A Novel Method to Culture Hepatocytes

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Background: Primary human hepatocytes (PHH) are an effective way to assess hepatotoxicity but PHH assays are unfeasible because of liver tissue scarcity. Stem cells can fill this void and generate viable hepatocytes but such cells are usually short-lived and dedifferentiate, losing activity of enzymes vital for drug metabolism like cytochrome P450 (CYP). These stem cells can be grown in three-dimensional cultures (spheroids) to maximize cytochrome P450 activity, but the inner cells become necrotic due to a lack of vascular structures unable to provide nutrients and oxygen. One novel solution is to culture these spheroids in 3D microvascular structures composed of biocompatible alginate hydrogels. The alginate microvascular structure, used in many studies to make robust bio-compatible structures, can supply the inner cells of the spheroids with nutrients and potentially prevent necrosis, and 3D printers can precisely place spheroids in the microvascular structures using a bio-ink composed of these aggregates. As this technology is still in its infancy, 3D printed cells in alginate microvascular structures have only been attempted with endothelial cells (Wang et al.). But there is potential for stem cell-derived hepatocytes to be cultured in a similar way. This warrants an investigation as this method has the potential to create lasting, viable hepatocyte cultures for testing hepatotoxicity.

Hypothesis: 3D printers can embed stem-cell derived hepatocytes in an alginate hydrogel microvascular structure that will induce cellular maturity and maintain function longer than conventional hepatocyte cultures.

Aim 1: To prevent necrotic cores in stem cell-derived hepatic spheroids, by embedding them in hydrogel microvascular structures using a 3D printer

Aim 2: Investigate the effects of extracellular matrix (ECM) proteins on cellular maturity in stem cell derived hepatocytes

Aim 3: Optimize cell culture conditions to augment P450 activity

Methodology: I will use induced pluripotent stem cells (iPSCs), by reprogramming human fibroblasts using Yamanaka factors, or human embryonic stem cells (hESC) (Sick Kids Embryonic Stem Cell Facility). The iPSCs will be programmed into hepatocytes using Si-Tayeb’s method or the hESCs using Keller’s technique. Then spheroids (500 cells, 200µm diameter) will be added to a bioink composed of alginate, arginylglycylaspartic acid (RGD peptide), and varying extracellular matrix proteins (collagen, fibronectin, laminin). Both the bioink and the microvascular structure are composed of alginate (to allow for efficient bonding of spheroids to the structure) with varying concentrations (8,10,15%) of alginate. Then, the 3D printer will place the spheroids in an alginate microvascular structure made of vessels (20µm diameter), in an interconnected checker-board pattern, with vessels 300µm apart. Each spheroid will be precisely deposited at the intersection of two channels using a computer algorithm, allowing cells maximum vascular exposure with 16-20 cells in between vessels (max. 140µm diffusion distance). The vessels will be created using polydimethylsiloxane (PDMS) mold. After printing, I will connect the cell culture to a cell perfusion system and degrade the PDMS, leaving behind hollow vascular alginate channels and embedded hepatic spheroids. Then each cell culture will be exposed to a variety of exogenous growth factors (HGF, FGF, BMP, OSM, Dex) and cultured for one week. Subsequently, viability and hepatic functionality assays for urea, albumin, and PCR for P450 genes will be performed and the results of these assays will be the basis of the analysis.

Significance: This investigation will lead to greater understanding as to how best to culture and mature stem cell-derived hepatic cells for longer periods of time. This will have great implications in the field of toxicology, where in vitro human hepatic cells are the best models of drug effects, and in the field of regenerative medicine where it can aid in fabricating functional hepatic tissue for liver transplants.
Existing Knowledge and its Significance

Introduction

Primary human hepatocytes (PHH) are an effective way to assess hepatotoxicity but PHH assays are unfeasible because of liver tissue scarcity. Stem cells can fill this void and generate viable hepatocytes but such cells are usually short-lived and dedifferentiate, losing activity of enzymes vital for drug metabolism like cytochrome P450. These stem cells can be grown in three-dimensional cultures (spheroids) to maximize cytochrome P450 activity, but the inner cells become necrotic due to a lack of vascular structures unable to provide nutrients and oxygen. One novel solution is to culture these spheroids in 3D microvascular structures composed of biocompatible alginate hydrogels. The alginate microvascular structure, used in many studies to make robust bio-compatible structures, can supply the inner cells of the spheroids with nutrients and potentially prevent necrosis, and 3D printers can precisely place spheroids in the microvascular structures using a bio-ink composed of these aggregates. As this technology is still in its infancy, 3D printed cells in alginate microvascular structures have only been attempted with endothelial cells. But there is potential for stem cell-derived hepatocytes to be cultured in a similar way. This warrants an investigation as this method has the potential to create lasting, viable hepatocyte cultures for testing hepatotoxicity.

