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The Response of 3D Printed Breast Cancer Cells to Chemotherapy Drugs

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ABSTRACT

3D culture of breast cancer cells gives researchers a better understanding of how cells behave in the human body and a better representation of their response to cancer therapy compared to 2D. Breast cancer cells also interact with other cell types in the body, including endothelial cells in the blood vessels. While endothelial cells have been co-cultured with breast cells to study tissue growth/development, cancer metastasis, and angiogenesis, 3D breast structures have not been used to study cancer metabolism- accomplished with this project. The purpose of this experiment is to determine how cell metabolism changes in A) 2D vs. 3D culture; B) monoculture vs. co-culture; and C) no treatment vs. Paclitaxel treatment. I hypothesize that Paclitaxel will have a greater effect on cell metabolism when breast cells are co-cultured with endothelial cells. Data was collected with three varying techniques, 2D Monolayer Printing, Manual 3D Hydrogel, and 3D Printing. The 3D Printed data displayed an increased efficiency between trials of the same cell type and could be used to develop new drugs in a low cost and efficient manner. The most important finding is that with both 3D techniques, the absorbance had a further decrease for the Human Umbilical Vein Endothelial Cells (HUVEC) co-culture with cancerous cells than the 2D data. The metabolism of cancer cells is approximately eight times greater than normal cells, so decreasing the absorbance in cancer cells over noncancerous cells has a greater impact. 3D cell structures are essential to create co-cultures to study and develop drugs with cell interactions using endothelial cells.

Introduction

There are many varying types of chemotherapy drugs, with the four main types being: antimetabolites, plant alkaloids, alkylating agents, and antitumor antibiotics.[1] Antimetabolites are chemotherapy drugs which act as mimic proteins the cancer cell needs to survive and when the cell consumes the mimic, it starves.[2] Plant alkaloids block the ability for the cancer cell to grow and reproduce.[2] Alkylating agents are chemotherapy drugs which bind directly onto the DNA and kill cancer cells at certain stages of the life cycle.[3] Lastly, antitumor antibiotics bind to DNA and stop-RNA from, so cancer cells are not able to reproduce.[3]

Paclitaxel, is an anti-cancer chemotherapy drug which can be classified as a plant alkaloid, and more specifically as a 'antimicrotubule agent' and 'taxane'.[4] Paclitaxel is used in the treatment of cancers such as breast, ovarian, prostate, bladder, esophageal, melanoma, and other categories of solid tumor cancer.[4] Plant alkaloids are 'cell-cycle specific', so they attack the cancer cell during various phases of its division.[3] Paclitaxel also falls under the label of antimicrotubule agent, which works to inhibit the microtubule structures within the cancer cell.[4] Microtubules are an essential part of the cell's framework for dividing and reproducing, so inhibition of these results in death of the cell.[5]

Endothelial cells are cells which line the inside surface of blood vessels as well as lymphatic vessels.[2] This forms a communication system between circulating blood or lymph in the lumen and the surrounding vessel wall. Endothelial cells which are in direct contact with blood are called vascular endothelial cells while those in contact with lymph are called lymphatic endothelial cells.[5] Vascular endothelial cells are located along the circulatory system,

and have interesting functions in biology such as hemostasis, fluid filtration, and blood vessel tone.[6] Endothelial cells are also involved in angiogenesis, inflammation, blood clotting, vasoconstriction, as well as barrier function.[6]

Over the past few years, there has been much growth in the study of key molecules in targeting tumor angiogenesis for human therapy. Current research efforts are concentrated on analyzing the origin and functional properties of endothelial cells in various tumors.[5] An in-depth understanding of the mechanisms regulating the properties and function of endothelial cells during tumorigenesis is resulting in the development of many bold approaches for cancer treatment, and the involvement of endothelial cells in treatments such as chemotherapy.

Endothelial cells have been highly studied in cancer with respect to angiogenesis and metastasis, but not in respect to cancer metabolism.[5] The MDA-MB-231 cell line is an epithelial, human breast cell line which was isolated from a 51 year-old woman suffering from a metastatic mammary adenocarcinoma, and is one of the most common breast cancer cell lines in medical laboratories for research.[7] MDA-MB-231 is a highly invasive and aggressive triple-negative breast cancer cell line, and its invasiveness is controlled by proteolytic degradation of the extracellular matrix.[7]

Triple negative breast cancer is an extremely aggressive form of breast cancer with very limited options for treatment. Comprehending the molecular establishment of triple-negative breast cancer is essential for the development of new drugs.[7] This is why many scientific studies on agents for breast cancer are conducted with the MDA-MB-231 cell line.

