Cell Physiology Lab Report

The individual and synergistic or antagonistic effects of testosterone and caffeine on cell migration of Ea.hy926 endothelial cells using scratch wound assay

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Abstract

Endothelial cells are commonly use research and drug trials in order to understand the effects of various substances and chemicals on the survival, proliferation and migration of these cells. Determining the interactions of different drugs in a system is important to understand how they may result in synergistic or antagonist interactions in the body. In this experiment, caffeine, which acts as an inhibitor of cell migration and testosterone, which acts as an inducer of cell migration, were used independently and simultaneously in order to understand the effects of these drugs on wound healing using a cell migration assay. A scratch wound assay was used and cells were monitored for four days following the initial scratch to monitor cell migration. Our results were consistent with previous studies, determining that caffeine acted as an inhibitor of cell migration and testosterone acted as an inducer of cell migration. When the drugs were tested simultaneously, our results demonstrated that cell proliferation was reduced and the scratch wound became larger with each time period the wound was monitored. Future work includes optimizing the concentrations of caffeine and testosterone used to determine at what concentration of each drug do we see an effect on cell proliferation.

Introduction

Endothelial cells are vital for our bodies functioning and play a role in regulating and controlling numerous bodily functions such as blood coagulation and pressure (Majewska et al. 2021). Endothelial cells create a very thin layer of cells that line all blood vessels and help control the movement of substances and white blood cells in and out of the bloodstream (Alberts et al. 2002). Issues or disorders associated with endothelial cells are known to be associated with multiple diseases such as cancer, cardiovascular disease, hypertension and diabetes (Wang et al. 2021). Immortal cell lines, such as EA.hy926, are commonly used in endothelial cell assays due to their increased stability and can be cultured and maintained more easily than primary endothelial cells which have not been immortalized (Wang et al. 2021).

Performing different assays using endothelial cells can also help us better understand mechanisms of and developing tools to mitigate and treat these diseases (Mjewska et al. 2021). Endothelial cells can be used in various assays to monitor cell proliferation, migration and apoptosis. For example, endothelial cells play a role in cancer cell proliferation. Methods such as

angiogenesis and cell proliferation assays can be used to understand how potential drug therapies can affect cancer cell growth (Majewska et al. 2021).

As discussed earlier, multiple different assays can be performed on endothelial cells to monitor the effect of various drugs or targets on different cellular activities. The wound healing assay, or scratch test, is used to study the migration of cells following the deliberate creation of a wound or gap within the cell culture (Jonkman et al. 2014). The gap healing rate is typically monitored using different drugs or substances to monitor the effect the substance used has on cell migration (Jonkman et al. 2014).

Testosterone is a sex hormone found in both men and women which has been found in previous studies to be associated with decreased risk of cardiovascular disease in men with higher levels of testosterone (Liao et al. 2013). As discussed by Liao et al. in 2013, sex hormones, specifically testosterone, increase endothelial cell migration via activation of moesin, causing actin rearrangement and potentially contributing to lower cardiovascular disease and death in men. Testosterone is the human body can have a range of concentrations in blood serum varying by sex, age and genetics. In a paper by Liao et al. published in 2013, testosterone concentrations of 50nM had positive effects on cell proliferation and migration. Testosterone targets androgen receptors within the body to regulate gene expression (Lucas-Herald and Touyz, 2022).

Conversely, caffeine has been found to inhibit cell proliferation and migration (Ojeh et al. 2014). Caffeine products include antioxidants, theobromine and xanthine, which have been found to increase wound healing; however, caffeine has been found to decrease wound healing (Ojeh et al. 2014). Caffeine is a known adenosine receptor antagonist and may induce cell death and angiogenesis (Li et al 2013; Ojeh et al. 2014). To study the effects of caffeine on various cellular activities, low concentrations (0.1mM and 450um) of caffeine are favoured due to high concentrations of caffeine having harmful effects on humans and would not be applicable to human physiology (Li et al 2013; Ojeh et al 2014). Caffeine may function through various mechanisms of action in the body, including, antagonism of adenosine receptors and intracellular calcium release (Institute of Medicine 2001).

Synergism of drugs and chemicals is a current topic of research to understand how different drugs/chemicals may have an overall increased effect when combined. This term often refers to drugs that have a similar effect on cells or organisms and combined in the organism

have an increased effect than either compound could have alone (Tallarida 2011). However, antagonistic effects of drugs refers to two or more drugs which have opposite effects and therefore block the effect of either drug (Yin et al. 2014). It is important for researchers to understand the synergistic and antagonistic effects of combined drugs to determine which drugs are not compatible (Yin et al. 2014).

In this paper we will investigate the individual and combined effects of testosterone and caffeine on cell migration of endothelial Ea.hy926 cells using a scratch wound assay. We will determine if caffeine and testosterone have antagonistic or synergistic effects on cell migration.