Hepatic stem cells – iPSC

Induced pluripotent stem cells (iPSC), discovered by Yamanaka et al. in 2006 (1), are adult somatic cells that have been “reprogrammed” into stem cells. Many techniques have been developed, each with varying success, but the most common one involves exposing specific somatic cells to a chemical cocktail of certain “Yamanaka factors” (2) of four genes: Oct3/4, Sox2, Klf4, c-Myc (2) which convert the cells into stem cells. A variety of somatic cells have been used like adipose-derived stem cells (3), renal tubular epithelial cells (4) but generally, skin fibroblasts are used (5) because they induce cellular maturity (6). These Yamanaka factors are introduced into the somatic cells by means of a murine leukemia retroviral vector (7) that affects dividing cells (7). But others like Picano-Castro et al. have even used lentviruses (8), another class of retroviruses. Although this method is very reliable (9), one major issue that exists with reprogramming somatic cells is that some cells can retain some of their original genes (9) and this leads to variation and a loss in function (9,10). Similarly, using retroviruses can increase the risk of activating oncogenes (7) and increasing the risk of tumor development (8). Although Yamanaka et al. (11) have developed plasmids that do the same thing as retroviruses (12,13), there has been varied success (12,13). Some groups like Wang et al. (12,13) have even used microRNA clusters, as an alternative, to overexpress somatic genes in a coordinated manner (13) but the RNA transcription mechanism is not fully known (13).

Now, multiple protocols have been developed to convert these iPSCs to hepatocytes, but most of them try to replicate embryonic developmental conditions by exposing the reprogrammed iPSCs to a variety of growth factors (9,11,14). Song et al. (10) was the first to come up with such a procedure by using slightly different combination of Yamanaka factors (10) and subjecting fibroblasts to a four step differentiation protocol using FGF4 (fibroblast growth factor) (10) and BMP2 (bone morphogenetic protein) (10) to promote maturity. But compared to primary human hepatocytes (PHHs), there was a significant lack of essential hepatic genes (10). Even albumin, an important protein secreted by hepatocytes, was ten times lower than PHHs (10) and cytochrome P450 (CYP) activity was barely expressed despite exposure to multiple maturity factors (10). Similarly, Chen et al. (15) used a similar cocktail but used HGF and Activin A (15) to achieve iPSCs with genetic profiles and albumin and urea secretion levels very similar to PHHs but only one cytochrome P450 enzyme, CYP3A4, was expressed
(15). Finally, Si-Tayeb et al. (16) used the original Yamanaka factors with an addition of NANOG and Lin-28 (16) and generated hepatocyte-like cells that not only had a very similar profile to PHHs but had comparable levels of CYP activity and were capable of glycogen storage (16), a key feature of liver cells. Although most protocols successfully generate hepatic cell from iPSCs, the data can be misleading as assays performed on these cells are compared to PHH assays. Generally these PHHs are immortal, tumour cell lines, like HepG2 (17) which are known to lack metabolic activity (17). Or they are simply donor PHHs but after in vitro culturing, they also lose function (9). Simply put, iPSC-derived hepatocytes show varied success but show immense potential if external cell culture conditions are controlled (16).

**Hepatic stem cells – hESC**

Human embryonic stem cells (hESCs), first isolated from mouse blastocysts in 1981 (18) by Evans et al. also have immense potential to differentiate into hepatocytes. Hamazaki et al. (19) was the first to generate hepatic-like cells, using a multistep protocol that involved adding specific growth factors, like HGF (human growth factor) (19) to induce maturity. However, only two hepatic genes, ALB and TAT, were expressed, albeit weakly, and the cells secreted some albumin and urea (20). These cells were even transplanted in mice (20), and although they were not rejected by host cells, a much smaller number of cells produced albumin (20). Similarly, hESCs isolated from human blastocysts (21) have better function as they tend to have normal karyotype and high telomerase levels (21). Generally, hESCs develop into 3 germ layers with an intermediate layer expressing AFP and albumin. Earlier attempts to differentiate hepatocytes involved isolating this layer and further culturing them, but the primitive hepatic-like cell lacked substantial function (22). So, other strategies have been developed that focus on exposing the developing endoderm (one of the internal germ layers) after gastrulation (blastocyst after developing for seven days) (23) to growth factors. One very effective protocol by Kenneth et al. (24) exposes the endoderm to Activin A, a protein complex crucial in differentiation (23), that binds to specific receptors (24,25), initiating hepatic differentiation, resulting in 80% differentiated cells (26). To mature the cells, they have to be exposed to specific growth factors like BMP and FGF (27) which can become problematic as certain combinations of growth factors, like Activin A and FGF2 (28) can enhance endoderm differentiation (28), but other combinations can reduce hepatic differentiation (27). Although, dimethyl sulfoxide was observed by multiple studies (14,29) in small concentrations to improve hepatic differentiation by modifying histone acetylation (30). Presently, these chemical cocktail combinations are not properly understood (29). Although Hay et al.(31) discovered a much more efficient protocol that developed faster and better functioning hepatic cells (29) using Wnt3a and Activin A, many additional factors can affect hepatic differentiation including the timing (28), concentration (28) and specific combination of growth factors (28), as well as the methods of generating definitive endoderm (28). Recently, Keller et al. (32) has used Wnt3a and Activin A in very precise combinations with HGF (hepatocyte growth factor), Oncostatin M (cellular growth factors) and dimethyl sulfoxide (32) to get highly mature hepatic-like cells in a 3D hydrogel culture with very high levels of CYP activity.

