The Interplay of Cholesterol and Glucose on Wound Healing

Submitted by

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Abstract

Heart disease has long been on the rise in the United States, bringing with it a host of comorbidities and additional complications. To combat these developments, physicians may prescribe a statin drug to lower a patient's cholesterol; a key component of both heart disease and cell membrane formation during wound healing. The goal of this project is to determine the role of statin treatment in the wound healing process in human dermal fibroblasts. The migration of cells to the wound environment will be observed with and without the conditions of elevated glucose, elevated cholesterol, and application of statin treatment. Cell migration will be evaluated through the monitoring of the enzyme deubiquitinase USP47 and its substrate β-catenin. These results will provide a more complete idea of the factors that prevent wound healing and contribute to chronic wound development, such as those found in diabetics, as well as give a starting point for potential treatments for these wounds. In this experiment it is hypothesized that compared to the wells not given any treatment, the wells treated with statins and glucose will show decreased cell migration and proliferation, as well as decreased levels of USP47 and β-catenin; and wells treated with LDL (low density lipoprotein) cholesterol will show increased cell migration and proliferation and increased protein levels.

Current Research

A well-known side effect of diabetes is slowed wound healing. Over time, uncontrolled blood sugar can damage the peripheral blood vessels, causing poor circulation to areas such as the legs and feet. Without proper blood flow, the wounds are not able to heal as quickly or effectively. Combined with neuropathy causing numbness in the injured area, the diabetic may not even know that they have hurt themselves and delay seeking out medical treatment. It is for these reasons that diabetes is a common topic selected for researching the correlation between obesity and slow wound healing (Monami et. al, 2008). However, not every obese patient suffers from diabetes. Another aspect to consider is the impact of high cholesterol on wound healing; something that has been researched very little.

For a wound to heal properly, there must be the migration of cells into the wound environment. This process requires cholesterol in order to create the additional cell membrane for new cells. Cholesterol is a molecule used in cell membranes by increasing fluidity and decreasing permeability. Statins are a family of drugs that are commonly prescribed to patients with chronic high cholesterol because they block its creation in the liver. If cholesterol plays a role in wound healing, then inhibiting it would result in slower wound healing. This is supported by a cell culture study testing the effects of Lovastatin on the signaling pathways of epidermal growth factor and insulin growth factor (Vincent et. al, 1991).

However, there is also evidence to suggest that statin therapy is beneficial to wound healing in studies done on living systems. Diabetic mice who were treated daily with Simvastatin had higher levels of vascular endothelial growth factor mRNA and protein expression than mice in the control group. Similar results were obtained with non-diabetic mice (Bitto et. al., 2008). To

determine if statins directly affect cell signaling, deubiquitinase USP47 will be investigated. This enzyme is responsible for deubiquitinating β-catenin, a protein involved in the migration and proliferation of cells. If USP47 levels are elevated, then the signaling pathway with β-catenin would also be elevated, leading to increased cell migration and proliferation in the wound environment. Research is needed to investigate this relationship between cholesterol inhibition through statins and wound healing in hyperglycemic environments, particularly focused on the enzymatic pathway of USP47 and β-catenin.

Objectives

Introduction

To best evaluate wound healing under conditions of increased glucose, cholesterol, and statins, this project will look at multiple aspects of this process including cell migration, cell signaling, and protein expression.

Objectives

1. Examining skin cell migration after the statin, glucose, and cholesterol treatments and comparing the results to the control samples. This will provide information on the overall effects that these conditions have on the progress of wound healing.
2. Evaluating the effects of the treatments on the proteins that stimulate wound healing provides a better understanding of which factors of wound healing they affect and to what extent. This will give insight into the relationship between statins and cholesterol's ability to synthesize cell membranes.
3. Examining the expression of USP47 and its correlation with β-catenin. This will show the effects of the treatments on cell signaling pathways stimulating cell migration during wound healing.

Methodology

The first stage of the project was to culture the chosen cell line, neonatal human dermal fibroblasts, to 100% confluence in a 12.5cm^2 culture flask. The vial of stock fibroblasts was taken from cryostorage at -80℃ and thawed in a 37℃ water bath. 1mL of the stock vial contents were taken and diluted with 4mL of Medium 106 supplemented with 10% bovine. The contents were then centrifuged and the separated media was replaced. The cell pellet was then resuspended and transferred into the culture flask. The flask media was changed after 24 hours with subsequent media changes occurring approximately every 48 hours. Between media changes the cells were stored in a humidified cell culture incubator at 37℃ with 5% CO2.

After 7 days, the cells reached approximately 90% confluence in the flask and were subcultured to two six well plates. 2mL total of Trypsin/EDTA was used to detach the cells from the flask surface and then 2mL of Trypsin Neutralizer was added to prevent degradation of the cells. The cells were transferred to a conical vial and an additional 3mL of cell culture medium was added to the original flask and then also transferred to collect any remaining cells. Then the vials were centrifuged after which the supernatant was replaced with 6mL of fresh medium used to resuspend the cells. The solution was then transferred to the well plates and returned to the incubator. Cell culturing in the well plates continued for the next 9 days before the cells had once again reached 90% confluence. Media changes were then performed approximately every 24 hours for the next 7 days. All steps involving the manipulation of the cells or their media were done in the laminar fume hood after the equipment was sprayed with 70% ethanol to prevent contamination.

At this point the cells were ready for the treatment phase. To test wound healing in high glucose conditions, we included two wells with a glucose treatment. To test high cholesterol conditions, three wells were treated with LDL cholesterol. To test the effects of statins, Pitavastatin was added to three wells. The two six well plates were divided as follows:

Plate 1:

No Wound (1)	No Wound (2)	Glucose (1)
No Treatment (1)	No Treatment (2)	Glucose (2)

Plate 2:

LDL (1)	LDL (2)	LDL (3)
Pitavastatin (1)	Pitavastatin (2)	Pitavastatin (3)

To mimic wounded tissue, the layer of cells in each well was scratched with a sterile 200µL pipette tip. The "No Wound" wells were not subjected to any treatment nor the simulated wounding and served as a control for cell growth without trauma. The "No Treatment" wells were wounded and served as a control against the effects of any of the treatments. The "Glucose" wells received 5µLs of 1M glucose stock solution. The "LDL" wells were given 10µLs of low-density lipoprotein from human plasma 2.5mg/mL stock. The "Pitavastatin" wells received 2.2µLs of Pitavastatin calcium from 2.27mM stock for a final concentration of 1mM.

After wounding and treating the appropriate wells, all wells were imaged at the 0 Hour mark; except for the "No wound" group which was simply monitored for contamination or other

potential disruptions to the growth that would be challenging to identify in the wounded wells. Further images were taken at 24 hours, 48 hours, and 72 hours included below.