Materials and Methods

Preparation of Complete Media

Leibovitz's L-15 complete media with 10% fetal bovine serum (FBS) was prepared. To prepare 100ml of complete media, 1.2282 g of Leibovitz's L-15 powder was added to 89ml of sterile, distilled water. In a beaker with a magnetic stir bar, the solution was mixed until the powder was dissolved. The beaker was removed from the stir plate and 10ml of 10% FBS was pipetted into the media. 600ml of penicillin was added to the beaker and then returned to the stir plate for 5 minutes. In a sterile workspace with flame, complete media was added to a pre-sterilized 250ml glass container. The media was sterilized using 50ml syringes and dispensed through a 0.22um filter into the pre-sterilized container. The glass container was appropriately labelled and placed into the fridge.

Preparation of Phosphate-Buffered Saline

To prepare 250ml of 1x solution, add 2.5g of PBS powder and 250ml of sterile, deionized water into a glass beaker with a magnetic stir bar. Place beaker on stir plate and allow to mix until PBS powder is dissolved. Calibrate pH meter using standard solutions. Determine pH of PBS solution, the final PBS solution should have a pH of 7.2. Add HCl or NaOH to adjust the pH of the PBS solution. Add PBS solution into a 500ml bottle and place cap loosely on bottle. Appropriately label bottle and place autoclave tape on glass bottle. Autoclave the PBS media, close the cap tightly and store at room temperature.

Preparation of Endothelial Cells

Materials:

- Frozen Ea.hy926 endothelial cells
- Warm, Leibovitz's L-15 complete media
- 15ml centrifuge tube
- T25 flask
- Inverted microscope to observe cells

Procedure:

1. After putting on lab coat and gloves, sterilize workspace using 70% EtOH, allow to evaporate and then light bunsen burner. Place Leibovitz's L-15 complete media into 37^{o}C water bath to warm.
2. Remove vial of Ea.hy926 endothelial cells from freezer and warm in hands. Once cells are thawed, gently invert the Eppendorf tube to mix the cell suspension.
3. Remove media from water bath and add 5ml of warm, complete medium to a 15ml centrifuge tube. Gently add the cell suspension, dropwise into the centrifuge tube containing the complete medium.
4. Centrifuge at 300 RCF for 5 minutes.
5. Pour off supernatant and add 3ml of complete medium. Gently resuspend the pellet using a pipette.
6. Using a T25 flask, add the 3ml of cell suspension into the flask and close the flask. Observe the cells using an inverted microscope.
7. Incubate cells in 37^{o}C incubator and feed every other day by aseptically removing half of the media in the flask and replacing with fresh, warm, Leibovitz's L-15 complete medium to maintain the volume at 3ml.

Preparation of Testosterone Solution

Materials:

- Testosterone, $C_{19}H_{28}O_2$, 288.42 g/mol
- Sterile, diH2O
- Sterile glass bottle
- Weigh boat

- Spatula
- Magnetic stirrer and stir bar
- 400 mL clean beaker

Procedure:

1. To make a 0.01 mg/mL testosterone stock solution, 2.5 mg of testosterone powder were weighed with an analytical balance and transferred to a 400 mL beaker.
2. Dissolved testosterone powder in 250 mL deionized water using a magnetic stirrer until no visible powder was observed.
3. Transferred solution to a labeled autoclaved bottle for storage in the fridge.

Preparation of Caffeine Solution

Materials:

- Caffeine anhydrous, $C_8H_{10}N_4O_2$, 194.2 g/mol
- Sterile, diH2O
- Sterile glass bottle
- Weigh boat
- Spatula
- Magnetic stirrer and stir bar
- 400 mL clean beaker

Procedure:

1. To make a 0.01 mg/mL caffeine stock solution, 2.0 mg of caffeine powder were weighed with an analytical balance and transferred to a 250 mL beaker.
2. Dissolved caffeine powder in 200 mL of deionized water using a magnetic stirrer until no visible powder was observed.
3. Transferred solution to a labeled autoclaved bottle for storage in the fridge.

Preparation of Scratch Wound Assay

Materials:

- Razor
- Hemocytometer
- Inverted microscope

- Phosphate buffered saline
- 24 well plate
- Ea.hy926 endothelial cell cultures
- Trypsin EDTA
- Leibovitz's L-15 complete medium
- 15ml centrifuge tube
- Trypan blue
- Eppendorf tube

Procedure:

1. Wearing all appropriate PPE, sterilize workspace using 70% EtOH. Once evaporated, light flame and wipe down the workbench again with 70% EtOH.
2. Place trypsin EDTA, complete medium and PBS in water bath.
3. Observe flask under inverted microscope to observe cell growth.
4. Remove all medium from the flask and replace with 3ml of warm, PBS solution and gently swirl.
5. Remove all PBS and repeat wash. Remove PBS solution once ready to add trypsin EDTA.
6. Add 2ml of trypsin EdTA to T25 flask and gently swirl the flask to ensure the trypsin EDTA has covered all of the cells.
7. Place flask into incubator for 2 minutes and then use inverted microscope to observe cells. Cells should be round and no longer adhered to flask. If cells are still adhered to flask, incubator for another 2 minutes and observe again.
8. Once cells are round and detached, firmly tap the flask to dislodge cells.
9. Quickly add 3ml of complete medium to deactivate the trypsin EDTA.
10. Transfer cells to a 15ml centrifuge tube and centrifuge at 300 RCF for 10 minutes.
11. Gently pour off supernatant and then add 1ml of complete media to the tube. Resuspend cells by gently vortexing for a few seconds or by using a pipette to dislodge the pellet.
12. To determine the cell concentration, remove a 20ul aliquot of the resuspended cells and add to an Epindorf tube containing 20ul of trypan blue. Count the cells using a hemocytometer and observe using a microscope.
13. Add 2ml of complete media to the 15ml centrifuge tube

14. Using the razor, score the underside of the 24 well plate to create 4 quadrants in each well
15. Add 500ul of cell suspension to each well and incubate on 37°C until cells had ~90% or full confluence. Maintain wells at 500ul by adding complete media as needed.

