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Does Docosahexaenoic Acid (DHA) Inhibit Metastasis in B16 Cell lines by Altering Cell Adhesion Potentials and Cell Motility?

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Does Docosahexaenoic Acid (DHA) Inhibit Metastasis in B16 Cell Lines by Altering Cell Adhesion Potentials and Cell Motility?

Abstract

Docosahexaenoic acid (DHA, an ω-3 fatty acid) has dramatic effects on many cancer cell lines including the ability to reduce their metastatic capacity. Though the details of the mechanisms of many of these effects remain unclear, DHA may alter the adhesion potential and motility, both of which contribute to the overall metastatic capacity of tumor cells. This research tests the hypothesis that DHA reduces metastatic capacity of cells by altering cell adhesion potentials and cell motility. Two isolation variants of the B16 mouse melanoma cell line will be used, one that is aggressively metastatic (B16-F10) and one that is mildly metastatic (B16-F1). Two independent assays were performed. One used a cell-entrapped fluorescent dye to measure the dose-dependent rate of cell adhesion to a substratum. The other microscopically measured dose-dependent changes in the rate of cell movement across the substratum. The results of the adhesion assays showed dramatic, DHA dose-dependent changes in the adhesion parameter, yet inconclusive data for the motility assay. Treatment with DHA reduces the adhesion percentage of both cell lines however, the effects are more pronounced in the more aggressive cell line. These results support the hypothesis that DHA reduces the metastatic potential of B16 cells by altering their ability to adhere to the substratum.

Introduction

DOCOSAHEXAENOIC ACID

Docosahexaenoic acid (DHA) is an Ω-3 fatty acid that has a broad range of health benefits that has even been described by some as "magical" (Stillwell, 2008). DHA is the most saturated, as well as the longest fatty acid found in biological systems in substantial quantities

(Stillwell, 2008). DHA is produced by algae as well as many cold-water fish and first drew attention when it was noticed that there was dramatically lower rate of thrombotic diseases in Greenland Eskimos, who have a mainly fish-based diet (Stillwell, 2008). Of the many health benefits of DHA, its anticancer properties are some of the least understood. DHA is known to inhibit the growth of cancerous tissue and induce dose dependent apoptosis in many cell lines (Gleissman, Johnsen, & Kogner, 2010). DHA is also known to dramatically reduces the metastatic capacity of many cell types, including the B16 line of mouse melanoma cells (Rose & Connolly, 1999; Iigo et al., 1997). DHA is also known to alter both cell membrane fluidity and cell adhesion potentials (Bourguignon et al., 1995; Collie-Duguid and Wahle; Rockett et al., 2012., 1996;). These alterations may be due to the structure of DHA in that its bent shape makes it an imperfect fit in the cell membrane, which may alter properties in the chain order as well as membrane fluidity (Gleissman et al., 2010).

CANCER

Cancer is a group of deadly diseases that affects as many as one in three people in their lifetime (Mulryan, 2010). There are many different types of cancers each affecting organs and tissues in different ways. However, many of the fundamental mechanisms and progression of the disease remains the same (Mulryan, 2010).

In the simplest sense, cancer can be defined as "an overgrowth of cells that have developed an ability to grow and divide outside the normal controls that usually regulate the growth of cells" (Mulryan, 2010). What this means is that the cell lifecycle is changed. A normal cell goes through its normal function in the G_0 Phase, replicates, and finally goes through apoptosis, or cell death. What occurs in cancerous cells is that instead of apoptosis occurring after replication, these cells keep replicating without cell death and bypass the G_0 phase, which

causes a mass to form known as a neoplasm, or more simply, a tumor (Mulryan, 2010). However, not all neoplasms are cancerous. Neoplasms can be classified into two different categories. Benign tumors are just a localized growth of cells that are slow-growing and pose little threat (Mulryan, 2010). The other classification is malignant, which refers to a neoplasm that exhibits several detrimental characteristics. These characteristics include rapid, uncontrolled tissue growth, variable appearances, and losing the ability of apoptosis, becoming immortal. Arguably, the most detrimental trait that these cells express is the ability to spread out from the original tumor and invade other tissue in the body, also known as metastasis (Mulryan, 2010).

For cells to become cancerous, they must undergo a change that alters them. This causes them to act outside of their intended role and become the malignant tumors described above. The mechanism of change in cells that causes them to become malignant occurs in the nucleus, more specifically in the DNA that provides the basic instructions for the cell over its lifespan (Mulryan, 2010). A cell copies its DNA so that after replication, there is copies of the DNA in both new cells. There is a chance that an error will be made during the copying of the DNA which will cause the DNA in the daughter cell to be slightly different. The chance of DNA being mutated is controlled by a variety of internal, external, controllable, and uncontrollable factors ranging from inherited genetic factors, working condition, lifestyle choices, exposure to chemicals, etc. (Mulryan, 2010). Organisms have safeguards to protect against these mutations, which either induce apoptosis in the abnormal cells, or the immune system of the organism recognizes an abnormal cell and phagocytizes it (Mulryan, 2010). Malignant tumors can form when these abnormal cells bypass the safeguards, take hold, and begin to divide in the body.

More than just one single mutation is needed for cancerous cells to form. They accumulate multiple chromosomal and genetic abnormalities through many generations (Van

Noorden, 1998). Some cells develop these abnormalities over a span more than 10 years, gradually building up these genetic changes, losing regulation on cellular growth and apoptosis, until there is unrestricted growth (Van Noorden, 1998). First, these cells locally divide forming a cluster of identical cells; however, once this mass grows to approximately two millimeters angiogenesis occurs (Van Noorden, 1998). At this point, the cells in the center of the cluster become starved for oxygen and release signals that enlist connective tissue as well as vascular cells to create a blood supply for the cluster (Van Noorden, 1998). The cluster becomes a neoplasm with its own blood supply that provides nutrients as well as an escape route for cancerous cells.

One singular tumor is not usually what causes an organisms death. What makes cancer so deadly is a property of some cancerous cells known as metastasis, which is the ability of a cancerous cell to detach itself from the original tumor, invade the blood stream or the lymphatic system, and then take root somewhere else in the body (Mulryan, 2010). These secondary tumors are just as debilitating as the original and when spread throughout an entire organism, ultimately causes death.

METASTASIS

Several processes go into transforming normal, stationary, stable cells into malicious, invading, malignant, cancerous cells. As noted above, many gene mutations contribute to the development of cancer cells, and even more changes occur in cancerous cells that become metastatic, where they develop the ability to spread throughout the body. Cancer cells are much more undifferentiated than normal cells and lose the ability to perform the function for which they were originally intended (Van Noorden, 1998). As cancer cells become metastatic, they take on new functions and characteristics that make them even more different from the original

noncancerous cell (Van Noorden, 1998). Three major changes occur in a cancerous cell that allow them to go from stationary nonmetastatic cells, to malicious metastatic cells that have free reign to traverse through an organism.

The first major change in the cell involves a change to a specific structure in the cell membrane called the adherence junction (Van Noorden, 1998). There are two types of cellular adhesion interactions; homotypic, involving cancer cells interacting with themselves; and heterotypic, whereby cancer cells interact with host cells (Elvin, 1984). There is a large protein called E-cadherin that extends out of the cell membrane and forms interlocking bonds with E-cadherin proteins on other cells. The internal portion locks into the cells cytoskeleton through catenin proteins (Van Noorden, 1998). This network of connections holds the cells together and allows tissue formation. In metastatic cancer cells, there is a dramatic change to this system. Down regulation of E-cadherin has been associated with reduced metastatic potential in murine cancer cells (Evans, 1992). There are many different changes that can occur that disrupt these interactions. It is seen in many different metastatic cancer cell lines that there is a change in the in the structure of the E-cadherin protein causing it to either be less effective, less abundant or less functional (Van Noorden, 1998). It has also been seen in human gastric carcinoma cells that the loss of E-cadherin is associated with the progress of the disease. There can also be changes in the catenin linking proteins causing them to be nonfunctioning or less abundant (Van Noorden, 1998). This nonfunctioning of the cellular adherence junction makes the connection very weak or nonexistent between cancer cells which contributes to a cancer cell's ability to separate from of the main tumor. This change also increases the overall internal disorganization of the cancer cell, which also results in poorly differentiated cancer cells changing the shape and size of the cancer cell (Van Noorden, 1998).

Changes to the catenin protein, specifically the ß-catenin protein, a cytoskeleton protein not only plays a large role in cellular adhesion, but also plays an integral role in the cells internal signaling network, where it interacts with adenomatous poliposis coli (APC), a protein that causes a cell to induce apoptosis if mutations are discovered (Van Noorden, 1998). The ß-catenin protein alone is able to instruct the cell to divide, however the APC protein can form a complex with it that prevents cell growth (Van Noorden, 1998). When a mutation occurs that disrupts either of these proteins, uncontrolled cancerous cell growth can occur.

The changes in the cell adherence junction greatly weaken the adhesive nature between adjacent cells, however another trait that metastatic cancer cells often develop makes them even more deadly. Integrins link normal cells to proteins in the extracellular matrix (Van Noorden, 1998). These trans-bilayer proteins are also imparted in the cell membrane and have a portion inside the membrane as well as a portion that protrudes out into the matrix (Van Noorden, 1998). A mutation reduces the ability of integrins to form connections with extracellular connective tissue, however the synthesis of a different integrin is greatly increased (Van Noorden, 1998). The mutated integrins produced by the metastatic cancer cells allow the cells to extend into the tissue matrix until it comes in contact with proteins that these new integrins bind to and then pull the metastatic cell around (Van Noorden, 1998). This new ability allows the cells to be motile whereby they are no longer locked in the place that they originated. This also changes the shape of the cells going from cuboid, flattened, or cylindrical shapes, to a sleeker elongated shape (Van Noorden, 1998). They are able to move around freely under their own power, a stark difference from their non-motile predecessors.

