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Varying culture conditions to enhance tissue formation

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Varying Culture Conditions to Enhance Tissue Formation

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Abstract

For severe muscle damage, tissue engineering has become a viable option to culture muscle tissues with the ultimate goal of implantation. However, the tissues are typically cultured on permanent scaffolds which would elicit a foreign body response if placed in the body. To avoid this, biological scaffolds are ideal for tissue culture and limiting any negative reactions. A sacrificial scaffold can be used to gather extracellular matrix (ECM), which can then be used as the primary scaffold for culturing tissues for implantation. To reach the ultimate goal of implanting muscle tissue, an understanding of how to produce ECM on a larger scale must also be gained. Supplementing culture media with pro-fibrotic molecules is an option to consider for enhancing ECM formation.

Two conditions, molecular crowding and the addition of transforming growth factor beta 1 (TGF-β1), were considered. In addition, the effect of the concentration of TGF-β1 on material yield was also analyzed. In the first experiment, molecular crowding macromolecules, TGF-β1, and a control with no additives were considered. . For the second experiment, concentrations of TGF-β1 at 0.5, 1, and 5 ng/mL were added to culture media and material yield was compared to a control set with no TGF-β1. Cells were cultured for three weeks on sacrificial scaffolds under varied culture conditions, the scaffolds were removed, and the mass of ECM collected was measured. The material yields were then analyzed using an analysis of variance (ANOVA) and linear regression.

The results from the first experiment indicated that neither molecular crowding nor TGF-β1 increase the material yield. In the second experiment, it appeared that the addition of TGF-β1 at all concentrations increased material yield. This could indicate even less TGF-β1 could be

added to culture media to increase material yield. Additionally, the amount of fetal bovine serum (FBS) added to the culture media was different between the two experiments, which could explain the conflicting results regarding the effect of TGF-β1 on material yield in the two experiments. Based on the results of the data, ECM formation can be enhanced by supplementing the culture media with TGF-β1 at a concentration as low as 0.5 ng/mL.

1. Introduction

For minor muscle damage, the recommended treatment is time, rest, and physical therapy. The body has its own mechanisms of healing muscle damage at the cellular level. However, if the muscle damage is severe enough, the body will not be able to repair the damage on its own. Tissue engineering has become a viable option to create muscle tissues in lab settings with the ultimate goal of implanting the tissues in patients that have suffered severe muscle damage. Typically, tissues are cultured on permanent scaffolds. If permanent scaffolds were implanted, a foreign body response could be triggered. A biological scaffold is ideal for limiting foreign body responses. A sacrificial scaffold can be utilized to collect the extracellular matrix (ECM) secreted by cells, which can then be used as the primary scaffold for culturing tissues for implantation. The ECM is the primary structural biological material in all tissues and organs [1]. Therefore, using it as a scaffold would better replicate true biological conditions while decreasing the risk for a foreign body response.

In lab settings, engineering muscle tissues on a small scale is a common occurrence. However, to reach the ultimate goal of implanting muscle tissue, an understanding of how to produce ECM on a larger scale must be gained. Supplementing the culture media with pro-

fibrotic molecules is an option to consider for enhancing the production of ECM. However, under standard media conditions, ECM formation in vitro is a slow process.

In this study, two fibrogenic culture additives, molecular crowding and the addition of transforming growth factor beta 1 (TGF-β1), were considered.

Molecular crowding is the concept of using macromolecules in the culture media to crowd the cells [2]. In the process of culturing tissues, cells are harvested and seeded onto scaffolds while being "bathed in a large volume of non-crowded aqueous medium" [1]. To better represent an *in vivo* environment, macromolecules are added to the culture media to provide more crowded surroundings. Ficoll (Fc) and Dextran Sulfate (DxS) are macromolecules used to simulate molecular crowding conditions (Fig. 1). Both molecules have large molecular weights, but the main difference between the two is Fc is neutral and DxS has a negative charge. Crowding the media with macromolecules like Fc and DxS has been shown to increase the chemical activity of the cells, which would promote collagen and ECM formation.