**Hepatocyte cell culture – monolayer**

Currently, one of the most common ways to culture stem cell derived hepatocytes is to simply grow them on a petri dish, pretreated with extracellular matrix (ECM) proteins like collagen (33) in a two-dimensional (2D) monolayer (34). Although it is the cheapest and quickest way, there are several drawbacks. For instance, PHHs start to de-differentiate and lose morphology and metabolic capability within a few hours (34), specifically, decreased albumin levels (33,35) and loss of phase I and II detoxification enzymes (36), an advanced class of CYP enzymes. Although it is possible to add supporting liver cells like Kupffer or stellate cells (37), they can be very difficult to buy due to scarcity (38), further rendering the cell culture useless. Moreover, the 2D structure forces the hepatic cell cytoskeleton to become flattened and this change in shape limits cell-cell interactions that ultimately cause a loss of important signaling pathways necessary for normal hepatocyte function (39). Finally, the
ability to detoxify xenobiotic (foreign chemical agents) decreases (35), (36), (40). Even HepG2, an immortal and rugged hepatic cell line (41), shows decreased albumin secretion (17) in monolayer.

**Hepatocyte cell culture – sandwich**

In a sandwich cell culture, a monolayer (discussed above) is sandwiched between two extracellular matrix layers, typically made of collagen (42). Generally, much better levels of protein are generated (43) than a 2D monolayer. Even some transporter proteins like BSEP (a pump for bile salt transport), MDR1 (another pump for pumping foreign agents out of cell), and MRP4 (regulates cAMP, a messenger protein) are expressed (44). Also, most sandwich cultures (35,44,45) promote the formation of bile capillaries (44) which allows for quantitative bile assays (38). It even promotes polygonal hepatocyte morphology for extended culture periods that allows them to maintain their function better than monolayer cultures (46) as cell-matrix interactions reduce flattening of cytoskeleton and promotes cell-cell contact which is necessary for function. Adding matrix proteins can further increase viability (47) and CYP activity (48). But, sandwich culture still can’t avoid hepatocyte dedifferentiation (49). Also, the ECM layers at the top and the bottom can significantly limit gas exchange (38), which is fatal as hepatocytes consume a significant volume of oxygen (50). This can lead to necrosis (38) that can compromise the entire cell culture.

**Hepatocyte cell culture – spheroid**

Spheroid culture is a three-dimensional aggregation of cells in a spherical arrangement. They promote maximum hepatic function (17) and even maturity (38). Hepatic cells in spheroid cultures have very high and stable cytochrome P450 (CYP) gene expressions (51). Biliary capillaries have even been reported in rat and human hepatic spheroids (52) with high intracellular ATP levels (38) with CYP enzymes active for more than seven days (38). Moreover, the close cell-cell interactions in a three-dimensional culture can minimize the need for an extracellular matrix (53), which is generally considered as an important influence on spheroid formation and function (54). But the spheroid culture is riddled with complications and intricate problems. For instance, HepG2 is a cancerous hepatic cell line that can form into spheroids and is capable of glycogen storage (17), bile salt transportation (17), and forms biliary capillaries (17), but the cell culture has limited function as cancerous hepatic cell lines lack substantial metabolic activity (55). Similarly, immunofluorescent scans (56) have revealed that albumin (a key hepatocyte protein) levels are generally high around the periphery and virtually non-existent in the core cells (56) which die as there are no vessels to supply nutrients or oxygen. Finally, spheroid cultures attain the best result in hydrogels like alginate but the effects of the hydrogel components, that have a substantial impact on hepatic cell morphology (45), are not properly understood (38,45).

**Use as toxicity testing**

Hepatocytes are particularly good to detect toxicity of drugs as they contain cytochrome P450 (CYP) enzymes that can detect and metabolize 50% of drugs (38). To determine drug effects, hepatic uptake and biliary excretion (57) can be monitored. Even reactive intermediates in hepatic cells, phospholipids, and cholestasis (a condition where bile cannot flow from the liver to the duodenum) (58) can be good indicators (58). Primary human hepatocytes can be used, if available, but due to their undependable nature in vitro (59) they typically dedifferentiate and lose P450 activity (59) resulting in false positive toxicity tests (60). Moreover, in monolayer, which is the most common way to culture PHHs, CYP enzymes are greatly reduced (58). Even in sandwich cultures, the less commonly used culture, despite the cell-cell/cell-ECM interactions, Albumin and CYP activity still decreases (46). Although porous membranes like silicon nitride can be used to aid liver functions, specifically Acetaminophen sensitivity (61), but after 2 weeks (58) cells are no longer viable. Also, due to biliary
secretion (in sandwich cultures), and genetic factors (31), metabolites cannot be metabolized (62), which further decreases utility for drug toxicity testing. Spheroids can be used as the cells are more sensitive to drugs (58), but 3D hepatocyte cultures are rarely used (58). Generally, PHHs are not used in drug testing because of poor lifespan and function and existing stem cell-derived hepatic cell lines are useless as they have decreased metabolic and CYP activity (59,63). But they show potential as it is possible to physiologically and chemically induce CYP activity (59) and activate important metabolic enzymes if cell culture is exposed to maturity factors (59), but this is not clear.