Motility gives the cells the ability to move around freely, however most of the time the cells are trapped within a matrix of connective tissue around the organ from which they

originated (Van Noorden, 1998). The final component needed to enable these cancerous cells to spread through the organism is a way to escape the confines of the connective tissue matrix so that they end up the blood stream or the lymphatic system. The cancer cells actually develop a way to digest through the proteins of the connective tissue, allowing them to squeeze through the tiny hole, thus giving them a route to freedom. The metastatic cells use proteases to break down proteins, to disintegrate the final barrier (Van Noorden, 1998). The proteases are synthesized in an inactive form, with a portion that needs to be cleaved off by a different protease in order to expose the protein digesting active site (Van Noorden, 1998). In normal cells, there is a delicate balance of protease and protease inhibitors, however this balance is upset in a metastatic cancer cell and either the amount of protease is increased, or the amount of inhibitor is decreased (Van Noorden, 1998)

Once the metastatic cells have gained access to the blood stream, they circulate until taking hold in another part of the body although, the journey is nowhere near easy. There are natural stresses in the blood stream that make it very difficult for a cell that is not native to the blood stream to survive, and only around one in 10,000 cells is actually able to reach another part of the body (Van Noorden, 1998). Further protection is provided by the immune system, which identifies the cell as cancerous and destroys it (Van Noorden, 1998). Cancerous cells are found in high proportions in the bloodstream of patients that have advanced carcinomas, which is the intermediate step between primary tumors and secondary tumors (Chaffer, 2011). The cancer cells have adapted to travel in clusters as well as surround themselves with platelets as a sort of disguise (Van Noorden, 1998).

The cells reach their final destination when the specific carbohydrate pattern on the cancer cells interact with a selectin, a receptor on endothelial cells, which forms a weak

interaction with the cancer cells (Van Noorden, 1998). Furthermore, this interaction causes the cancer cells to roll along the blood vessel wall until integrin proteins from the cancer cell are able to bond with the new cells that it adhered to. The integrin binding ligand ICAM-1 correlates with increased metastatic potential and can increase the adhesive ability of a cancerous cell (Evans, 1992). There is also a cellular adhesion molecule, VCAM-1, that can act as a receptor for cancer cells; and these molecules tend to be in higher concentration on the endothelial cells and can promote cell adhesion to the endothelium (Evans, 1992). At this point the cancer cell uses the same process to eat, squeeze, and crawl its way into the new host organ and begin the same rapid cell division needed to perpetuate a tumor (Van Noorden, 1998). In essence, for these cells to be maximally metastatic, they must contain poor homotypic adhesive ability so that they can detach from the primary tumor, while having maximal heterotypic adhesiveness to crawl around, as well as adhere to the distant site and begin secondary tumor growth (Elvin, 1984). There is also evidence that in highly metastatic tumors, there could be a decrease in cell-cell and/or cell/matrix adhesion (Cavallaro, 2001). It is also suggested that the cells may detach from the primary tumor in clusters, but potentially only one will attach to the new host area and begin the secondary tumor (Cheung, 2016). This could explain the opposing adhesive ability of the cancer cells, as cells with low adhesive ability could detach as part of a cluster, while the cells with high adhesion could be the ones that break off and are able to adhere to the host site. These cancer cells can latch on all over the body and the multitude of tumors cause the vital organs of the host to shut down and ultimately die.

THIS EXPERIMENT

There are many different factors that contribute to increased metastatic ability of a cancerous cell. It has been suggested that the major changes a cell can undergo involves changes

to adhesive ability, motility, deformability, invasiveness, growth rate, cellular communication, angiogenesis, enzyme production, immunogenicity, and differentiation (Evans, 1992). The focus of this thesis is on testing two of these factors, adhesion and motility, to determine whether they are affected by an omega-3 fatty acid, docosahexaenoic acid, which as mentioned above, is known to reduce the occurrence of metastatic lesions via an unknown mechanism. This experiment will test this hypothesis in two isolation variants of the B16 cell line. The B16-F1 and B16-F10 cell lines have very different metastatic capacities; B16-F1 is moderately metastatic whereas B16-F10 is aggressively metastatic. Oleic acid was used as the control fatty acid for this study because it is the most abundant fatty acid in many cell types, it is not toxic to B16 cells (Williams, 2015) and because in other cell types and in model membranes, it neither induces apoptosis nor influences membrane raft function or structure (Kishida et al. 2006; Shaikh et al. 2009; Shaikh et al. 2009a).

Methods

PREPARING MEDIUM

B16 cells achieved optimal growth in Dulbecco's Modified Eagle Medium (DMEM) comprising of a sterile filtered, high glucose medium with added L-Glutamine (ATTC cat no. 30-2002). The medium was further modified with the addition of 5% fetal bovine serum (30-2021). The prepared medium was stored between 2°C and 8°C. The fetal bovine serum was stored below 0°C.

PREPARING STOCK CELLS

The cell lines were bought frozen and stored in a liquid nitrogen dewar until ready to be grown. Cells were thawed by running them under cold water and then warmed further by holding in hands until completely thawed. Ten ml of prepared medium was added to a $25cm^2$

Thermo Scientific sterile disposable cell culture flask, and cells were cultured at 37°C and 5% CO_2 in air. Cells were checked daily for healthy growth, confluency, and were passaged once healthy growth was achieved, usually every other day.

PASSAGING CELLS

Cells were passaged routinely every other day and cells were reduced to $1/6^{th}$ density with culture medium to maintain appropriate volumes, confluence, and nutrients. Healthy cells had adhered to the base of the flask so the medium above was pipetted out and the cells were washed with 1 ml of Trypsin (0.25%) at 37°C. After the wash was removed 1 ml trypsin was again added and the flask was rocked until cells detached. Once cells had detached, 5ml of prepared medium at 37°C was added and the solution was aspirated. One ml of this solution was added to new flasks along with 5 ml of prepared medium. Cells were incubated at 37°C until experiment or future passaging.

CELLULAR ADHESION ASSAY

On the day of an experiment, cells were prepared following a modification of the passaging procedure described above. Trypsinized cells from a single confluent flask were added to a 15 ml Falcon tube and centrifuged at 500g for five minutes. The supernatant was gently aspirated off the cell pellet and six ml of pre-warmed Dulbecco's phosphate-buffered saline (DPBS) was added. The cell suspension was gently aspirated to break up the pellet before it was again centrifuged at 500g for five minutes. This process was repeated one final time. The supernatant was gently aspirated, and the final cell pellet was resuspended in two ml DPBS containing 1mM calcein-AM (CAM). The cell suspension was aspirated gently until homogenous and incubated at 37°C for 20 minutes inverting the flask four times every 5 minutes

to prevent cell adherence to the tube. During this period the CAM was processed by living cells and converted to a florescent state.

After incubation, the cell solution was split between two 1.5 ml microcentrifuge tubes and centrifuged at 12,000g for 2 minutes. The supernatant was gently aspirated off and 1 ml medium was added to each tube and the solution was aspirated until homogenous. The tubes were centrifuged again at 12,000g for 2 minutes. Again, the supernatant was aspirated off and one ml of medium was added to each tube and the solution was aspirated and centrifuged. Then, the final supernatant was aspirated off, 1 more ml of medium was added, and the solutions were gently aspirated to break up the pellets. The homogenates were added to a sterile 15 ml Falcon tube and brought up to four ml with prepared medium and aspirated gently. The final suspension was plated on a sterile 24-well flat bottom plate. The plate was incubated for 20 minutes at 37°C and then plated in a spectrophotometer at 37°C to read kinetic fluorescence. The wavelength excitation bandwidth was set to 492 nm and emission bandwidth was 517 nm. The plate was read from the bottom. The total run time was five minutes with 30 second intervals. After the determination of total cell florescence, the medium in each well was pipetted out leaving only the adhered fluorescing cells; the plate was read again under the same settings. The fraction of total florescence remaining in the wells after aspirations of non-adherent cells was calculated. For each well, fluorescent measurements were made at 21 different spots and the entire plate was read 10 times. Thus, for this experiment every well was sampled 210 times.

TREATMENT OF CELLS

DHA and oleic acid were delivered to cell cultures in 30µl 100% ethanol at final concentrations between 0-100 µg/ml. Carrier controls included 30 µl (.086M) of ethanol alone. In all cases, cells were treated for six hours before the cell adhesion assay was performed. All

treatments were carried out in a single-blind format (i.e., the individual carrying out the assay will not know which treatment was being measured).

MOTILITY ASSAY

Cells were passaged two days prior to experiments and were ~90-100% confluent to the flask at the time of the experiment. A sterile pipette tip was used to make a 400μm scratch according to the method of Guan, Park and Liang (2007). The existing medium was removed from the cells and replaced with six ml of fresh medium. This cleaned up the scratch and removed dead and broken cells, as well as gave the cells fresh medium to grow and be motile. A fine point marker was used to draw a line perpendicular to the scratch on the outside of the flask to create an intersection that was the zone of interest, a rectangle approximately 300μm by 500μm. The width of the scratch was immediately measured using the IC measure program with The ImagingSource DMK23UX249 microscope camera attached to a Nikon Diaphot microscope. In the zone of interest, seven different measurements were taken and recorded. Following the measurement, the flask was incubated at 37˚C until the next measurement could be taken for a minimum of one hour. Measurements were taken for up to 18 hours. After all measurements had been taken, the measurements from each time interval were averaged and plotted against time to calculate the rate at which the cells closed the scratch.

TREATMENT OF CELLS

DHA and oleic acid were delivered to cell cultures in 100% ethanol at final concentrations of 50μg/ml. Carrier controls included 30 μl (.086M) of ethanol alone. In all cases, cells were treated right before the cell motility assay was performed.