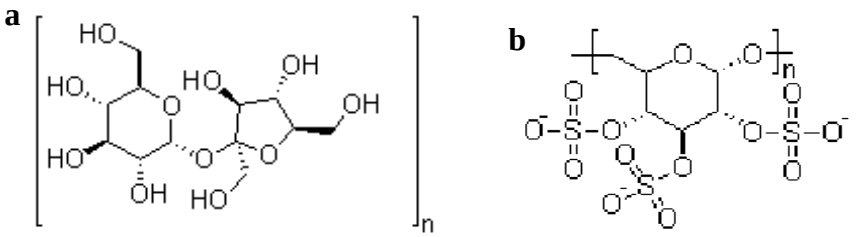

Fig. 1 – (a) Chemical structure of Ficoll [3] (b) Chemical Structure of Dextran Sulfate. [4]

TGF-β1 is a protein that is commonly used to induce cells to synthesize and contract ECM. It is a cytokine that has been believed to be a central mediator of the fibrotic response and enhancement of collagen expression [5]. Adding TGF-β1 to the culture media will increase ECM formation in the tissues, which will increase the amount of tissue formed.

To determine whether molecular crowding or the addition of TGF-β1 will significantly increase the amount of ECM formed, tissues were cultured in these conditions and the average material yields were examined. Also, there has been speculation about whether or not material yield is sensitive to TGF-β1 concentration. To answer this question, another study was conducted to determine the effect of TGF-β1 concentration on the material yield.

2. Materials and Methods

2.1. Scaffold Preparation

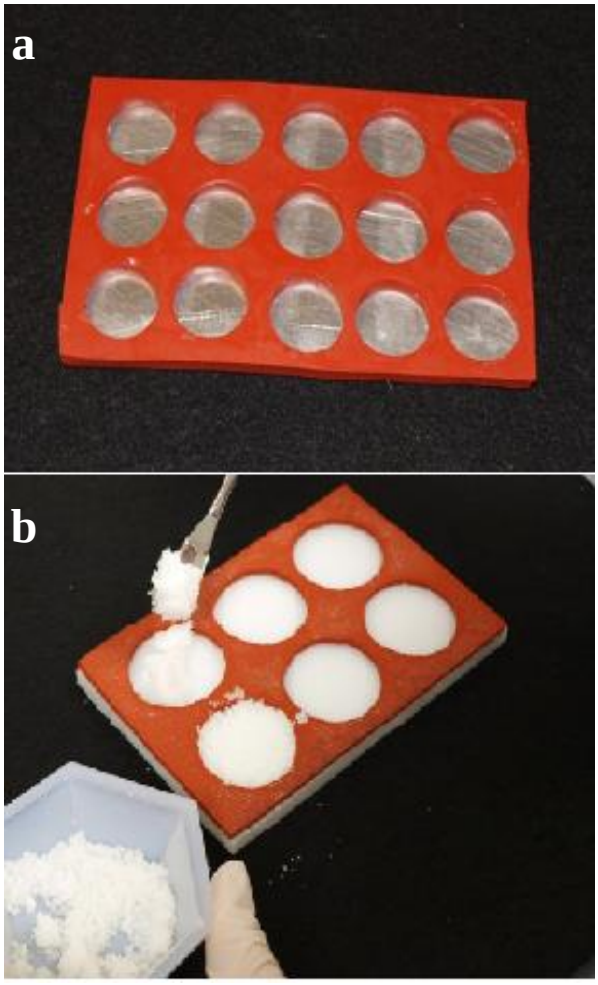

Fig. 2 – (a) The mold for scaffolds
(b) Addition of sugar mixture to mold

To produce the sacrificial scaffolds, a mold was made by using a small metal plate covered with a 0.3 millimeter thick soft rubber. A 1 centimeter diameter hole was cut through the rubber and the circle was removed leaving the metal plate visible. This step was completed until there was not any more space for holes. The mold is shown in Figure 2. The holes were then filled with a moist sugar mixture that provided the backbone of the scaffold, as shown in Figure 2. Using a spatula, sugar was mixed with water at a ratio of 10 g of sugar to 200 µL of water until the water is evenly distributed. When filling the holes, it is important to not pack the sugar in

too tight. Once all of the holes were filled with the moist sugar mixture, the entire mold was placed in a drying oven overnight.

A 10% polyurethane solution is used to make the foam scaffolds. The solution was prepared by dissolving 2 g of polyurethane pellets (Tecoflex, SG-80A, Lubrizol, Cleveland, OH) in 20 mL of dimethylacetamide (DMAC) (Sigma, St. Louis, MO) in a 100 mL round bottom flask. A stirring pellet was then added and the mixture was placed on a hot plate. The solution was heated to 60°C and left overnight. Once the moist sugar mixture was dry and the polyurethane pellets were dissolved in DMAC, the 10% polyurethane solution was drizzled over each sugar-filled hole as shown in Figure 3. Particular attention was given such that there was not any solution pooled at the surface of the sugar mold.