The MCF10A human mammary epithelial cell line is used commonly *'in vitro'* for studying breast cell function and transformation.[7] This is a non-tumorigenic epithelial cell line and was derived from adherent cells in a population.[7] MCF10A cells exhibit 3D growth in collagen, and also form domes in confluent cultures.[7] This cell line is responsive to insulin, glucocorticoids, cholera endotoxin, and EGF and they also express breast specific antigens.[7]

HUVEC, or human umbilical vein endothelial cells, are cells which come from the endothelium of veins from the human umbilical cord.[7] They are mainly used in labs for the study of function as well as pathology of endothelial cells such as tumor-associated angiogenesis, oxidative stress, hypoxia, inflammation related pathways, and cardiovascular-related complications.[7] They are used for their simple technique of isolation from umbilical cords after childbirth and relatively low costs, and can easily proliferate in the laboratory.[7]

The specifics of the cellular microenvironment are just as crucial as biochemistry itself in managing cell behavior. Alginate hydrogel is a very popular biological material in 3D bioprinting, mostly extrusion-based printing.[6] While, the alginate material system is the most popular material system in use, however, there are some concerns over the outcomes of alginate studies. Alginate systems are useful for technology development purposes but are unlikely to have any long-term negative impact because of the poor cellular adhesion that has been observed.[6] Also, cells cannot degrade the surrounding alginate gel matrix, so they remain located specifically in their original deposited position during the entire culture period, limiting their capacity to proliferate.[6]

Collagen has been used in many tissue-developing applications for skin, bone and cartilage because of its biocompatibility and low antigenicity, but its use in 3D-bioprinting has limitations.[6] Collagen is most often used in inkjet bioprinting, which prints materials with low viscosity but is rarely used in extrusion bioprinting. In extrusion bioprinting, gelatin rather than collagen, has often been used as a bioink because its property fits the requirement mentioned above and it gels at room temperature.[6] Very recent scientific studies have proven that using a hydrogel with collagen and alginate had a clear and stable structure with connected channels and networks created by the cells.[6] The fibers of the 3D printing constructs were uniform and smooth.The thickness could be controlled by regulating the thickness of one layer or printing different layers.The printing material has mechanical properties to self support for specific layer-by-layer fabrication and is fitting to be used in extrusion bioprinting as done in this project.

Breast cancer is the second most common cancer diagnosed in women in the United States.[1] Every two minutes a woman is diagnosed with breast cancer and one woman dies of breast cancer every thirteen minutes.[1] Survival rates are increasing, and deaths are declining due to key factors such as new methods of treatment along with a better understanding of the disease through modern diagnoses and research.[1]

3D printing has the ability to revolutionize medicine and can aid millions suffering from diseases such as cancer worldwide, since 3D printed tissue models give cancer researchers a better understanding of how cells behave in the human body and a better representation of how they respond to treatment such as chemotherapy drugs.[8] For example, a recent study showed that 3D printed cells demonstrated a higher resistance to chemical treatment than the same cancer cells grown in 2D.[8] Monolayered cell culture with enhanced drug agents have previously failed to mimic *in vivo* tumor characteristics.[8] It has been demonstrated that 3D Printing showed enhanced resistance to anti-tumor drugs compared with a 2D planar cell culture.[8]

Additionally, 90% of preclinical drugs fail when introduced to the human body, so 3D printing provides more data that would decrease drug failures.[8] Printing 3D cells allows researchers to manipulate the tumor microenvironment to understand cancer better and increases precision and lessens room for error which adds to costs.[8]

Chemotherapy is a method of treating cancer which uses drugs to kill the cancer cells.[1] Chemotherapy is meant to target cells that grow and divide at a fast pace, which cancer cells do.[1] Chemotherapy drugs impede the cancer's ability to reproduce and divide.[1] Chemotherapy drugs can trigger a number of outcomes in cells including: the ability to prevent mitosis, target the cancer cell's source of food, start apoptosis, prevent the growth of blood vessels which supply the tumor.[1] This project will use a chemotherapy drug called Paclitaxel or Taxol. Paclitaxel is used in the treatment of cancers such as breast, ovarian, prostate, bladder, esophageal, melanoma, and other categories of solid tumor cancer.[1] It works to inhibit the microtubule structures within the cancer cell, resulting in death of the cell.[1]

The 3D bioprinting breast cancer model is made of endothelial cells, either normal or cancerous breast epithelial cells, and a collagen alginate hydrogel.[2]

The hypothesis in this experiment is, "Paclitaxel will have a greater effect when cancer cells are co cultured with endothelial cells." The greater effect is further decreasing the glucose absorbance as glucose starvation activates a metabolic and signaling amplification loop that leads to cancer cell death because of the toxic accumulation of oxygen species.

