 200 μL of trypsin blue for a 1:5 ratio. The mixture was dispensed into a hemocytometer to count how many cells appeared in the 50 μL sample. The concentration of cells was determined to be 12.5×10^6 cells/mL.

For the cell density experiment, 18 polyurethane foams were removed from the fibronectin and divided evenly between 3 Petri dishes. Foams were seeded at concentrations of 1×10^6 cells, 2×10^6 cells, 4×10^6 cells / foam. For the foams containing 1×10^6 cells, 40 μL of L6 and DMEM/F12 mixture was coated on one side of the foam. The foams were then incubated for 30 minutes. Forty μL of the L6 and DMEM/F12 mixture was added to the other side of each foam. This cell-seeding process was repeated for the other polymer foams to contain 2×10^6 cells and 4×10^6 cells. However, for the foams containing 2×10^6 cells and 4×10^6 cells, 80 μL and 160 μL of the cell and DMEM/F12 mixture covered each side of the foam, respectively. 13.5 mL of DMEM/F12 media, 1.5 mL of 1 mM ascorbic acid, and 3 μL of TGF β 1 were added to each Petri dish containing foams following seeding for cell cultivation.

For the time study, 20 PU foams were evenly divided between 3 Petri dishes. One Petri dish would be cultivated for a period of 2 weeks, one for a period of 4 weeks, and one for a period of 6 weeks. The foams in each dish were each initially seeded with 2×10^6 cells utilizing the process described in the previous paragraph for the cell density experiment.

Cell Cultivation

After seeding with cells, the polymer foams were cultured for a time period of 3 weeks for the cell density experiment. For the time study, the polymer foams were cultured for two different time periods: 2 and 4 weeks. For both experiments, each Petri dish containing the cell-seeded scaffolds were cultivated in DMEM/F12 media supplemented with 10% FBS, 1 mM ascorbic acid (AA) and TGF β 1 (4 ng/mL) (R&D Systems, Minneapolis, MN). The media was changed every 2-3 days using sterile technique.

To prepare 50 mL of 1 mM AA, 75 mg of L-AA 2-Phosphate (Sigma-Aldrich, St. Louis, MO) and 25 mg of L-AA (Sigma-Aldrich, St. Louis, MO) were added to a 50 mL sterilized tube. In a sterile hood, 50 mL of DMEM/F12 were added to the L-AA 2-P/L-AA mixture in the 50 mL sterilized tube. The mixture was sterile filtered using a 30 mL syringe (BD, Franklin Lakes, NJ) and a sterile syringe filter (VWR International, Radnor, PA) then aliquoted into 15 mL sterilized tubes. The tubes containing 1 mM ascorbic acid were stored in a 4 °C freezer until needed for replacing cell media.

Cell media was replaced every 2-3 days. DMEM/F12 media and 1 mM AA were placed in a hot water bath at approximately 36 °C for 10 minutes to allow thawing. After thawing, the petri dishes containing the cell-seed polymer foams were retrieved from an incubation chamber and placed in a sterile hood. In the sterile hood, the existing media was removed using a sterilized pipette. To ensure the cells were alive, the existing media had a yellowish hue due to pH change. In 13.5 mL of DMEM/F12 media, 1.5 mL of 1 mM AA and 3 μ L of TGF β 1 were added to the Petri dishes using sterile technique. The Petri dishes were then placed back into the incubation chamber until the next replacement of media.

For the cell density experiment, after the 3 week time period, the media was removed, and the PU foams from one Petri dish were placed in a 50 mL tube using sterile technique. The 50 mL tube containing the foams was stored in a -80 °C freezer until further testing. The same process was performed for the time study at the end of each time period (2 and 4 weeks).

Polyurethane Removal

At the end of testing, the 50 mL tubes were removed from the -80 °C freezer and placed in a warm water bath at 37 °C to expedite thawing. 4% paraformaldehyde (EMD Millipore, Darmstadt, Germany) was added to each 50 mL tube, covering each foam. The foams were incubated in 4% paraformaldehyde to fix the proteins, creating a more cohesive structure. After approximately 30 minutes of soaking, the individual foams were removed with tweezers and placed in individual vials. In the fume hood, 3 mL of DMAc was carefully added to each vial enveloping the foams. The DMAc rinsed the foams of polyurethane for about 5 hours and was then carefully removed. The rinsing was repeated with 3 mL of DMAc added to each vial, but the foams were instead rinsed for a period of 24 hours. The foams underwent 3 DMAc rinses for complete polyurethane removal from the foams.