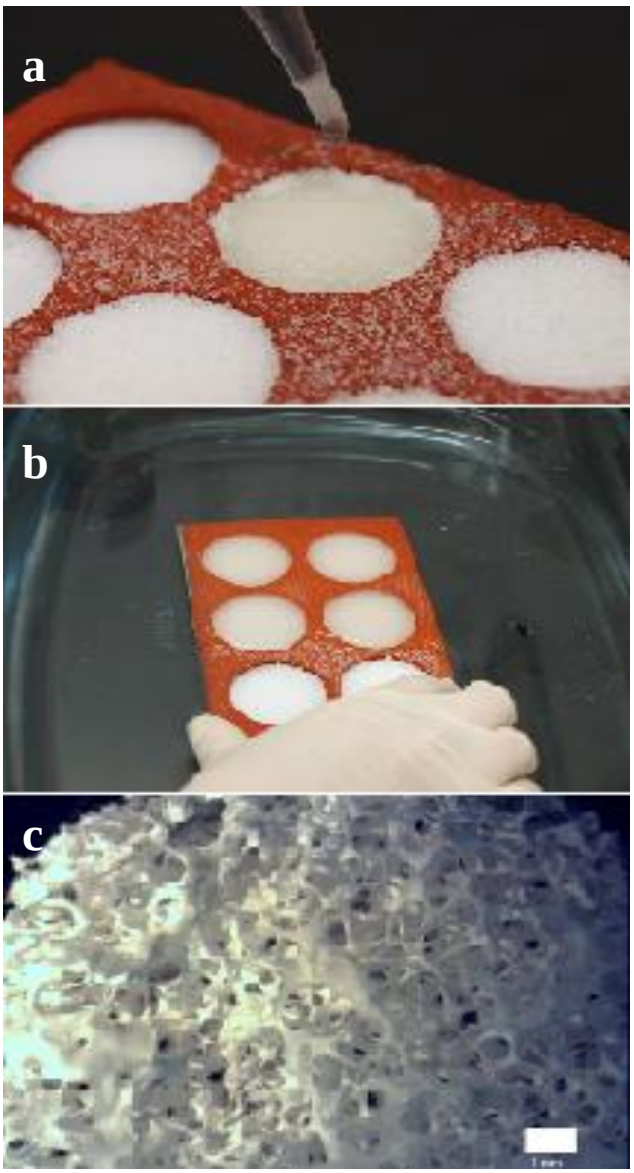

Fig. 3 – (a) Addition of polyurethane solution to sugar (b) Placement of mold with sugar and polyurethane solution into water container (c) Structure of polyurethane scaffold

The final step in preparing the scaffolds relied on solubility principles. Since sugar was water-soluble and polyurethane was not, the entire mold was placed in a small container of water and left over night. When

completed, only polyurethane foam scaffolds remained in each hole. The foams were removed from the mold, placed in a clean beaker filled with DI water, and stirred to ensure all of the DMAC and sugar was rinsed from the scaffolds.

2.2.1. Media Preparation – Molecular Crowding

For the molecular crowding experiment, four different conditions were analyzed: Fc and DxS as the molecules used for molecular crowding, TGF-β1, and a control group with no molecular crowding or TGF-β1. Each condition had different additives in the media. Since molecular crowding is the focus of the experiment, it was decided to use the media mixture that is suggested for Fc and DxS [2]. The media mixture (0.5% FBS media) contained Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F12) (Gibco, Grand Island, NY), Glutamax (10 µL/mL) (Gibco, Grand Island, NY), Gentamicin (1 µL/mL) (Gibco, Grand Island, NY), and Fetal Bovine Serum (FBS) (5 µL/mL) (Gibco, Grand Island, NY), where Glutamax is used to improve growth effiiciency, Gentamicin is an antibiotic, and FBS is a growth supplement. The mixture was prepared in sterile conditions and stored at 4°C for use as needed.