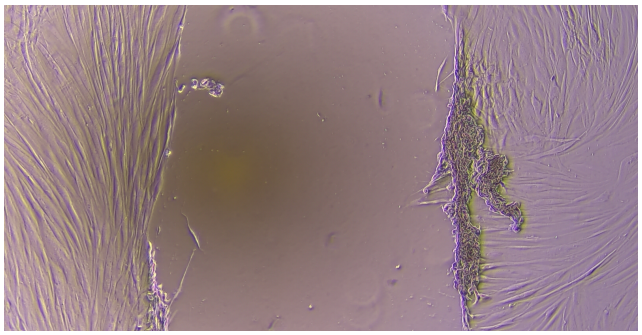


Figure 1: No Treatment Well 1 at 0 Hours

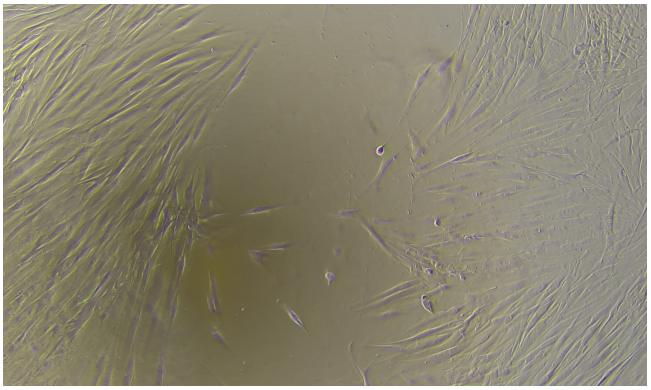


Figure 2: No Treatment Well 1 at 24 Hours

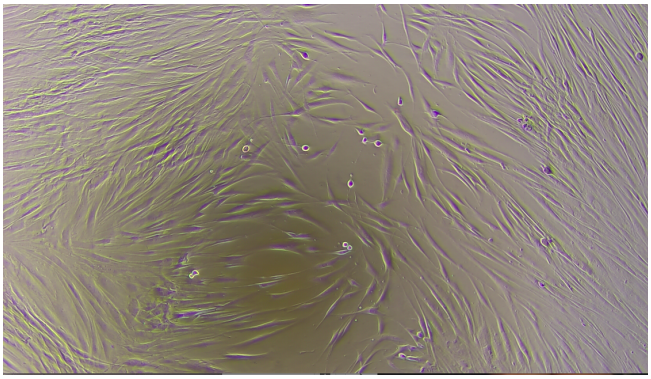


Figure 3: No Treatment Well 1 at 48 Hours

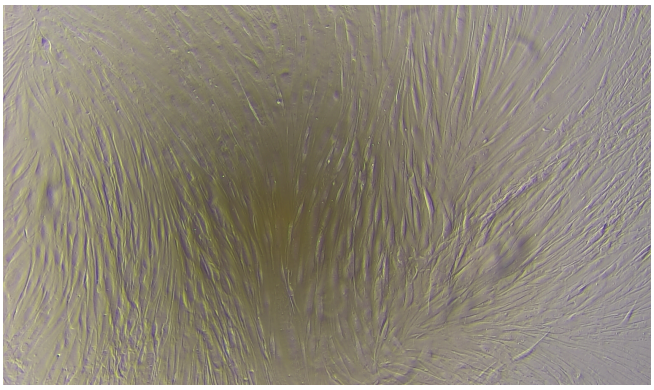


Figure 4: No Treatment Well 1 at 72 Hours

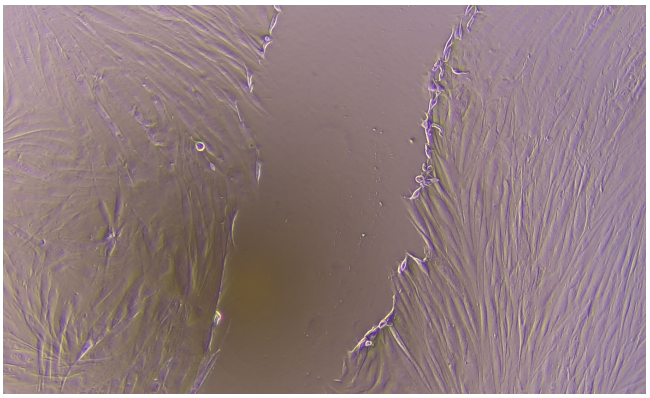
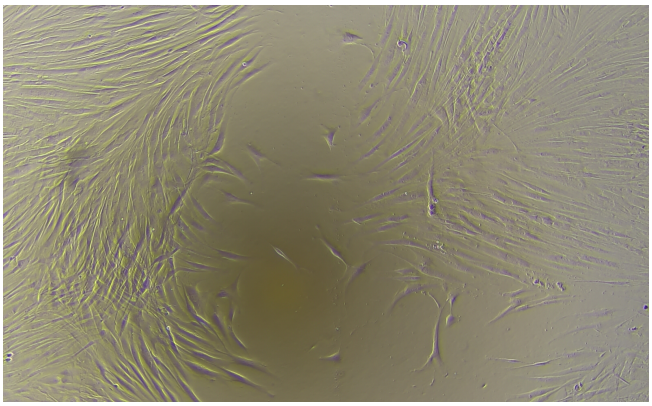


Figure 5: No Treatment Well 2 at 0 Hours



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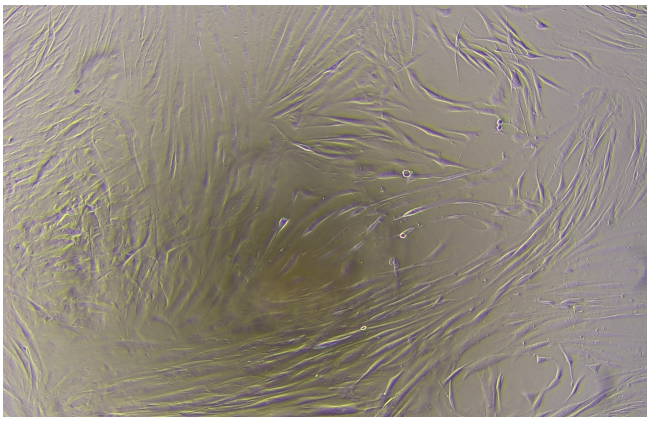