**Alginate and 3D Printers**

Alginate, derived from brown algae (64), is a bio-compatible material (65) that is excellent for culturing three-dimensional cell cultures. It is highly porous with (100um) wide pore sizes (66) that allow diffusion of nutrients and oxygen (67). Hepatocytes function particularly well in alginate (68) as alginate scaffolds can support aggregation, cell-cell interactions, and even improve hepatic functions like CYP enzyme levels (48). The concentration of alginate also seems to have a noticeable effect on hepatocytes (69). Arginylglycylaspartic acid (RGD peptide) can be added to alginate to increase cell adhesion (70). Typically, hepatocytes cultured in alginate survive for more than a month (71) and even form cell-cell connections (71) that allow cytochrome P450 enzymes to remain high for 7 days (72). Hepatocytes in alginate have different morphology and have greater viability (73); compared to monolayer cultures, where cell mortality was 66%, only 25% of cells in alginate lost viability. Alginate is not without problems either. Alginate even shows potential to be used as a “biological ink” (composed of cells/tissue suspended in an alginate solution) for 3D printers (74) to be precisely printed in specific patterns. It is even strong enough (in high enough concentrations (75)) to be molded into vascular channels, like artificial capillaries, that are capable of handling high flow rates (74) with minimal leakage. Although alginate has been used to culture cells, like hepatocytes spheroids, in blob-like aggregates, they have never been used to construct vascular channels and culture hepatic spheroids in those vascular structures. But 3D printers have been used to test viability of hepatocytes in alginate hydrogels. Essentially, there is massive potential for alginate to be used in 3D printing technology to make vascular structures to culture these cells in vitro (76).

**Oxygen/gas exchange**

Hepatocytes are one of the few mammalian cells that use a very high amount of oxygen (77). Also, hepatic cells have a relatively small diffusion gradient (9-15µm). This can be expected from stem cell-derived hepatocytes as well. Generally, when hepatocytes are cultured in vitro, the diffusion gradient is generally ignored (17). The problem becomes worse when 3D aggregates are cultured where the core cells are further away from any vascular structures. The oxygen gradient is what results in necrotic hepatic spheroids.

**Summary & Rational**

Currently, multiple protocols have been developed to generate hepatocytes from stem cells and induced pluripotent stem cells. But the biggest challenge is preventing early mortality and function of these cells. Even if such cells remain viable long enough, they are generally cultured in monolayers or sandwich between extracellular matrix proteins that either change cellular morphology or function. Although function can be maximized by culturing these stem cell-derived hepatic cells in three-dimensional aggregates (spheroids), the small oxygen gradient causes necrosis in core cells. It is possible to use porous hydrogels like alginate to allow for effective gas exchange and induce maturity and maintain viability, but this has not been attempted before. This warrants an investigation as this can create viable stem cell-derived hepatic cells ideal for drug toxicity testing.
Research Proposal

Working Hypothesis:
3D printers can embed stem-cell derived hepatocytes in an alginate hydrogel microvascular structure that will promote cellular maturity and maintain function longer than conventional hepatocyte cultures.

Specific Objectives:
1. To develop a cell culture device for stem cell-derived hepatocytes with an alginate microvascular system that maximizes hepatic spheroid viability
   a. To determine the optimum concentration of alginate for maximum strength and minimal degradation
   b. To determine the optimum spacing and number of channels in the microvascular structure that promotes hepatic cell-cell connections
2. To develop the ideal concentrations of extracellular matrix proteins in the 3D printer bio-ink that will result in maximum maturity in stem cell-derived hepatocytes
   a. To determine the optimal combination of Extracellular matrix proteins
3. To identify exogenous growth factors that will lead to maximum activity of cytochrome P450 enzymes
   a. To determine the optimal combination of exogenous growth factors

Long Term Aims:
1. This investigation will lead to a better understanding of how stem cell-derived hepatic spheroid cultures function in vitro by enhancing the conditions in the inner cells.
2. This study will also clarify the ideal concentration of extracellular matrix proteins and exogenous growth factors needed to maximize cellular maturity

Significance:
This study is very important as it will elucidate previously unknown in vitro behavior of stem cell-derived hepatocytes and how best to culture such cells in three-dimensional aggregates (spheroids) in such a way that hepatic functions like cytochrome P450 enzyme activity is maximized. Essentially, this study expects to discover an optimum way to culture stem-cell derived hepatocyte spheroids so that they can be used as reliable drug toxicity models.
Experimental Design