Media was also supplemented with 1mM ascorbic acid. To prepare a 10 mM ascorbic acid stock solution, 75 mg of L-Ascorbic Acid-2-Phosphate (Sigma, St. Louis, MO) and 25 mg of L-Ascorbic Acid (Sigma, St. Louis, MO) is mixed in 50 mL of media. Once everything was dissolved, the ascorbic acid was sterile filtered using a sterile filter syringe. Ascorbic acid is very unstable and rapidly oxidizes in aqueous solutions. To maintain the ascorbic acid properties, the ascorbic acid was separated into 6 mL aliquots and kept at -80°C. The aliquots were thawed and used as needed.

To avoid weighing Fc and DxS for each media change, Fc and DxS solutions were prepared and stored for the duration of the experiment. For the Fc solution, Fc 70 (GE Healthcare, Pittsburgh, PA) at 37.5 mg/mL was mixed with Fc 400 (GE Healthcare, Pittsburgh, PA) at 25 mg/mL [2]. At this ratio, 18.75 g of Fc 70 and 12.50 g of Fc 400 were mixed with 500 mL of the 0.5% FBS media and stored for use throughout the experiment. The DxS solution was prepared at 100 µg/mL [2]. Since this is such a small mass, it was decided to prepare the DxS solution as a 10X concentrated solution. To mix the 10X solution, 60 mg of DxS 500 kDa (pK Chemicals, New York, NY) were mixed with 60 mL of the 0.5% FBS media. Each solution was sterilized by filtration to decrease the chance of contamination. In addition, TGF-β1 (Sigma, St. Louis, MO) was added at a concentration of 4 ng/mL. The media composition for each well is described in Table 1.

Table 1: Cell-Feeding Formula for each Condition (per well)

Control	TGF-β1	Fc	DxS
0.5% FBS media 1 mM Ascorbic Acid	0.5% FBS media 1 mM Ascorbic Acid 4 ng/mL TGF-β1	0.5% FBS media 37.5 mg/mL Fc 70 25 mg/mL Fc 400 1 mM Ascorbic Acid	0.5% FBS media 1 mM Ascorbic Acid 100 µg/mL DxS

2.2.2. Media Preparation – TGF-β1

Four TGF-β1 conditions were examined during this experiment. The four conditions were the control with no TGF-β1, 0.5 ng of TGF-β1 per mL of media, 1 ng of TGF-β1 per mL of media, and 5 ng of TGF-β1 per mL of media. The media (10% FBS media) consisted of the standard mix of DMEM F12 with Glutamax (10 µL/mL), Gentamicin (1 µL/mL), and Fetal Bovine Serum (FBS) (10 µL/mL).

Since the amount of TGF-β1 used was such a small amount, it was deemed very difficult to add TGF-β1 to each individual well. Because of this, the media was mixed with ascorbic acid and TGF-β1 for the entire six-well plate and then distributed to each individual well at the time of changing the media. In 30 mL plastic vials, 27 mL of 10% FBS media and 3 mL of ascorbic acid were placed. Then, a micropipette was used to add the required amount of TGF-β1 for each condition. The TGF-β1 mixtures for each six well plate are listed in Table 2.

Table 2: TGF-β1 Mixture for 6-Well Plate		
TGF-β1 – 0.5 ng/mL	TGF-β1 – 1 ng/mL	TGF-β1 – 5 ng/mL
27 mL 10% FBS media 3 mL Ascorbic Acid 3 µL TGF-β1	27 mL 10% FBS media 3 mL Ascorbic Acid 6 µL TGF-β1	27 mL 10% FBS media 3 mL Ascorbic Acid 30 µL TGF-β1

It was assumed that the media, ascorbic acid, and TGF-β1 had a constant concentration throughout the 30 mL of solution. An auto pipette was used to distribute 5 mL of solution to each well for each TGF-β1 condition. The cell feeding formulas for each well is listed in Table 3.

Table 3: Cell-Feeding Formula for each Condition (per well)			
Control	TGF-β1 – 0.5 ng/mL	TGF-β1 – 1 ng/mL	TGF-β1 – 5 ng/mL
10% FBS media 1 mM Ascorbic Acid	10% FBS media 1 mM Ascorbic Acid 0.5 ng/mL TGF-β1	10% FBS media 1 mM Ascorbic Acid 1 ng/mLTGF-β1	10% FBS media 1 mM Ascorbic Acid 5 ng/mL TGF-β1

2.3. Tissue Culture

For both experiments, rat skeletal muscle myoblasts (L6, ATCC, Manasas, VA) were used. They were expanded in flasks and the media was changed 3 times per week. Once confluent, the cells were split into two flasks and continued until it was believed there were enough cells to seed 2 million cells per scaffold. This procedure was continued until sufficient numbers of cells were produced such that scaffolds were incubated with 2 million cells per

scaffold. To confirm the approximate number of cells, the cells were removed from the flasks and counted using a hemocytometer.