After the final DMAc rinse, the DMAc was removed from each vial and 3 mL of water was added instead to ensure DMAc did not remain. The foams were immersed in water for 15 minutes, after which the water was discarded and 3 mL of fresh water added. The foams were soaked for another 15 minutes. The water rinsing was repeated three times. At the last rinse, only 1 mL of fresh water was added to each vial. The vial lids were taken off, and the tops of the vials were covered with Kimwipes and secured with rubber bands. The vials containing the

ECM foams were frozen in the -80 °C freezer and then placed in a lyophilizer (Cat No: 7382020, Labconco, Kansas City, MO) for freeze drying for about 24 hours.

Yield Calculation

Following freeze-drying, the samples were removed from the lyophilizer and weighed on a microbalance (XA204DR, Mettler-Toledo International, Columbus, Ohio). Material yield was calculated as the ratio of material mass (in mg) to PU foam volume (in cm³).

Statistical Analysis

Using the cell density results, a t-test with a level of significance of 0.05 was performed in Microsoft Excel 2010 for the time studies that contained only two data sets. For the cell density experiments with three data sets, an ANOVA test with a level of significance of 0.05 was executed in Microsoft Excel 2010.

Results

Cell Density Experiments

For scaffolds seeded with an initial density of 1×10^6 cells/foam, 1.99 +/- 0.95 mg of material was harvested for every cc of foam seeded with cells. Scaffolds initially seeded with a density of 2×10^6 cells/foam resulted in 2.09 +/- 0.29 mg of material collected for every cc of foam. For the initial cell density of 3×10^6 cells/foam, 2.60 +/- 0.85 mg of ECM material was collected for every cc of foam seeded.

When an ANOVA statistical analysis was performed, the resulting p-value between groups was 0.4072. Because this value does not meet the standard criteria of $p \leq 0.05$, the cell density experiments did not produce results that are statistically significant.

Time Studies

The average ECM yield after a two-week period was 2.07 +/- 0.75 mg of material for every cc of foam seeded with cells. After a 4 week period, average ECM yield for every cc of foam was 5.39 +/- 2.12 mg. The values were proven to be statistically different by performing a t-test analysis and resulting in a p-value of 0.000228 meeting the standard scientific criteria of $p \leq 0.05$ suggesting that lengthening the culture period from 2 to 4 weeks significantly increased material yield.

Discussion

The technique of using porous polyurethane sacrificial foams to collect ECM material from cells allows for greater accumulation of material due to additional surfaces on which ECM can accumulate [3]. A current challenge is accelerating the in-vitro synthesis and yield of ECM

material [3]. Various growth factors, such as TGF β -1, have shown an effect on the amount of ECM accumulation on porous scaffolds; however, the effect of initial cell density and cultivation period on subsequent ECM yield has not been tested [3,4].

The results for the cell density experiment show a gradual increase in the average ECM density produced as the initial cell seeding density increases. However, after statistical analysis was performed, this increase was determined statistically insignificant using a standard 95% confidence interval. Therefore, it cannot be concluded in this experiment that initial cell seeding density affected the amount of resulting ECM. Vu et al. had similar findings where expansion capacity in 3D Ca-alginate hydrogel culture was found to be independent of initial hematopoietic cell seeding densities [5]. As a result of these findings, a possible inference is the rapid proliferation of cells when space was available in the porous polyurethane scaffold as in the case of the foams seeded with a density of 1×10^6 cells/foam. Also, the surface area of the porous foams for possible cell adhesion was difficult to determine; therefore, the maximum cell density capacity was unknown. This could have resulted in lower initial cell densities on the foams than believed, with not all cells initially adhering to the fibrous structure of the polyurethane. Future testing is encouraged to test initial cell densities lower than 1×10^6 cells/foam to conclude how few cells can be seeded on the polyurethane foam to produce similar ECM yields.

