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Caffeine effects on endothelial cells

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Abstract

The effect of caffeine on cell growth was determined using scratch assay. Scratch assay is a commonly used cellular biological technique that is simple and easily reproducible. The assay is used to measure basic cell migration by inducing a thin scratch along a confluent growth of cells[5]. The distance within the scratch is used to measure the speed at which the cells are able to "heal" the "wound". This technique does not involve the use of expensive equipment, and requires only a microscope with the ability to measure the very small width of the scratch. This protocol allowed for the use of scatter plots of distance versus time to analyze the effect that various concentrations of caffeinated Leibovitz's L-15 with 10% FBS complete medium had on endothelial cells. The experiment resulted in a growth rate decrease when fed with caffeine media, however, due to the cells becoming radioactive halfway through the experiment, no further assumptions could be made from the data.

Introduction

The most widely taken psychoactive stimulant is the naturally occurring central nervous system stimulant, caffeine[1]. It is commonly consumed through natural beverage sources such as coffee and teas, but is also found in chocolate (from the cacao beans) and in soft drinks and energy drinks (as an additive). Caffeine is a fat and water soluble molecule that readily crosses the blood-brain barrier[1]. It acts on adenosine receptors as an antagonist in and outside of the brain[2]. This action increases sympathetic nervous system stimulation[1] resulting in the increased feeling of wakefulness, along with the increase in blood pressure and heart rate associated with caffeine use. In previous studies caffeine has been reported to affect cell cycle regulation, proliferation, support cancer cell growth, induce apoptosis and mutate regulatory proteins[3]. Caffeine also has positive effects, its ability to inhibit kinase activities allows it to be useful in suppressing phosphoinositides 3-kinase. The intent of this experiment was to determine the effect of caffeine on endothelial cells. The effect of caffeine tends to be complicated with respect to endothelial cells as it may improve or diminish the viability of the cells. A large part of the effect of caffeine on endothelial cells in-vivo is dependent on the interaction with vasodilation, thrombosis, inflammation, and anti-oxidation/pro-oxidation. Because the experiment performed here is in-vitro, oxidation is the main significant factor, and due to the lack of supplementation of the cells with antioxidants, pro-oxidation is suspected to be significant. For this reason, the cells are expected to experience oxidative stress resulting in an inhibition of cell proliferation.[8] The scratch test was performed because of its simplicity as well as cost effectiveness, and the gap closure rate being an accurate representation of cell health.

Experimental

Preparation of Leibovitz's L-15 complete medium

100 mL of Leibovitz's L-15 with 10% FBS complete medium was prepared using the materials outlined in table 1.

Table 1. Materials required for the preparation of 100 mL of Leibovitz's L-15 with 10% FBS complete medium.

Leibovitz's L-15 (HiMedia)	1.3293 g
antibiotic/antimycotic	0.6 mL
Fetal growth serum	10 mL
Sterile water	89 mL
Filter syringe	50 mL
Sterile TB-50 x 2	50 mL
1 M HCl	Added dropwise
1 M NaOH	Added dropwise

After combining the Leibovitz's L-15, antibiotic/antimycotic, Fetal growth serum and sterile water, the solution was mixed using a magnetic stir bar. Once the contents were fully mixed, they were separated into 2 TB-50 tubes, the media was then pH corrected using the 1 M HCl and NaOH in a dropwise manner. With the pH of the media falling at 7.3245 which is within the optimal range of 7.3 - 7.5. The media was then filtered through the 50 mL syringe filter during the transfer.

Preparation of Phosphate Buffered Saline

200 mL of 10x Phosphate Buffered Saline (PBS) was prepared using the materials outlined in table 2.

Table 2. Materials needed for 200 mL of 10x Phosphate Buffered Saline (PBS).

PBS powder	2 g
Autoclaved DiH_2O	200 mL
Glass bottle	Approximately 500 mL
1 M HCl	Added dropwise
1 M NaOH	Added dropwise

In a 250 mL sterile flask, the PBS powder was mixed into the DiH_2O using a magnetic stir bar. Using a pH meter, the pH of the solution was then corrected to 7.2 using 1 M HCl and 1 M NaOH in a dropwise manar. The solution was transferred to the glass bottle, in which it underwent autoclaving.

Growing cells and subculturing

The cells were acquired on March 18th 2024, in a 15 cm^2 tissue culture flask in approximately 3.5 ml of Leibovitz's L-15 complete media. They were left for 48 hours to continue to grow in an incubator held at 37 ˚C, then subcultured into 2U well plates on March 20, 2024, following the procedure outlined below.