Results and Discussion

ADHESION RESULTS

Overall, 43 successful adhesion assays were performed with the B16-F10 cell line and 50 successful assays with the B16-F1 line (Appendix A). Many of experiments were in two to three a day staggered in one-hour intervals. The first major difference seen involved the large difference in percent adherence between the B16-F1 cells and the B16-F10 cells. The more metastatic B16-F10 cells had an average of 81.8% cells adhered after 20 minutes in control conditions. The less metastatic B16-F1 cells only had 54.3% of cells adhere on average under control conditions. Another notable difference was noticed when comparing the difference in ethanol effect between the two cell lines. Figure 1 shows the average percent adhesion between the control groups and the ethanol control groups. Figure 2 shows the proportional difference between the control group within each cell line respectively.

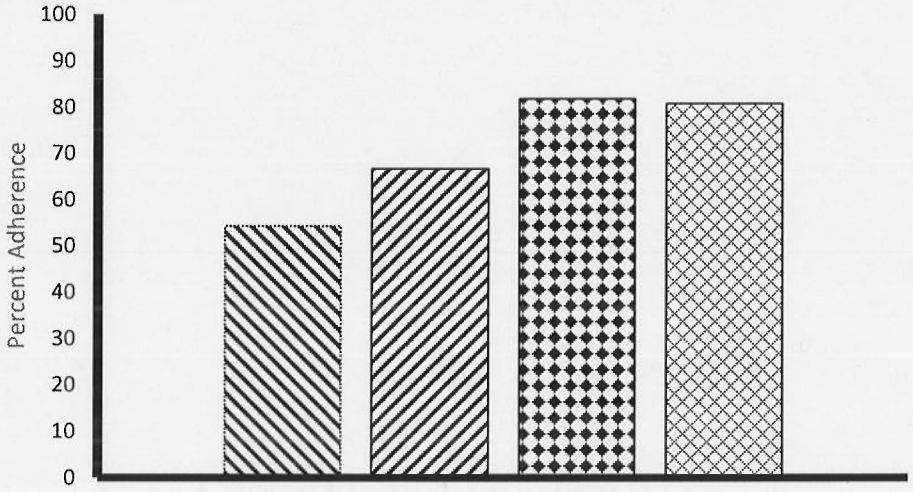

Figure 1. Percent Adhesion of both B16-F1 control (downward stripe) as well as B16-F1 ethanol control group (upward stripe), B16-F10 control (dark checkered) as well as B16-F10 ethanol control (Light checker)

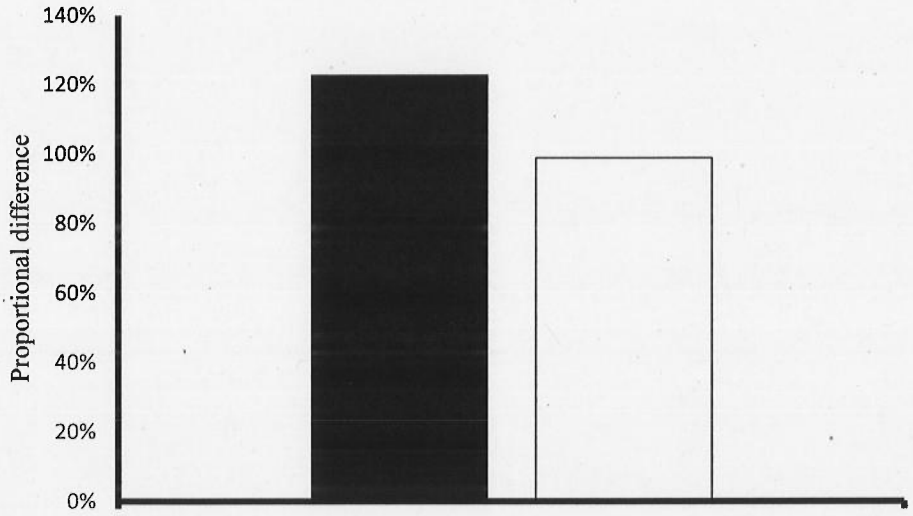

Figure 2. Percent difference between control trials and ethanol trials between B16-F1 (filled) and B16-F10 (open)

Ethanol positively affected the B16-F1 cell line increasing its adhesive capability from 54.3% to 66.7%. Proportionally, the ethanol trial adhered 122.8% as often compared to the control in the B16-F1 line, representing a 22.8% increase. The B16-F10 line was very slightly hindered by the ethanol by a nearly unnoticeable amount, adhering 98.8% as often as the control group. The data collected with the B16-F10 cell line shows that 30µl (.086M) ethanol does not have a significant effect on the adhesive ability of the cells.

The adhesion rates of DHA treated cells as well as oleic acid treated cells were compared for percent adherence. Then they were compared to the individual ethanol controls for each cell line. As seen in figure 3, the B16-F1 cell line does not experience a drastic change in adhesive capability up until all of the cells died at the 100µg/ml concentration, which proved to the lethal dose in both cell lines. The B16-F10 line (Figure 4) exhibits a relatively small decrease from 0 to 23.2µg/ml, yet had a much more noticeable decrease in adherence from 23.2 to 100µg/ml.

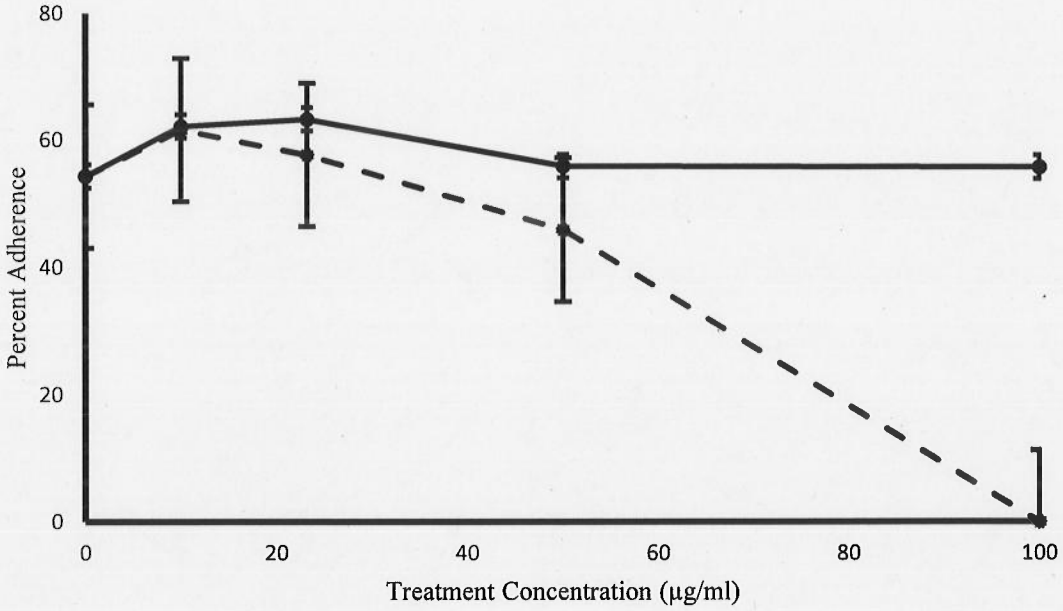

Figure 3. B16-F1 adhesion of average percent adherence for the DHA treated samples (dashed line) and oleic acid treated samples (solid line) at every concentration in the B16-F1 cell line.

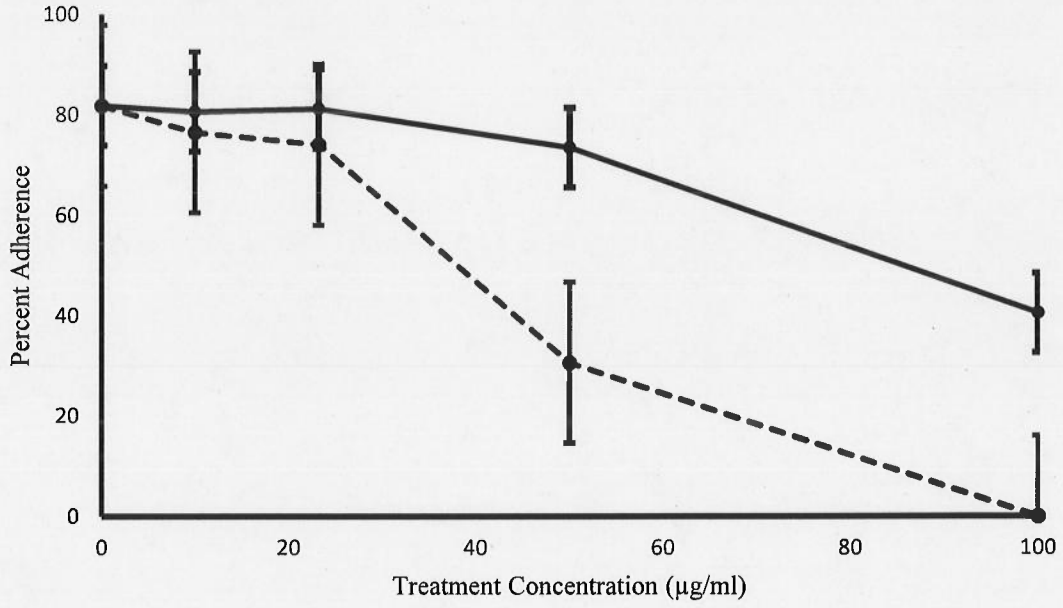

Figure 4. Side by side comparison of average percent adherence for the DHA treated samples (dashed line) and oleic acid treated samples (solid line) at every concentration in the B16-F10 cell line.