Objective 1: Fabrication of cell culture device for maximum hepatic viability

First, the microvascular structures will be fabricated according to the method described by Wang et al. (78). I will purchase all reagents and components, unless specified otherwise, from Sigma-Aldrich. Polydimethylsiloxane (PDMS) will be used as a mold and patterned, using conventional photolithography and soft lithography techniques (78), to create a 5x5 mm board that I will pattern in a checker-board style pattern mold with the squares measuring 300x300µm. The space between the squares will be kept consistent at 20µm and will be 5µm lower than the squares. The PDMS mold will be sandwiched between two porous polycarbonate membranes 20µm thick which will be further sandwiched between two additional PDMS molds of the same template (important note: the 3 PDMS molds will create a 3D mesh network of 2 channeled vascular system. See Fig. 1). I will sterilize this “stack” of membranes and PDMS molds with 75% ethanol and treat it with UV light overnight (for stability). Then sodium alginate and a 10% calcium chloride solution will be injected into the PDMS layered structure for 30 seconds. The concentration of the sodium alginate will be 8, 10, or 15%. Sigma-Aldrich will provide stock solutions with the required concentrations of alginate. I will then separate the PDMS mold and the resulting 3D alginate mesh network of microvascular channels, and this will be immersed in ethanol for 30s to increase stiffness. I will also make “control” devices with no microvascular structures for each alginate concentration. In total, I will fabricate six templates, one control and one with vessels for each concentration, in which viability will be tested.

<table>
<thead>
<tr>
<th>Alginate Structure #</th>
<th>Type and Alginate concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8% control</td>
</tr>
<tr>
<td>2</td>
<td>8% experimental</td>
</tr>
<tr>
<td>3</td>
<td>10% control</td>
</tr>
<tr>
<td>4</td>
<td>10% experimental</td>
</tr>
<tr>
<td>5</td>
<td>15% control</td>
</tr>
<tr>
<td>6</td>
<td>15% experimental</td>
</tr>
</tbody>
</table>

Table 1 highlights all the alginate structures fabricated in this experiment

I will then make a bio-ink composed of stem cell-derived hepatocytes (iPSC or hESC), RGD peptides (to promote cell adhesion (79)), and alginate. The alginate will be of the same concentration as the microvascular structure (8,10,15%). If iPSCs are used, then I will reprogram human fibroblasts into hepatocytes using Si-Tayeb’s method (16) (see Hepatic Stem Cells - iPSC). If hESCs are used, I will program human embryonic stem cells form blastocysts into hepatocytes using Gordon Keller’s method (32) (see Hepatic Stem Cells – hESC). I will create multiple bio-inks by adding iPSC or hESC spheroid aggregates to varying concentrations of sodium alginate (mentioned above) and 1% RGD peptide solution. To prevent degradation, the bio-inks will be kept at -20°C in a general purpose freezer (Thermo Scientific) until needed. Each bio-ink will be loaded into a syringe and connected to a Palmetto piston-driven printer (purchased from the Medical University of South Carolina). The printer will be connected to a computer and I will write an algorithm for it to embed hepatic spheroids in the alginate microvascular structure of the same concentration. The algorithm will enable the printer to embed the spheroids (500 cells, 200µm in diameter) at the intersections of the microvascular channels allowing cells maximum vascular exposure (max. 140µm diffusion distance). After embedding, the alginate microvascular structure will be treated with 40 mM ethylenediaminetetraacetic acid for less than 30s to reinforce the structure and subsequently treated with 10% CaCl₂ solution for 30s to finally increase stiffness and elasticity of vessels. Subsequently, I will insert glass capillary tubes and create an inlet and outlet which will be connected to a cell perfusion system (Thermo Scientific) by means of stainless steel needles and elastic tubing and cultured for 2 days. The cell perfusion media will maintain a steady temperature of 36°C, with a pH of 7.4, isotonic conditions, 4.5 g/L glucose, and an oxygen concentration of 5-10%. The spheroids will remain embedded in the microvascular structure because the bio-ink will aid in bonding the
cellular aggregates to the channel (6,17,51). To test hepatic spheroid viability, I will perform fluorometric MTT assay (In Vitro Toxicology Assay Kit), ATP assay, and 3D Spheroid Evaluation assay using a fluorescence microscope (EVOS XL, Thermo Scientific).

Figure 1. Highlights the alginate microvascular structure with an embedded hepatic spheroid.

Expected Results

I expect the spheroids in alginate with microvascular structures will not have necrotic cores (revealed by high assay results) as the vessels will provide nutrients and oxygen to the core cells. The spheroids can even be expected to proliferate and expand around the vascular channels. The 3D spheroid orientation will most likely be maintained as the shear flow from perfusion system induces cells to aggregate further forming close cell-cell connections that will further promote essential hepatic pathways (62). In other words, the MTT and 3D Spheroid Evaluation assays should reveal high viability and high intracellular ATP activity in alginate microvascular structures and very low in the control alginate structures. Finally, multiple studies (64,65,68) have shown that the 8% alginate solution to be particularly robust in that it degrades very slowly and has maximum stability and strength in vascular structures. So, I expect that particular concentration to have the highest viability and albumin/urea levels (65).