Scratch Wound Assay

Materials:

- Sterile P200 pipette tips
- Seeded 24 well plates
- Phosphate Buffered Solution

Procedure:

1. Done all appropriate PPE and sterilize workbench using 70% EtOH and light bunsen burner. Warm PBS solution, caffeine solution and testosterone solution in 37°C water bath.
2. Observe wells using an inverted microscope to ensure cell growth is between ~90 to full confluence to begin experiment.
3. Working near the flame, use a sterilized P200 pipette tip to make a vertical scratch to each well from top to bottom in a swift, continuous movement.
4. Wash each well with PBS and then replace with appropriate media. For caffeine and testosterone independently treated wells, add 250ul of 0.01 mg/mL stock solution to each well and 250ul of complete media. For co-treated wells with caffeine and testosterone, add 125ul of each stock solution and 250ul of complete media. For the wells treated with negative control, add 500ul of sterile, diH2O. For wells treated with positive control, add 500ul of complete media. See Figure 1.
5. Observe the wells using an inverted microscope to ensure the scratches are all similar. Take images of each well and determine the distance of each scratch.
6. Incubate wells at 37°C. Observe scratches 8 hrs, 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs, and 72 hrs after scratch and treatment were applied. Take images of each well and record distance of scratch wound.

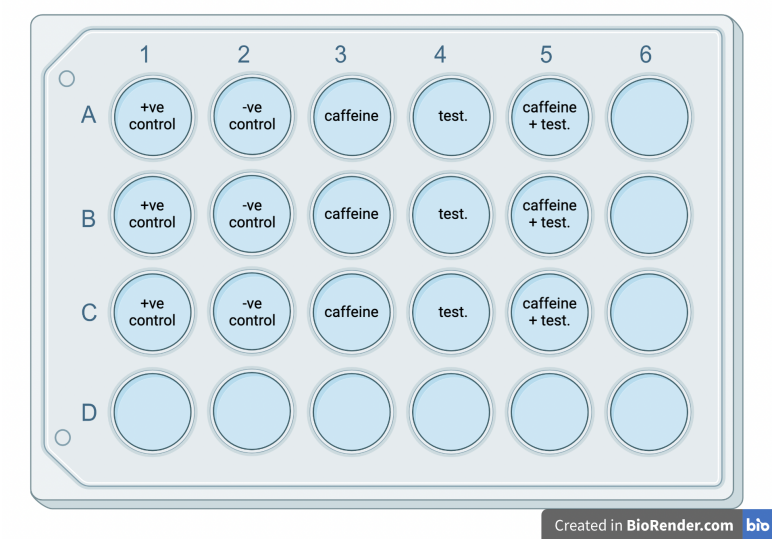

Figure 1**.** Example of treatments used for scratch wound assay using a 25 well plate to determine the individual and synergistic effects of caffeine and testosterone on endothelial cell migration. +ve control = 500ul Leibovitz's L-15 complete media, -ve control = 500ul sterile, diH2O, caffeine = 250ul 0.01 mg/mL caffeine solution plus 250ul Leibovitz's L-15 complete media, test. = 250ul 0.01 mg/mL testosterone solution plus 250ul Leibovitz's L-15 complete media, caffeine + test. = 150ul 0.01 mg/mL caffeine solution plus 150ul 0.01 mg/mL testosterone solution plus 250ul Leibovitz's L-15 complete media. Created with BioRender.com

Results

Table 1. Scratch wound width measurements for March 29 (first day) at 8:00 AM (immediately following scratch), 4:00 PM, and 8:00 PM.

Time	Well #	Positive control	Negative control	Caffeine	Testosterone	C + T
8:00 AM	1	346.0 µm	399.1 µm	419.0 µm	485.2 µm	389.6 µm
	2	357.0 µm	379.5 µm	458.0 µm	329.2 µm	339.3 µm
	3	341.0 µm	508.7 µm	457.9 µm	410.6 µm	299.6 µm
4:00 PM	**Well #**	**Positive control**	**Negative control**	**Caffeine**	**Testosterone**	**C + T**
	1	331.6 µm	434.0 µm	538.8 µm	453.7 µm	390.4 µm
	2	302.9 µm	427.3 µm	455.4 µm	310.6 µm	367.1 µm
	3	273.2 µm	450.8 µm	554.0 µm	353.2 µm	313.0 µm
8:00 PM	**Well #**	**Positive control**	**Negative control**	**Caffeine**	**Testosterone**	**C + T**
	1	282.0 µm	471.0 µm	564.7 µm	390.5 µm	403.5 µm
	2	272.0 µm	478.3 µm	489.2 µm	280.6 µm	389.5 µm
	3	233.4 µm	545.3 µm	567.6 µm	326.8 µm	323.1 µm

For each positive control and testosterone well, the scratch distance decreased, while for the negative control, caffeine, and caffeine + testosterone well, the distance increased. There was no pattern or trend observed in the width changes.