Figure 7: No Treatment Well 2 at 48 Hours

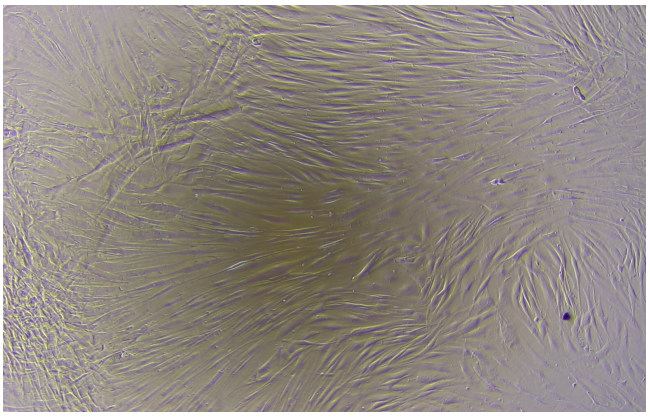


FIgure 8: No Treatment Well 2 at 72 Hours

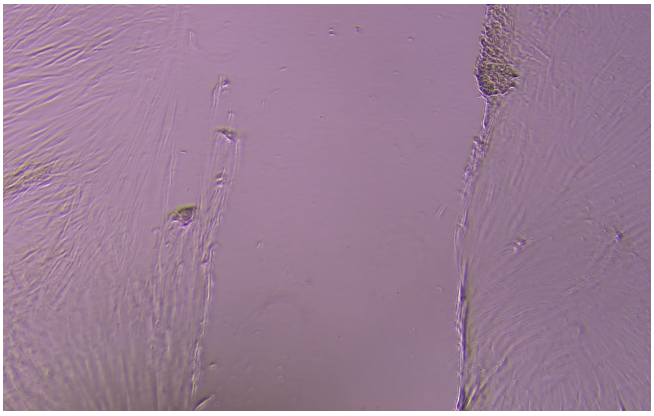
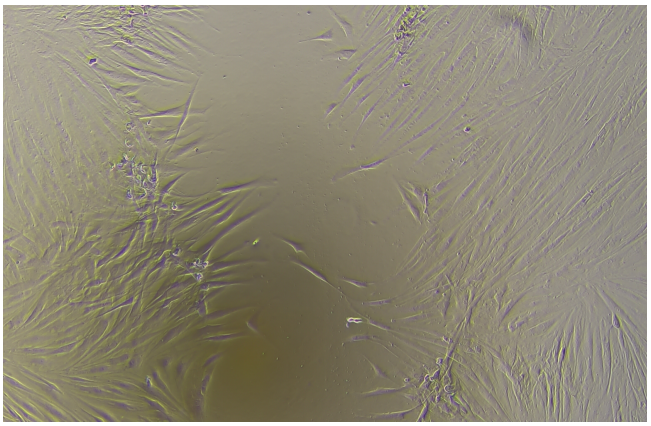


Figure 9: Glucose Well 1 at 0 Hours



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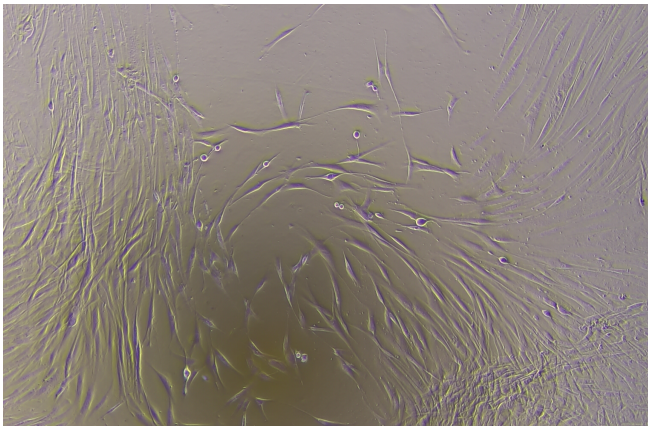

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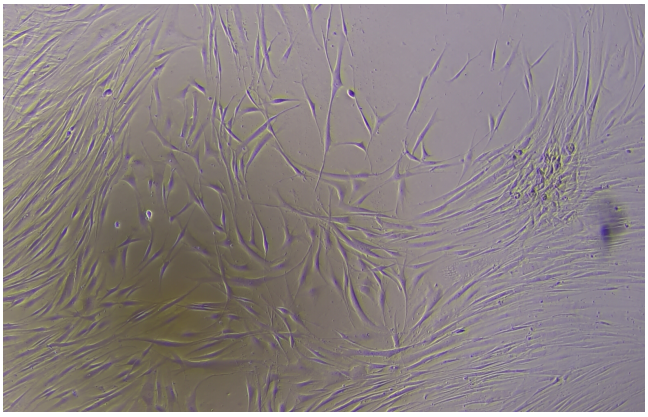
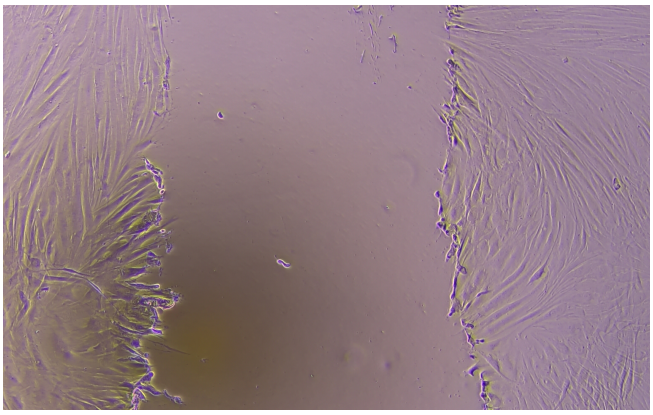


Figure 12: Glucose Well 1 at 72 Hours



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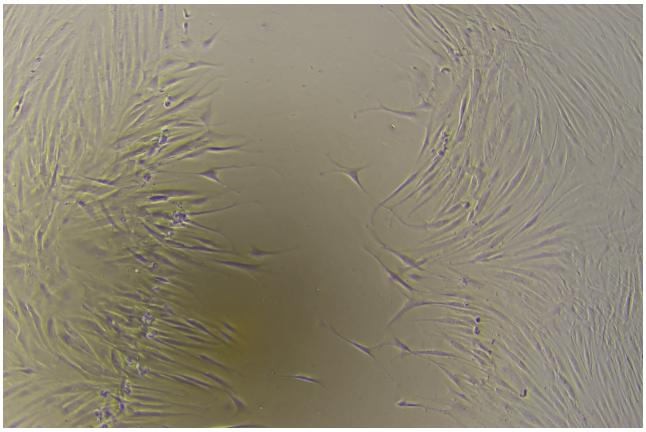

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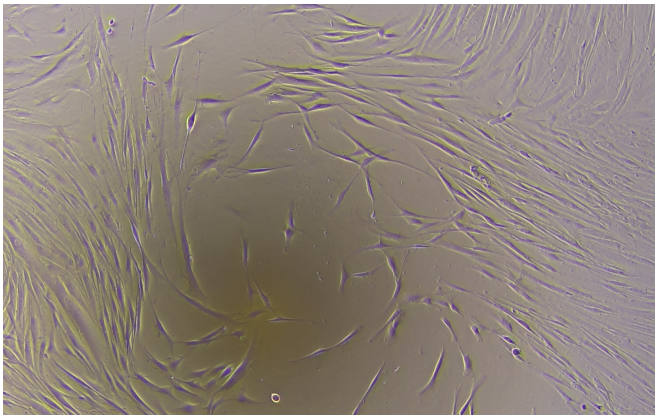

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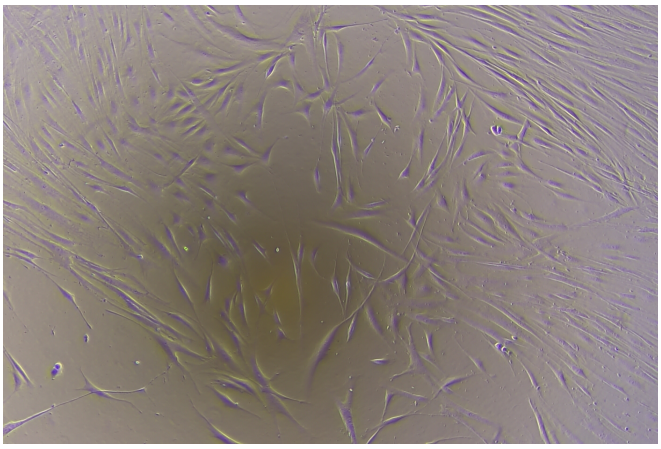