Statistical Analysis

In this experiment, the null hypothesis is: the microvascular structure has no effect on hepatic spheroid viability. The alternate hypothesis then is: the microvascular structure does have an effect on hepatic spheroid viability. Since continuous data of discrete samples will be collected from the assays (quantified fluorescence levels from MTT and 3D Spheroid Evaluation assays), an analysis of variance (ANOVA) can be performed using Statistical Analysis Software (SAS). The data for the control and experimental groups will be used and if there is a significant effect, the P value (calculated from the ANOVA F value) will be less than 0.05, and the null hypothesis can be rejected.

Limitations and weakness of method

One particular limitation in this experiment is with the 3D printer. Since the 3D printer used is a rudimentary one that I will assemble, it may not be as accurate as a laser-controlled 3D printer. That might be rectified by improving the controlling algorithm (allow for more precise movement). Similarly, the iPSC/hESC-derived hepatocytes may not fully differentiate, so in that case, I will use another protocol (for iPSCs: Song et al.’s method (10) and for hESCs: Hay et al.’s method (31)). Finally, the shear flow rate of the cell perfusion system may have to be determined experimentally, so initially parts of the spheroids may get “washed away” due to high flow rates.

Potential errors and complications

A multitude of things can go wrong in this experiment. For instance, the alginate pores in the microvascular structure may be less than 100µm and that can make gas and nutrient exchange difficult.
This can be rectified by decreasing the concentration of alginate to 5% which should increase the size of the pores (64) and allow more room for gas exchange. Similarly, the spheroids can become too clustered together inhibiting efficient vascular flow. This can be rectified by increasing the 20µm vascular channel width to 30µm allowing more space for the large hepatic cells, but there is a trade-off between channel width and oxygen gradient as increasing it can potentially cut off gas exchange from cells further away from channel (because diffusion distance will increase and hepatic oxygen gradient is very small (80)). Likewise, the spheroids can fail to aggregate altogether. Increasing the alginate concentration in the bio-ink and keeping it at a steady 10% can help aggregate the cells (71) and if that does not work, then adhesin, an “adhesive” protein, can be added to the bio-ink. On the other hand, ATP assays may reveal low intracellular ATP levels. This can be caused by two factors. The simpler reason may be that cells are not getting enough glucose to complete Krebs cycle in which case, I will increase glucose concentration in the cell perfusion media (from 4.5 g/L to 9 g/L) to increase cellular glucose uptake, and increase citric acid cycle rate. The other explanation is that core cells might be dying due to lack of oxygen which is resulting in low ATP levels. In that case, I will increase the PDMS/porous membrane stack size so more vascular channels can be made (basically, a taller 3D mesh network) that can sustain the core spheroid cells. Even the assays might be misleading if they are unable to measure cellular activity in the core cells. In that case, more sophisticated immunostaining methods, like fluorescent-tagged ELISA assays, may have to be employed.

**Alternatives**

If iPSCs are not available, then hESCs will be used and differentiated according to the protocol discussed previously. Additionally, if available, human umbilical vein endothelial cells (HUVECs) can be used by adding to the bio-ink. This will promote vascularization (81), leading to more vessels that will supply nutrients and oxygen to hepatic core cells. Finally, if available, I will add kupffer and stellate (secondary liver cells) cells that will help improve hepatic cell viability significantly (3,6,10,23).

**Objective 2: Synthesis of Extracellular matrix protein-based bio-ink**

Once an optimum structure has been determined for cell viability from the previous experiment I will use it for subsequent investigations. Using it, I will then investigate the effects of extracellular matrix proteins on hepatic spheroid culture maturity. For this experiment, the alginate structure will be made the same way as in the previous section but the bio-ink will be different. In this case, the bio-ink will be made by adding iPSC or hESC spheroid aggregates to the optimum concentration of sodium alginate (found previously), RGD peptides and varying concentrations of three extracellular matrix proteins: type I collagen (Sigma-Aldrich C5483), fibronectin (Sigma-Aldrich F2006), and laminin (Sigma-Aldrich L4544). A control bio-ink will also be made that will have no ECM proteins (a general saline solution like Ringer’s Lactate solution (Moore Medical) will be used instead). The table below highlights the ECM protein concentrations for the bio-inks that will be made for this experiment.

<table>
<thead>
<tr>
<th>Bio-ink #</th>
<th>Collagen Concentration</th>
<th>Fibronectin Concentration</th>
<th>Laminin Concentration</th>
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<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
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<tr>
<td>3</td>
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<td>High</td>
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</tr>
<tr>
<td>6</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>7 (control)</td>
<td>-</td>
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</table>

Table 2 briefly summarizes the ECM protein concentration in the bio-inks for this experiment.  
**High = 20% Low = 5%**
Each of the seven bio-inks will be loaded into the 3D printer the same way as in the previous section and will be printed on seven reproductions of the optimum alginate structure determined previously. I will connect the structures to a cell perfusion system (same equipment/media as in previous experiment) and culture for 2 days. Subsequently, I will perform albumin and urea assays (both essential functions of hepatocytes) using kits purchased from Sigma-Aldrich. Also PCR will be performed to check the RNA levels of the following genes considered essential for liver function (15): AFP, ALB, HNF-4, TAT, CK-18, CYP3A4, and GAPDH. The PCR reagents will be purchased in a PCR kit (Sigma-Aldrich) and the tests will be done in a PikoReal PCR system (Thermo Scientific). The gel electrophoresis results will be compared to values in the literature.