Methodology

3D Printing

First, the bioprinter was UV treated for at least an hour. To do this, it was exposed to UV light. Next, the collagen-alginate matrix was prepared. To do this, 0.5mg/ml Collagen was neutralized with the following materials: 1N NaOH, water and 10x PBS. Collagen now has a pH of 7.4. Then, a solution of 3% sodium alginate in 0.9% NaCl solution was made. Next, low viscosity alginate salt was added to medium viscosity alginate salt in a 3:1 ratio and the salts were mixed in a 0.9% sodium chloride solution(sodium chloride salt in distilled water). It takes over 4 hours to dissolve the alginate at 37-40 degrees Celsius with continuous stirring so make it in advance (alginate is stable so it can be made days in advance). Then, a P100 ~80% confluent dish of breast cells was washed with 10ml warm 1x PBS, aspirate followed by 2ml warm trypsin. When cells were rounded, trypsin was aspirated. Then 5 ml of DMEM 1x were added and counted to 5 million cells/ml. Next, the matrix cell mixture was created as described above by mixing in a P35 dish and was loaded onto a 10ml sterile syringe. Then, the 3% CaCl2 solution was loaded in another sterile syringe. Next, the coaxial needles were assembled in the printer and made sure there was no clogging. Also, a glass slide was prepared with equally sized kimwipe, drenched with 3% CaCl2. To create the grid structure, 6 intersecting layers of the breast cell/ HUVECs were printed at vertical increments of 0.015 cm by forced extrusion at a speed of 3 mm/sec (using the code). Next, the grid structure was transferred to 3% CaCl2 in a P35 dish and it was allowed to crosslink completely for 2min. Then, the grid structure was transferred to 1X DMEM in a P35 dish with 2% CaCl2 Incubated at 37 C for 24hrs. After equilibrating for 24 hrs, it was replaced with DMEM1x with no phenol red. Then, 200nM paclitaxel was added in the appropriate test wells (at least 3 replicates for each breast cell type). Next, it was incubated

for 48hrs. After 48hrs, the media was collected into another 96 well plate and 5% (v/v) Resazurin was added; it then incubated at 37 degrees C for 2hrs. Proceeded with L/D assay for the cells in the 96-well plate immediately. Finally, absorbance was read at 570 nm using plate reader, plot samples (+/-) Paclitaxel.

To measure Cell metabolic activity using Resazurin in extruded/printed structures

First, 2D cells were printed as described above in a 96-well plate (if testing with a monolayer, seed ~5000 cells per well in 96-well plates because over-confluent cells can give varying and inconsistent absorbance values). After equilibrating for 24 hrs, cells were replaced with DMEM1x with no phenol red. Next, 200nM paclitaxel was added in the appropriate test wells (at least 3 replicates for each breast cell type). Then, the mixture incubated for 48hrs. After 48hrs, the media was collected into another 96 well plate and 5% (v/v) Resazurin was added; the mixture then incubated at 37 degrees Celsius for 2 more hrs. Then, proceeded with L/D assay for the cells in the 96-well plate immediately. Finally, absorbance was read at 570 nm using plate reader, plot samples (+/-) Paclitaxel.

Results

Abbreviations or short names used to represent different 'Cell Types' in below tables providing results -

Key	All samples in three replicas
Coll/Alg	Only material
HUVEC	mono-culture
MCF10A	mono-culture
MDA-MB-231	mono-culture
HUVEC+10A	co-culture
HUVEC+MDA-MB-231	co-culture
Coll/Alg	Only material

Extrusion by Pipetting:

Table 1: Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of MCF 10A non-cancerous cells in mono-culture vs. co-culture with endothelial cells (250,000 cells for each).

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

	No treatment				200 nM Paclitaxel			
Cell Type	Coll/Alg	HUVEC	MCF10A	HUVEC+MCF10A	Coll/Alg	HUVEC	MCF10A	HUVEC + MCF10A
Absorbance Value at 570nM	0.291	1.115	1.145	1.019	0.329	0.521	0.627	0.893
Absorbance Value at 570nM	0.249	1.722	1.904	1.663	0.3	0.417	0.532	0.8
Absorbance Value at 570nM	0.147	1.435	1.503	1.475	0.394	0.451	0.408	0.784

Table 2: Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of MDA-MB-231 cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each).

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

	No treatment				200nM Paclitaxel			
Cell Type	Coll/Alg	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231	Coll/Alg	HUVEC	MDA-MB-231	HUVEC+ MDA-MB-231
Absorbance Value at 570nM	0.261	1.421	1.462	1.559	0.542	0.432	0.348	0.762
Absorbance Value at 570nM	0.199	1.191	1.425	1.448	0.328	0.466	0.583	0.704
Absorbance Value at 570nM	0.247	1.324	0.922	1.444	0.415	0.429	0.334	0.721

Table 3: Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of non-cancerous cells mono-cultured vs. co-cultured with endothelial cells (450,000 for each).

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment at higher cell count

	NO Treatment				200nM Paclitaxel			
Cell Type	Coll/Alg	HUVEC	MCF10A	HUVEC+MCF10A	Coll/Alg	HUVEC	MCF10A	HUVEC+MCF10A
Absorbance Value at 570nM	0.274	1.337	1.382	1.509	0.047	0.699	0.656	0.682
Absorbance Value at 570nM	0.241	1.218	1.679	1.752	0.238	0.466	0.419	0.69
Absorbance Value at 570nM	0.265	1.47	1.725	1.726	0.119	0.539	0.499	0.82

Table 4: Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of cancerous cells mono-cultured vs. co-cultured with endothelial cells (450,000 for each).

