Wound Healing Assay (Scratch Test) on Endothelial EaHy.926 Cells Using Caffeine

Written by Jessica Giles, Paula Arango, and Feliks Iliusha

Thompson Rivers University

Abstract:

This study investigates the effects of caffeine on endothelial wound healing using a scratch test on EaHy.926 cells, a widely used model for studying vascular endothelial function. Endothelial cells play a critical role in maintaining vascular integrity and responding to injury, making wound healing an essential process in cardiovascular health. We aim to determine whether low to moderate doses of caffeine can enhance cell migration and accelerate wound closure, while higher doses may inhibit this process. To test this hypothesis, endothelial cells will be cultured until they reach confluence, after which a standardized mechanical scratch will be introduced to simulate a wound. Cells will then be treated with varying concentrations of caffeine (0, 0.5, 1.0, 2.5, and 5.0 mM), and wound closure will be monitored over a 48-hour period. Phase contrast microscopy (4x magnification) will be used to capture images of the wound at specific time intervals, allowing for the quantification of migration rates and closure efficiency across different treatment groups. We will be analyzing these results using ANOVA to determine whether there are statistically significant differences in wound closure percentages among the multiple caffeine treatment groups. ANOVA analysis revealed no significant effect of caffeine concentration on wound closure rate ($p = 0.799$), suggesting that caffeine did not have a statistically significant impact on the overall wound healing process. We have identified an optimal caffeine concentration range (0.5–1.0 mM) that promotes endothelial wound healing, suggesting potential applications for therapeutic strategies targeting vascular repair. Conversely, excessive caffeine intake may have adverse effects on endothelial function. By analyzing these images, we aim to identify potential dose-dependent effects of caffeine on endothelial cell behavior. The findings of this study could provide valuable insights into the impact of caffeine on vascular repair mechanisms, potentially informing therapeutic strategies for conditions involving endothelial dysfunction.

Introduction:

Our Background literature contains three major articles. In-depth study conducted by Ojeh et al. (2014), examined the effects of caffeine on wound healing. They utilized human epidermal keratinocytes and HaCaT cells that were cultured in complete medium and placed in an incubator to control temperature levels. The MTT assay was used to measure proliferation. The cells were treated with varying caffeine doses ranging from 0.1 to 5 mM. The controls they used included untreated cells, positive with EGF (epidermal growth factor) and KGF (keratinocyte growth factor), and negative controls used to detect contaminations and unexpected results. The cells were grown to confluence and scratched with a pipette tip. Caffeine was applied and the wound closure was monitored over 27 hours. Jonkman et al (2014) published an article on wound healing assay and identified the different ways to statistically graph and analyze the wound healing rate. This article will be used as a guide when determining how to resent our data. Another article written by Wang et al studied how Caffeine (50 μM) promotes endothelial cell motility through the signaling pathways of cAMP/PKA/AMPK. This article mainly focused on the physiological aspects of cells undergoing the scratch wound healing assay with caffeine.

We would like to discover if low/moderate doses of caffeine will enhance cell migration and accelerate wound closure, and if higher doses will inhibit this process. Therefore, this study aims to determine how different concentrations of caffeine influence endothelial cell migration and wound closure. By using a well-established scratch assay on EaHy.926 cells, we can simulate endothelial injury and assess caffeine's dose-dependent effects on the repair process. Understanding these effects could provide insight into the role of dietary caffeine consumption in vascular health and tissue regeneration. Moreover, this research may have clinical implications for wound healing in patients with cardiovascular conditions, where endothelial function is already compromised. By performing a

scratch assay, we can determine whether caffeine enhances, inhibits, or has no effect on endothelial wound healing.

We hypothesize that caffeine will influence endothelial cell migration as well as wound healing rates based on the concentration of stimulant applied. Therefore, low/moderate doses of caffeine will facilitate the process of wound recovery by enhancing proliferation and migration of endothelial cells. Moreover, we anticipate that elevated caffeine concentration will ultimately decrease the rate of cell migration.