**Expected Results**

Since fibronectin is present in large amounts in adult liver extracellular matrix (82), I can assumed that the bio-ink with high fibronectin concentration will result in a genetic profile and urea/albmin levels very similar to primary human hepatocytes. Similarly, collagen and fibronectin have been found in comparable levels in fetal liver ECM (82), so it can be expected that the bio-ink with high fibronectin and collagen levels will also lead to more mature hepatic spheroids with a comparable genetic profile and urea/albmin levels to PHHs. The control bio-ink should have no effect on maturity.

**Statistical Analysis**

In this experiment, the null hypothesis is: the extracellular matrix (ECM) proteins have no effect on hepatic spheroid maturity. The alternate hypothesis then is: the ECM proteins do have an effect on hepatic spheroid maturity. Since continuous data of discrete samples will be collected from the assays and PCR, an analysis of variance (ANOVA) can be performed using Statistical Analysis Software (SAS). I will use the data for the control and experimental groups and if there is a significant effect, the P value (calculated from the ANOVA F value) will be less than 0.05, and the null hypothesis can be rejected.

**Limitations and weakness of method**

The exact concentrations of ECM proteins have to be determined experimentally as the literature does not state exact values (6). This means that it will be very difficult to get high gene maturity gene expression initially. It also means that I will have to modify ECM concentrations in the bio-ink several times and expect a significant effect every time. Also, upon addition of extracellular matrix proteins, the alginate concentration may change and that can be problematic as it may have to be tweaked.

**Potential errors and complications**

Again, a multitude of factors can lead to unpredictable and erroneous results. The optimum concentration of alginate determined in the previous experiment, might change upon the addition of ECM proteins. In that case, that optimal concentration of alginate may have to be tweaked (increased or decreased). The spheroid size could also be changed, so instead of 500 cells/spheroid I will use 1000 cells/spheroid as a higher cell density shows higher maturity potential (10) or if need be even 250 cells/spheroid. The ECM proteins may not be in the optimum concentration and that will have to be tweaked multiple times. In high enough concentrations, ECM proteins may even dedifferentiate (49) the stem cell-derived hepatocytes and negatively affect the PCR results by repressing hepatic gene expression (49). But in low concentrations, the cells may not be affected at all, so this can be rectified by increasing concentrations. Essentially, there is probably a trade-off between ECM protein concentration and gene maturity so I will have to figure that out by fine-tuning the ECM concentrations. Finally, the addition of extra ECM proteins may hinder microvascular structure flow rates. I will attempt to solve that by increasing the channel size in the optimum alginate structure, determined in the previous experiment, from 20µm to 30-35µm. Again, a trade-off probably exists between channel size and gas exchange rate (that might affect viability by increasing oxygen gradient (80)) and that will have to be taken into consideration. Even, culture time can be increased to 3 days or decreased to 1 day to precisely gauge the effects of ECM proteins if the initial culture time does not result in meaningful results. Finally, when
calculating ANOVAs for gene expression, it may become difficult to get significant results as the standard deviations might vary from study to study and that may affect how I will interpret a statistically significant result.

**Alternative**

Instead of type I collagen, type IV collagen may be used because it is also present in comparable (but smaller) concentrations in liver extracellular matrix (82). Similarly, entactin and proteoglycans may be used instead of fibronectin and laminin as they are also present in developing liver embryos (again, much smaller concentrations) (82) so they may also influence gene expression.

**Objective 3: Exogenous growth factors and CYP maturity**

After an optimum combination of ECM proteins in the bio-ink is identified from the earlier experiment, it will be printed on the optimum alginate microvascular structure (determined in the first experiment). Then the cell culture device will be connected to a cell perfusion system (cell culture conditions will still be the same as for experiment 1) which will expose it to varying amounts of four growth factors: HGF, FGF, BMP, OSM, and Dex. A control cell perfusion media will also be made which will contain no growth factors (a general saline solution like Ringer’s Lactate solution (Moore Medical) will be used).