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment at higher cell count

	No Treatment				200nM Paclitaxel			
Cell Type	Only Coll/Alg	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231	Only Coll/Alg	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231
Absorbance Value at 570nM	0.274	1.337	1.609	1.182	0.047	0.699	0.566	0.773
Absorbance Value at 570nM	0.241	1.218	1.698	1.802	0.238	0.466	0.502	0.449
Absorbance Value at 570nM	0.265	1.47	1.732	2.049	0.119	0.539	0.628	0.449

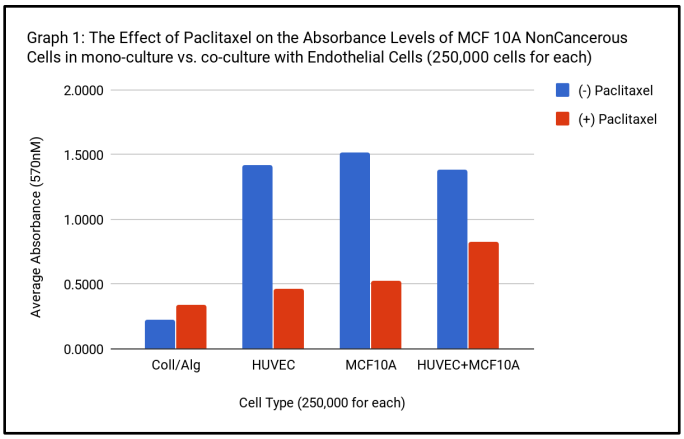

Figure 1: Graph1 - Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of MCF 10A non-cancerous cells in mono-culture vs. co-culture with endothelial cells (250,000 cells for each).

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

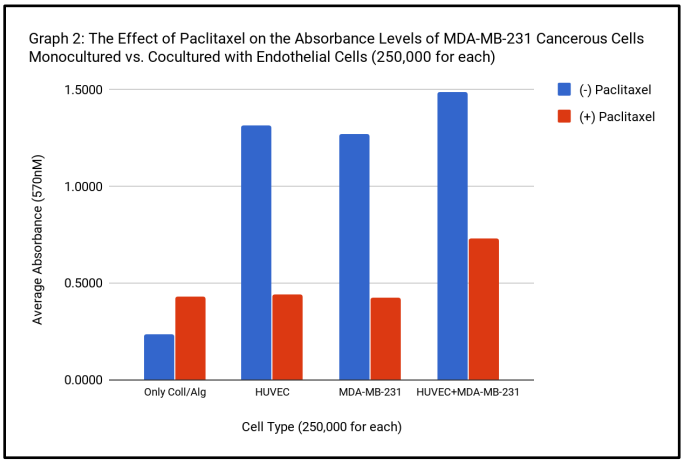

Figure 2: Graph 2 - Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of MDA-MB-231 cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each).

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

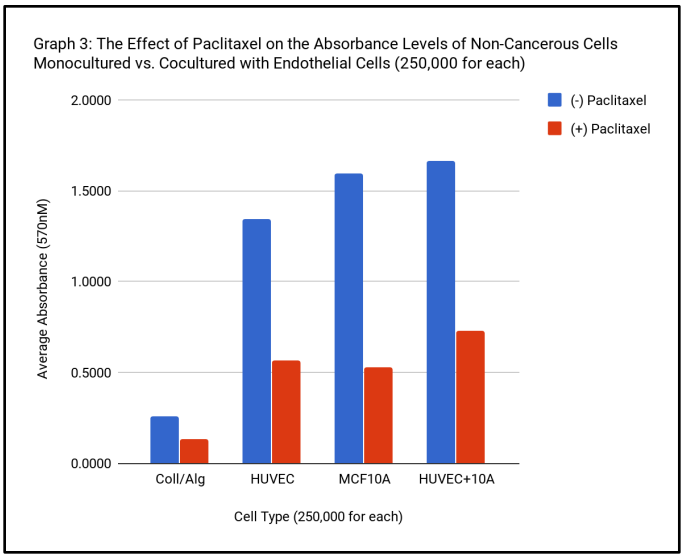

Figure 3: Graph 3 - Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of non-cancerous cells mono-cultured vs. co-cultured with endothelial cells (450,000 for each).

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment at higher cell count

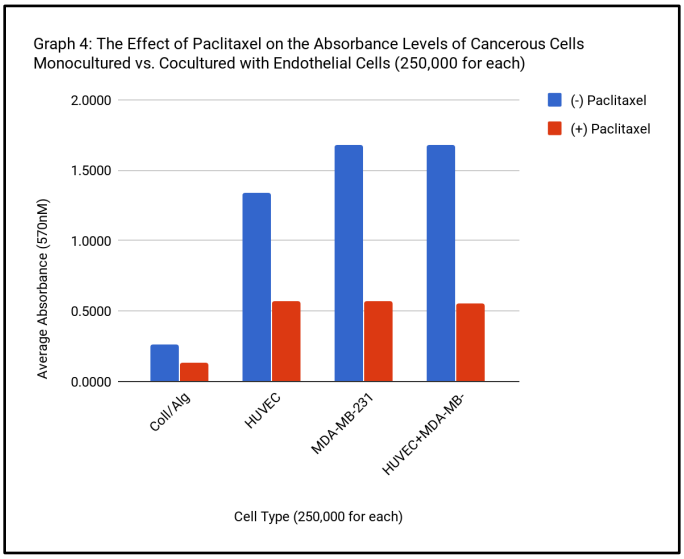

Figure 4: Graph 4 - Extrusion by pipetting - The effect of Paclitaxel on the absorbance levels of cancerous cells mono-cultured vs. co-cultured with endothelial cells (450,000 for each).