Before cells could be seeded on the scaffolds, the scaffolds had to be prepared. Thus far, the scaffold preparation has not been sterile. Therefore, the scaffolds were sterilized to prevent contamination. To sterilize the scaffolds, the required number of scaffolds were placed in a Petri dish, covered with 70% alcohol, and placed in a vacuum desiccator for 20-30 minutes. Then, the Petri dishes were removed from the container and the alcohol was aseptically aspirated from the Petri dishes. The scaffolds were then rinsed with phosphate buffered saline (PBS) (Sigma, St. Louis, MO) three times for 2-3 minutes per rinse. Finally, the scaffolds were covered with fibronectin (Sigma, St. Louis, MO) and left overnight in the 4°C cooler with parafilm sealing all edges of the Petri dishes.

The sterilized scaffolds were placed in six-well plates, with one scaffold per well. To seed the cells onto the scaffolds, the amount of cell solution that contains 1 million cells was determined based on the number of cells counted. Using a micropipette, 1 million cells were seeded onto the scaffold by carefully dropping the cell solution onto the scaffold. Once all of the scaffolds were seeded, the six-well plates were placed in the incubator for 30 minutes. Then the scaffolds were flipped, another one million cells were seeded on the scaffolds, and the six-well plates were placed incubator for another thirty minutes. Finally, the scaffolds were covered with 10% FBS media and left in the incubator overnight.

Media was changed three times per week for three weeks. Each six-well plate contained the six samples for each condition. The feeding outline followed the protocols previously mentioned in sections 2.2.1. and 2.2.2.

2.4. Scaffold Removal

After three weeks of culturing the tissues for each experiment, the tissues were removed from the six-well plates and placed in plastic vials. All six tissues from each condition were placed in the same vial, and the vials were then placed at -80°C. To preserve the material, the tissues were freeze-dried.

After the tissues were freeze-dried, the tissues were thawed and covered with a 4% paraformaldehyde (Sigma, St. Louis, MO) solution and left overnight at 4°C. The paraformaldehyde solution fixed the tissues and cells by terminating any ongoing biochemical processes. The next day, each tissue was removed from the plastic vials and placed individually in a scintillation vial.

To remove the scaffolds from the tissues, DMAC was used to dissolve the polyurethane foam scaffolds by carefully rinsing the foams. A micropipette was used to add and remove each rinse being careful not to tear the tissue. Four rinses were performed to ensure the entire polyurethane scaffold was dissolved. For the first rinse, 3-5 mL was used to cover the tissue in the scintillation vial. After 20-30 minutes, the first rinse was removed and a second rinse was left overnight at room temperature. The third and fourth rinses were also left overnight. Figure 4 displays a tissue being rinsed with DMAC to remove

Fig. 4 – Tissue during rinse of sacrificial scaffold

the sacrificial foam.

After the fourth rinse, the polyurethane scaffold was removed and only the muscle tissue remained. However, to rinse the DMAC from the muscle tissues, DI water was used. The rinse process for the DI water was similar to the rinse process with DMAC. After the fourth rinse, one mL of DI water was added to each scintillation vial and placed in -80°C. The tissues were then freeze-dried.

2.5.1. Data Analysis – Molecular Crowding

To measure tissue formation, the material yield for each tissue was measured after the tissues had been freeze dried. The mass of each tissue was weighed to the nearest tenth of a milligram, and the average material yield was determined for each condition. To determine any significant differences between conditions of the molecular crowding experiment, an analysis of variance between groups (ANOVA) was used. It is important to note that ANOVA will only report a difference between groups. To determine which groups were significantly different, post-hoc testing was conducted. The post-hoc testing used was the Tukey Test.

2.5.2. Data Analysis – TGF-β1

The average material yield for each concentration of TGF-β1 was measured by weighing the mass of each tissue. An ANOVA test was used to determine if there was a difference in the material yield for each condition, and the Tukey test was used as post-hoc testing. Additionally, linear regression analysis was used to determine the relationship between the concentration of TGF-β1 added to the culture media and the material yield. Using the output data of the linear

regression analysis, the R-squared value and the significance F value (p=0.05) were used to determine significance.