Figure 16: Glucose Well 2 at 72 Hours

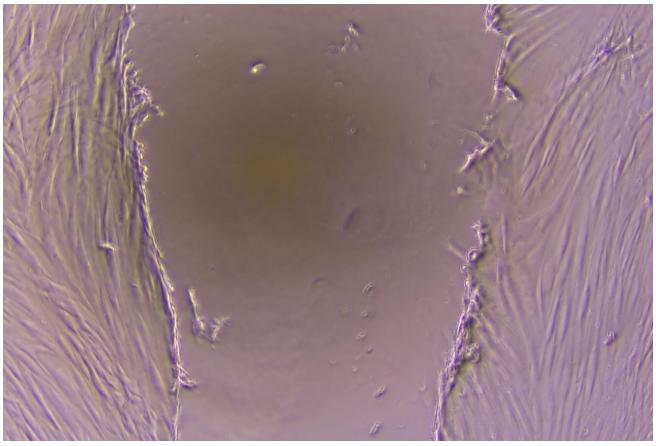
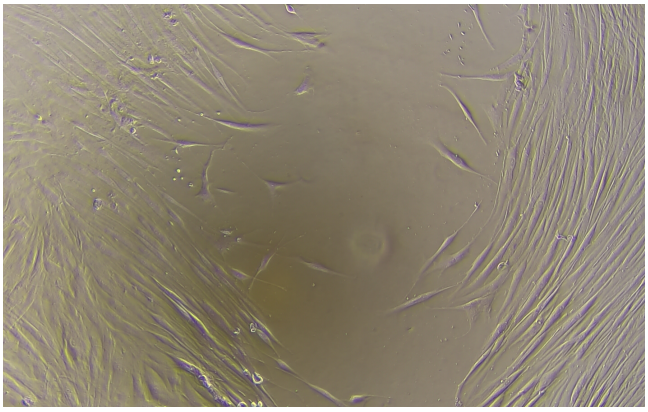


Figure 17: Pitavastatin Well 1 at 0 Hours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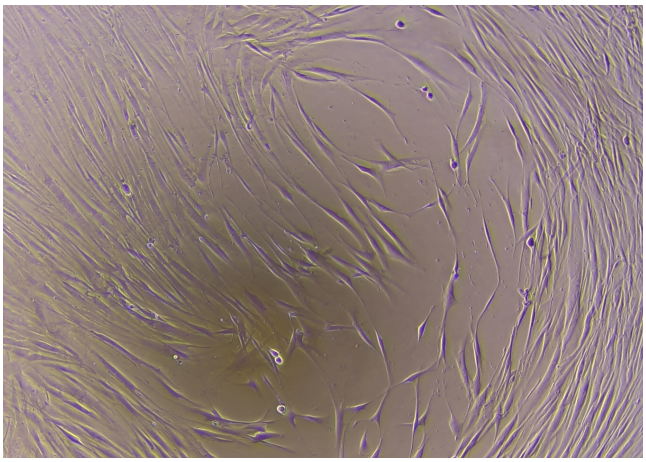
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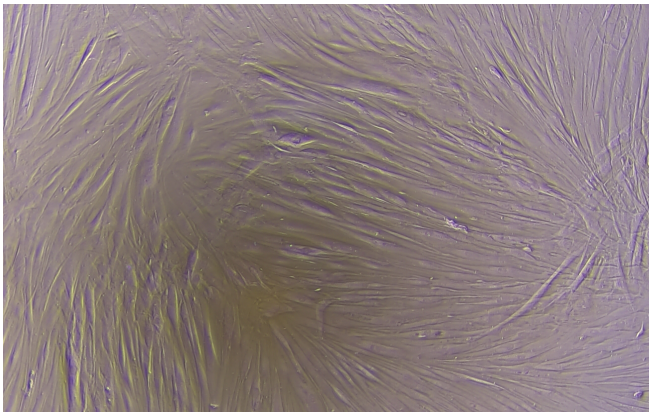
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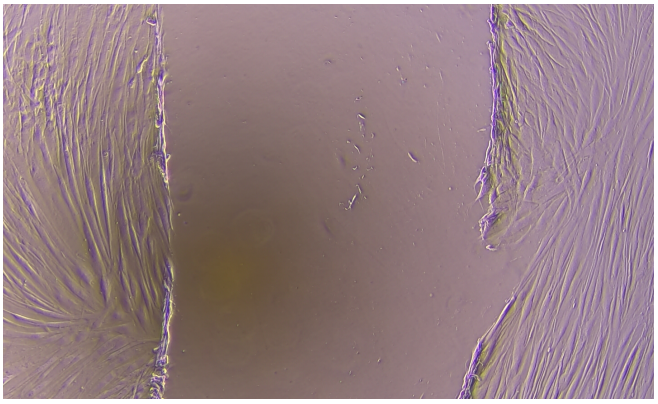
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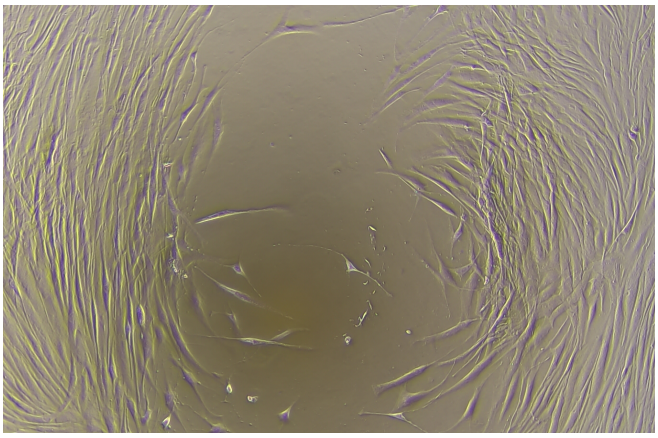
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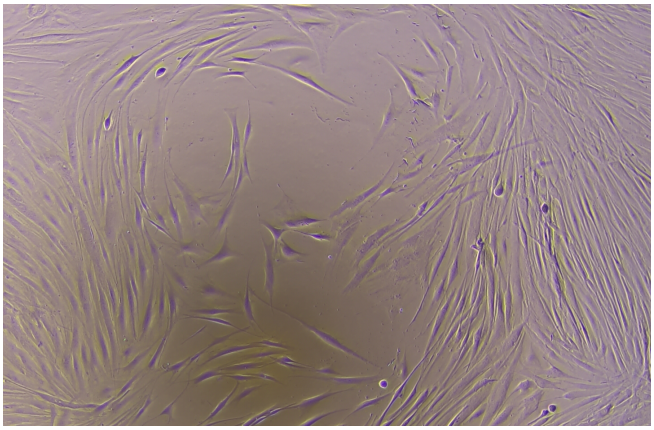
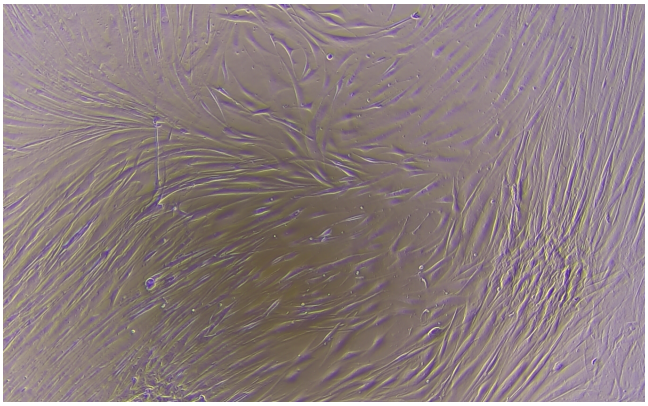