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment at higher cell count

2D Monolayer Data:

Table 5: 2D Monolayer - The effect of Paclitaxel on the absorbance levels of non-cancerous cells and cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

	NO TREATMENT				PACLITAXEL-200nM			
Cell Type	DMEM	HUVEC	MCF10A	HUVEC+10A	DMEM	HUVEC	MCF10A	HUVEC+10A
Absorbance Value at 570nM	0.175	2.009	2.727	2.618	0.179	0.479	0.476	0.988
Absorbance Value at 570nM	0.109	2.228	2.689	2.758	0.182	0.521	0.681	1.018
Absorbance Value at 570nM	0.164	2.146	2.561	2.906	0.167	0.593	0.525	0.974

Table 6: 2D Monolayer - The effect of Paclitaxel on the absorbance levels of non-cancerous cells and cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

	NO TREATMENT				PACLITAXEL-200nM			
Cell Type	DMEM	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231	DMEM	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231
Absorbance Value at 570nM	0.175	2.009	2.966	2.869	0.179	0.479	0.649	1.344
Absorbance Value at 570nM	0.109	2.228	3.017	2.986	0.182	0.521	0.623	1.428
Absorbance Value at 570nM	0.164	2.146	2.973	3.099	0.167	0.593	0.762	1.349

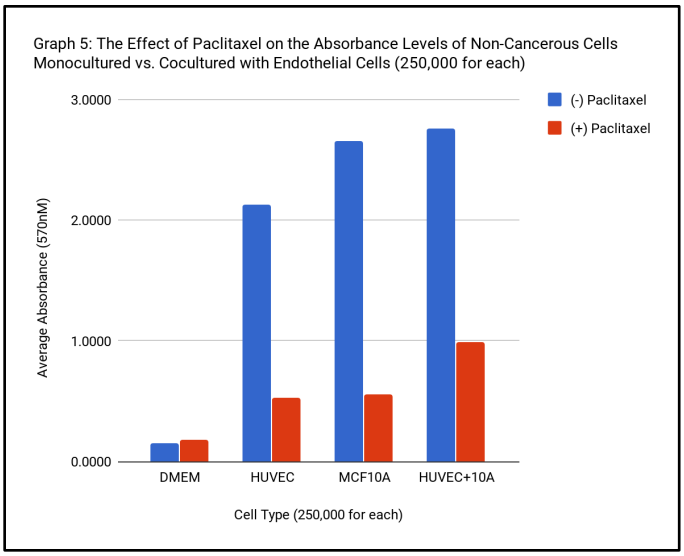

Figure 5: Graph 5 - 2D Monolayer - The effect of Paclitaxel on the absorbance levels of non-cancerous cells and cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

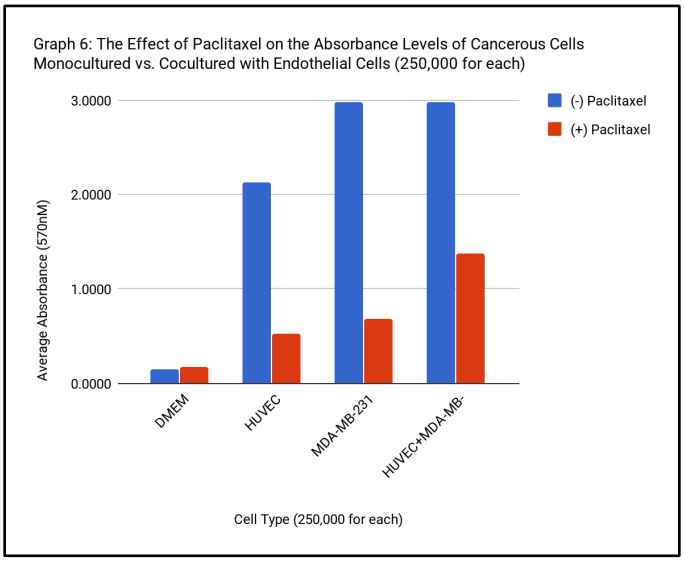

Figure 6: Graph 6 - 2D Monolayer - The effect of Paclitaxel on the absorbance levels of non-cancerous cells and cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)
Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

3D Printed Data:

Table 7: 3D Printed - The effect of Paclitaxel on the absorbance levels of non-cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