In order to factor out the increase in adhesive capability because of the ethanol in the B16-F1 cell line, the adhesion rates for the DHA and oleic acid trials were compared their respective ethanol carrier control. There is an even more dramatic decrease seen in the B16-F10 cells (Figure 6) than in the B16-F1 cells (Figure 5). This decrease is seen most from the 23.2 to 50μg/ml concentrations. While the DHA visibly hinders the proportional adhesive capability of the cells, the oleic acid trials appear to show very little, if any hindrance.

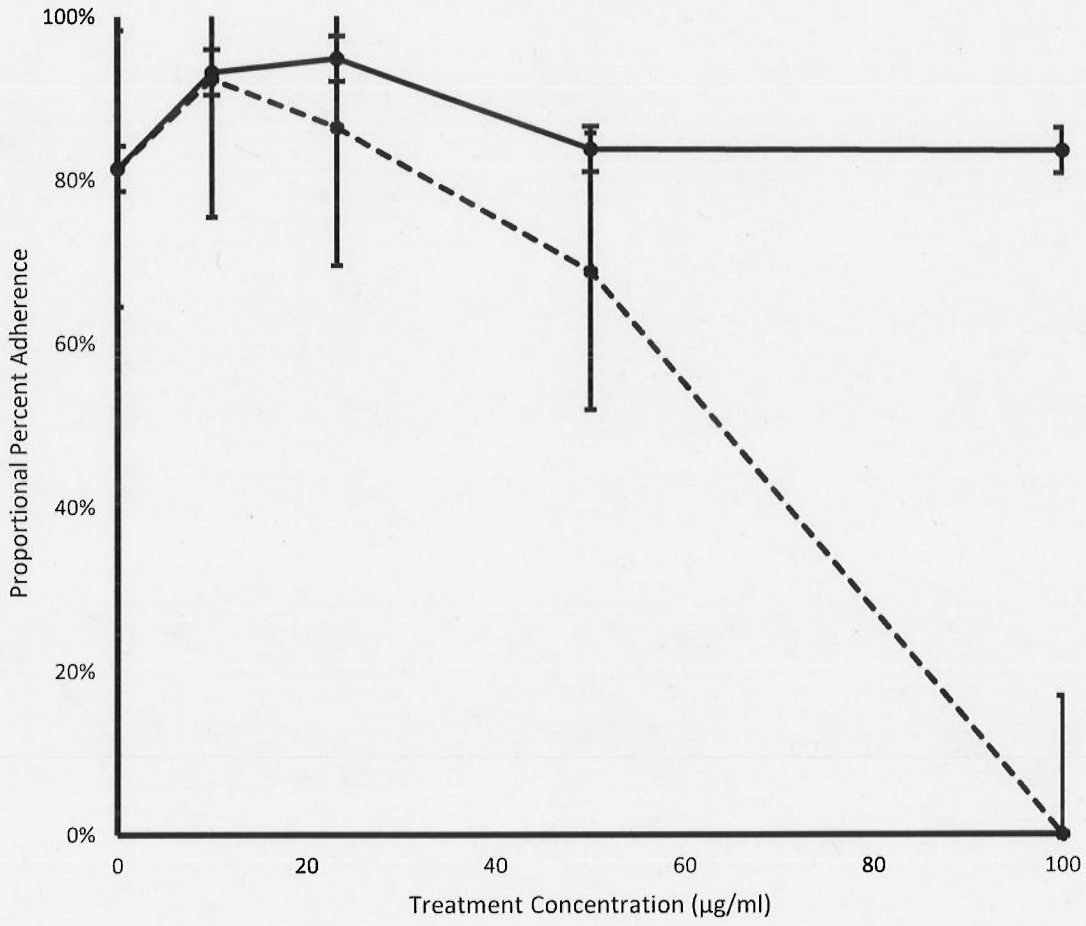

Figure 5. The percent adhesion in the B16-F1 line (Fig. 3) was compared proportionally to the ethanol carrier control average adhesion to balance out the increase in adhesion in just the B16-F1 ethanol carrier control. The oleic acid trials are represented by the solid line while the DHA trial are shown with a dashed line.

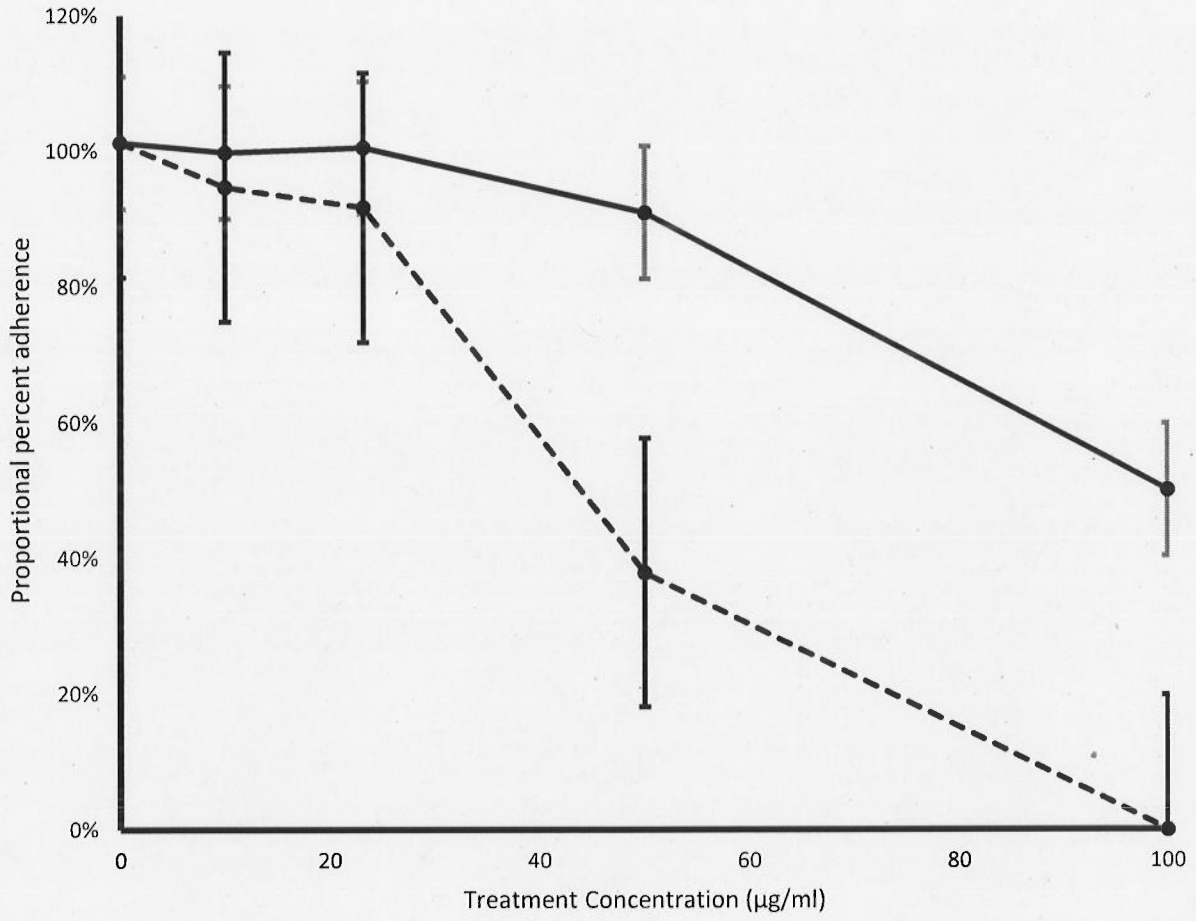

Figure 6. The percent adhesion in the B16-F10 line (Fig. 5) was compared proportionally to the ethanol carrier control average adhesion to balance out the increase in adhesion in just the B16-F1 ethanol carrier control. The oleic acid trials are represented by the solid line while the DHA trial are shown with a dashed line.

After the effect of ethanol is negated, the hindrance of adhesive capabilities in both cell lines can directly be compared to each other. In each respective cell line, the DHA hindered adherence is proportionally compared to the oleic acid hindered adherence. The cell lines were then compared directly to each other (Figure 7). Because it is the lethal dose in both cell lines, the 100µg/ml concentration was not included as the cells cannot be hindered by the effects of DHA if they are not living. There is very little difference in hindrance between the two lines in

the concentrations below 23.2μg/ml. However, at the 50μg/ml level, the percent hindrance between the two cell lines becomes drastically different with the B16-F1 line only being hindered 17.8% and the B16-F10 line being hindered 58.3%, a very dramatic change.

Table 1. Quantitative chart comparison of the percent hindrance of each cell line at all concentrations except 100μg/ml.

