Three-Dimensional Cell Culture of Hepatocytes using Gel Molds

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Abstract

Currently in vitro drug, alcohol, and detoxification tests of the liver are either conducted on live mice models, excised liver slices, or on monolayer hepatocyte (liver) cell cultures. Recently, there has been an expansion into three-dimensional (3D) cell cultures for testing because the most often used monolayer cell culture is not an accurate representation of the liver. The micro-structure of the liver is composed of hepatocytes that form small spherical micro-tissues, also called spheroids, and this structure is essential to the metabolic and detoxification functions of the liver. This project proposes that hepatocytes self-assemble into three dimensional micro-tissues, where cells attach to each other and proliferate as a small mass under certain specified conditions. In order for cells to self-assemble and proliferate as a 3D tissue, the cells must be cultured in a precise micro-environment where there are no flat surfaces for the cells to attach to, the cells must be able to easily move towards each other, and there should be no extracellular matrix (ECM) present in the initial cell seeding. To provide these conditions for the hepatocyte cell culture a sub-microliter reservoir was created to house the cells. These reservoirs or microtest-tubes were molded with a hydrogel, 2% agarose. After multiple trials of 3D cell culture, spheroids or microtissues were successfully produced from the H35 rat hepatoma cells.

Keywords: Hepatocyte, H35 Rat hepatoma Three-Dimensional culture, Spheroid, Micro-tissue, Hydrogels

1. Introduction

Lab testing of different drugs and lab research of different diseases has become very common in academic as well as commercial settings. Currently tests are usually either run on live animals, excisions of organs or tissues from animals, or monolayer cell cultures. Although animal use has been deemed acceptable in the interest of human kind, when there are other options available it does not seem necessary to proceed in this fashion. Of course in order to harvest cells for use some animals have to be sacrificed, however the minimization of the number of sacrifices would be beneficial. Tests run on monolayers of cell culture can be helpful in initial testing stages; however monolayer cell cultures are unable to accurately fully mimic the functions of cells in the body (Napolitano et al. 2007). In order to improve this process and provide more realistic models in testing, researchers have begun expounding on the idea of three-dimensional cell culture models. Although many advanced labs in academic and pharmaceutical settings have accomplished reliable three-dimensional models, it has been a struggle for many small scale labs with limited resources. In order to address this problem this project proposes that cells will begin to self-assemble when put into a microenvironment with specific conditions. The desired microenvironment must prevent cells from attaching to its surroundings, must allow the cells to move freely, and must be initially cultured in the absence of extracellular matrix (ECM) (Brophy et al. 2009). Three-dimensional micro-tissue cell cultures, better known as spheroid cell cultures, have been formed with many types of cells including, dermal fibroblasts, cardiomyocytes, chondrocytes, and hepatocytes (Kelm and Fussenegger 2004).
1.1 structure and function of the liver
The liver is an important part of the body. It has many functions, but most importantly it plays a large role in metabolizing and detoxifying the substances that enter the body. These functions contribute to how the liver is structured: like a pipe system in which there are many channels devoted to different purposes. For example there is a network of vessels that bring in nutrient filled blood from the intestines for the liver to process. Another example would be the bile producing system within the liver, where bile can be stored in the gall bladder and a pipe-like network through the liver releases the bile into the small intestines when necessary for digestion (Friedman 1997).

A large part of the liver is made up of clusters of hepatocytes which function as the “workhorses” of the liver by storing nutrients, manufacturing proteins, removing toxins, and producing bile (Malarkey et al 2005). The liver functions in digestion by releasing bile and enzymes when needed to break down fats and cholesterol. After breaking down these substances the liver is in charge of processing the glucose that is a product of that digestion. If there is excess glucose, it is often stored in the liver as glycogen, an alternate form of glucose. The liver also plays a huge role in detoxification of the body; some would even refer to the liver as a “toxin filter”, where enzymes break down poisonous substances (Friedman 1997). The liver also helps process the over the counter, prescription, and recreational drugs that people take. Even if a drug is in slight excess for the body the liver determines the appropriate amount and disposes of the excess that can be toxic (Kaplowitz 2004).

1.2 pathological conditions of the liver
Since the liver provides so many functions for the body, it would be detrimental to one’s health if it were not functioning properly. Scarring of the liver unfortunately occurs often and can lead to many serious diseases. Fibrosis is a minor scarring of the liver that occurs when liver cells produce more ECM when they are inflamed impeding regular cell function in the liver. Although fibrosis can be somewhat combatted, it can lead to a more complex disease called cirrhosis, where the cell functions are halted completely and that portion of tissue becomes a permanent scar (Poynard et al. 2003). Fibrosis can be caused by many diseases that can be genetic, acquired, or self-inflicted. In fact about half of the people who are diagnosed with chronic liver disease or cirrhosis are cases of alcohol or drug induced disease. In 2010 about 32,000 people died of chronic liver disease in the United States, where about 16,000 were alcohol related liver disease (CDC 2014). Because the liver is such a good toxicity filter it deals with all of the drugs that are put into the body, and often if taken in excess these drugs can wear out the liver cells and begin to cause scarring. Another prevalent form of liver disease is attributed to hepatitis infections, where human immunodeficiency virus is the largest contributor to liver disease (Poynard et al. 2003).