The time studies produced results that showed the duration of cell cultivation affected the subsequent ECM accumulation. After performing a statistical analysis, the resulting amount of ECM material harvested was proven statistically significant based upon a 95% confidence interval. However, future testing is encouraged using longer cultivation periods to determine a maximum yield of ECM. In these experiments, two cultivation periods of 2 and 4 weeks were tested with an initial cell density of 2×10^6 cells/foam. Due to contamination during the

experiment, cells were not allowed to cultivate for a 6 week period. Therefore, testing should be done using longer cultivation periods of 6 or more weeks to speculate if the cells exhibit a rate plateau of ECM production.

Although more research is needed to determine the optimal standard for cell cultivation that maximizes the accumulation of ECM, this project has shown trends based upon initial cell density and cultivation period. With knowledge of these trends, the cell cultivation process can be improved accelerating the accumulation of ECM to create ECM-derived biomaterials for tissue engineering and possible commercial purposes [4].

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Figures

Table 1. Cell Density Experiment Results

Initial Cell Density (Million/Foam)	1	2	4
Density (mg/cc)	1.972386588	2.275830678	2.427552723
	2.124108633	1.668942497	2.427552723
	3.641329085	2.275830678	2.579274769
	0.910332271	2.124108633	3.641329085
	1.213776362		3.337884995
	2.124108633		1.213776362

Table 1. The Resulting ECM Densities (mg/cc) for the PU Scaffolds Seeded at 1×10^6 , 2×10^6 and 4×10^6 cells/foam and Cultured for 3 Weeks in the Cell Density Experiments.

Figure 2. Cell Density Experiment Results

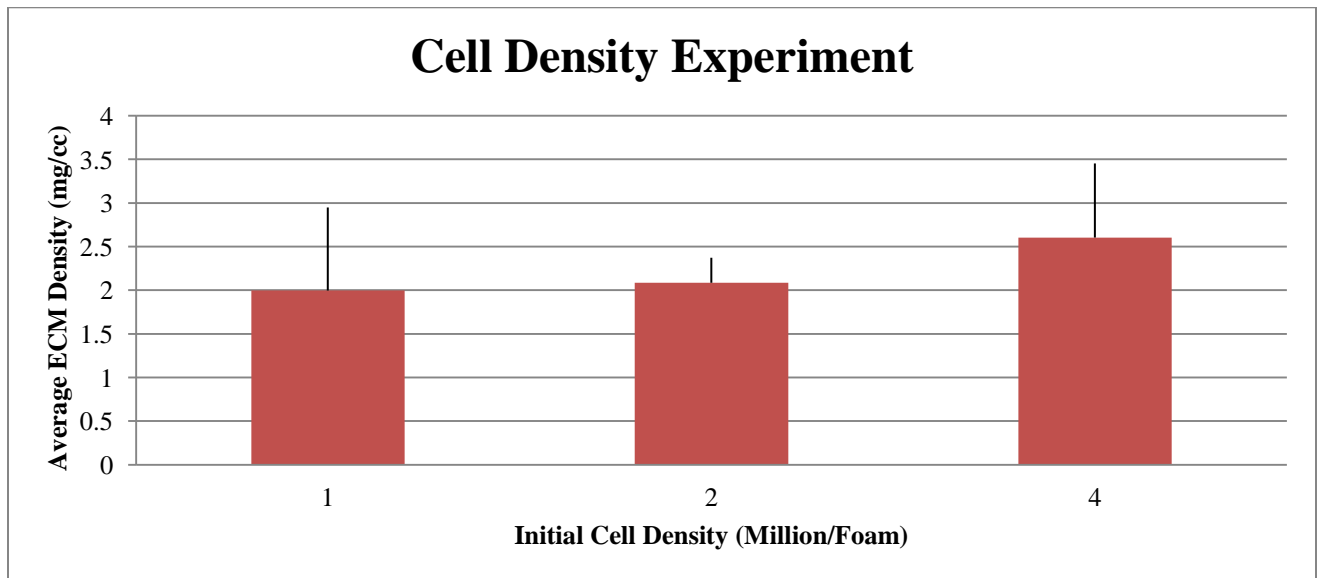


Figure 2. Resulting Average ECM Densities (mg/cc) Graph for each Initial Cell Density (1×10^6 ; 2×10^6 ; 4×10^6 cells/foam) in the Cell Density Experiments.