- All materials were brought to room temperature
- All medium in the flask was removed
- Using 3 mL of PBS, the flask and cells were washed twice, ensuring that all remaining PBS was removed afterwards

- 2 mL of trypsin EDTA was then added to the flask, and the cells were incubated for 2 minutes.
- A firm tap was used to dislodge any remaining cells, then the trypsin EDTA was neutralized with 4.5 mL of complete medium
- The cells were transferred to a 15 mL tube, then centrifuged at 300 rcf for 10 minutes.
- The supernatant was removed, and the pellet was resuspended in 1 mL of complete medium.
- The well plate was scratched horizontally on the bottom outside of the plate using a razor blade
- 3 mL were added to the suspended cells, and the solution was dispersed into 6 wells in a 3 x 2 grid, with each well containing 0.5 mL of the cellular solution.

After the subculture process, the cells were left to grow at 37 ˚C for 48 hours. On the morning of the 22nd, the following procedure was conducted in order to test the effects of caffeine on the cells.

- Two concentrations of caffeine were created out of complete medium from tube 1. The amounts of caffeine powder added to each are summarized in table 3.
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Table 3. Amounts of caffeine added to complete medium AHH and their corresponding concentrations

Caffeine amount (mg)	Complete Medium amount (mL)	Concentration (µM)
0.1	20	26
1.0	20	260

- Using a different 20 µL micropipette tip for each well, the wells were scratched once horizontally.
- The remaining media, along with the scratched off cells within each well, were removed.
- The wells were then washed twice with about 0.5 mL PBS.
- 0.5 mL of medium were added back into the well in correspondence to table 4. Each concentration was completed in duplicate.

Table 4. outline of the medium added to each well

Well Row	Caffeine concentration medium (µM)
1	0
2	26
3	260

The distance between the gap in the cells (caused by the scratch) in each well were measured using the ruler feature on the microscope. These distances were recorded periodically over a stretch of 48 hours to analyze the speed at which the cells were growing. The data was charted using EXCEL.

Data and Results

After completing the scratch assay, the cells were observed and measured periodically over a 9 hour period. It was planned to carry out the experiment further over a 48 hour period, however, every well except for well A1 went radioactive pink between the 9 hour and 24 hour measurements. Further experimental analysis was thus terminated because of this. Over the 9 hours that the cells were observed, measurements of cell growths were recorded for each well. These results are summarized in tables 5 through 7 below.

Table 5. Distance between cells for row 1 (0 μM Caffeine) over the 9 hr time period.

Time (hours after scratch and addition of media)	Distance of well 1 (μM)	Distance of well 2 (μM)	Average distance (μM)
0	350	450	400
1	350	400	375
3	350	400	375
5	200	300	250
7	200	250	225
9	150	200	175

Table 6. Distance between cells for row 2 (250 μM Caffeine) over the 48 hr time period.

Time (hours after scratch and addition of media)	Distance of well 1 (μM)	Distance of well 2 (μM)	Average distance (μM)
0	400	400	400
1	400	400	400
3	350	350	350
5	300	350	325
7	300	350	325
9	300	300	300

Table 7. Distance between cells for row 3 (7500 μM Caffeine) over the 9 hr time period.

Time (hours after scratch and addition of media)	Distance of well 1 (μM)	Distance of well 2 (μM)	Average distance (μM)
0	500	550	525
1	500	550	525
3	450	450	450
5	450	450	450
7	400	400	400
9	400	400	400

After the data was collected over the 9 hour period, it was plotted into EXCEL to determine the cell growth rate as a function of gap closure. The concentrations and their corresponding rates are summarized in table 8 below.

Table 8. Rate of scratch healing for all treatment types over the 9 hour period

Caffeine Concentration (µM)	**Average gap closure rate (µm/h)**
0	25
26	11
260	14

The following figures were made using R studio and the statistical analysis was calculated using ANOVA tests in R studio as well. Figure 1 shows the growth rate of the different treatments with control growing at an average rate of 25µM/Hour, 26µM at an average rate of 12µM/Hour, and 260µM at an average rate of 14µM/Hour.