Concentration (μg/ml)	B16-F1	B16-F10
0.0	0.0%	0.0%
10.0	0.9%	5.1%
23.2	8.8%	8.7%
50.0	17.8%	58.3%

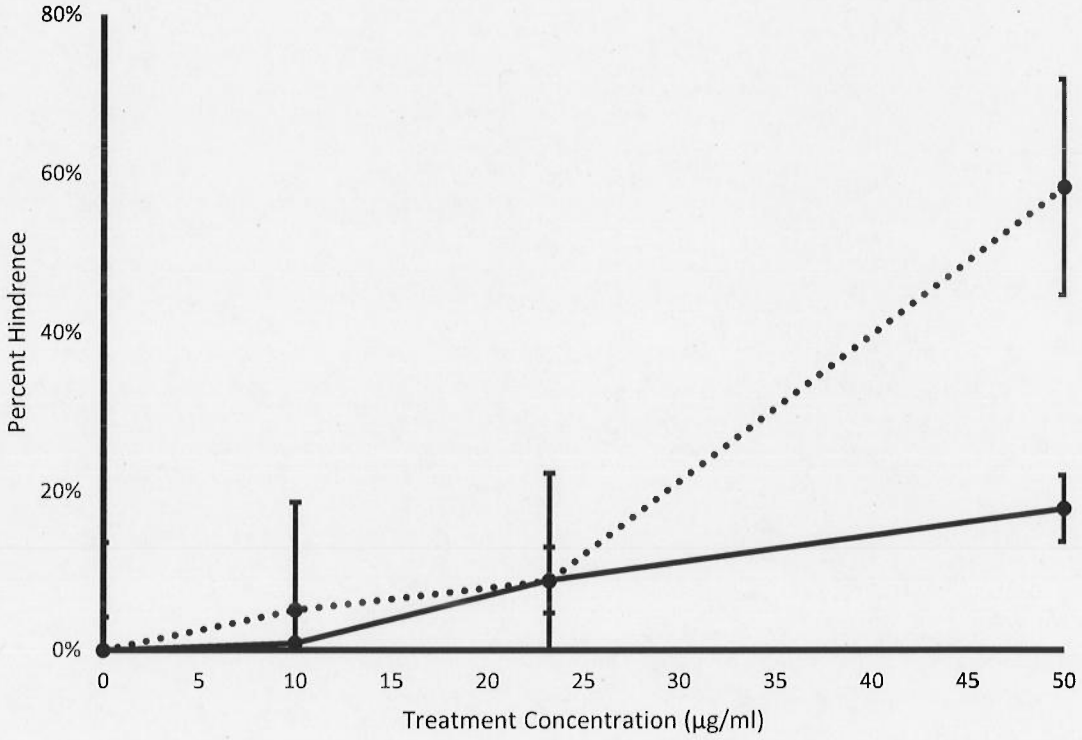

Figure 7. Plotted percent hindrance of B16-F1 (solid) and B16-F10 (dotted) compared side by side.

ADHESION DISCUSSION

As the treatment concentration increased, the difference in adherence of the oleic acid control group and the DHA experimental group also increased. However, this is not noticeable from the 0μg/ml to 23.2μg/ml and is only of an observable difference in the 23.2 μg/ml to 100μg/ml concentrations. Based on the data gathered the null hypothesis that DHA has no effect on adhesion can be rejected because DHA does presents a significant amount of hindrance to adhesion when compared to the effects of oleic acid and ethanol. Furthermore, the difference is greater in higher concentrations and in the highly metastatic B16-F10 cell line. The addition of DHA to the cell lines makes the B16-F10 cells look much more like the B16-F1 cell line. In the future, additional concentrations of fatty acid should be tested as there is not much of a change below 23.2μg/ml and the exact lethal concentration is not known other than it is somewhere between 50 and 100μg/ml. Future concentrations will include 36.7μg/ml, 65μg/ml, and 80μg/ml to determine if the relationship is consistent. That is, will a higher concentration of DHA hinder the adhesion of the B16-F10 cells much more than the B16-F1 cells. In addition to this more thorough statistical analysis will be conducted once the future concentrations are tested.

This research could make a large contribution to the science community. The adhesion data shows that DHA has an impact on the cell adhesion potentials of cancer cell, and specifically, hinders the adhesive capacity in the more metastatic cell line, B16-F10 more dramatically than the less metastatic B16-F1 cell line. Additional concentrations should be tested to consider the minimum concentration of DHA needed to affect the adhesion properties, as well as the minimum lethal dose of DHA. Four concentrations between these two limits should be used so that linear relationships between concentration and percent hindrance can be

determined and compared across cell lines. Pictures of the varying levels of confluence are seen in Appendix C.

MOTILITY RESULTS

Overall, there were 21 successful motility assays performed where the motility rate (μm/min) was assessed and quantified with a high degree of accuracy (Appendix B). Although, the four treatments assayed, control, ethanol carrier control, oleic acid control, and the experimental group, DHA, had more variance within each group rather than between groups. All motility assays were performed using the B16-F1 cell line. General noted trends were that both the control and the DHA experimental group had the highest rates of motility, with oleic acid being next lowest, followed by the ethanol control (figure 8).

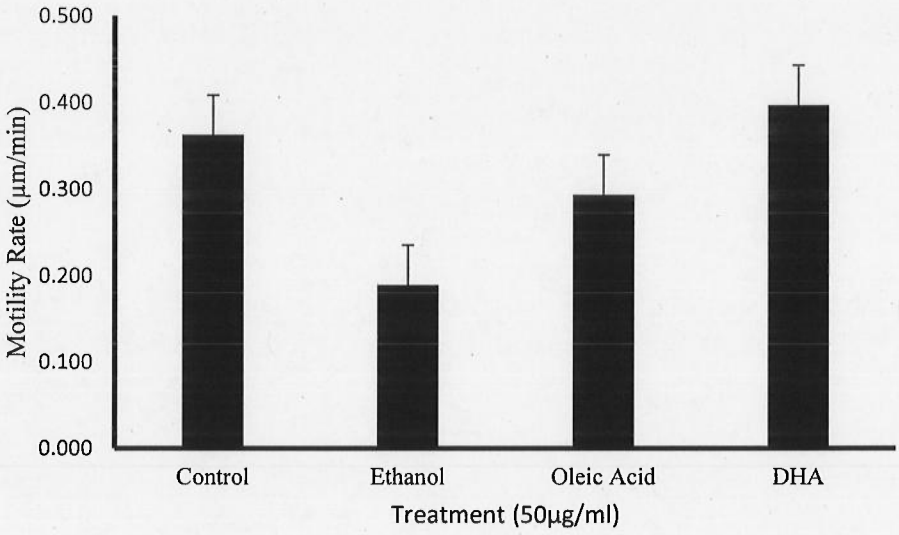

Figure 8. Average motility rate of each group with standard error bars plotted. Large variability was discovered within each group.

MOTILITY DISCUSSION

Significant issues were discovered with the consistency of data during the motility trials and it appears that there is more variability in motility rate within each group than across

different groups. No conclusions were drawn from the data gathered, however important information was learned. Flasks were chosen on the date and time of last passage, however small variances in confluence appear to have more of an effect on motility than treatment with a fatty acid or ethanol. Looking forward, motility rates will be retested with a slightly different procedure involving eight flasks all passaged from the same flask and grown to achieve 95% confluence. Then, two flasks will be randomly assigned to each group and tested using the same procedure. Rather than directly compare motility rates, rates will be calculated as a proportion of the control for the day and those percentages will be compared across different test days. This change to the procedure should allow much more replicability to the assays and allow the effect of the treatment to be more accurately measured and conclusions to be drawn. Pictures of motility assays are in Appendix D.

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Appendix A

B16-F1 Adhesion Data

Date	Run	Experiment	Percent Adherence	Standard Deviation
3/7/18	A	100 DHA	0.0	0.0
3/7/18	B	Control	64.3	3.7
3/7/18	C	50 OA	64.3	12.4
3/9/18	A	36.7 DHA	66.5	2.1
3/9/18	B	Ethanol	55.6	3.8
3/12/18	A	Control	64.8	2.6
3/12/18	B	10 OA	66.2	1.8
3/12/18	D	Ethanol	68.5	2.88
3/12/18	E	50 DHA	61.6	0.89
3/13/18	A	100 OA	26.7	9.8
3/13/18	B	23.2 OA	69.9	3.7
3/13/18	C	23.2 DHA	49.8	1.61
3/13/18	D	10 DHA	68.3	1.39
3/14/18	A	Control	38.3	5.15
3/14/18	B	Ethanol	65.5	1.61
3/14/18	C	10 DHA	60.7	1.25
3/14/18	E	50 DHA	42.6	1.97
3/15/18	A	100 DHA	0.0	0
3/15/18	B	36.7 OA	58.3	3.03
3/15/18	C	23.2 DHA	51.7	1.27
3/16/18	A	Control	43.1	0.6
3/16/18	B	36.7 DHA	54.8	3.3
3/16/18	C	10 OA	58.1	3.9
4/2/18	A	100 OA	74.6	2.16
4/2/18	B	50 OA	81.8	2.85
4/2/18	C	23.2 OA	57.9	6.3
4/2/18	D	23.2 DHA	0.2	1.6
4/2/18	E	10 DHA	0	0
4/5/18	A	10 OA	0.0	0
4/5/18	B	Ethanol	67.8	2.7
4/5/18	C	36.7 DHA	54.8	1.48
4/5/18	D	100 OA	53.6	1.3
4/6/18	A	100 OA	DNE	DNE
4/6/18	B	Control	64.1	3.01

4/6/18	C	Control	48.5	3.71
4/6/18	D	50 DHA	37.7	3.24
4/9/18	B	23.2 OA	44.4	2.51
4/9/18	C	50 DHA	57.4	1.54
4/10/18	A	Ethanol	76.0	1.11
4/10/18	B	50 OA	21.5	17.9
4/10/18	C	36.7 OA	62.9	1.14
4/10/18	D	10 DHA	55.8	0.91
4/10/18	E	10 OA	0	0
4/11/18	A	Control	70.4	2.68
4/11/18	B	100 DHA	0	0
4/11/18	C	100 OA	67.8	1.3
4/11/18	D	23.2 DHA	71.5	1.24
4/11/18	E	23.2 OA	81.4	1.79
4/12/18	A	Control	51	5.93
4/12/18	B	23.2 OA	62.7	2.37

Figure 9. Daily results from adhesion assay on B16-F1 cell line conveying experiment title, average adhesion as well as standard deviation between wells.