Table 2. Scratch wound width measurements for March 30 at 8:00 AM and 8:00 PM.

Time	Well #	Positive control	Negative control	Caffeine	Testosterone	C + T
8:00 AM	1	246.5 µm	526.0 µm	572.4 µm	382.5 µm	410.2 µm
	2	185.8 µm	529.3 µm	517.8 µm	269.9 µm	433.3 µm
	3	216.8 µm	567.0 µm	582.0 µm	289.0 µm	330.7 µm
8:00 PM	**Well #**	**Positive control**	**Negative control**	**Caffeine**	**Testosterone**	**C + T**
	1	235.9 µm	563.9 µm	572.6 µm	320.6 µm	413.0 µm
	2	157.7 µm	578.0 µm	534.4 µm	264.0 µm	477.2 µm
	3	186.2 µm	581.0 µm	597.0 µm	258.4 µm	343.4 µm

Positive control and testosterone wells continued to decrease in width with no particular trend. Negative control, caffeine, and caffeine + testosterone scratches kept increasing in distance. A slower change in width was observed throughout the day compared to March 29 measurements, which within twelve hours increased/decreased respectively by a considerable amount.

Table 3. Scratch wound width measurements for March 31 at 8:00 AM and 8:00 PM.

Time	Well #	Positive control	Negative control	Caffeine	Testosterone	C + T
8:00 AM	1	167.5 µm	dead cells	603.2 µm	dead cells	414.0 µm
	2	135.0 µm	dead cells	573.8 µm	dead cells	495.2 µm
	3	150.0 µm	dead cells	603.1 µm	dead cells	dead cells
8:00 PM	**Well #**	**Positive control**	**Negative control**	**Caffeine**	**Testosterone**	**C + T**
	1	148.0 µm	dead cells	605.0 µm	dead cells	414.2 µm
	2	111.4 µm	dead cells	602.6 µm	dead cells	518.8 µm
	3	119.5 µm	dead cells	610.1 µm	dead cells	dead cells

Scratch wound width for the positive control cells continued to decrease, while caffeine and caffeine + testosterone treated cells continued to slowly increase in width. There were no viable cells remaining in the negative control and testosterone wells, as well as for the third well for caffeine + testosterone.

Table 4. Scratch wound width measurements for April 1 at 8:00 AM. (TBTM = too big to measure).

Time	Well #	Positive control	Negative control	Caffeine	Testosterone	C + T
8:00 AM	1	dried out	dead cells	TBTM/dead	dead cells	415.5 µm
	2	closed scratch	dead cells	TBTM/dead	dead cells	dead cells
	3	closed scratch	dead cells	TBTM/dead	dead cells	dead cells

Wells 2 and 3 for the positive control had their scratch wound completely closed, while well 1 had dried out. The cells with caffeine treatment had died and their wound was too wide to measure with the inverted microscope. Wells 2 and 3 for the caffeine + testosterone treatment had also died, but well 1 showed a slight increase in width.

