Analysis of the Ideal Conditions for the Differentiation of Embryonic Stem Cells

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Abstract

Embryonic stem (ES) cells have the potential to differentiate into any type of body cell. For our experiment, we used mice embryonic stem cells. We aimed to create the ideal two-dimensional film to model a future three-dimensional scaffold that could be implanted into humans to repair damaged bone. We created a film that could endure normal human bone stress and strain while making a surface where the cells could attach and eventually differentiate.

We made a solution of chitosan and sodium alginate, two nontoxic, biodegradable polymers, and combined it with hydroxyapatite, an inorganic human bone salt. Using this solution, we made two-dimensional films which served as sites of attachment for stem cells.

The film was tested for its strength using an Instron tensile strength tester. Young’s Modulus, which is a mechanical property for evaluating the elasticity of a material, was calculated. Our average Young’s modulus value was 3.3 gigapascals and the greatest stress was 70 megapascals. Thus, our substitute was strong enough to act as human bone.

To test cell attachment, we used the same solution used to create films which we placed on two six-by-four welled plates. We examined two factors that affect cell-to-film adhesion: media and cell concentration. One plate contained cells in growth medium, which promoted stem cell differentiation. The other plate contained culture medium, which optimized stem cell proliferation. Half of each plate contained 10,000 cells per well, while the other half contained 20,000 cells per well.

The cells were then stained and fixed after one week of incubation. Although only one trial was performed, we concluded that the optimal tested condition for cell adhesion to film was the 20,000 cell concentration combined with a culture medium. While further research must be conducted before conclusions may be drawn, our results show promising results for the future of stem cell bone research.

1 Introduction

In the past, bone splits have been one of hardest problems to treat. Recently, artificial transplants have been utilized to heal split bones. Transplants of actual bone cells have also been attempted. However, such processes can be very expensive and surgically complicated. Therefore, naturally regrowing bone would be the most desirable solution. It not only minimizes the effects of bone surgery, but also reduces the possibility of host rejection. This solution becomes feasible with the use of embryonic stem cells.

Embryonic stem cells, or unspecialized cells, have the ability to differentiate into any type of body cell. This unique ability is known as pluripotency. It makes stem cells a great resource for engineering cells into tissue for transplants.

Scaffolds serve as a vital step in regenerating bone. They act as three-dimensional homes for cells to attach to and grow on when inserted into human bodies. Furthermore, scaffolds contain many pores through which nutrients can flow. For our experiment, we created films as two-dimensional models for scaffolds because films simplify the process of counting stem cells while accurately showing a representation of the growth.

Our goal for this experiment was to test ideal conditions for stem cell adhesion and growth. We used two different media and varied cell concentrations to find the optimal conditions. We also tested the mechanical strength of the films to test their elasticity.

2 Background

Embryonic stem cells have opened up a new avenue through which scientists may find success in syn-
thesizing bone. [6] Mice embryonic stem cells were used in our experiment. Stem cells are pluripotent; therefore, through control of environmental factors, chemicals, and other surrounding features, stem cells can differentiate into specific, desired cell types. [2] Furthermore, stem cells have the ability to replicate indefinitely since they do not respond to the concentration of surrounding cells. [4] However, despite the limitless promise of stem cell technology, all practical applications require that the cells be properly manipulated and specified.

Scaffolds are temporary structures that allow for the colonization of cells that can replace missing tissue. They act as three-dimensional matrices for the stem cells to attach to. Inside of these scaffolds, the stem cells differentiate into new bone cells. Biopolymers, materials that are biodegradable and non-toxic, are ideal for promoting the growth of new bone tissue. [5] Scaffolds are made of biopolymers so that they will dissolve into the injured bone during the healing process, effectively letting the bone heal itself over time.

Despite their advantages, biopolymers are weaker than other rehabilitative methods and therefore must be properly placed in the body to avoid stress-related fractures. [7] The biopolymer structures must be able to withstand the amount of pressure placed on a normal bone; this involves the concept of stress shielding. For example, if the material supports too much weight, the surrounding bone and the regenerated structure will be too weak. However, if the material is too brittle, the bone will collapse.

Scaffolds must also be porous to allow for the exchange of nutrients between the cells through a newly formed web of blood vessels. [5] Lastly, it is important to note that the strength of the scaffold is dependent on the severity of the injury. To evaluate the scaffold’s mechanical properties, a tensile test can be performed on the films to characterize strength and elasticity. Young’s modulus (See equation 3), the measure of these properties, is calculated as the value of the change in stress over the change in strain of the material. Stress is defined as the ratio of the force applied to the cross sectional area of the film (See equation 1). Strain is defined as the change in length divided by the original length (See equation 2). Young’s modulus is also useful in evaluating the film’s ability to replace bone.

\[ \text{stress} = \frac{\text{force}}{\text{cross sectional area}} \]  
\[ \text{strain} = \frac{\text{displacement}}{\text{length}} \]  

\[ \text{Young’s Modulus} = \frac{\Delta \text{stress}}{\Delta \text{strain}} \]  