B16-F10 Adhesion Results

Date	Experiment	Percent Adherence	Standard Deviation
10/11/17 A	control	89.0	1.926
10/12/17 A	control	82	2.555
10/13/17 A	control	88	2.209
10/17/17 A	control	87	2.412
10/18/17 A	control	85	2.492
10/25/17 A	Control	72	11.5
10/27/17 A	100 DHA	0	0
10/31/17 A	50 OA	72	8.5
11/15/17 A	Ethanol	80	4.27
11/16/17 A	50 DHA	0	0
12/1/17 A	50 OA	78	5.67
12/1/17 B	23.2 DHA	87	2.02
12/5/17 A	Ethanol	78	8
12/7/17 A	10 DHA	20	11.5
1/8/18 A	100 OA	85	2
1/8/18 B	100 DHA	0	0
1/8/18 C	10 DHA	76	3
1/9/18 A	23.2 OA	79	8
1/9/18 B	23.2 DHA	81	3
1/10/18 A	Control	70	2
1/10/18 B	50 DHA	0	0
1/11/18 A	Control	84	1
1/11/18 B	10 OA	85	2
1/11/18 C	100 DHA	0	0
1/23/18 A	100 OA	77	3
1/23/18 B	36.7 DHA	83	1
1/23/18 C	10 DHA	81	3
1/23/18 D	23.2 OA	83	3
1/24/18 A	50 OA	73.4	2.1
1/24/18 B	10 OA	74	4.1
1/25/18 A	100 OA	0	0
1/25/18 B	Control	79	2.3
1/25/18 C	23.2 OA	81.5	4.5
1/25/18 D	36.7 DHA	74.6	3.6
1/25/18 E	50 DHA	58.7	7.4
1/30/18 A	Ethanol	84.3	3.4

1/30/18 B	10 OA	82.8	2.2
1/30/18 C	50 DHA	74	3.6
2/1/18 A	50 OA	70.4	8.6
2/13/18 A	100 OA	0	0
2/13/18 B	10 DHA	72.5	5.1
2/13/18 C	23.2 DHA	54.2	6.5
2/13/18 D	50 DHA	20.4	5.4

Figure 10. Daily results from adhesion assay on B16-F10 cell line conveying experiment title, average adhesion as well as standard deviation between wells.

Appendix B

Scratch Data Overview B16-F1

Date	Test	Treatment	Rate (μm/min)	R squared
2/28/18	A	Control	0.455	0.97
3/9/18	A	50 DHA	0.173	0.75
3/12/18	A	Control	0.297	0.98
3/14/18	A	50 Oleic Acid	0.245	0.95
4/5/18	A	Control	0.539	0.9
4/8/28	A	Control	0.491	0.83
4/8/18	B	Control	0.356	0.92
4/8/18	C	Control	0.235	0.91
4/8/18	D	Ethanol	0.145	0.85
4/8/18	E	Ethanol	0.181	0.97
4/8/18	F	Ethanol	0.083	0.94
4/9/18	A	Control	0.277	0.86
4/9/18	B	Control	0.247	0.75
4/9/18	C	Control	0.246	0.98
4/11/18	A	50 Oleic Acid	0.281	0.89
4/11/18	B	50 Oleic Acid	0.212	0.94
4/18/18	A	Control	0.484	0.98
4/18/18	B	50 Oleic Acid	0.436	0.97
4/18/18	C	50 DHA	0.539	0.95
4/18/18	D	50 DHA	0.425	0.99
4/18/18	E	50 DHA	0.451	0.99
4/18/18	F	Ethanol	0.349	0.92

Figure 11. Daily motility rates of different trials on the B16-F1 cell line. Because inconsistent data was collected, and new procedures created, motility tests were not conducted on the B16-F10 line but will be at a future date under revised protocol.

Appendix C

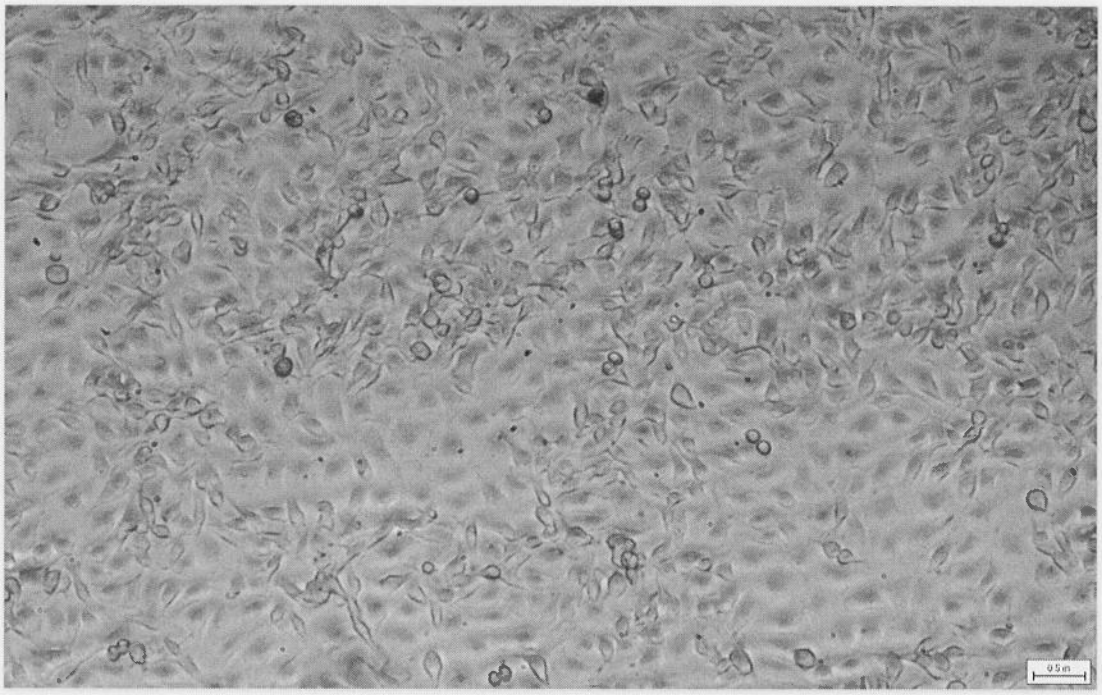

Figure. 12. Healthy B16-F1 cells 6 hours after being treated with 50μg/ml oleic acid

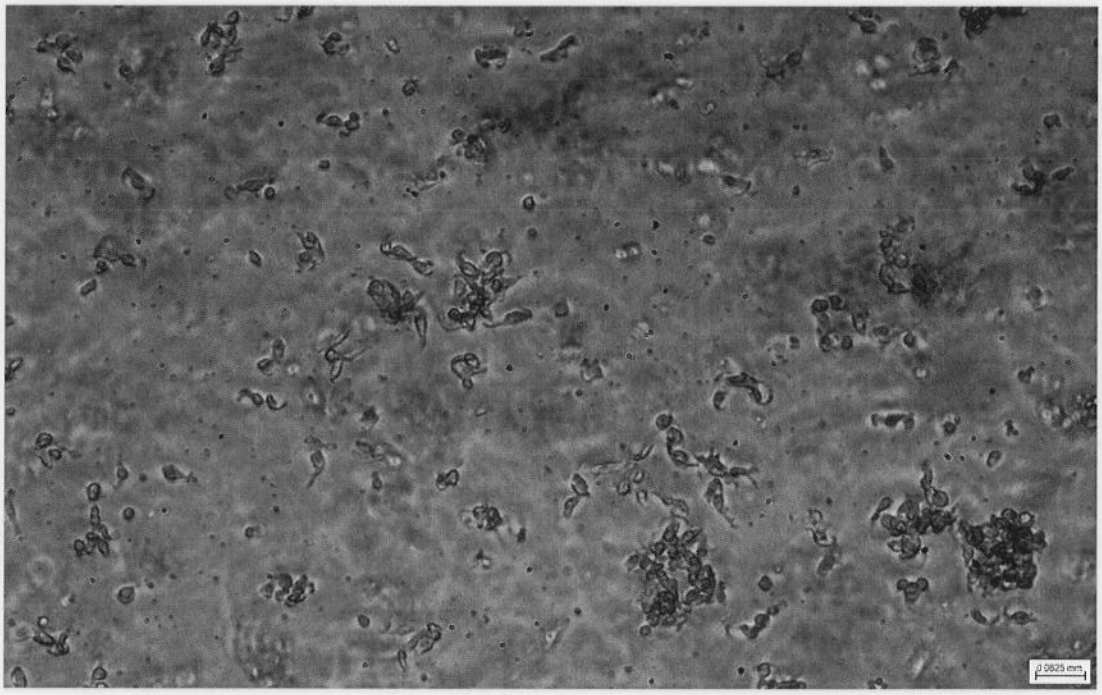

Figure 13. Mostly dead and some non-adhering cells 6 hours after a 50μg/ml treatment of DHA in the B16-F1 line

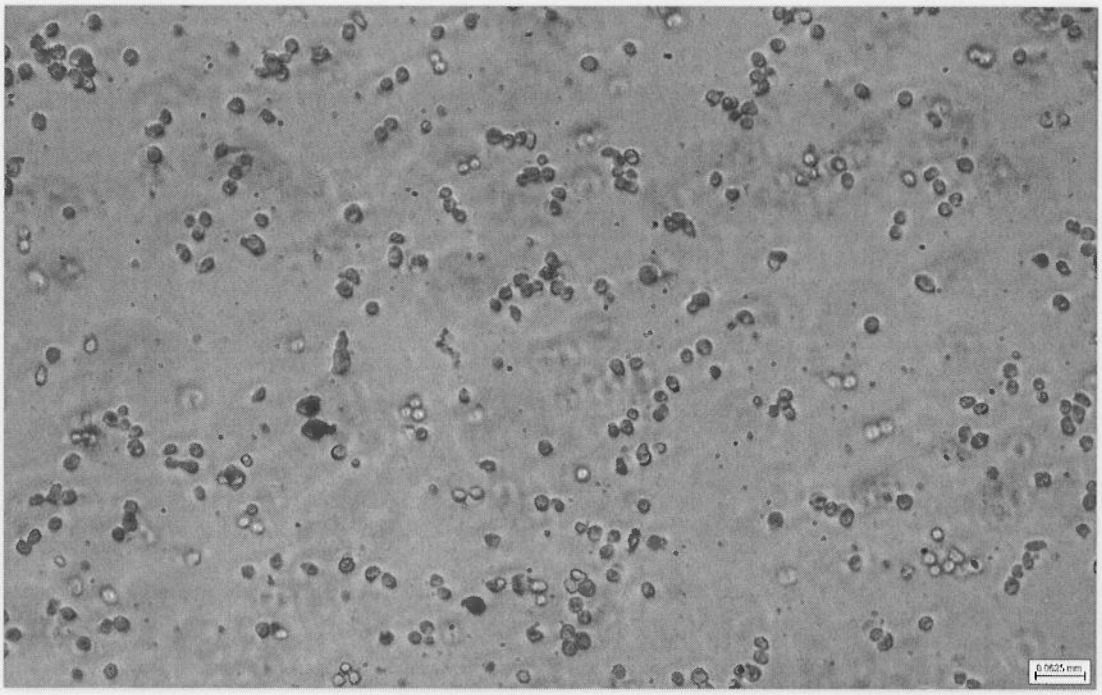

Figure 14. Dead cells 6 hours after a 100μg/ml treatment of DHA in the B16-F1 line.