Methods and the design of the study:

We will prepare a caffeine stock solution to ensure accurate dosing. Since our highest concentration is 5.0 mM, we will prepare a 10 mM stock solution to make dilutions easier. Dissolve 19.42 mg of caffeine (FW = 194.2 g/mol) in 10 mL of sterile water to make a 10 mM caffeine stock solution. Please see table 1 in the Results section. Endothelial cells will be cultured under standard conditions in a complete medium within 4×6 well plates, with an 'X' mark made on the bottom of each well using a razor for reference. Three T75 flasks containing confluent cell growth will be used for seeding. To prepare the cells, the complete medium will be removed from each flask, followed by two washes with 5 mL of phosphate-buffered saline (PBS) to eliminate residual medium. Next, 2.5 mL of trypsin-EDTA will be added to detach the cells, ensuring full coverage of the flask surface. The flasks will then be incubated until detachment is confirmed under an inverted microscope, which typically takes around five minutes. To ensure complete detachment, the flasks will be tapped firmly before adding 8 mL of complete medium to deactivate the trypsin. The cell suspension will then be transferred into a 15 mL tube and centrifuged at 300 rcf for nine minutes to pellet the cells. The supernatant will be removed by pipetting, and 1 mL of complete medium will be added to resuspend the pellet, followed by gentle shaking before increasing the volume to 3 mL. This process will yield

three tubes with a total volume of 9 mL of resuspended cells. Using a pipette, 0.5 mL of this cell suspension will be dispensed into each well of an 18-well plate, where the cells will be allowed to adhere for two days at 37°C. Once the monolayers reach 90–100% confluency, a 200 µL pipette tip will be used to create a straight-line scratch through the cell layer to simulate a wound. Following a predetermined treatment chart, the required amount of complete medium will be removed and replaced with the appropriate concentration of stock solution in each well, maintaining a final volume of 0.5 mL of cells and medium. The plates will then be incubated at 37°C, and wound closure will be monitored over a 48-hour period. This time frame was selected based on previous laboratory results from February 3, 2025, which demonstrated that untreated endothelial cells completely heal within 48 hours. Images of the wound areas will be captured using a phase contrast inverted microscope with a 4× objective at specific time points: 9:00 AM, 1:00 PM, 5:00 PM, and 9:00 PM on the first day, followed by 9:00 AM on the second, third and fourth day. These images will be analyzed to quantify cell migration and wound closure rates across different time intervals using the statistical method ANOVA. Finally, the healing rates will be compared among the different treatment groups to determine the effects of caffeine on endothelial wound repair.

Results:

Wound closure was measured across different caffeine concentrations (0 mM, 0.1 mM, 0.5 mM, 1.0 mM, 2.5 mM, and 5.0 mM) at multiple time points (9 AM, 1 PM, 5 PM, 9 AM next day, March 16, and March 17). Figure 1 illustrates the mean wound width (µm) over time for each caffeine concentration. The data indicate that wound widths remained stable at 9 AM and 1 PM, but significantly increased by 5 PM, reaching peak values across all caffeine conditions. This increase may reflect early migration and proliferation responses following the initial scratch. After this peak, wound width decreased substantially by 9 AM the next day, suggesting active wound closure.

However, by March 16 and March 17, wound widths stabilized, with the highest caffeine concentration (5.0 mM) exhibiting a slower rate of closure compared to lower concentrations.