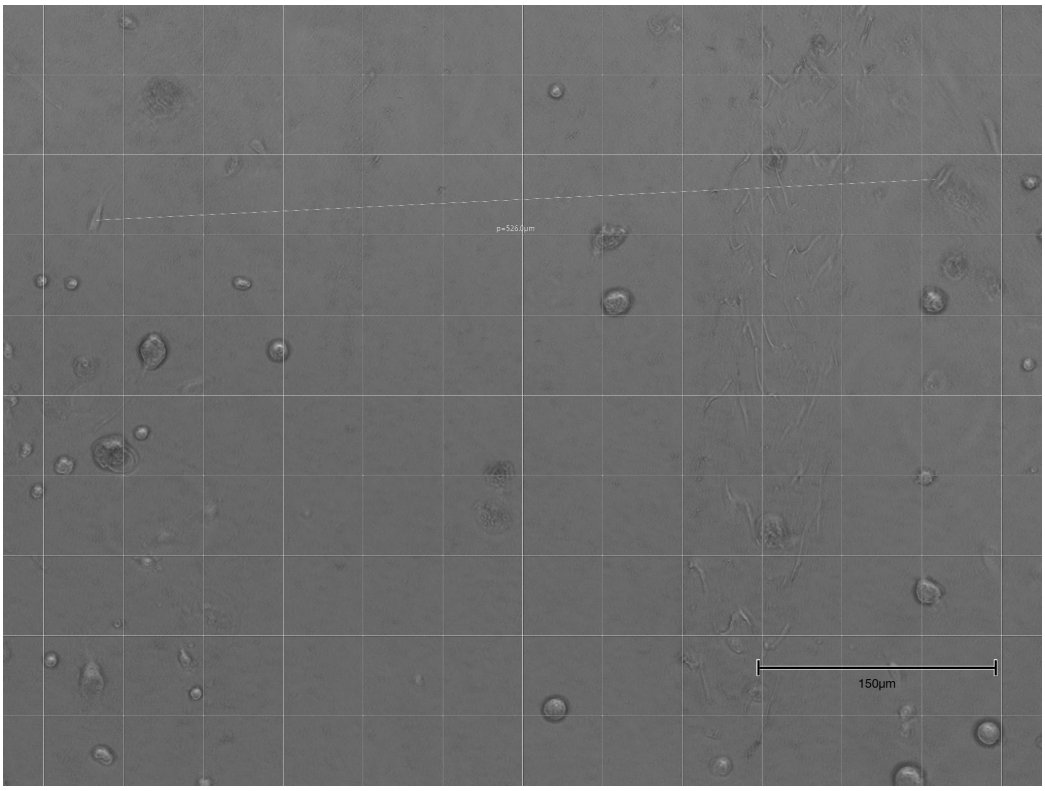

Figure 2. Negative control well 1 post scratch. Image taken on Wednesday, March 29th at 08:00.

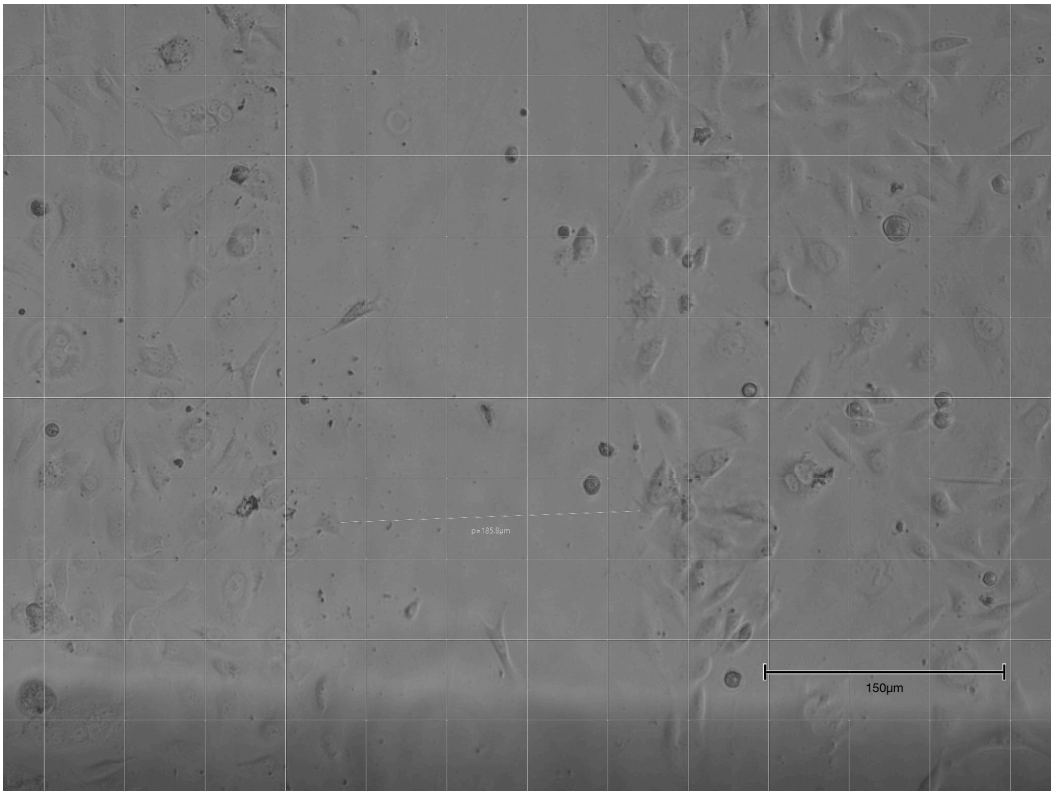

Figure 3. Positive control well 2 post scratch. Image taken on Thursday, March 30th at 08:00.