Appendix D: 04/11/18 Motility Assay

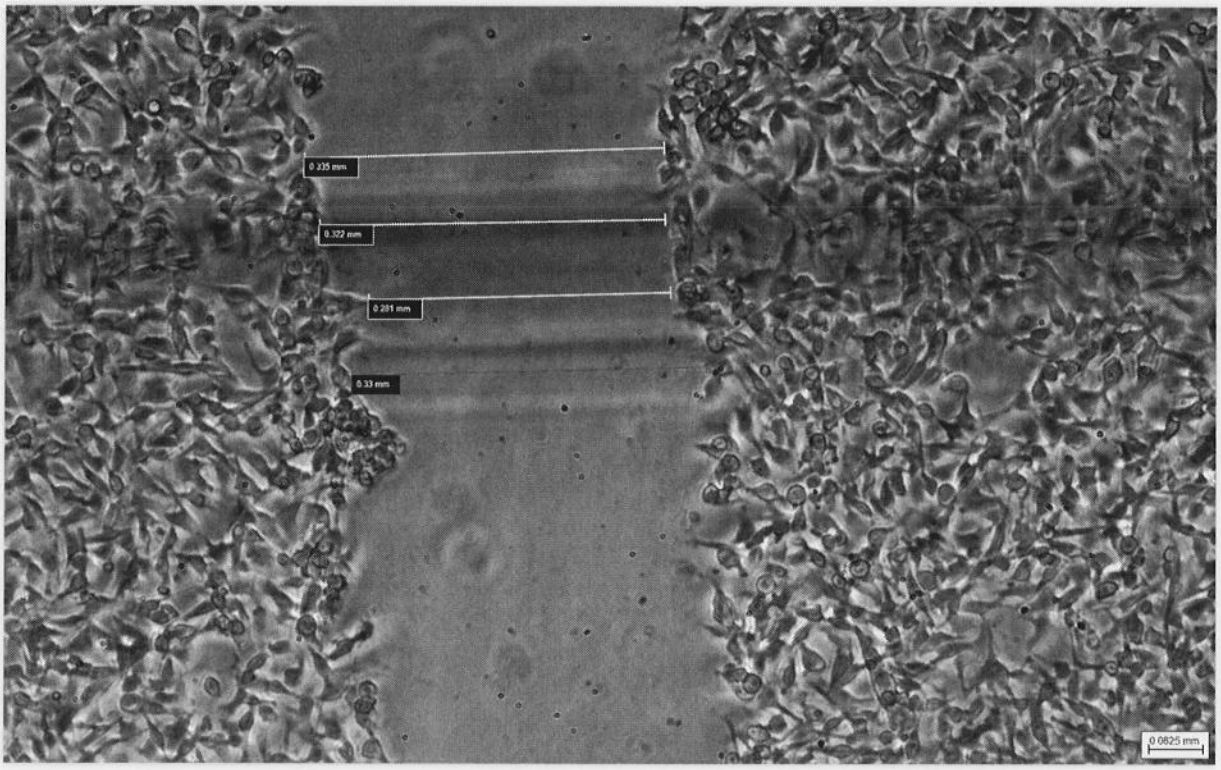

Figure 15. 50μg/ml treatment Oleic Acid Treatment. Time: 0 min. Average distance: 317μm

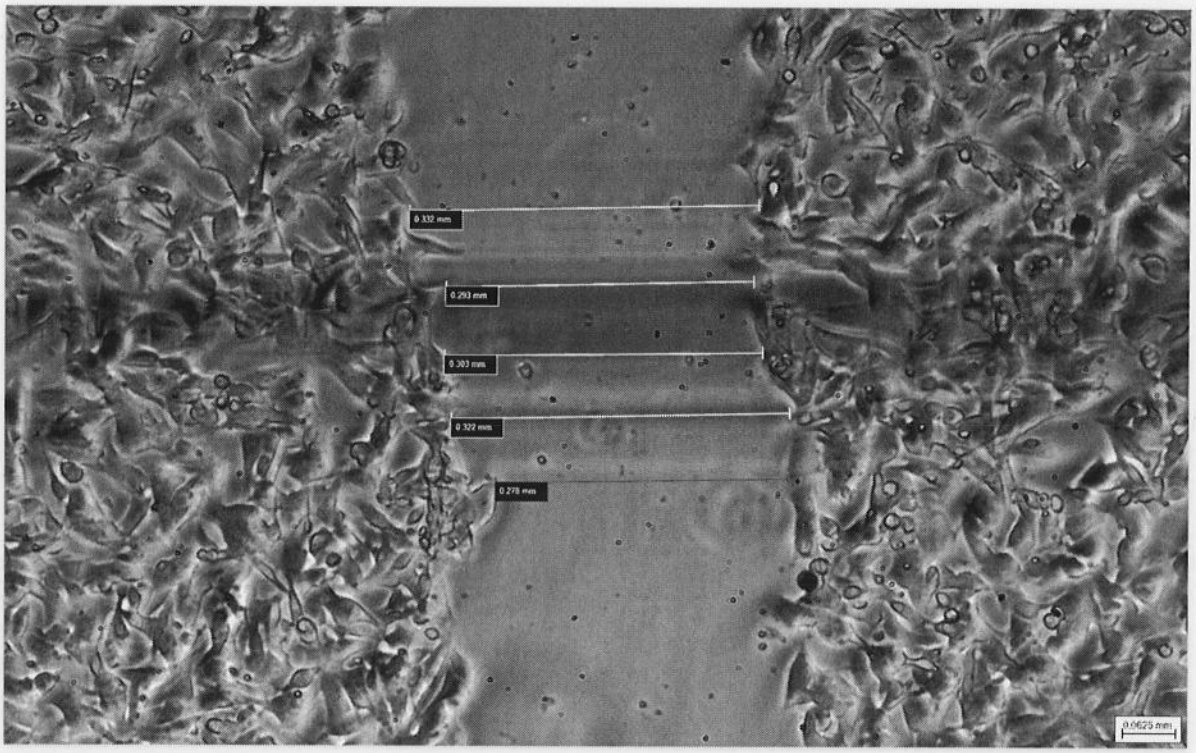

Figure 16. 50µg/ml treatment Oleic Acid Treatment. Time: 179 min. Average distance: 305µm

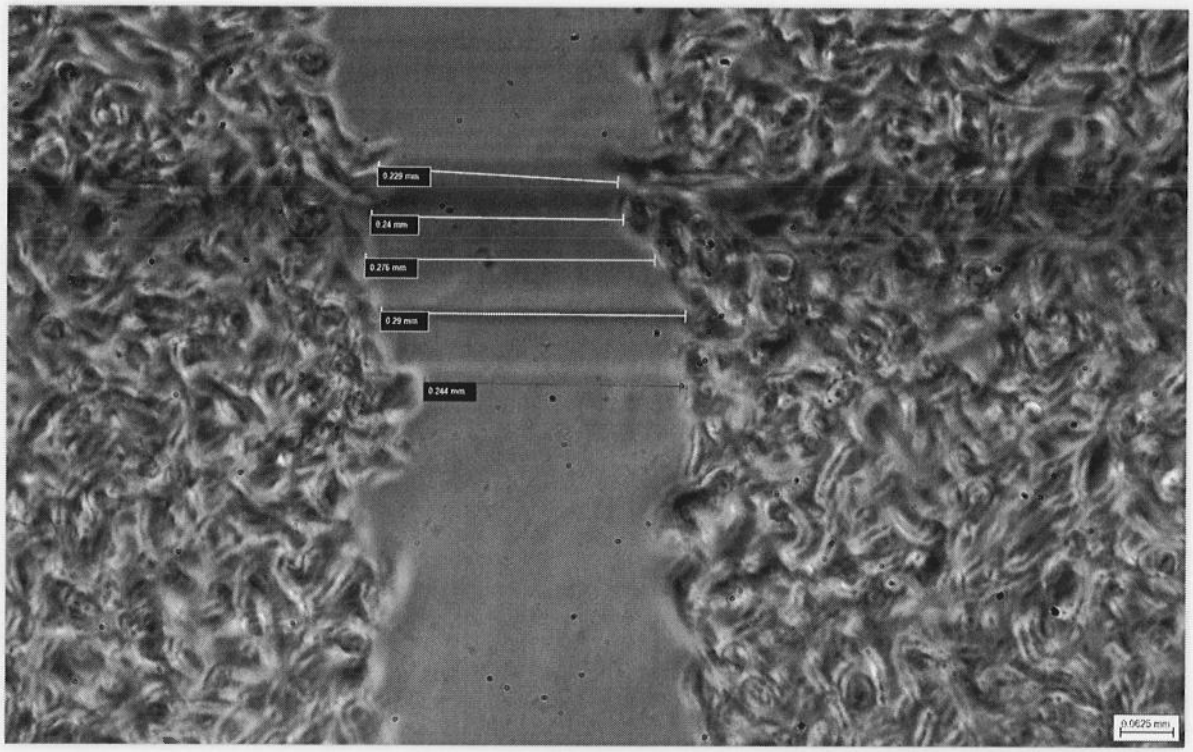

Figure 17. 50µg/ml treatment Oleic Acid Treatment. Time: 284 min. Average distance: 254µm