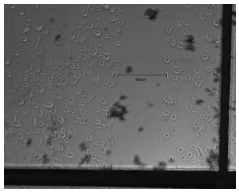
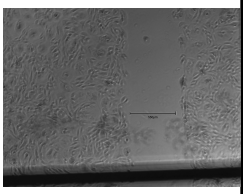
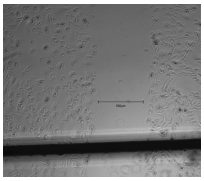
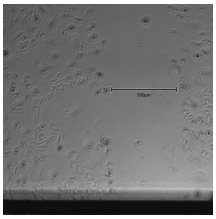
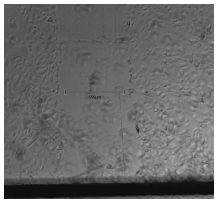
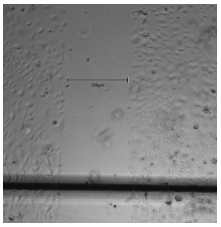
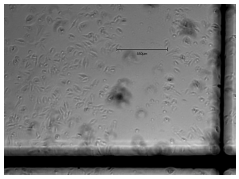
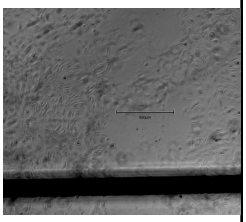
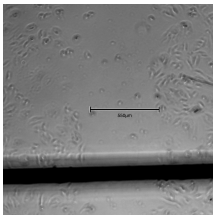
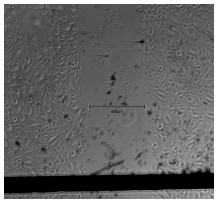
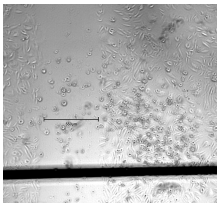
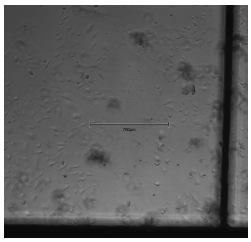
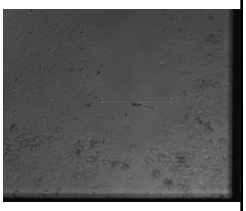
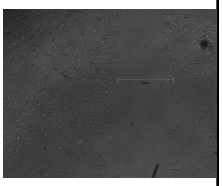
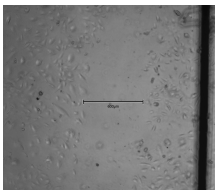
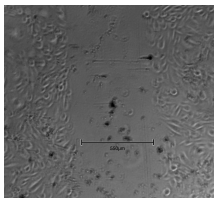
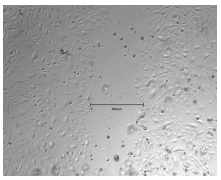
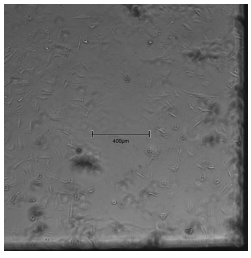
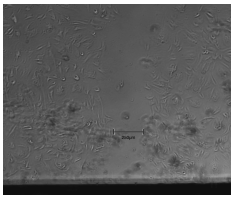
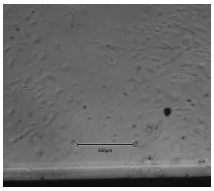
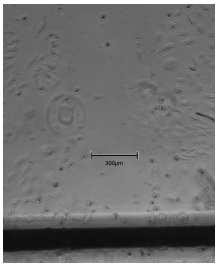
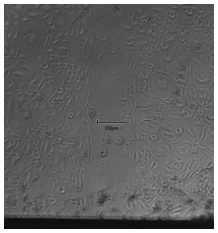
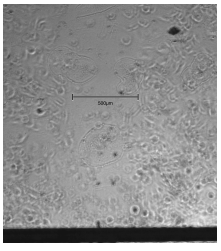
The preparation of caffeine treatments is detailed in Table 1, which outlines the volume of 10 mM caffeine stock added to each well to achieve the desired final concentrations. These dilutions ensured consistency in exposure across wells and allowed for comparisons between different caffeine levels.

A one-way ANOVA analysis revealed no significant effect of caffeine concentration on wound closure rate ($p = 0.799$), suggesting that caffeine did not have a statistically significant impact on the overall wound healing process. However, visual trends indicate that higher caffeine concentrations (5.0 mM) may delay wound closure compared to lower concentrations. While the mean wound width in the 5.0 mM condition remained wider than others at later time points, this difference was not statistically significant.