1.3 current methods
As three-dimensional cell culturing techniques have recently been of great interest, there have been many successful methods of doing so. One of the first approaches toward three-dimensional cell culture was putting the cells into a solution of hydrogel that could be poured into a mold that also contained a structural scaffold (Dvir-Ginzberg et al. 2004). Although this is effective it cannot accurately model tissues from the body. There are three methods that have proven to form spheroids, the hanging drop method, the pellet method, and the rocker method, however there are many drawbacks. The hanging drop method works by having a plate arranged in a way that a drop of cell solution mixed with media can be placed in a hole through the plate, and the drop will hang. The cells will then be able to self-assemble since they have no surface to adhere to and since they have the freedom to move around within the drop (Kelm and Fussenegger 2004). This method is difficult to scale up due to complexity. In the pellet method cells are centrifuged down into a pellet and just cultured from the cell pellet. This method stresses the cells making them more prone to death; however some cells will remain alive and begin to adhere to each other just because of their close proximity (Tare et al. 2005). The rocker method involves placement of a cell culture flask on a see-saw rocker such that the cells would be continuously moving and self-assemble into spheroids as quickly as twelve hours. This method, although effective, produced inconsistent spheroid sizes (Brophy et al. 2009). However this study attempts to simplify and refine 3D cell culture techniques to grow cells reliably.

1.4 possible uses
If this new method of forming spheroids of the liver is possible there could be many ways to use this new method of three-dimensional cell culturing. One of the most impactful would be the possibility for automation of the formation of liver or even other cell types of spheroids, which could be used in large pharmaceutical settings for drug screening and discovery, where they would be able to easily scale up the 3D cell culture. Another use would be to test the effects of different drugs on healthy and diseased tissues, as well as run tests on alcohol induced cirrhosis. This would also provide an inexpensive way for academic labs to be able to keep up with the progression of cell culture. An ultimate application of this project could be using human tissue biopsies to start a 3D culture to test different drugs.

2. Methodology
For the purpose of the experiment the cell type chosen was H35 Rat Rueber Hepatoma cells (Eton BioScience), since they are so detrimental to liver function, and are relatively easy to grow in vitro. Here, the cells were first cultured in monolayer settings, and then cultured in a gel that would promote three-dimensional self-assembly. The gel was created by using agarose and polyacrylamide hydrophilic hydrogels with a rapid prototyped part. The growth of spheroids within the gels was determined by daily microscopic quantification.

2.1 mold
The mold used was designed by 2014 Milwaukee School of Engineering (MSOE) Research Experience for Undergraduates (REU) participant, Justin Clough (Clough 2014). The Rapid Prototyping Center’s Selective Laser Sintering machine was used to make Duraform Polyamide molds that were modeled in solid works. The molds were made so that hydrogels could be cast with microtest-tubes with a height of 1100 microns and a diameter of 600 microns. The bottom of the test tube was designed to be completely spherical, and is shown in Figure 1.

![Figure 1. Microtest-tube](image1)

2.2 hydrogels
The hydrogels used were 2% agarose and 14% polyacrylamide. These hydrogels, often used in electrophoresis applications, were chosen because of their hydrophilic nature. They were also chosen because of previous success rates in other cell culture molds used in three-dimensional cell culture studies (Napolitano et al. 2007). The 2% agarose was made by a simple weight by volume percent mixture. The 14% polyacrylamide was prepared using a recipie from a previous study, (Napolitano et al. 2007). After running many trials of preparing sterile hydrogels and comparing the polyacrylamide and agarose, agarose proved to be the better option with cell culture. Many times the polyacrylamide failed to cure, as well as stuck to the mold, as shown in Figure 2. Therefore, agarose was used in the 3D cell culture procedures.

![Figure 2a Polyacrylamide adhering to mold.](image2a)
![Figure 2b Polyacrylamide (top) verses agarose (bottom)](image2b)

2.3 monolayer cell culture
Cell culture is the process of growing living cells in a lab setting. In order for any cells to grow well they need to be provided with an aseptic environment, media that contains food and nutrients, and plenty of room to spread when growing. The H35 cell culture line was bought from Eton BioScience, and cultured as a monolayer pictured in
Figure 3. The cells were cultured in 1:1 media mixture of Dulbecco’s Eagle Medium (DMEM) and Ham’s F12 Medium (F12). This media also contained 10% fetal bovine serum (FBS) and 1% antibiotics containing Penicillin, Streptomycin, and L-Glutamine. Cells were grown in standard tissue culture conditions of 37 degrees Celsius and 5% CO₂. H35 cells were passaged every thirty-six hours. Passaging was done by adding a small amount of trypsin-versene to the cell plate, which detaches the cells from the flask, and centrifuging that mixture of cells then replating them in lower concentrations.