<table>
<thead>
<tr>
<th>Cell Perfusion Media #</th>
<th>HGF concentration</th>
<th>FGF concentration</th>
<th>BMP concentration</th>
<th>OSM concentration</th>
<th>Dex concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
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<tr>
<td>2</td>
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<tr>
<td>4</td>
<td>Low</td>
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<td>High</td>
<td>High</td>
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<tr>
<td>5 (control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 3 highlights the concentration of growth factors in each cell media.*

- **High = 100ng/ml**
- **Low = 30ng/ml** (6)

Each culture system will be exposed to a cell media for 1 week (same cell perfusion media, physiological conditions, and equipment as previous sections) and then I will do PCR tests (PCR Kit, Sigma-Aldrich) for the following cytochrome P450 genes: CYP3A4, CYP3A7, CYP1A1, and CYP1A2 (enzymes essential for metabolism of most drugs (30,38,58)). The PCR will be done using a PikoReal PCR system (Thermo Scientific) and a PCR kit (Sigma-Aldrich). The gel electrophoresis results will be compared to values in the literature, particularly of primary human hepatocytes (PHHs).

**Expected Results**

Since the exact effect of growth factors is not particularly known, it is hard to predict which cell culture will be best. But, the media with high HGF and high BMP will most likely lead to greater expression of cytochrome P450 gene expression as evidence by Keller et al. (32) and Chen et al. (83). Although it is less likely, but it is possible that cell media with high OSM and Dex may also exhibit increased detoxification enzyme levels.

**Statistical Analysis**

In this experiment, the null hypothesis is: the exogenous growth factors have no effect on cytochrome P450 gene expression. The alternate hypothesis then is that: exogenous growth factors do have an effect on cytochrome P450 gene expression. Since continuous data of discrete samples will be collected from the PCR, an analysis of variance (ANOVA) can be performed using Statistical Analysis Software (SAS). I will use the data for the control and experimental groups and if there is a significant effect, the P value (calculated from the ANOVA F value) will be less than 0.05, and the null hypothesis can be rejected.
Limitations and weakness of method

Again, this experiment faces the same limitations as the previous experiment. Although the effects of growth factors like HGF or BMP are known to cause some effect, the effects of multiple concentrations of ECM on maturity is not properly documented (82). So certain concentrations of HGF/BMP/FGF etc. might reduce cytochrome P450 (CYP) enzyme gene levels and some might increase it significantly. So even if the concentrations of growth factors are fine-tuned, there is no way to accurately predict how the CYP enzyme activities may change. Again, there is probably a trade-off between growth factor concentration and CYP gene expressions and that will be problematic.

Potential errors, complications, and alternatives

A wide variety of errors can result in unexpected results and even complications. For instance, the exogenous growth factors can drastically change the gene expression of the stem cell-derived hepatic spheroids. If tweaking the concentrations has no effect, then I will try different combinations and if that does not work either, I will use a different set of growth factors namely: insulin, FGF2, and EGF (Epidermal growth factor) (6,17,71), that have a similar effect on CYP gene expression. Finally, if none of the aforementioned modifications work, then I will change iPSC to hESCs or if already done so from previous experiments, change hESCs to iPSC. If there are very low mRNA levels for cytochrome P450 genes, then they might not express correctly or they might need more time, so I will probably increase culture time to 1.5-2 weeks and perform PCR tests again.

Acknowledgements

I wish to extend my sincerest gratitude to Dr. Michael Sefton for his invaluable advice in understanding the complexities of tissue engineering and the intricacies of technological limitations. I would also like to thank Dr. Andrew Baines for being patient with me and for answering my numerous inquiries.

Legal Disclaimer

Human embryonic stem cells will be purchased from the Sick Kids Embryonic Stem Cell Facility and all research involving such cells will be in compliance with Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2), Chapter 12, section F (2014). All other cell lines and any controversial reagents will be bought ethical and will comply with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2014) outlined by the Government of Canada Panel on Research Ethics.

Proposed Budget

<table>
<thead>
<tr>
<th>Item</th>
<th>Predicted Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal Investigator (Dr. Sami Ul Haq)</td>
<td>1 year ($242,000) * 2 = $484,000</td>
</tr>
<tr>
<td>Graduate Students (2)</td>
<td>$50,000</td>
</tr>
<tr>
<td>Lab technicians</td>
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</tr>
<tr>
<td>Lab equipment</td>
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<tr>
<td>Laboratory set-up + rental</td>
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<tr>
<td>Reagents and materials</td>
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<tr>
<td>Miscellaneous expenses</td>
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<tr>
<td>Total</td>
<td>$894,000 (to be taken with a grain of salt)</td>
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Proposed Timeline

1st month: Set up of laboratory and purchase of reagents and equipment

2 – 12 months: Development of 3D printer microvascular structure (Objective 1)

13 - 18 months: Develop extracellular matrix protein-based bio-ink (Objective 2)

19 - 26 months: Formulate optimum exogenous factor combinations (Objective 3)

27 – 30 months: Publication of results (Nature, Cell, PNAS)

References


(28) New Approaches in the Differentiation of Human Embryonic Stem Cells and Induced Pluripotent Stem Cells toward Hepatocytes. Stem Cell Reviews (3): 748.


(38) Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. Expert Opinion on Drug Metabolism & Toxicology. 2010 06/01;6(6):733-746.


Sami Ul Haq

Grant Proposal: A Novel Method to Culture Hepatocytes


(58) Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. Expert Opinion on Drug Metabolism & Toxicology. 2010 06/01;6(6):733-746.