Figure 2 displays representative microscopic images of wound closure at different caffeine concentrations over time, providing visual confirmation of the trends observed in Figure 1. These images highlight the gradual reduction in wound width and the variations in closure patterns among different caffeine treatments.

Table 1. Preparation of caffeine concentrations for the scratch test. Different final concentrations (0 – 5.0 mM) were prepared by diluting a 10 mM stock solution in water. The final volume for each well was adjusted accordingly.

Final Concentration (mM)	Volume of 10 mM Stock (µL)	Volume of Water (µL)	Total Volume (µL)
5.0	150	150	300 → take 250uL for each well
2.5	75	225	300 → take 125uL for each well
1.0	30	270	300 → take 50uL for each well
0.5	15	285	300 → take 25uL for each well
0.1	3	297	300 → take 5uL for each well
0 (control)	0	0	0

Time	Control	0.1mM	0.5mM	1.0mM	2.5mM	5.0mM
9am	 Control top	 Second well	 Top	 Top	 Third well	 Top
1pm	 Control top	 Second well	 Top	 Top	 Third well	 Top
5pm	 Control top	 Second well	 Top	 Top	 Third well	 Top
9pm						
9am next day	 Control top	 Second well	 Top	 Top	 Third well	 Top

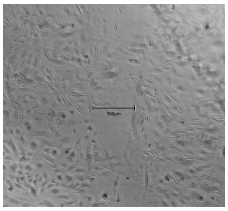
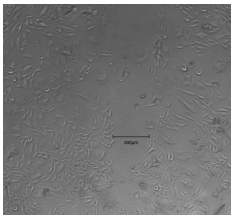
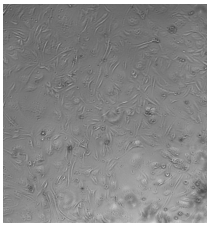
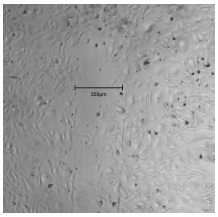
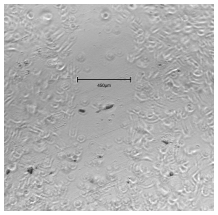
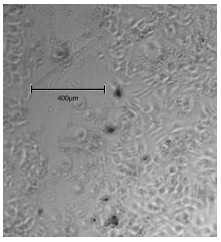
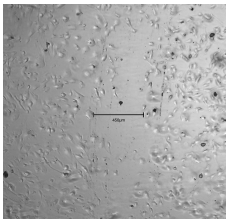
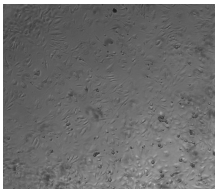
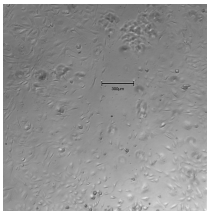
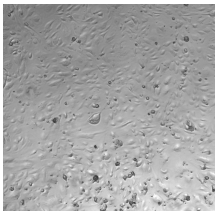
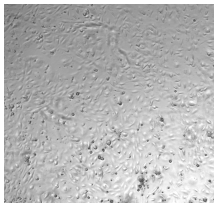
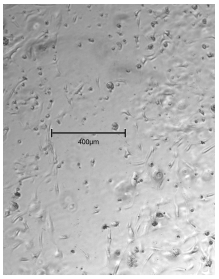
March 16 9am	 Control top	 Second well	 Top	 Top	 Third well	 Top
March 17 9am	 Control top	 Second well	 Top	 Top	 Third well	 Top

Figure 2. Representative images of wound closure over time. Microscopic images showing wound healing progression at different caffeine concentrations (0 – 5.0 mM). Images were taken at 9 AM, 1 PM, 5 PM, and subsequent days.