Figure 23: Pitavastatin well 2 at 48 Hours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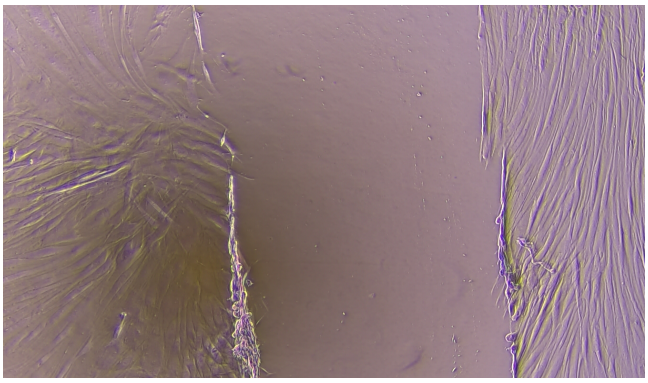
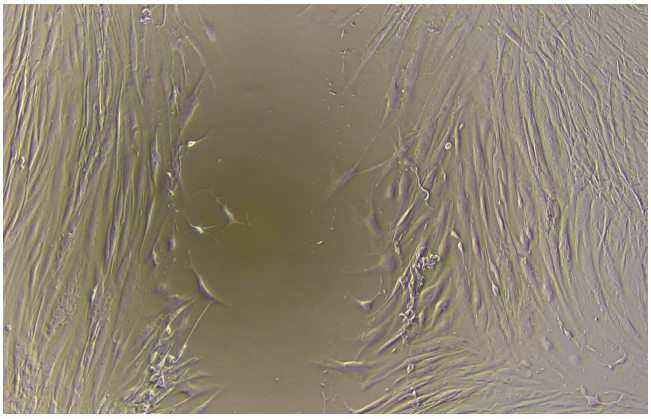


Figure 25: Pitavastatin Well 3 at 0 Hours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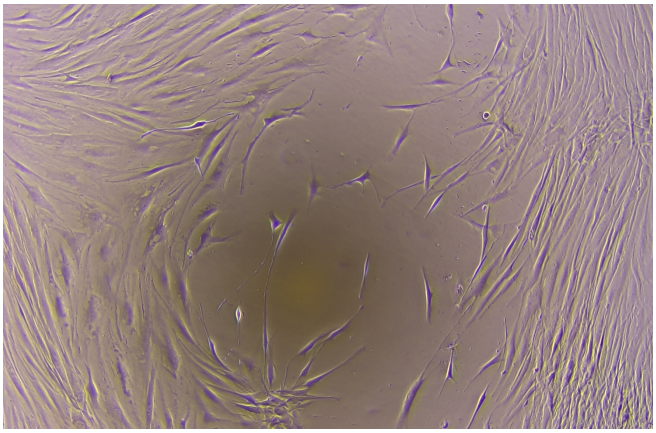


Figure 27: Pitavastatin well 3 at 48 hours

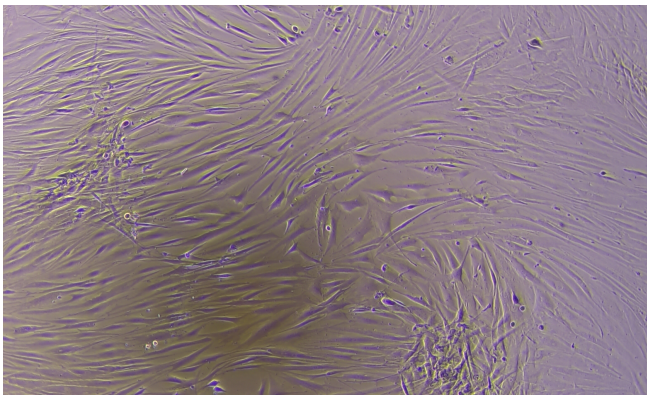


Figure 28: Pitavastatin well 3 at 72 Hours

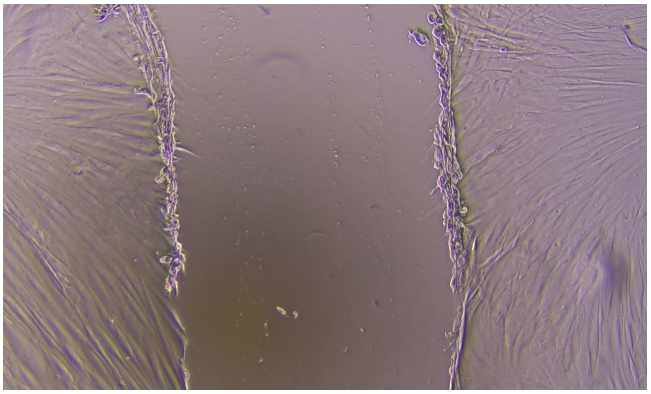
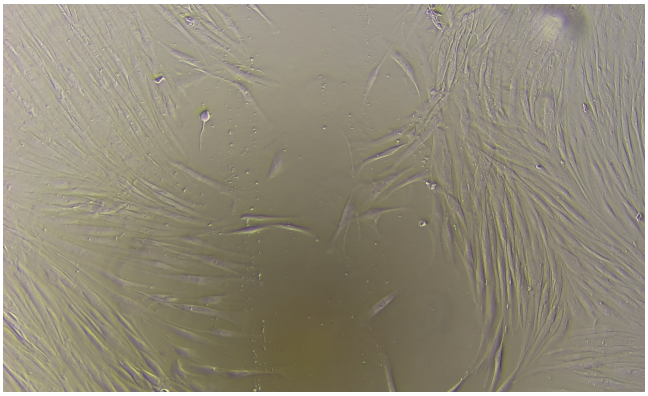


Figure 29: LDL Well 1 at 0 Hours



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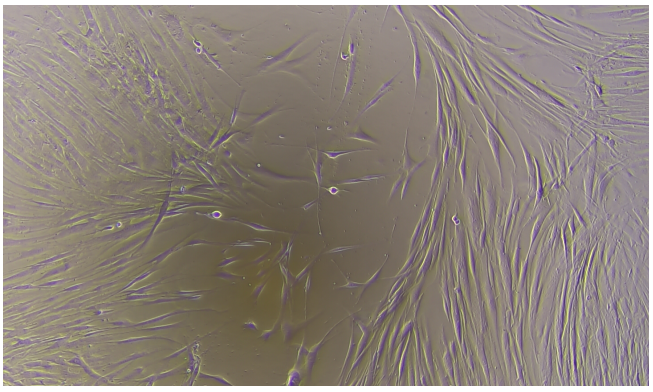
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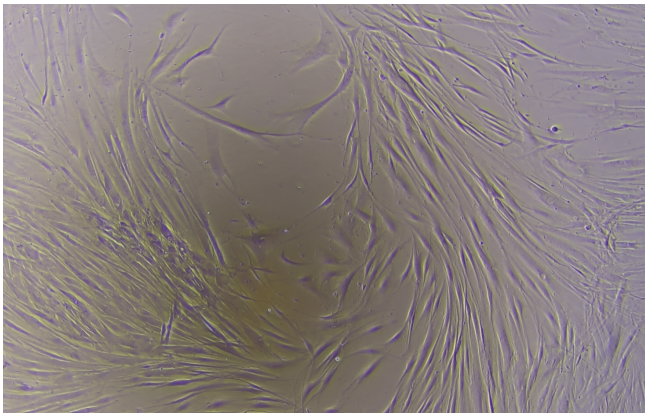