Figure 3. ANOVA Statistical Analysis Results

Anova: Single Factor						
SUMMARY						
Groups	Count	Sum	Average	Variance		
1	6	11.98604157	1.997674	0.906204		
2	4	8.344712486	2.086178	0.082487		
4	6	15.62737066	2.604562	0.722047		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.243536841	2	0.621768	0.963555	0.407182976	3.805565
Within Groups	8.388718248	13	0.645286			
Total	9.632255089	15				

Figure 3. The Resulting Values for the ANOVA Statistical Analysis Test Performed for the Cell Density Experiments.

Time Studies

Table 4. Cultivation Duration Experiments Results

Time (Weeks)	2	4
Density (mg/cc)	1.517220452	8.799878622
	3.337884995	5.917159763
	1.820664543	5.006827492
	2.124108633	8.496434532
	2.124108633	5.917159763
	3.48960704	3.416149068
	1.552795031	3.97515528
	1.49068323	2.857142857
	1.677018634	4.099378882
	1.552795031	

Table 4. The Resulting ECM Densities (mg/cc) for the PU Scaffolds Seeded at 2×10^6 cells/foam and Cultured for 2 and 4 Weeks in the Cultivation Duration Experiments.

Figure 5. Cultivation Duration Experiments Results

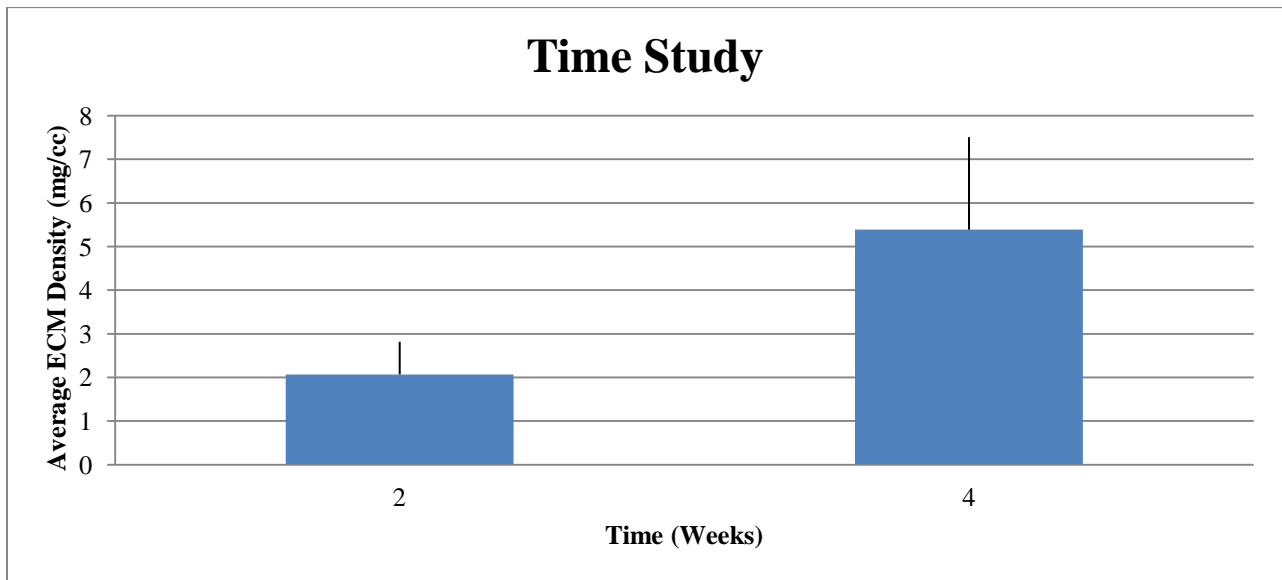


Figure 5. Resulting Average ECM Densities (mg/cc) Graph for each Culture Period (2 and 4 Weeks) in the Cultivation Duration Experiments.