Assessing the Effects of Beetroot Powder Supplementation on Endothelial Cell Migration

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Abstract

The purpose of this study was to examine the effects of beetroot on endothelial cell migration. Based on previous research, it is predicted that beetroot will increase migration through increasing nitric oxide by the nitric oxide pathway. This experiment used 24-well plates which were subcultured with Ea.hy926 cells and treated with various concentrations of beetroot. A scratch assay was used to determine cell migration over a 48 hour time interval. The results show that beetroot inhibits cell migration when treated with higher concentrations of beetroot compared to control. The results of the analysis also concluded there was a statistical difference in migration rates for cells treated with 5 mg/ml of beetroot compared to control after 24 and 48 hours ($p = 0.0013$, $p = 0.0291$), and no statistical difference between cells treated with 0.1 mg/ml ($p = 0.551$). Percentage of wound closure also was statistically different when treated with 5 mg/ml of beetroot compared to the control after 48 hours ($p = 0.0179$). Overall, the results did not support this research's hypothesis that higher concentrations of beetroot powder administration would improve cell migration. However, this study illustrated a potential way to inhibit cell migration which could have therapeutic benefits and would require further study.

Introduction

Endothelial cells form a monolayer inside all blood vessels, regulating the blood flow between the bloodstream and the surrounding tissues (Alberts et al. 2016). Endothelial cells migrate during vasculogenesis (the process by which blood vessels are formed de novo) and angiogenesis (the growth of blood vessels from the existing vasculature), as well as to restore the integrity of a damaged vessel (Goldie et al. 2008; Adair and Jean-Pierre Montani 2010). These processes are fundamental in the formation of new blood vessels (Michaelis 2014). Nitric oxide (NO), a potent vasodilator and regulator of vascular homeostasis, is one of several variables that influence the migration of endothelial cells. NO is continually produced in endothelial cells from the amino acid L-arginine by nitric oxide synthase (NOS) (Tousoulis et al. 2012). NO stimulates the cGMP-dependent protein kinase and PI3K/Akt pathways, two signalling pathways that aid in endothelial cell migration (Kawasaki et al. 2003). Previous studies have shown that NO is crucial to endothelial cell migration and angiogenesis, both in vitro and in vivo (Papapetropoulos et al. 1997; Murohara et al. 1999). Additionally, Hendgen-Cotta et al. (2012) demonstrated that dietary nitrates in vegetables, such as beets, increases NO production while promoting endothelial function.

Beta vulgaris rubra, also known as red beetroot, is a root vegetable that has gained attention as a health-promoting functional food (Clifford et al. 2015). Beetroot is associated with numerous health benefits including lowering blood pressure, improving endothelial function, enhancing exercise performance, and increasing blood flow (Asgary et al. 2016). These benefits are associated with high levels of inorganic nitrates (NO_3^-), a compound naturally present in processed meats and vegetables. Following ingestion, anaerobic bacteria in the oral cavity use

the enzyme, nitrate reductase, to convert NO_3^- to nitrate (NO_2^-), which is subsequently converted to NO in the stomach (Domínguez et al. 2017). Nitrites not converted to NO or other reactive nitrogen oxides in the gut are taken up into the bloodstream and converted to circulatory NO to cause vasodilation, primarily in dysfunctional and poorly perfused tissues. Consequently, the consumption of beetroot can increase NO bioavailability through the nitrate-nitrite-nitric oxide pathway (Nogueira Soares et al. 2021). Although previous studies have shown that nitric oxide administration on endothelial cells improves migration, whether dietary beetroot supplementation would acutely improve cell migration remains to be fully investigated.

Based on the recently described effects of NO produced from dietary nitrate, this study investigated whether nitrate-rich beetroot powder supplementation would acutely improve cell migration in endothelial cells. In this research, it was hypothesized that higher concentrations of beetroot powder treatment would improve cell migration compared with baseline measure.

Methods and Materials

2.1 Broth preparations

Leibovitz's L-15 broth + 10% fetal bovine serum (FBS) was prepared in a sterile 500 mL flask using 89 ml of deionized water, 1.23 g of L-15 medium, 10 ml of fetal growth serum (FCS), and 0.6 ml of penicillin/streptomycin. To remove contaminants, the broth was filtered by a 0.45 μm syringe filter.