	NO treatment				200nM Paclitaxel			
Cell Type	Coll/Alg	HUVEC	MCF10A	HUVEC+MCF10A	Coll/Alg	HUVEC	MCF10A	HUVEC+MCF10A
Absorbance Value at 570nM	0.269	2.328	2.438	2.132	0.429	0.679	0.782	1.074
Absorbance Value at 570nM	0.248	1.983	2.195	2.775	0.372	0.732	0.948	0.896
Absorbance Value at 570nM	0.317	2.182	2.303	2.047	0.419	0.668	0.857	0.971

Table 8: 3D Printed - The effect of Paclitaxel on the absorbance levels of cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

	No treatment				200nM Paclitaxel			
Cell Type	Only Coll/Alg	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231	Only Coll/Alg	HUVEC	MDA-MB-231	HUVEC+MDA-MB-231
Absorbance Value at 570nM	0.347	1.965	2.642	2.522	0.458	0.562	0.934	1.267
Absorbance Value at 570nM	0.39	2.192	2.827	2.383	0.375	0.666	0.782	1.093
Absorbance Value at 570nM	0.328	2.138	2.329	2.465	0.462	0.713	1.033	1.227

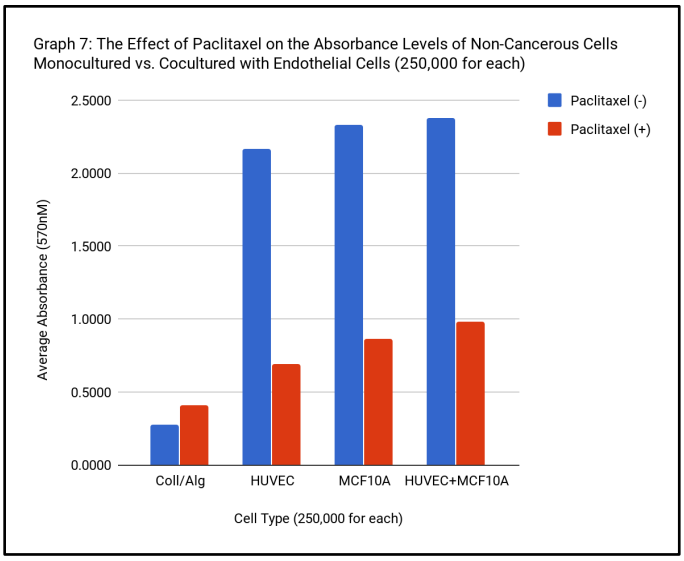

Figure 7: Graph 7 - 3D Printed - The effect of Paclitaxel on the absorbance levels of non-cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MCF10A, & HUVEC+MCF10A with Paclitaxel treatment

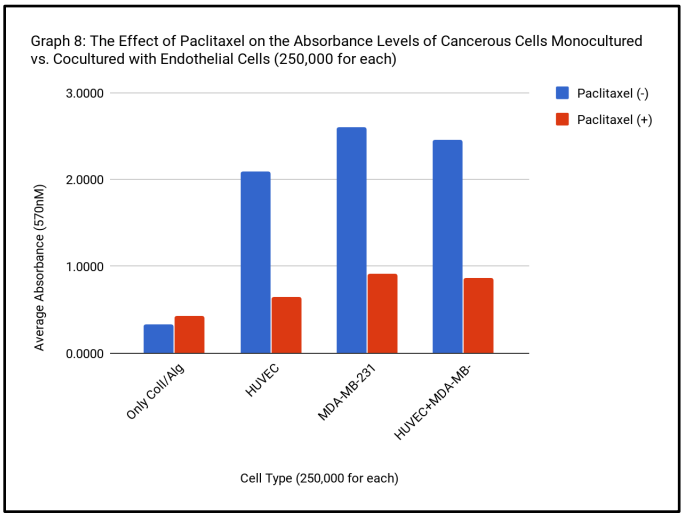

Figure 8: Graph 8 - 3D Printed - The effect of Paclitaxel on the absorbance levels of cancerous cells mono-cultured vs. co-cultured with endothelial cells (250,000 for each)

Decreased absorbance for HUVEC, MDA-MB-231, & HUVEC+MDA-MB-231 with Paclitaxel treatment

Discussion

The data has conclusively shown a very drastic difference in the absorbance level of all cells with the incorporation of the Paclitaxel drug. The hypothesis stated that Paclitaxel will have a greater effect when breast cancer cells are co-cultured endothelial cells, which was not clearly supported as it had a less effect in 2D and the same level of absorbance in 3D. Endothelial cells are extremely applicable in the area of metastatic tumors, but have been ignored, because of

the absence of 3D physiologic layouts. Most cancer researchers test cells alone, which does not represent a realistic tumor in the human body. Having at least two cells with a co-culture increases the tumor heterogeneity and the practical stage of tumors which is truly in the human body. In tumors, to stimulate the true environment, more than one cell is necessary and having the endothelial cell provides an extra layer of protection.