Scaffolds are usually composed of synthetic polylactic acid and polyglycolic acid. [3] Even though these synthetic materials have similar properties to materials found in humans, they do not encourage cell adhesion to the surface as well as organic materials do; therefore, natural materials are the best substances in which cells can proliferate. [7] Common biopolymer materials used to create scaffolds are chitosan and sodium alginate. We used these two substances to create our solution. Together, chitosan and sodium alginate have an overall positive charge. The positive charge promotes better cell adhesion because cells have negative charges. [2] Hydroxyapatite was added to the solution because it is a salt that is naturally found in bones and has shown promise in generating higher cell growth. [1] It also encourages the acceptance and growth of the newly generated bone cells into the damaged bone. Finally, hydroxyapatite was needed to strengthen the solution because the biopolymers by themselves are not strong enough to support the stress of daily human activity.

To find the ideal solution for the future three-dimensional scaffolds, tests were performed on two-dimensional films. Films simplify the experimental and analytical processes while serving the same purpose as scaffolds. Thus, for our experiment, we used films to model future three-dimensional scaffolds.

3 Methods: Stem Cell Culture Growth Process

3.1 Solution and Scaffold Preparation

To begin creating the films and scaffolds, a solution was mixed in a test tube by combining chitosan and sodium alginate in a 90:10 ratio. Acetic acid, which helped the chitosan dissolve more quickly, was added to the solution as well. Then, a sonicator, which uses ultrasonic waves to agitate solutions, was used to make the solution more uniform. 2 mL of the sonicated solution were removed from the test tube and replaced with an equal amount of hydroxyapatite. This new solution was sonicated again and frozen overnight at -20 degrees Celsius. To complete the scaffolds, the solution was freeze-dried for two days (See Figure 2).

To make the two-dimensional films, the solution was diluted with 10 mL of deionized water. 300 mL of the solution were inserted into each well of two
six-by-four-welled plates (See Figure 1). The wells were then dried overnight at 50 degrees Celsius (See Figure 3).

3.2 Cell Culture

The experiment proceeded with the testing of the films. Two factors, media and cell concentration, were tested to find the optimal combination for cellular attachment to films.

Stem cells were pipetted onto two different plates of varied media. One plate was treated with a culture medium while the other was treated with a growth medium. On each plate, one half of the wells were seeded with a 10,000 cell per well concentration while the other half was seeded with a 20,000 cell per well concentration. These concentrations were chosen since previous studies have been performed on higher concentrations such as 10,000,000; 20,000,000; and 100,000. These concentrations were all too high and the cell growth rate was too rapid; the new cells quickly depleted the nutrients and were therefore inefficient. Consequently, a lower number of cells needed to be tested. If a successful lower concentration can be found, it will not only be more effective, but also more cost-effective. Stem cells and media can be expensive, and an over-arching goal of stem cell research is to make new technology available to the public.

The two plates were then placed in an incubator to simulate human body environment. After a few days, the nutrient-depleted media was replaced with new media and put back in the incubator for two more days. The cells were then fixed with 4% paraformaldehyde in order to preserve them in a single state for observation. Next, they were stained with DAPI, which enabled us to observe the nuclei of the cells for analysis under a fluorescent microscope. The white pixels from the microscopic images correspond to the presence of cells.

The images from the microscope can be quantitatively analyzed to find the ratio of cells to the surface area, calculated as the percentage of the image that appears white. It is important to find what values could correspond to white or black. Adobe Photoshop Creative Suite 4 was used to analyze the boundaries of cell growth. The values of the pixels along these boundaries were used to differentiate between the presence and the absence of cells. Using the histogram feature, the number of pixels considered white was divided by the total number of pixels in the image to find the surface area coverage by cell growth.
3.3 Young’s Modulus: Instron Tensile Tester

The strips of film were tested for their material properties. Using an Instron tensile tester, the strips were stretched with a constant velocity. The displacement, or the length that the strip was stretched, and force values of the sample films were used to calculate the stress and strain.

After all the data was gathered from multiple strips, the linear portion of the graph, known as the elastic region, was extracted (Figure 6). The material is able to return to its original shape if it is not stretched beyond this region. The Young’s modulus of each strip was found by taking the slope of the best-fit lines of the graph for each trial. The slope was calculated by curve-fitting the graphs with linear functions to determine Young’s modulus.

4 Results and Discussion

4.1 Scaffold Preparation

The scaffolds were successfully freeze-dried; many pores covered the surface, which would allow for the easy exchange of nutrients. This permits the scaffold to mimic a healthy, living bone. No testing for cell attachment was performed on our scaffold because of time constraints.

4.2 Young’s Modulus

The results of the tensile test produced a graph like the one below in Figure 5 (graph for trial 1).