2.1.1 Beetroot medium

A 10 mg/ml stock solution was made by dissolving 1.0050 g of beetroot powder in 100 ml of Leibovitz's L-15 broth + 10% FBS. A magnetic stir bar was added to the flask and placed on a magnetic stirrer for five minutes on low heat to fully dissolve the powder. The broth was the divided into ten different test tubes for treatment with various concentrations of beetroot powder, including: 0.1 mg/ml, 0.5 mg/ml, 5 mg/ml 10 mg/ml, 20 mg/ml 40 mg/ml, 60 mg/ml, 80 mg/ml, 100 mg/ml, 120 mg/ml. The pH of the broth was then adjusted to 7.23 using 1M NaOH and 1M HCl.

2.2 Ea.hy926 endothelial culture

Immortalized Ea.hy926 endothelial cells were cultured four days before the experiment. Cells were cultured in phenol-red L-15 Leibovitz's medium supplemented with 10% FBS in a T75 flask. All cells were incubated at 37°C. Cells were also fed three times a week by replenishing the media.

2.3 Preparation of wells

Before harvesting cells, 24-well plates were scored into four quadrants on the underside of each well using a razor. Cells were subcultured from a T75 flask. The flask was washed twice using 9 ml phosphate buffer saline. This was followed by the addition of 6 ml of trypsin and a two minute incubation to dislodge adherent cells. Immediately, 9 ml of L-15 Leibovitz's medium supplemented with 10% FBS was added to deactivate trypsin. The cells were then transferred to a test tube to be centrifuged at 300 rcf for ten minutes. The supernatant was discarded by pouring it out of the tube, leaving only the concentrated cell pellet. The pellet was

resuspended in 2 ml of medium and vortexed. This was followed by the addition of 10 ml of media so that the cells could be harvested into the well plates.

The endothelial cells were then seeded in triplicate in 24-well plates at a density of 7000 cells per well (70,000 cells/ml) and incubated overnight at 37°C in 50 µl of Leibovitz's L-15 + 10% FBS medium. The endothelial cells were grown until a confluent cell monolayer was formed.

2.4 Scratch wound assay

Once a confluent monolayer was formed, an open wound area was created in the cell monolayer using a sterile 200 µL pipette tip and washed with 500 µL PBS. 500 µL of medium containing varying beetroot powder amounts described in 2.1.1 were added to the designated wells. Water was used as a negative control and untreated leibovitz's L-15 + 10% FBS media was added as a positive control. The cell culture plate was added to the incubator to allow the cells to grow and migrate into the scratch area.

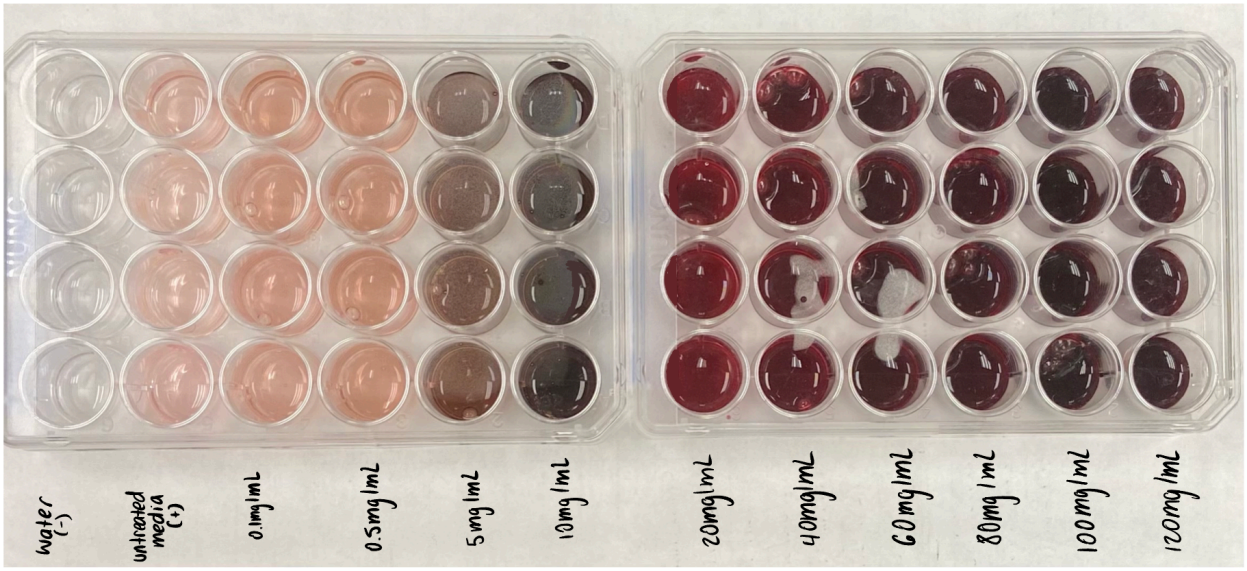

Figure 1. Sample of the 24-well plate in quadruplicate for the first scratch wound assay. Each column of wells was divided into 12 labeled sections, containing media concentrated with beetroot powder with either 0 mg/mL, 0.1 mg/ml, 0.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, 100 mg/ml, 120 mg/ml. The clear zone of the scratch was measured to the nearest µm. *(-) = negative control, (+) = positive control.*