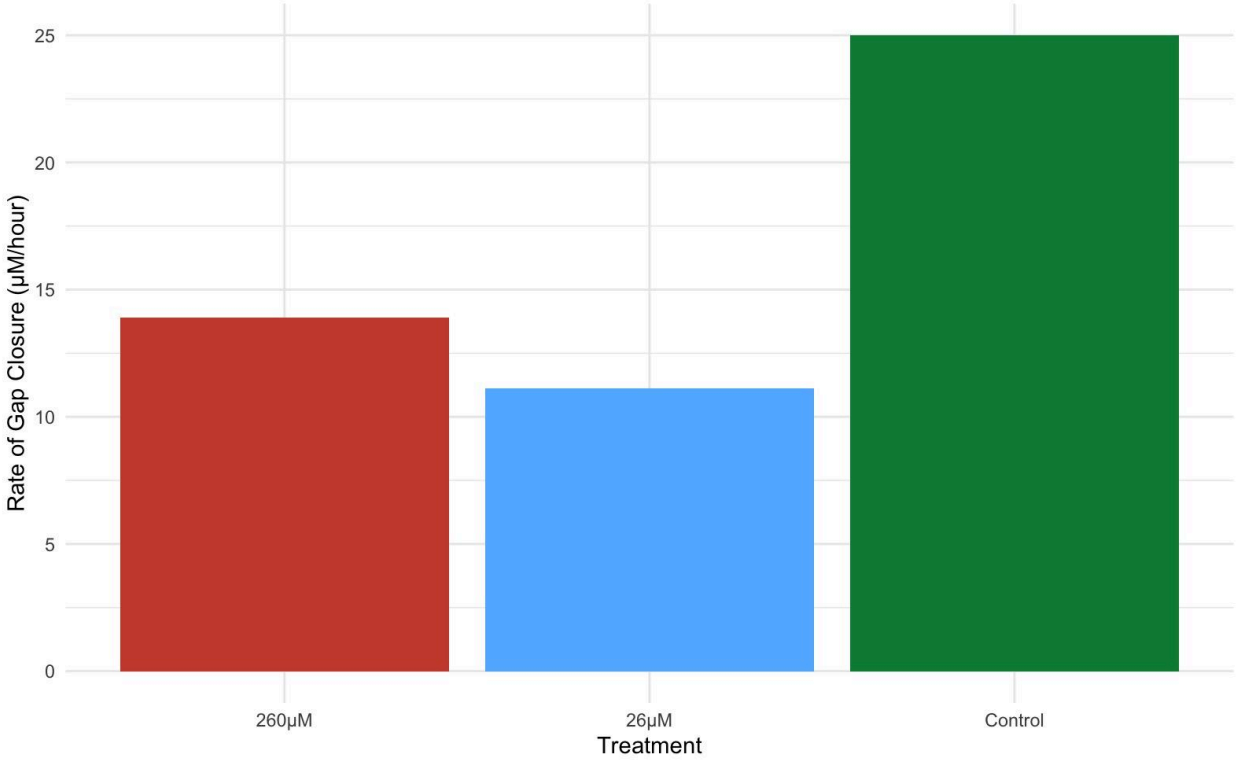

Figure 1. Average change in scratch width was averaged over the nine hour period. Highest change in scratch width occurred in wells with no added caffeine, and the lowest change in scratch width occurred in wells containing 26 micromolar caffeine content.

Figure 2 shows the width of the scratch over a period from 10:30 am, March 22nd, 2024 to 7:30 am March 22nd, 2024. After the initial scratch measurements, the scratch was measured every 2 hours with the exception of the first hour to check for immediate growth.