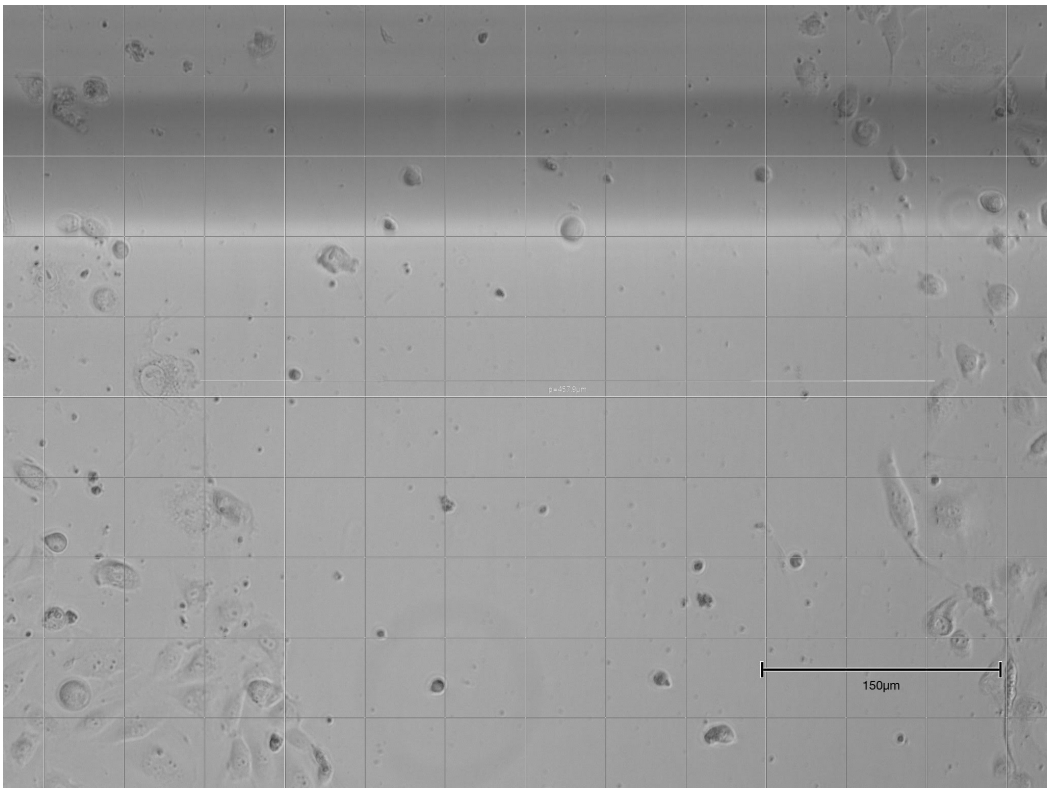

Figure 4. Caffeine well 3 post scratch. Image taken on Wednesday, March 29th at 08:00.

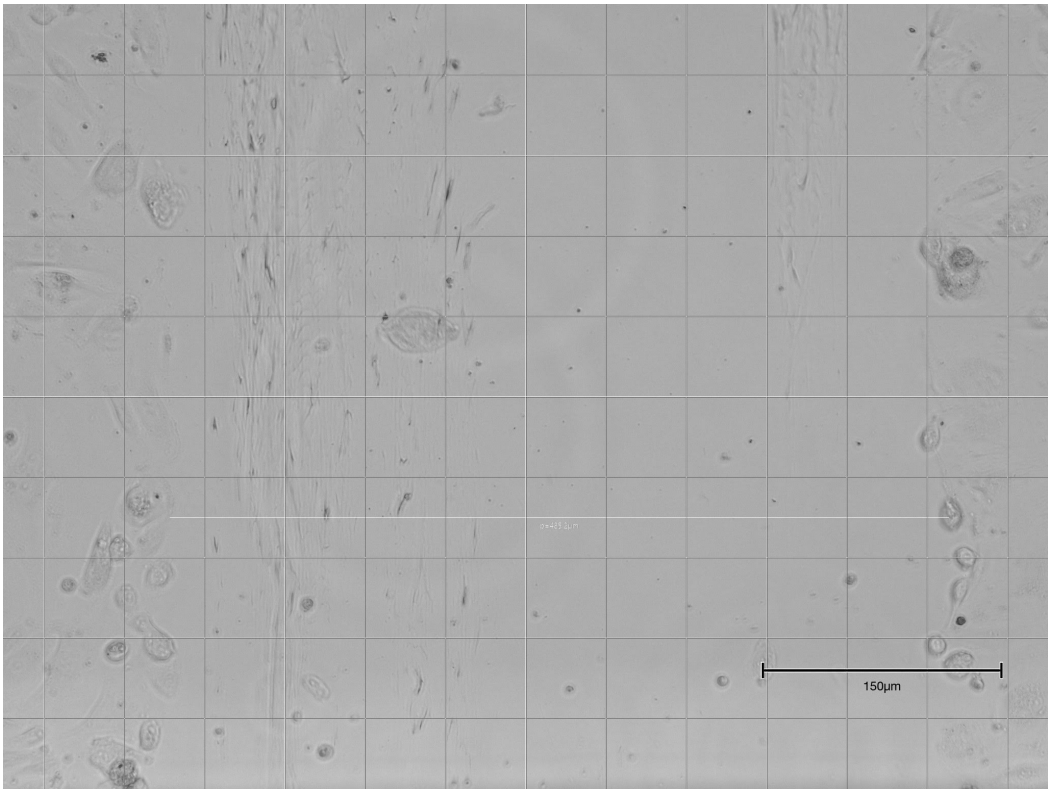

Figure 5. Testosterone well 1 post scratch. Image taken on Wednesday, March 29th at 08:00.