2.4.1 Data analysis

The scratch width was determined with the aid of the inverted microscope. Rate of migration (RM) was calculated through a ratio of average final width (W_f) subtracted from average initial width at 0 hours (W_0) divided by time (hours). Percentage of wound closure was calculated by subtracting the average wound length at time *t* from the original wound length at 0 h (W_0) and divided by W_0. W_0 and W_f are expressed in units of µm. Both formulas are included below (Suarez-Arnedo et al. 2020).

(Eq 1). $$R_M = \frac{\overline{W_0} - \overline{W_f}}{t};$$

(Eq 2). $$Wound\ closure\ \% = \left(\frac{\overline{W_0} - \overline{W_t}}{\overline{W_0}}\right) \times 100\%$$

Wound closure is expressed as a percentage, where 100% would confer full closure and 0% would confer no closure.

2.5 Image acquisition and processing

Images at baseline were taken immediately after creating the open wound area. Inverted microscope (20X) images were analyzed for wound width at the center of the scratch using a built-in measuring tool. Contrast between cell and background was maximized by using a bright field, high light intensity and nearly closed aperture. Brightness gradients were avoided to optimize a homogeneous light distribution and as such prevent image artifacts. The media was removed from each well and the cells were washed with PBS to remove beetroot powder residue. Cells were imaged after the initial scratch every 12 h for a total duration of 48 h.

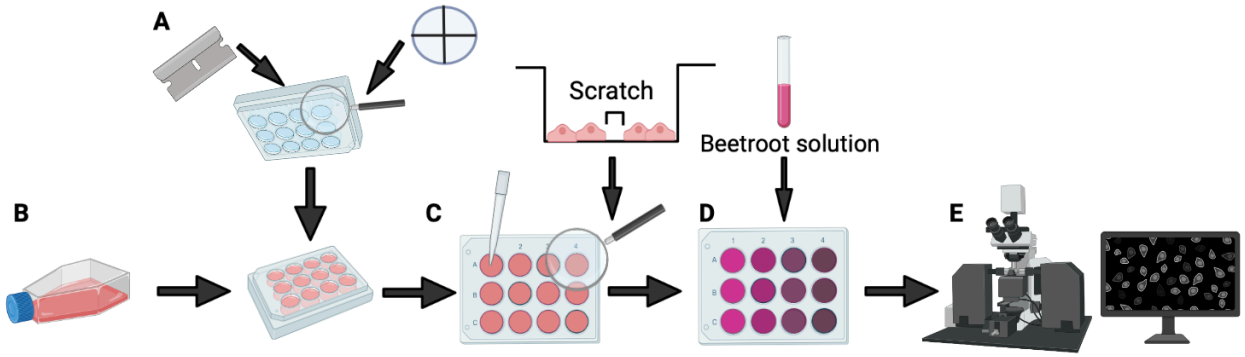

Figure 2. Procedure of scratch wound assay. (A) Well plates scratched into four quadrants, (B) cells cultured into well plate from T75 flask, (C) cells scratched along quadrant lines via a pipette tip, (D) beetroot concentrations of interest replace standard media, (E) cell migration monitored in inverted microscope. Image was created using Bio Render.

2.6. Statistical analysis

All statistical analyses were conducted using Excel software, Version 16.66.1 by Microsoft. All data are presented as means values ± the standard error of the mean and *n* indicates the sample size. Data analysis involved the Student's t-test conducted at a 95% confidence level to determine any difference between the beetroot treated cells and the control. $P < 0.05$ was considered significant.

3. Results

3.1 Cytotoxicity effect of beetroot

To find the best concentrations for testing, endothelial cells were plated with concentrations of beetroot in the range of 0.1-120 mg/ml. Low concentrations of 0.1 mg/ml, 0.5 mg/ml, and 5 mg/ml resulted in an increased number of endothelial cells in the denuded area compared to the higher concentrations (10mg/ml-120 mg/ml). Furthermore, higher concentrations of 10-120 mg/ml induced cell death (Figure 3 C and D). Therefore, 0.1-5 mg/ml of beetroot powder was chosen as the ideal concentration range for the scratch assay.