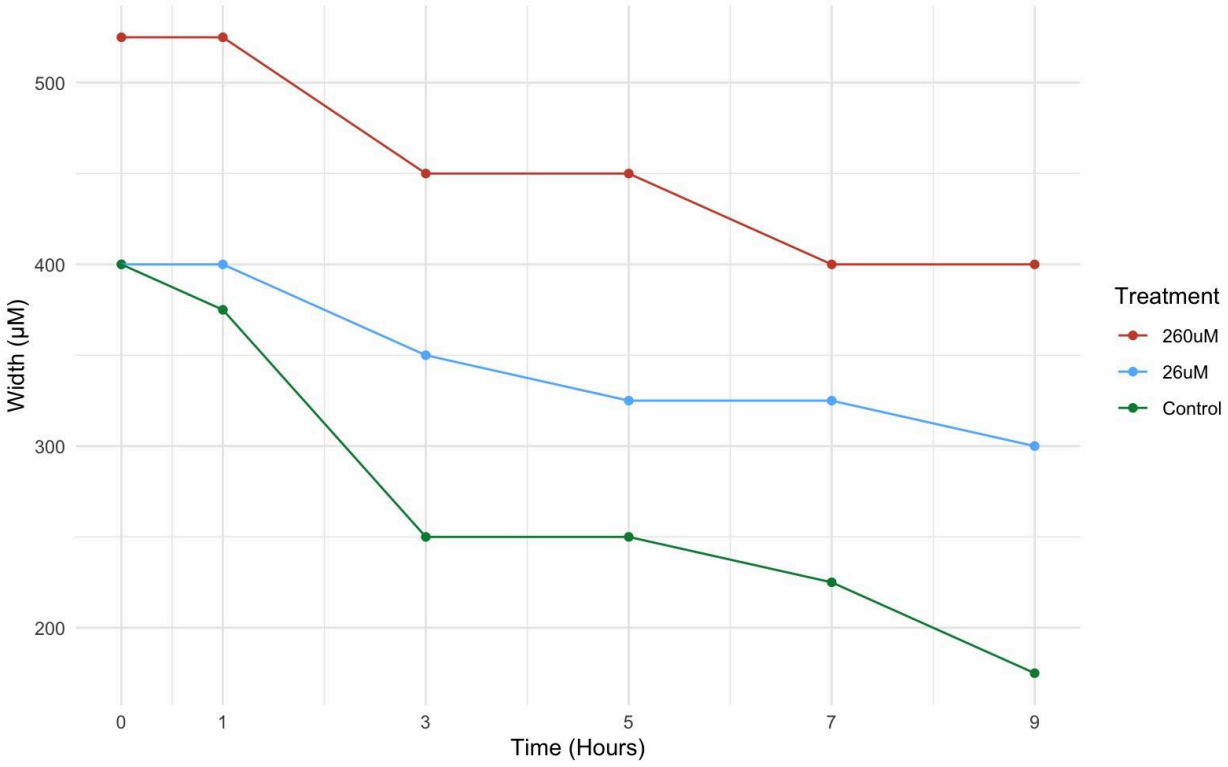

Figure 2. Change in the width of the scratch over 9 hours of testing. Using R studio an Anova was completed on data with a resulting p-value of $p<0.0001$ showing statistical significance in the data.

Discussion:

The scratch-wound assay is commonly used for the measurement of migration rate and is advantageous due to its low cost and simplicity. The assay involves the scratching of a confluent growth of adhered cells producing a line where the width can be measured under microscope. In order for the wound to be healed sheet migration must occur, sheet migration is dependent on the cell's ability to polarize toward the wound. This polarization involves a combination of mechanical forces, molecular interactions, and biochemical cascades which lead to proliferation into the wound.[7] If the cell's processes are interrupted by the contents of the medium, then so must the cell's ability to heal the wound. Therefore, the health of the cells can be interpreted from the rate of gap closure.

Scratch assay was performed on all the wells to gauge the ability of the cells to heal the wounds based on their caffeine interactions. The width of the gaps were measured six times over a period of nine hours and the average rate of gap closure was determined for each set of cells. It was observed that the control had a gap closure rate of 25 micrometers per hour, while a 26 micromolar concentration had a gap closure rate of 11 micrometers per hour. The highest concentration treatment was at a concentration of 260 micromolar and had a measured gap closure rate of 14 micrometers per hour. The results from the ANOVA statistical test showed a p-value < 0.0001, indicating that there is a significant relationship between cells growth in caffeine as compared to cells grown without. These results show that there is a significant reduction of the cell's ability to proliferate caused by caffeine. It was against expectations that the highest concentration caffeine treatment did not result in the slowest rate of gap closure. This may be due to an insufficient sample size as each treatment group only consisted of two wells. These results correlate with literature, as caffeine has previously been shown to cause oxidative

stress as well as lysosomal dysfunction in human cancer cell lines. These effects tended to lead to apoptosis of the cells.[8] The procedure performed here was on a single lineage of endothelial cells. In the future, the experiment may be repeated on other cell lines such as neuronal and cardiovascular cells. In addition to expanding the sample size, future research should involve other cell lines and for a longer period of exposure.

Conclusion:

A scratch-wound assay was performed on endothelial cells to determine the effect of caffeine on sheet migration. The gap rate closure was found to be acutely reduced in treatments containing caffeine, indicating a disruption of cell processes within the cells.

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