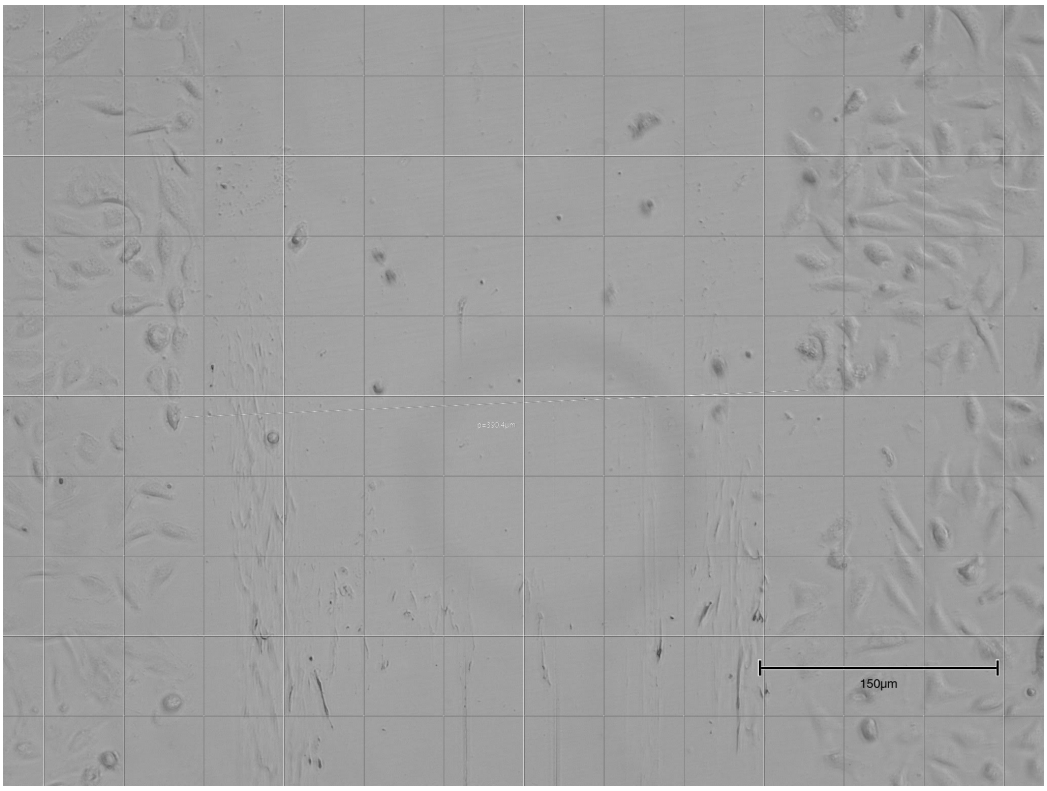

Figure 6. Caffeine and testosterone well 1 post scratch. Image taken on Wednesday, March 29th at 16:00.