3D tumor models with microenvironmental traits of interactions cell to cell and cell to matrix *in vivo* are becoming essential for drug testing and studying tumors. Compared with the 2D monoculture, the additional dimension of the 3D Printed culture leads to differences in functions of the cell including proliferation, morphology, and gene/protein expression.

This is demonstrated in Tables 5-6 (2D Monolayer testing), Tables 1-4 (Manual 3D Pipetting), as well as Tables 7-8 (3D Printing), while in detail each technique displayed varying characteristics. In the Manual 3D Pipetting test in Tables 1 & 2 with 250,000 cells and Tables 3 & 4 with 450,000 cells each, many interesting discoveries were made. First, the non-cancerous MCF-10A cells displayed a significant decrease in absorbance value, with the dose of Paclitaxel as well as the same occurrence with the co-culture. MCF10A with Paclitaxel: 0.5223, MCF10A alone: 1.52, MCF10A co-cultured with Paclitaxel: 0.83, MCF10A co-cultured without Paclitaxel: 1.39. The numbers remained the same for the control of only the collagen/alginate material. The fact that the co-culture of HUVEC and MCF10A lowered the absorbance value, set the tone for the rest of the experiments and for the baseline of cancerous cells. MCF10A is the founder cell line of a progressively aggressive family of breast cancer cell lines. It is used as a control in this experiment, with the co-culture of endothelial cells as it has been used before to see the role of the EGFR-DEL mutation in promoting gefitinib resistance in breast cancer cells.

As for the cancerous MDA-MB-231 cells, similar information was concluded as well, with the Paclitaxel reducing the absorbance value more than the MCF10A cells. Once again, the co-culture of HUVEC with the cancerous cells also fascinatingly showed a substantial decrease in absorbance value. MDA-MB-231 alone: 1.27, MDA-MB-231 with Paclitaxel: 0.422, MDA-MB-231 co-culture: 1.48, MDA-MB-231 co-culture with Paclitaxel: 0.73. What stood out in the cancerous cell testing of the pipette experiment is that the paclitaxel had a greater effect, further decreasing the absorbance of the cancer cells both mono-cultured and co-cultured than with the Monolayer experiment. Allowing replication is a major cancer trademark, however tumor cell proliferation in a 2D Monolayer is known to be hindered by the growth surface area. In the 3D culture environment, MDA-MB-231 displays an endothelial morphology, as it is distinguished by its phenotype, with projections of cell colonies, moreover the use of the co-culture measuring cellular metabolism is essential to the study of cancer as high rates of glucose metabolism have been effectively used to facilitate tumor imaging.

The Monolayered 2D printing data in Tables 5-6 was not as consistent as the two 3D experiments. Studies indicate that endothelial cells go through tubular morphogenesis in a 3D matrix and not in 2D conditions.[8] Interestingly, in the monolayer, the co-culture with HUVEC and noncancerous MCF10A cells showed a greater decrease in absorbance value as compared to noncancerous cells alone- both with Paclitaxel. MCF10A alone with Paclitaxel: decrease of 2.1, MCF10A co-culture with Paclitaxel: decrease of 1.9

For the third and final test, 3D Printing the cells, Tables 7-8 indicate the results were in the same category, with a sizable difference between the tests with Paclitaxel and without, and the results similar with the co-culture and monoculture. The biggest breakthrough to take from the three techniques of collecting data, is that with both 3D techniques(Hydrogel and 3D Printing), the absorbance value had a further decrease in absorbance values for the HUVEC co-culture with the cancerous cells than the Monolayer data. This is because the 2D cell cultures lack features such as the extracellular matrix, metabolic demand, and effects of the tumor microenvironment. The 3D structure additionally acts as a form of protection for the cells.

In cancer cells, measuring glucose uptake can monitor the overexpression of glucose transporters or identify glucose transporter inhibitors. Glucose is essential for tumors, being the tumor's main energy source. If enough glucose is not provided, the tumor will either die or become exceptionally weak. Breast cancer cells use most of their glucose in an efficient manner, and require great amounts of glucose, thus large amounts of glucose transporters on the membrane. By co-culturing with endothelial cells and showing a decrease in cellular metabolism, it is seen to be

inhibiting the cancer cell's ability to survive. Huntsman Cancer published in the journal *Proceedings of the National Academy of Sciences* said, "It's been known since 1923 that tumor cells use a lot more glucose than normal cells.[9] Glucose starvation, depriving cancer cells of glucose, activates a metabolic and signaling amplification loop that leads to cancer cell death because of the toxic accumulation of oxygen species.[9] Drs. Rainer Klement and Ulrike Kammerer state "Increased glucose flux and metabolism promotes several hallmarks of cancer such as excessive proliferation, anti-apoptotic signaling, cell cycle progression and angiogenesis".[9] The metabolism of cancer is approximately eight times greater than the metabolism of normal cells, so by decreasing the absorbance in cancer cells greater than non-cancerous cells it has a great impact.