Figure 32: LDL Well 1 at 72 Hours

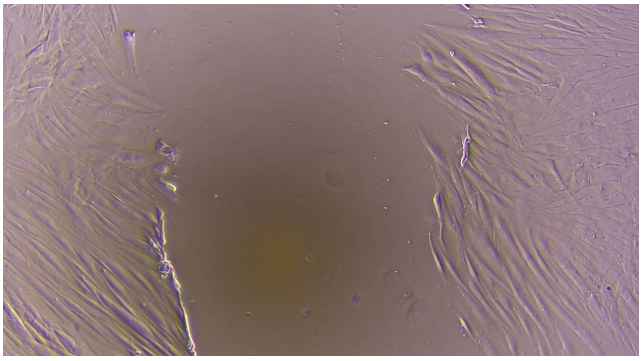


Figure 32: LDL Well 2 at 0 Hours

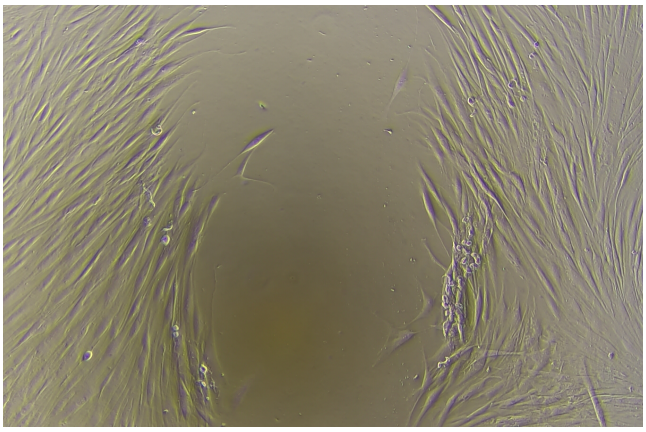


Figure 33: LDL Well 2 at 24 Hours

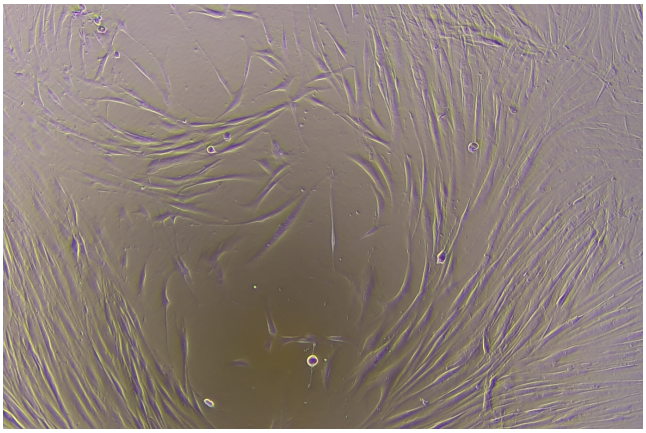


Figure 34: LDL Well 2 at 48 Hours

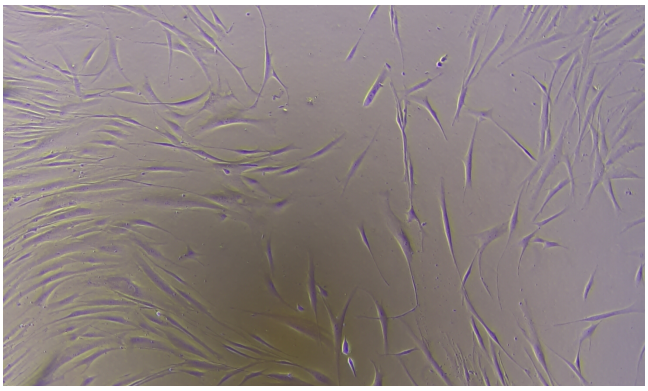


Figure 35: LDL Well 2 at 72 Hours

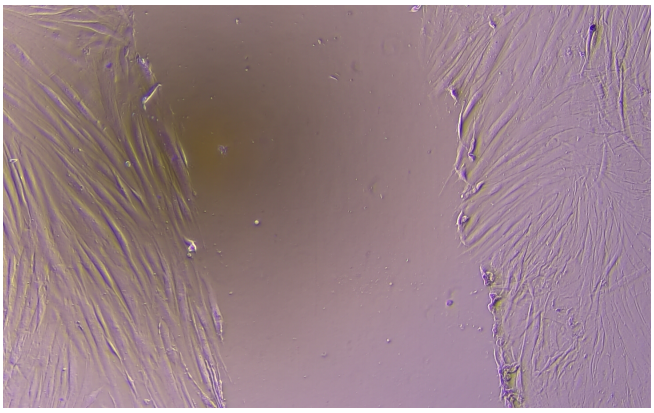
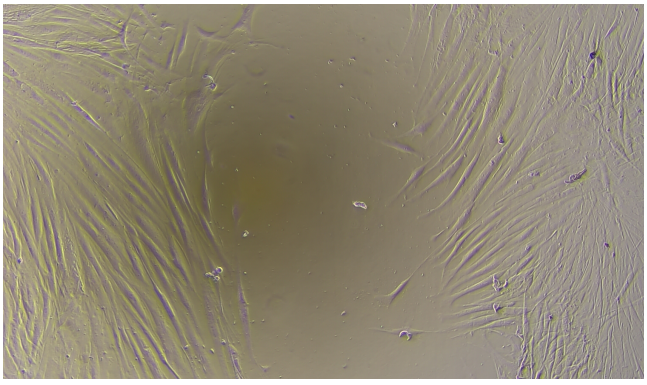


Figure 36: LDL Well 3 at 0 Hours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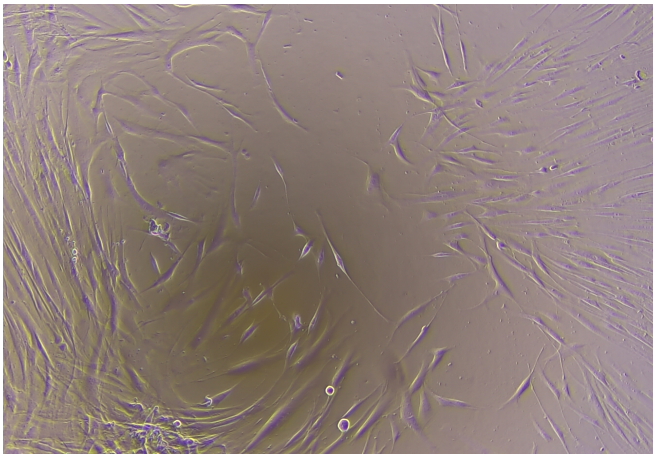


Figure 38: LDL Well 3 at 48 Hours

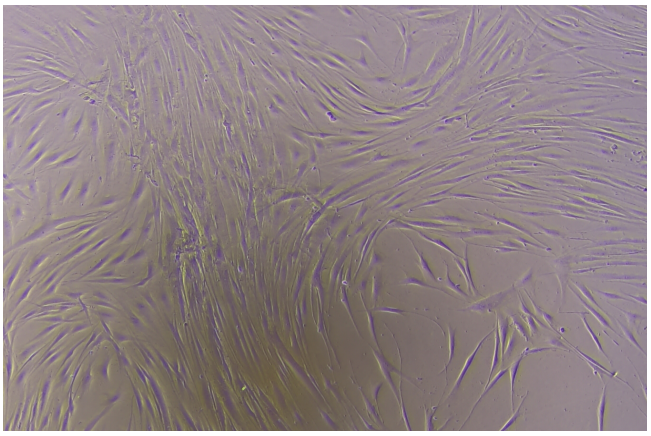


Figure 39: LDL Well 3 at 72 Hours

After 72 hours, the wells were washed twice with cold PBS. Then 1 tablet of Pierce Protease Inhibitor was dissolved in 10mL of cold RIPA lysis buffer, with 200 µLs of the solution allocated to each well. The plates were kept on ice for 5 minutes with occasional swirling for even coverage of the wells. The wells were then scraped to collect the lysate which was then centrifuged. The supernatant was then transferred to new tubes to be frozen at -20℃ where they were stored for 11 days until analysis.