Discussion:

Visual evidence supported by statistical analysis (ANOVA) revealed that different caffeine concentrations, indeed, influence the rate of endothelial cells regeneration caused by mechanical disruption. As hypothesized, low/medium doses of caffeine induced the wound recovery, while the excess of stimulant not only did halt the development of new cells, but also led to extensive death of cells in [5.0] and control well plates.

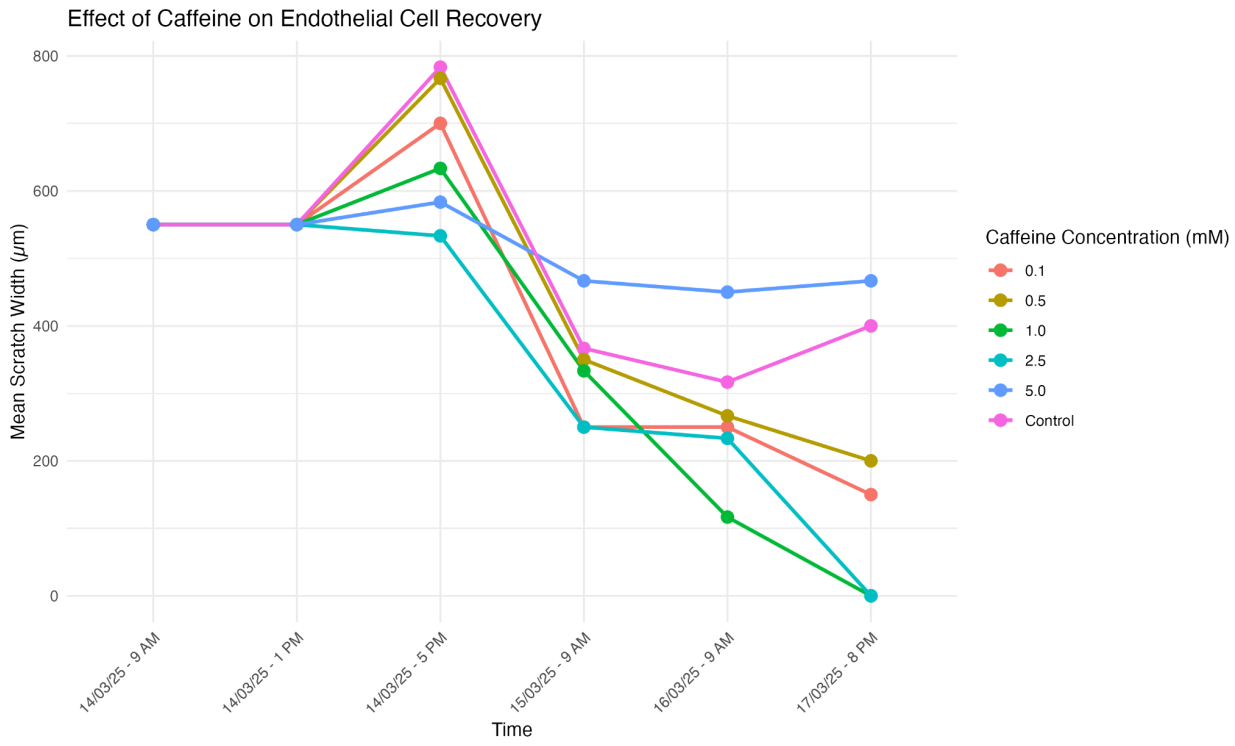


Figure 3. Line graph demonstrating the relationship between different caffeine concentrations (mM) and mean scratch width (μm) throughout the 4-day course. For each concentration a mean of 3 was taken; n = 18.