Every test (Monolayer 2D Printing, 3D Pipetting, and 3D Printing) had at least three trials and all three types of tests described above were definitely repeatable. Between trials, the highest difference between cells of the same trial was a mere 0.6nM, showing the accuracy of the results. For the extrusion by pipetting, it was known beforehand that pipettes allow for measuring liquids very precisely, by relating pipette volumes to mass measurements. Disposable tips were used after each test or when taken out of the sterile environment. All pipettes were calibrated and checked regularly for the manual pipetting test as well. All tips were filled, emptied, then refilled to reassure for no errors when transferring samples to other containers. Proper technique was used by aspirating and ejecting in a very smooth motion to ensure accurate results. The 3D Printing method has a written code with a specific velocity thus extremely minimal to no room for error. Bioprinting controls the shape, size, and interconnectivity of the cells.

This research project can introduce unique ways to potentially aid someone suffering from breast cancer and develop a new technique of treatment for the future of breast cancer treatment. It displays another versatile example of 3D printing in the world of medicine and potentially treat people with untreatable conditions. Scientists can accurately test drugs/observe cells/tissues much more similar to what is in the actual human body- alternate perspective when in 3D than 2D. Monolayered cell culture with enhanced drug agents have previously failed to mimic *in vivo* tumor characteristics. It has been demonstrated that 3D Printing showed enhanced resistance to anti-tumor drugs compared with a 2D planar cell culture. Through this project scientists are able to witness the results of breast cancer cells treated alone as well as with the aid of human endothelial cells to bring an amazing new treatment to the world of medicine to add to the over 3.3 million breast cancer survivors in the United States. Seeing if co-culturing with endothelial cells will enhance the benefits of paclitaxell, the same procedure can be adopted globally as HUVEC is a very low cost and efficient form of endothelial cells, perhaps aiding the hundreds of countries in need for treatment.

The data concluded has been supported by studies done patterning stem cells with human endothelial cells by bioprinting in the study of cardiac regeneration and studying the characterization of human pluripotent stem cells with arterial endothelial cells. The results were within expectations, as it was known that the presence of Paclitaxel would decrease the absorbance value, while co-culturing with endothelial cells did not further decrease metabolism, it produced a better representation of cancer metabolism in the human body. Subsequently, researchers can use endothelial cells and stimulate blood vessels, adding another layer to the tumor in the human body, allowing them to behave as they would in an intact organism. This result indicated the importance of dimensionality on the effectiveness of chemotherapy.

Conclusion

Bioprinting as well as Extrusion by Pipetting and Monolayer printing are excellent methods for determining the comparison between Paclitaxel's effectiveness on MCF10A, MDB-DB-231, and co-cultures between both, with HUVEC cells. This experiment shows the effectiveness in 3D printing to reduce the risk of error, increase efficiency, as well as accuracy. Both types of 3D and 2D data shows that co-cultures certainly have a great impact, but not continuously greater than Paclitaxel alone. This shows that the hypothesis of Paclitaxel having a greater effect with co-culturing is not always true, while it does add perspective to the model to benefit the researchers. co-cultures are extremely relevant for chemotherapy research as they provide an increased representative human *in vivo* quality as cell-to-cell interactions can be monitored. The endothelial co-culture data displayed a key pathway to aid in cancer initiation and progression.

3D printing can assemble cells with altered phenotypes, allowing the formation of 3D *in vitro* models with heterogeneous cells to then formulate a heterogeneous tumor microenvironment. This project may also have vast implementation in the inquiry of tumor heterogeneity. The data from all trials of all three techniques stated above indicates that Paclitaxel has about an equal effect for the majority of the monocultures and co-cultures for cancerous cells with a colossal decline in absorbance value without therapy and with. The biggest breakthrough is that with both 3D techniques(Hydrogel and 3D Printing), the absorbance value had a further decrease in absorbance value for the HUVEC co-culture with the cancerous cells than the Monolayer data. This data indicates that Paclitaxel, a plant alkaloid, 'antimicrotubule agent' and 'taxane' should be used in the treatment of additional cancers such as ovarian, prostate, bladder, esophageal, and melanoma. This experiment shows that paclitaxel's process to inhibit the microtubule structures within the cancer cell should be implemented by these drastic changes in all techniques of testing. Even patients diagnosed with the same disease have different tumor phenotypes, so the complexity of tumor biology must be explored as is done most efficiently with the 3D printed model. By co-culturing with endothelial cells and showing a decrease in cellular metabolism, it is seen to be inhibiting the cancer cell's ability to survive with even a small decrease in absorbance. The metabolism of cancer is approximately eight times greater than the metabolism of normal cells, so by decreasing the absorbance in cancer cells to a level greater than noncancerous cells causes an increased impact. The printed 3D models replicate tumor characteristics enhanced when compared to the 2D planar cell culture models. These 3D biological attributes from the printed tumor models *in vitro* along with the 3D bioprinting technology may help the advancement of 3D tumor biology and as stated lead to vast implementation in the inquiry of tumor heterogeneity.

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