3. Results

3.1. Molecular Crowding

The average material yield for the control, TGF-β1, Fc, and DxS was 0.65±0.085 mg, 0.88±0.060 mg, 0.8±0.097 mg, 0.17±0.071 mg, respectively (Fig. 5). According to the ANOVA (p=0.05) analysis, there was a significant difference between conditions. Post-hoc testing confirmed there was a significant difference in the average material yield between DxS and the control, TGF-β1, and Fc.

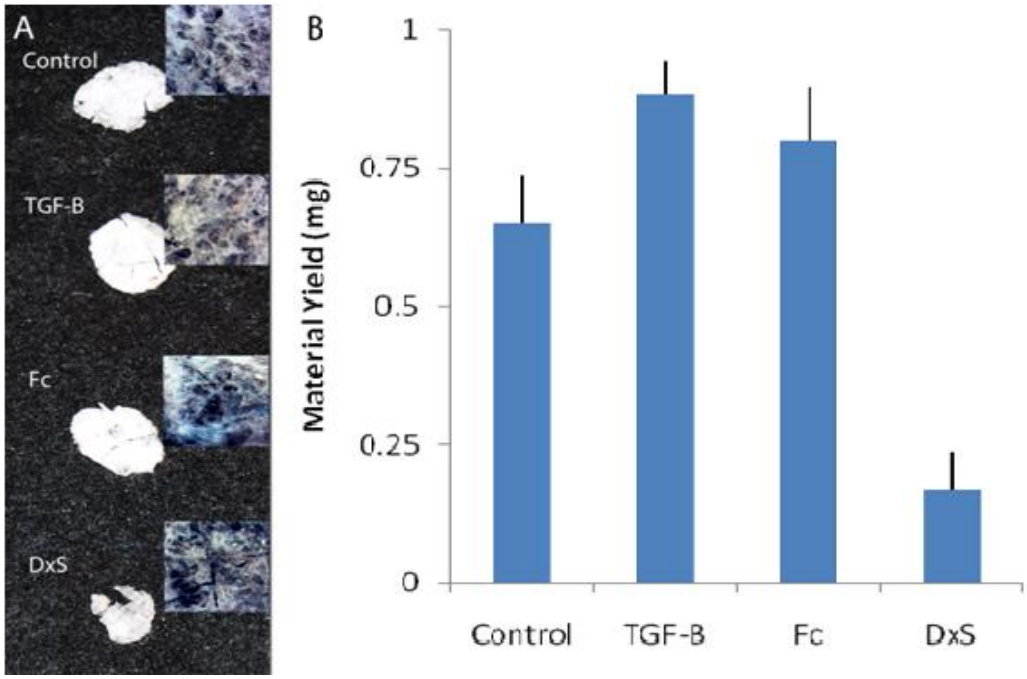

Fig. 5 – (A) Display of the relative size and the structure of material formed in each condition (B) Average material yield comparing TGF-β and molecular crowding

3.2. TGF-β1

The average material yield for the TGF-β1 concentrations of 0, 0.5, 1 and 5 ng/mL was 0.51±0.074 mg, 1.83±0.076 mg, 1.33±0.067 mg, and 1.53±0.163 mg, respectively (Fig. 6). The

ANOVA (p=0.05) confirmed there was a significant difference between the tested conditions. Post-hoc testing showed that adding TGF-β1 significantly increased the average material yield. In addition, it was also concluded that adding TGF-β1 at 0.5 ng/mL will significantly increase average material yield compared to adding TGF-β1 at 1 ng/mL. However, there is not a significant difference between adding TGF-β1 at 0.5 ng/mL and 5 ng/mL.

Linear regression analysis indicates the correlation between the concentration of TGF-β1 and the material yield was low. The R-squared value was 0.138, which specified that only 13% of the material yield was attributed to the concentration of TGF-β1 and did not signify a relationship between TGF-β1 concentration and the material yield. The significance F value was 0.62, which was far greater than the notable value of 0.05. Since the significance F value was greater than p = 0.05, it was determined that there was not a statistically significant association between the material yield and the TGF-β1 concentration. This also indicated there was not a linear correlation between the TGF-β1 concentration and the material yield.