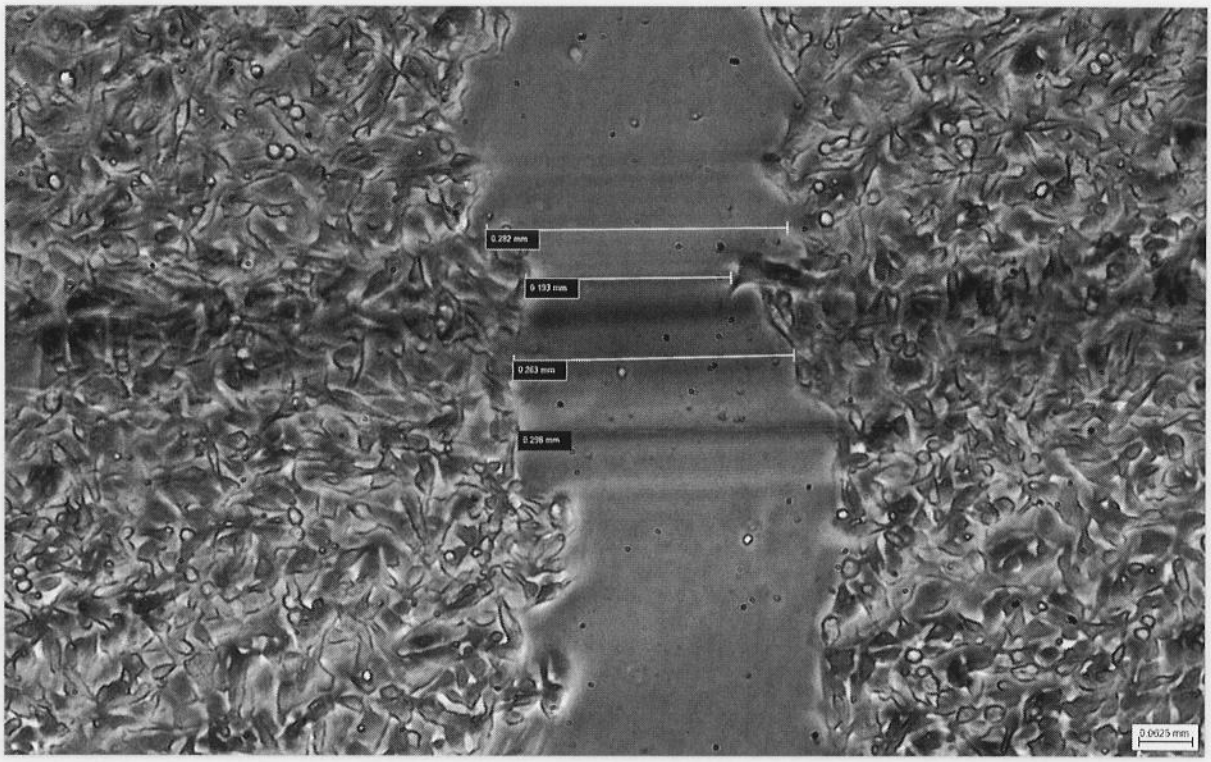

Figure 18. 50µg/ml treatment Oleic Acid Treatment. Time: 387 min. Average distance: 254µm

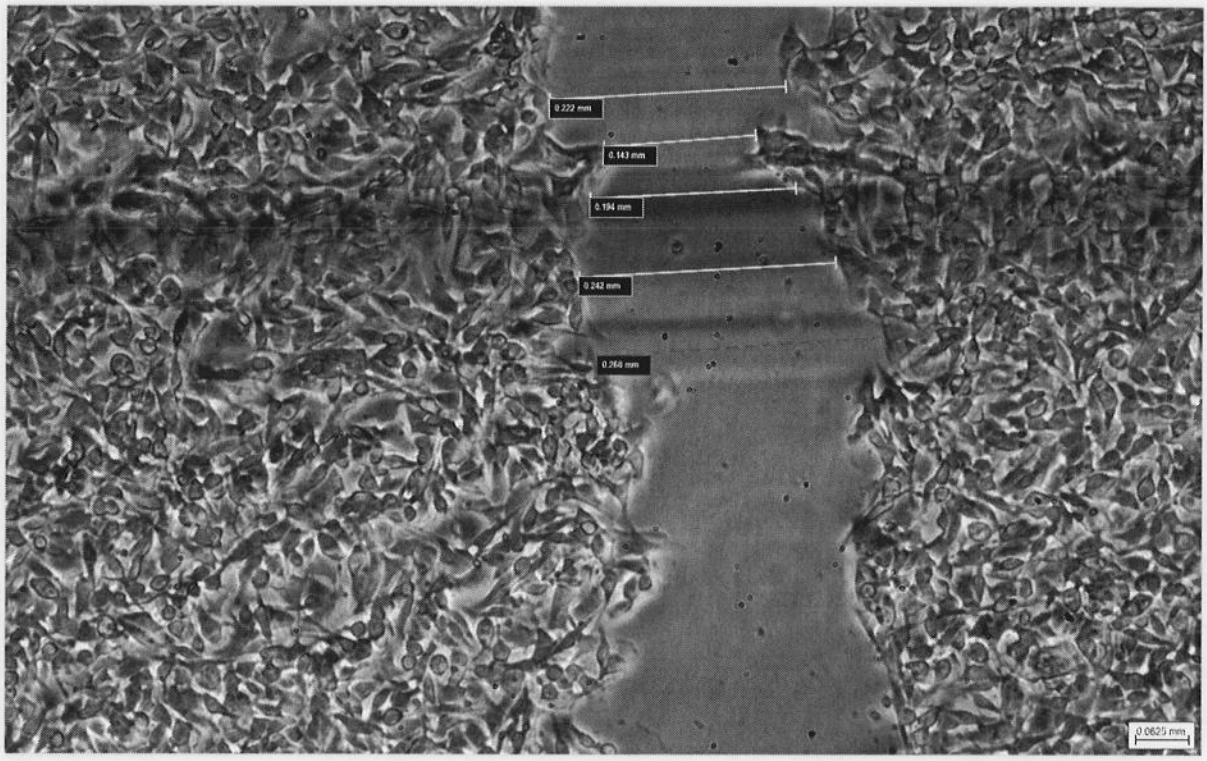

Figure 19. 50µg/ml treatment Oleic Acid Treatment. Time: 509 min. Average distance: 213µm

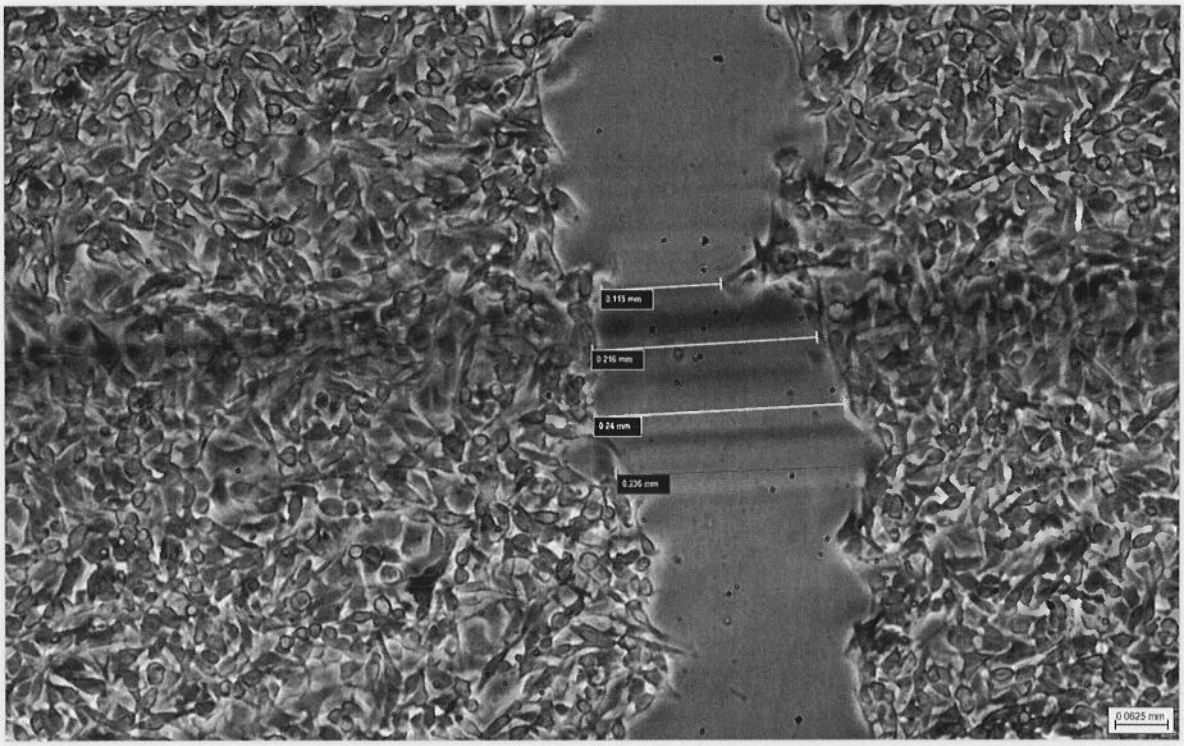

Figure 20. 50μg/ml treatment Oleic Acid Treatment. Time: 565 min. Average distance: 202μm