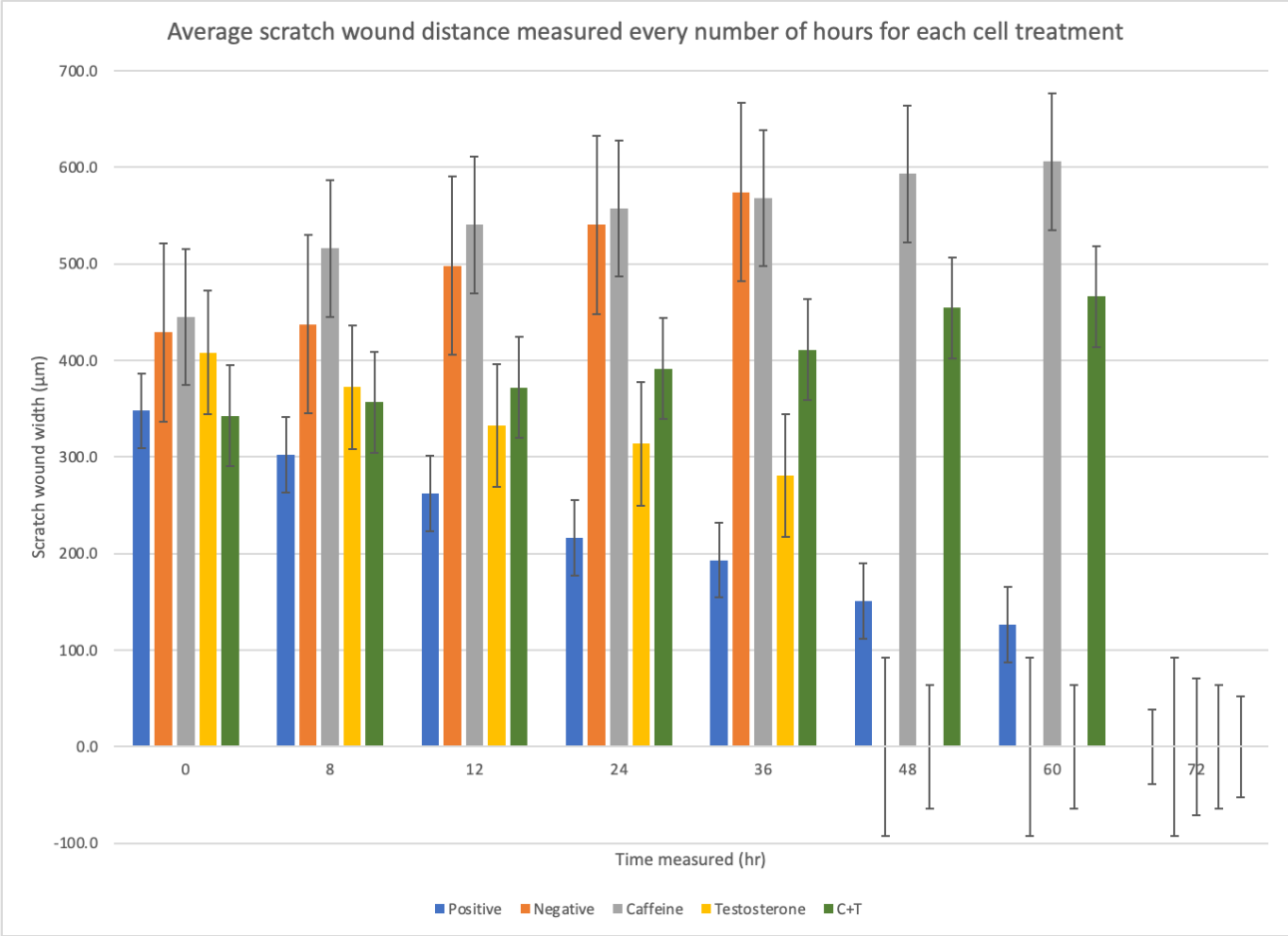

Figure 7. Bar graph for the average scratch would distance (in µm) measured every 8 to 12 hours from initial scratch for each cell treatment.

Discussion

For the positive and negative control wells, the outcomes were as expected. The positive control was treated with Leibovitz's L-15 media, which contains fetal growth serum (FGS) that helps the cells grow and proliferate to close the scratch wound, as seen by the decrease in scratch width in Figure 2. This decreasing trend continues until 72 hrs after the initial scratch, when the wound had completely closed for two of the wells and thus there is no bar for that time in Figure 2. The negative control wells were treated with sterile deionized water, which holds no nutritional value for the cells and does not help them grow. This is confirmed in Figure 2, where the bar for negative control increases in height for every measurement until the 48 hr mark when the cells had died because no nutrients were available to help proliferation and movement.

From previous research studies, it was established that caffeine acts as an inhibitor for cell proliferation, migration, and wound healing and as an adenosine receptor antagonist which may cause cell death and angiogenesis (Li et al 2013; Ojeh et al. 2014). These findings were confirmed through the results of this experiment. In Figure 2, an increase in bar height for the caffeine-treated cells indicated that the scratch wound widened every number of hours until 72 hours had passed from the initial scratch. By then, the wound was either too wide to measure or the cells had died, suggesting that caffeine acted effectively to prevent the growth of new cells post-scratch and the movement of any remaining ones to cover the surface. Other studies state that this inhibitory effect is observed even after caffeine withdrawal (Liu et al. 2017), which can be also confirmed with these results since no new caffeine was administered to the cells after the start of the test.

Testosterone is known to induce cell proliferation and cell cycle gene expression (Barbosa-Desonlges et al 2013). It does so by promoting protein synthesis, stimulating the production of growth hormones, and thus stimulating cell proliferation. Our findings confirmed this statement as seen from the steady decline in the width of the scratch from the first hour to 36 hours. There was a constant growth of cells seen in the scratch until hour 36. Two days (48 hours) from the initial scratch, the cells in the well started to die. This could be attributed to two explanations; firstly, since we did not re-feed the cells in the wells with more Leibovitz's media, perhaps the testosterone alone was not enough to allow the cells to grow. Secondly, we did notice a yeast infection in our wells around the 48-hour mark. These explanations might be able to answer why there were only dead cells present in the testosterone wells from 48 hours and onwards. Nevertheless, from the 36 hours where there were no dead cells we can conclude that there was a steady decline in the width

of the initial streak confirming that testosterone alone was able to induce Ea.hy926 endothelial cell growth, as confirmed by previous research.

Lastly, as described above, the effects of caffeine and testosterone are opposing. While caffeine decreases cell growth, testosterone increases cell growth. Since they have opposing effects, investigating their effect on Ea.hy926 endothelial cells was something that piqued our interest. We saw that, in conjunction, there was a steady increase in the scratch wound width as time went on. The caffeine-testosterone treatment was not able to induce cell proliferation. At the 48-hour mark, much like the testosterone only treatment, the wells treated with caffeine and testosterone showed some dead cells. This shows that when used together, the caffeine's capability of depleting cell growth dominates over testosterone's capability of inducing cell proliferation.

Future work for this experiment would include further testing of different concentration of caffeine and testosterone used in the treatment wells. This would be done in order to understand the lowest concentration of each drug needed in order to effect cell migration. Optimizing the concentration of each drug and varying the concentration of caffeine and testosterone used in the simultaneous wells would allow us to determine the optimal concentration in order to induce cell proliferation. In these experiments, equal concentrations of caffeine and testosterone were used in order be readily comparable. However, because these drugs work in opposing effects and through different mechanisms, each drug liekly has different optimal concentrations to produce their effect.

Conclusion

In conclusion, this experiment successfully shows the effect of caffeine, testosterone, and a combination of caffeine and testosterone on endothelial cell proliferation and migration through the use of a scratch wound assay. Using positive and negative controls as baseline for comparison, the effects of each treatment was observed and recorded every 8 to 12 hours through the measurement of the scratch wound width. Caffeine-treated wells showed decreased proliferation and migration, demonstrated by the continuous increase in wound length and the lack of coverage from any existing cells over the wound. Testosterone-treated wells showed an increase in cell proliferation indicated by the steady decline in the streak width. Lastly, the effect of caffeine and testosterone treated wells showed an antagonistic effect on Ea.hy926 endothelial cells, meaning that the opposing effects of caffeine and testosterone led to a decrease in cell proliferation.

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