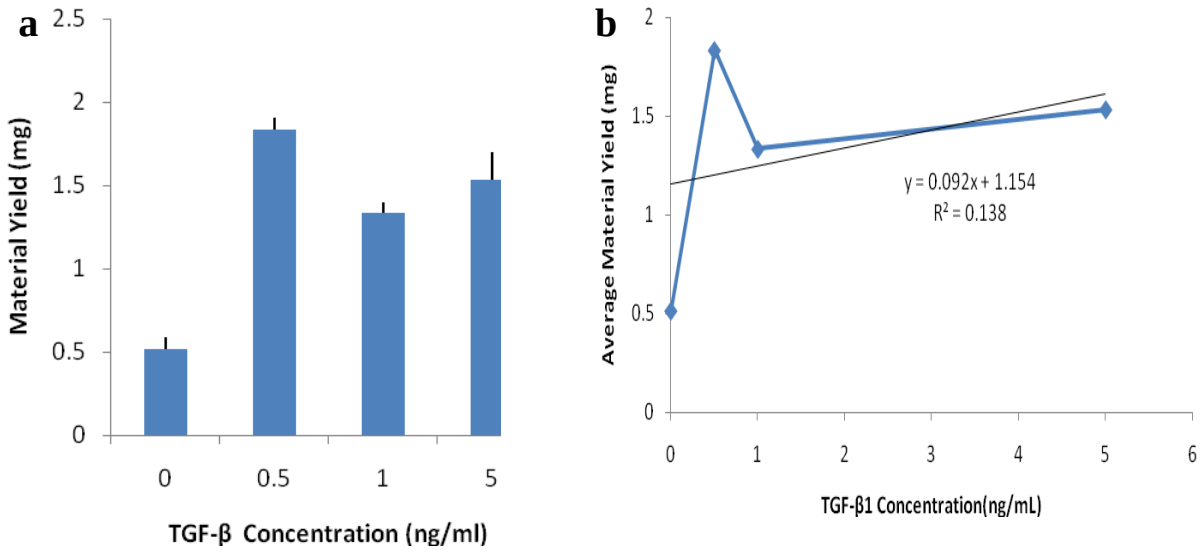

Fig. 6 – (a) Average material yield for the different TGF-β1 conditions (b) Scatter plot of average material yield versus TGF-β1 concentrations with trend line

4. Discussion

Even though there was an increase in the average material yield of the Fc and the TGF-β1 conditions compared to the control, the difference was not significant (p=0.05) under these conditions. It even appeared that DxS hindered material yield. This study displayed results that indicate molecular crowding will not increase the material yield of the ECM.

The TGF-β1 experiment demonstrated that there was not a linear relationship between the amount of TGF-β1 added to the media and the material yield. The concentrations of 0.5 ng/µL and 5 ng/µL yielded the highest amounts of tissue. It is unknown why the material yield at 1 ng/mL decreased. Since there is not a significant difference between concentrations of 0.5 ng/mL and 5 ng/mL of TGF-β1, 0.5 ng/µL is sufficient to add to the media to increase ECM yield. However, since the material yield for all concentrations of TGF-β1 were all similar and increased compared to the control, this could indicate that an even lower concentration of TGF-β1 could be used as a supplement to increase material yield. Determining this value could prove beneficial because TGF-β1 is an expensive product and using as little as possible while still maximizing results would be more cost efficient. Although there was not a linear correlation between material yield and TGF-β1 concentrations of 0.5, 1, and 5 ng/mL, there may be a linear correlation for concentrations between 0 and 0.5 ng/mL.

In the first experiment, adding TGF-β1 does not make a significant difference in the material yield. However, for the second experiment, adding TGF-β1 at any concentration yielded a significant increase in the amount of tissue formed. The only difference between the two conditions was the amount of FBS used in the media. This could suggest the importance of using a 10% FBS media versus a 0.5% FBS media when aiming for a high material yield.

Based on this study, it can be concluded that ECM formation can be enhanced with the addition of TGF-β1 at a concentration as low as 0.5 ng/mL. Although molecular crowding did not seem to increase the material yield, it would be interesting to see the outcome if a 10% FBS media is used rather than 0.5% FBS media. In addition, other conditions could be considered, such as supplementing molecular crowding conditions with TGF-β1 or increasing the length of the culture period.

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