The samples from each well were then analyzed through BCA protein assay using a 96 well plate (Table 1). Seven standard concentrations were prepared and labeled 'A-G' and given two wells each. The final standard concentrations from A to G were 2,000, 1,500, 1,000, 750, 500, 350, and 125 µLs respectively. A blank, with a concentration of 0 µLs was also included in two wells. The samples from the tubes were also distributed into two wells each with concentrations calculated to be within working range. Using the PowerWave XS2 spectrophotometer to read the absorbance of each well, I compared the absorbance and concentration of each sample to the values of the standards and determined an R^2 value of 0.73.

The samples were then calculated to provide an equal amount of protein in µgs/µL to be loaded into two 8% Tris-HCL-SDS-PAGE gels. The two gels were loaded as follows:

Gel 1:

		Glucose 1			No Treatment 2		No Treatment 1		

Gel 2:

No Wound 1		No Wound 2	Glucose 2	Pitavastatin 1	Pitavastatin 2	Pitavastatin 3	LDL 1	LDL 2	LDL 3

The gel plate sandwich was assembled, and the separating gel mixture was made using 100mL of Acrylamide Stock Solution with 30% acrylamide and 0.8% bis-acrylamide and 100mL of Separating Gel Buffer made of 75% 2M Tris-HCl and 0.4% SDS. The remaining volume of both solutions was obtained through the addition of distilled water. Next, 50 µLs of 10% ammonium persulfate and 5 µLs of TEMED were added and mixed, beginning the process of polymerization. The solution was then pipetted into the gel sandwich without forming bubbles, and then topped off with a 1.5cm layer of water. The gel polymerized over the course of one hour.

After the separating gel was complete, the stacking gel was added. The top layer of water was poured off of the separating gel and the Acrylamide solution was again added, this time combined with 100mL of Stacking gel buffer made up of 50% 1M Tris-HCl, 0.4% SDS, and 46% water. 30 µLs of Ammonium persulfate and 5 µLs of TEMED were then added. The stacking gel solution was then pipetted onto the separating gel. The comb was inserted to form the wells, and the gel was left to polymerize for 30 minutes. The comb was then removed and the gel sandwich was added to the electrophoresis chamber and electrophoresis buffer filled the chamber, immersing the gel.

20 µLs of each sample was combined with 5 µLs of the sample buffer. The samples were then heated at 100℃ for approximately 5 minutes before being centrifuged for 5 seconds. The samples were then loaded into the wells. The gels were then run with a voltage of 200 for about 90 minutes. They were then transferred to 8 x 10 cm sheets of nitrocellulose using polyacrylamide gel-membrane sandwiches. The gel-nitrocellulose sandwiches then underwent electrotransfer with a Trans-Blot apparatus in transfer buffer.

After a brief storage period, the blots were ready for immunodetection. Nonspecific binding sites were blocked through incubation in blocking buffer made from dry milk, Tween-20, and TBS. After removing the blocking solution and rinsing with TBS, the first primary antibody (USP47) was applied in a 1:1000 ratio in TBS with 2% Bovine Serum Albumin. After incubation, the membranes were washed 3 times in five-minute intervals with TBS. The membranes were then incubated with the secondary antibody (Universal Mouse Secondary:m-IgGk-HRP), also at a 1:1000 dilution. The washing process was then repeated. For imaging, the membranes were then lightly coated with Peroxide Solution and Luminol Enhancer Solution. The membranes were then placed into a Kodak ChemiDoc Imaging System and the images below (Figures 40, 41) were produced.

Figure 40: Blot 1, Nitrocellulose Sheet 1

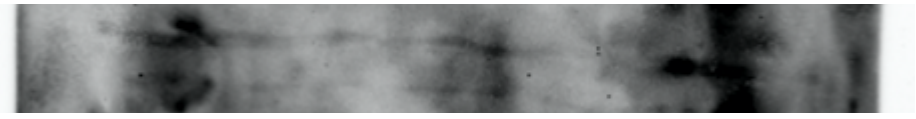


FIgure 41: Blot 1, Nitrocellulose Sheet 2

The antibodies were then removed from the blot. First the membranes were incubated in 5% powdered nonfat milk/TBS. Next the membranes were dried on absorbent paper and then incubated in Erasing Buffer. Then the membranes were washed twice in TBS for ten minutes each and incubated again with the powdered milk and TBS for 6 hours. The immunodetection process was then repeated with an additional primary antibody (phosphorylated **β**-catenin) at a concentration of 1:200, whose images were also included below.

Figure 42: Blot 2, Nitrocellulose Sheet 1

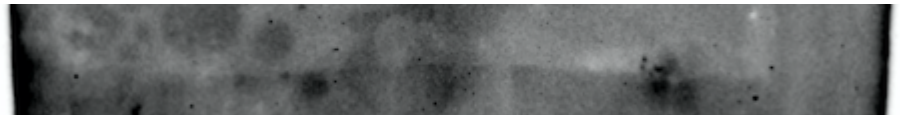
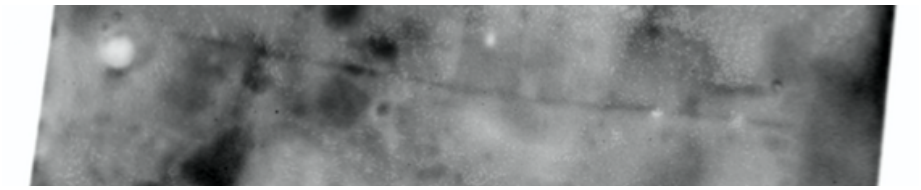


Figure 43: Blot 2, Nitrocellulose Sheet 2:



Normally when performing Western Blots, several months are taken to refine the antibody concentrations and minimize the background interference to get clear bands. However, given the limited time frame for this project, the standard primary and secondary antibody concentration recommendations were used with limited trials.