Figure 3. H35 hepatocyte cells in monolayer culture.

2.4 Three-dimensional cell culture
To prepare for the 3D cell culture the sterile 2% agarose was equilibrated for 24 hours with a solution that will promote cell growth. This equilibration process lasts for twenty-four hours. After the gel is made, a solution was added to the gel and put in the incubator. Two equilibration solutions were tested: 2% Bovine Albumin Serum (BSA) and the complete cell culture media used for the H35 cells. BSA was chosen as an equilibration solution because it is a protein often used in cell culture that promotes cell culture growth. Another reason for choosing BSA was the possibility that the BSA will crosslink together forming a mesh over the surface of the microtest-tube inhibiting the cells ability to attach to the surface. Equilibration with media was chosen as a control, and has been used in previous research.(Napolitano et al. 2007) The cells were then seeded into the microtest-tubes after the equilibration process. Three-dimensional growth was tested at many different concentrations within the microtest-tubes. The concentrations tested were 25,000, 50,000, 100,000, 250,000, 500,000, and 1,000,000 cells/mL. The cell solutions were placed into the microtest-tube with a P-10 pipette, and the cell solution contained the cell concentration desired as well as an extra 10% FBS to make the solution more dense and sink towards the bottom of the test-tube. Only the center nine test tubes were used, since those were the only openings that could be viewed with the microscope. An example of the cell layout with the cell concentrations are displayed in Figure 4. Cell growth was monitored daily with the use of digital microscopy imaging.

Figure 4. 3D cell culture layout in a 6 well plate
2.5 microscopy
The Motic Digital Microscope DMWB1-223ASC was used in taking pictures of the spheroid or micro-tissue formation. Pictures were taken every 24 days right after the initial seeding formation, then every 48 hours for the remaining days. Pictures were quantitatively analyzed using the program ImageJ.

3. Results and Data Analysis
3.1 images
There were many images taken with Motic during the 3D cell culture. Figure 5 displays the progression of 3D cell culture with differing cell concentrations.

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Figure 5. 3D cell growth of H35 rat hepatoma. Cells were grown in hydrogel microtest-tube equilibrated with (a) BSA or with (b) complete media. Images for day 0 and day 4 are shown.

3.2 quantitative analysis
The radii of spheroids were measured using the program ImageJ. Assuming that spheroids are spheres the volume of each spheroid was calculated. The comparison of media equilibration and BSA equilibration for each concentration of the initial seeding day and day 4 is shown in the graphs below, Figure 6.1 and 6.2.
Another comparison of the media and BSA is shown in Figure 7. A percent growth was done by comparing the initial seeding day and day 4. The lower concentrations of initial cell seeding solution grew more than those of higher concentrations. This could be because of the ample amount of room to grow for the lower concentrated cells. However regardless of the cell concentration spheroids did form and the cells proliferated in the spheroid.

4. Conclusion
These experiments suggest that when provided with the appropriate conditions rat hepatocytes can grow in 3D micro-tissue clusters known as spheroids. Another conclusion could be that microenvironments that have been equilibrated with 2% Bovine serum albumin (BSA) promote faster spheroid formation as compared to equilibration with complete media. Further, it was observed that regardless of the initial seeding concentration of hepatocytes, given enough time, the cells in the microenvironment can form a spheroid.

5. Future Research
Future research could include liver functionality tests on the micro-tissues to prove the benefits of using 3D tissue culture instead of monolayer culture and compare to liver function. Pathology tests on the micro-tissues could be beneficial for medical purposes, such as drug and disease testing with the 3D tissues. Future research could also include better microscopy techniques and methods of quantification to monitor spheroid growth and formation.

6. Acknowledgements
Special thanks go to Milwaukee School of Engineering, the Rapid Prototyping Center, and the National Science foundation for funding this project. Another thanks goes to Dr. Vipin Paliwal, Dr. Subha Kumpaty, Betty Albrecht, and the rest of the REU Staff. The author would also like to thank Lisa Kann, Rick Wolter, Dr. Anne Alexander, for helping with lab equipment and techniques, and Justin Clough—the REU student who made the rapid prototyped molds. This material is based upon work supported by the National Science Foundation under Grant No. EEC-1062621. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of The National Science Foundation.
7. References

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