The scratches of identical width (550 μm) were introduced to 18 well plates with confluently growing cells on March 14th at 9 AM following an immediate treatment by different caffeine concentrations, ranging from 0.0 (mM) to 5.0 (mM). After 8 hours, we started to observe the variation in the width of scratches. At first, control and low-concentration treatments significantly increased the size of wounds stretching from an average of 633 μm for [1.0] to 783 μm [control]. At the same time, the maximum concentration of caffeine [5.0] led to only a slight enlargement of an average wound size to 583 μm, while [2.5] application, unlike all the others, initiated the direct recovery of a mechanically distorted area with an average width of 533 μm. Over the night, on the 15th of March at 9 AM endothelial cells began rapid proliferation under most treatments, except for

the maximum, which did not appear to cause notable wound reduction width (466 μm). Cells were left in the incubator for another night to analyze their subsequent recovery rate. However, no remarkable observations were made, since wounds remained generally unaltered, with only [1.0] treatment continuing to heal the wound at a prompt rate of 65.2% (116 μm on the 16th of March). On the last day of the experiment, we found that [1.0] and [2.5] concentrations of caffeine fully "sealed" the wound by newly produced cells, [0.1] and [0.5] treatments were also effective, but the scratch still could be seen. Finally, [5.0] with control treatment (surprisingly) did not did not aid in healing. Furthermore, after viewing the corresponding wells under the microscope, we noted that a substantial number of cells were dead.

The ANOVA results reveal that both caffeine concentration and time significantly impact endothelial cell recovery. The p-value for caffeine concentration ($p = 1.46e-07$) is extremely small, indicating a strong effect on scratch width, with an F-value of 10.447 suggesting that variations in caffeine concentration contribute meaningfully to these changes. Similarly, time has a profound effect on recovery, as evidenced by the p-value ($< 2e-16$) and a large F-value of 81.420, indicating significant variation in scratch width over time. Additionally, the significant interaction between caffeine concentration and time ($p = 4.30e-05$, $F = 3.285$) shows that the rate of recovery is influenced by their combined effects, rather than by either factor independently. Consequently, our hypothesis is confirmed not only by graphic analysis but also by the statistical test.

There are some findings that we found worth mentioning. First of all, it was unexpected that instead of shrinking in width, wounds started to expand (except for [2.5] treatment). This could be caused by several factors, such as mechanical stress on the tissue, prolonged inflammatory response, microbial infections, or an imbalance in extracellular matrix remodeling. Additionally, variations in

treatment efficacy and differences in individual healing responses may have contributed to the observed wound expansion.

References:

CHEN Xing, LUO Fen, HUANG Zhizhong, PENG Yan, XIONG Renping, & LI Ping. (2021). Effects of caffeine consumption on cutaneous wound healing and scar formation in mice. Di-San Junyi Daxue Xuebao, 43(1), 75–82. https://doi-org.ezproxy.tru.ca/10.16016/j.1000-5404.202006248

Jonkman, J. E., Cathcart, J. A., Xu, F., Bartolini, M. E., Amon, J. E., Stevens, K. M., & Colarusso, P. (2014). An introduction to the wound healing assay using live-cell microscopy. Cell adhesion & migration, 8(5), 440–451. https://doi.org/10.4161/cam.36224

Ojeh, N., Stojadinovic, O., Pastar, I., Sawaya, A., Yin, N., & Tomic-Canic, M. (2016). The effects of caffeine on wound healing. International wound journal, 13(5), 605–613. https://doi.org/10.1111/iwj.12327

Wang, L. T., He, P. C., Li, A. Q., Cao, K. X., Yan, J. W., Guo, S., Jiang, L., Yao, L., Dai, X. Y., Feng, D.,

Supit, T., Susilaningsih, N., & Prasetyo, A. (2021). Effects of Caffeine Consumption on Autologous Full-Thickness Skin Graft Healing in an Animal Model. Indian Journal of Plastic Surgery, 54(3), 314–320. https://doi-org.ezproxy.tru.ca/10.1055/s-0041-1734573

Xu, Y. M., & Tan, N. (2021). Caffeine promotes angiogenesis through modulating endothelial mitochondrial dynamics. Acta pharmacologica Sinica, 42(12), 2033–2045. https://doi.org/10.1038/s41401-021-00623-6