The films showed varied success. During the tensile strength testing, the films exceeded expectations. The films proved to be much stronger and more durable than needed by regular human use. The four trials of the dry tests yielded graphs with Young’s modulus values of 4.59, 3.42, 2.59, and 2.58 gigapascals, respectively. The results of the Young’s Modulus show an average of 3.42 gigapascals, with a standard deviation of 1.02 gigapascals. Since the elastic regions of the materials show strong values for the elasticity modulus and can sustain a maximum stress of 70 megapascals, they show promise in being able to provide enough support to act as a replacement for any bone tissue since normal human bone strain averages less than 100 megapascals. Therefore, our film can endure any daily human usage and stress.

In this case, the slope, or the value for Young’s Modulus, is 4.59 gigapascals.

4.3 Cell Culture

While the films performed exceptionally well in tensile strength testing, they did not promote enough cell adhesion for any of the four combinations of cell concentration and media, as seen in Figure 4. Figure 4 displays microscopic images of each of the environments and concentrations in which the cells were kept. The white spots indicate the areas stem cell attachment.

Overall, the film helped increase stem cell adhesion. A contrast can be seen between the wells with film and our control wells which had no film.

Minimal cell attachment for both the 10,000 and 20,000 cell concentrations was observed in the growth medium plate. The culture medium yielded better results overall. While the 10,000 cell concentration also showed little stem cell attachment, the 20,000 cell concentration exhibited considerably more attachment. It was concluded that the optimal conditions for cell attachment and growth were the culture medium combined with the 20,000 cell concentration.

These results are not favorable since our goal was cell attachment leading to stem cell specialization into bone cells. The most successful medium was culture, which does not promote cell differentiation. Furthermore, not enough of the seeded stem cells
survived. Less than 1% of the cells remained after the one week of incubation. Improvement must be made to increase cell attachment rate and specialization of stem cells.

After performing the additional quantitative analysis of the images found in Figure 4, the qualitative results were confirmed. Growth medium with 20,000 cells had a 19.45% cell concentration. The control medium with 20,000 cells had 0.18%. The well with 20,000 had a 0.03% cell growth. The growth medium seeded with 10,000 cells had 1.11% surface area coverage. The control medium with 10,000 cells returned a 0.29% cell growth. The control medium with 10,000 cells returned 0.02% cell yield on the surface.

5 Conclusion

The chitosan-sodium alginate films, combined with hydroxyapatite, were tested for their uses as possible replacements for damaged bone tissue.

The cells grown in a culture medium were compared to those grown in a growth medium. Development was observed only in the growth medium. Two different cell concentrations were applied to each of the media. The wells with a 20,000 cell concentration showed more cell growth than did those with a 10,000 cell concentration. Overall, according to our observations, the best way to attach stem cells onto the chitosan-sodium alginate-hydroxyapatite films is to seed 20,000 stem cells in a culture medium.

With the data gained from our one trial, we recommend using a culture medium to first promote cell growth and adhesion onto the film; a growth medium can then be added to catalyze cell specialization. Together, the two media may work better to create more cell attachment onto the film and higher success for differentiation.

The mechanical properties of the biopolymer films were analyzed for their tensile strength. The calculated Young’s Modulus had an average value of 3.33 gigapascals. We concluded that our films have the mechanical properties of an acceptable substitute. Therefore, chitosan and sodium alginate, combined with the added hydroxyapatite, is a viable matrix to use for future scaffolds.

6 Future Research

Two-dimensional films only mark the beginning of many possibilities within bone repair research. More trials of our experiment must be run to find the optimal surface attachment of stem cells for more conclusive results. Furthermore, wet testing of the film for tensile strength must also be conducted to imitate physiological conditions in the human body. Other experimentation with different combinations of cell concentrations and media can be performed to test for optimal cell attachment.

Afterwards, the idea of differentiating stem cells into bone cells must be explored. This once again goes back to studies of types of media. Three-dimensional scaffolds would be the next step since films cannot be practically implemented into the body as replacement bones. Scaffold research would open up a large field since scaffold implants, length, host body acceptance, and duration, would all have to be closely examined and tested before put into public use. However, if accomplished, the perfect solution for bone injuries, and possibly many other diseases, may be found.

7 Acknowledgements

We would like to thank Dr. Devandra Verma, our project mentor, and Malov Desai and Namrata Kulkarni, Rutgers undergraduates who aided us in the biomedical laboratory. We would also like to express our great appreciation to Alison Russell, our RTA mentor, for her constant support and guidance. Also, we acknowledge Blase E. Ur, GSET Program Coordinator, Ilene Rosen, GSET Program Director, Kristin Frank, Head RTA, and Jameslevi Schmidt, Research RTA, and the Governor’s School Board of Overseers: Marguerite Beardseley, Chair, and Laura Overdeck, Vice Chair, who all deserve our sincere thanks as well. Lastly, without the sponsors of the 2010 Governor’s School, particularly Rutgers University, the Rutgers University School of Engineering, Morgan Stanley, the State of New Jersey, Lockheed Martin, PSEG, the Tomasetta family, the Provident Bank NJ Foundation, Silver Line Building Products, and the families of Governor’s School alumni, none of this summer would have been possible.

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