Results Interpretation

Based on the cell culture imaging, I have ranked the relative cell migration and proliferation of each well. The wells in order from highest amount of cell migration (i.e. fastest and most complete wound healing) to lowest are as follows:

1. Pitavastatin Well 1
2. No Treatment Well 1
3. No Treatment Well 2
4. Pitavastatin Well 3
5. Pitavastatin Well 2
6. LDL Well 3
7. Glucose Well 1
8. LDL Well 1
9. Glucose Well 2
10. LDL Well 2

It is important to note that with this imaging, certain areas of the wounds healed faster or slower than others. The small sections that the images capture do not give a complete picture of the overall healing process and are therefore only used as preliminary evidence. From this data we can infer that Pitavastatin treatment may have a limited effect on wound healing because the progression is closest to the untreated wells, with one well showing improved healing and the other two showing poorer healing. It can also be inferred that LDL and glucose treatments may have similar levels of decreased wound healing.

Next, I interpreted the results from the Western Blots. Using ImageJ software, I was able to quantify the amount of protein in each band on the nitrocellulose based on the size of the band and its intensity, which I then graphed as shown in Figures 44 and 45.

Figure 44:

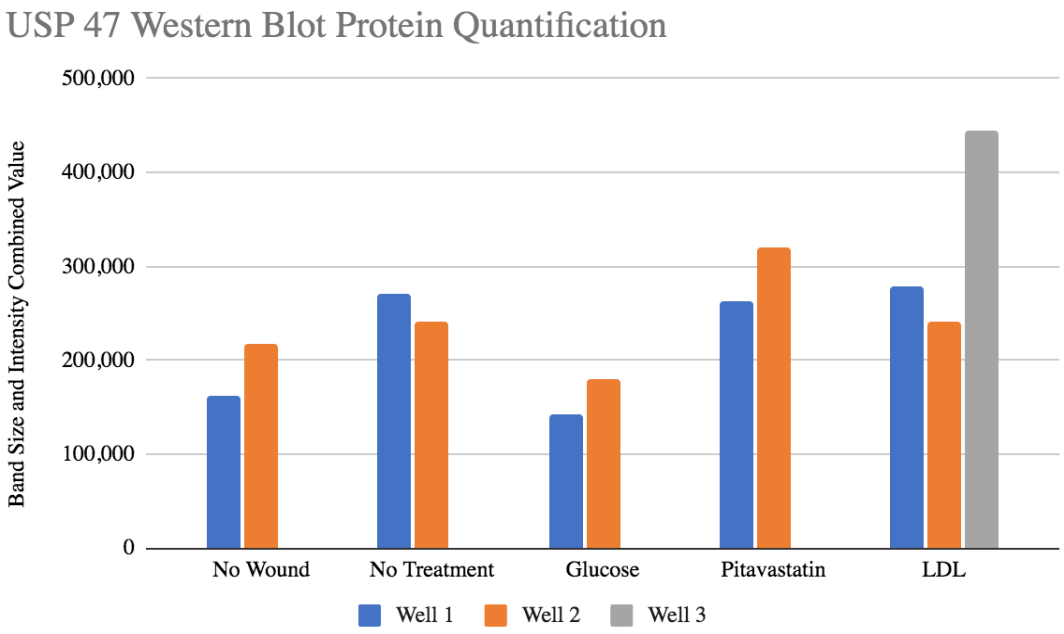
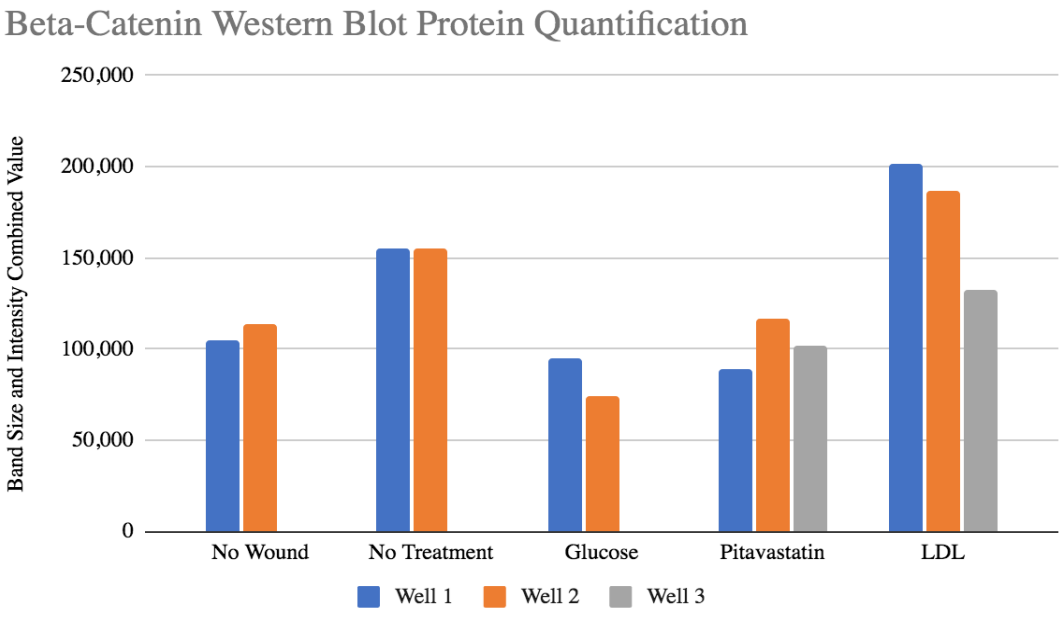


Figure 45:



The relative values were then analyzed using Microsoft Excel's T-Test function to determine statistical significance in the variation from the No Treatment wells to the treatment wells in the form of P values.

Table 1: USP 47

No Wound	No Treatment	Glucose	Pitavastatin	LDL
161,252	270,000	142,560	N/A*	278,208
218,400	241,080	179,605	262,656	241,020
			319,748	444,220
P values:		0.057	0.381	0.479

Table 2: **β**-catenin

No Wound	No Treatment	Glucose	Pitavastatin	LDL
104,960	155,181	94,530	88,704	201,240
114,070	154,752	73,920	116,714	186,405
			101,640	132,300
P values:		0.021	0.015	0.546

*The Pitavastatin Well 1 band was lost due to background interference

P values less than 0.05 indicate that the differences in the categories of values are statistically significant and a significant effect was observed in the data. Based on these values, the amount of **β**-catenin in the No Treatment wells compared to the Glucose and Pitavastatin wells was significantly higher. The difference between the No Treatment wells and LDL was not found to be significant. Looking at USP47, the comparison of Glucose to No Treatment appears to be approaching significance. Comparison to Pitavastatin and LDL shows no significance.

Discussion

Overall, some results met expectations, while others did not. The results that met expectations were that of the significantly decreased **β**-catenin expression in the glucose and Pitavastatin treated wells. Although not proven to be significant, the increase in expression of both proteins in the LDL treated wells was also expected.

Future research would be useful for running additional trials to expand the data pool to increase the certainty of the significance (or insignificance) of these results. Additionally, future work in this area may involve the examination of other similar factors that influence wound healing. In particular, studying alternate cell signaling pathways that are important for the wound healing response under similar conditions, such as the JNK pathway, would be of interest. Other cell type models or the use of mouse models may also be adapted to bring conditions closer to those in human tissue. Another avenue of research may involve looking into the effects of varying cholesterol and glucose levels in the environment on other cellular processes.

From a clinical standpoint, these studies may lead to a reevaluation of the treatment of high cholesterol in diabetic patients and others who are suffering from chronic wounds. A better understanding of the relationships between high blood sugar, LDL cholesterol, and statin drugs on wound healing could also alter both long-term and short-term care approaches for patients experiencing more than one of these conditions at once.

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