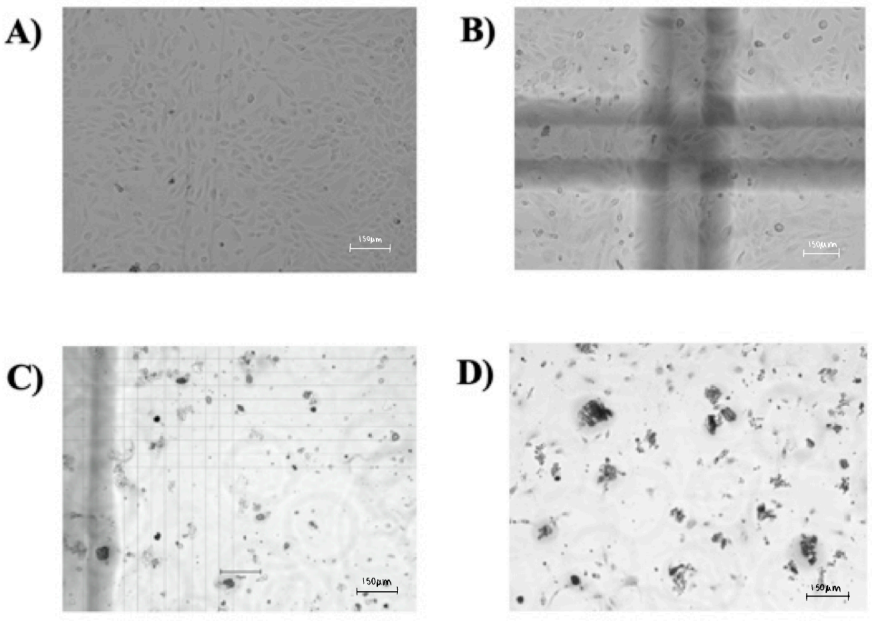

Figure 3. Images of endothelial cells in inverted light microscopy after exposure to low and high concentrations of beetroot and incubated for 24 hours at 37°C. From 'A' to 'D' where A) shows cells treated with untreated cell culture media, B) shows cells treated with 5 mg/ml concentration of beetroot, C) shows cells treated with 40 mg/ml concentration of beetroot and D) shows cells treated with 120 mg/ml concentration of beetroot. After incubation of 24 hours, beetroot displayed toxicity when the concentration was beyond 10 mg/ml.

3.2 Beetroot Decreased Endothelial Cell Migration

Figure 4b shows that scratch width was smaller in the beetroot (5mg/ml) supplemented condition compared to that of the vehicle control after 48 h (107.1 ± 38.3 μm vs. 0 ± 0 μm , significant effect for condition $p < 0.0001$). Scratch width was also smaller after only 24 h of exposure when treated with 5 mg/ml of beetroot compared to control (113.1 ± 30.14 μm vs. 8.57 ± 8.57 μm, significant effect for time $p < 0.05$). Scratch width of the endothelial cell monolayer was smaller when treated with 0.5 mg/ml of beetroot compared to vehicle control after 24 h (90.46 ± 5.43 μm vs. 8.57 ± 8.57 μm, significant effect for time $p < 0.001$). Lower concentration of beetroot (0.1mg/ml) did not alter wound-healing rate and had no difference after 48 h (0.0 ± 0.0 μm vs. 0.0 ± 0.0 μm).

Percentage of wound closure at different time intervals in untreated, 0.1 mg/ml, 0.5 mg/ml and 5 mg/ml beetroot treated cells have been represented in Figure 5. Beetroot powder did not induce the migration of Ea.hy926 cells. The percentage of wound closure at 12 h after wounding was 37.4 ± 7.68% in control cells, whereas in 0.1 mg/ml beetroot-treated cells the percentage was 51.3% ± 12.1%. The percentage of wound closure in cells treated with 0.5 mg/ml and 5 mg/ml was 48.4 ± 3.3% and 32.8 ± 9.1% respectively. In the untreated, 0.1mg/ml, and 0.5mg/ml treated cells, 100 ± 0% of the gap was closed at 48 h. Wound closure percentage was lower, at 53.4 ± 12.0%, when treated with 5 mg/ml of beetroot compared to vehicle control at 48 h (significant effect $p < 0.01$). Percentage of wound closure was also lower in cells treated with 0.5 mg/ml of beetroot at 24 h (96.3 ± 3.8% vs. 64.9 ± 4.3%, significant effect $p < 0.05$).

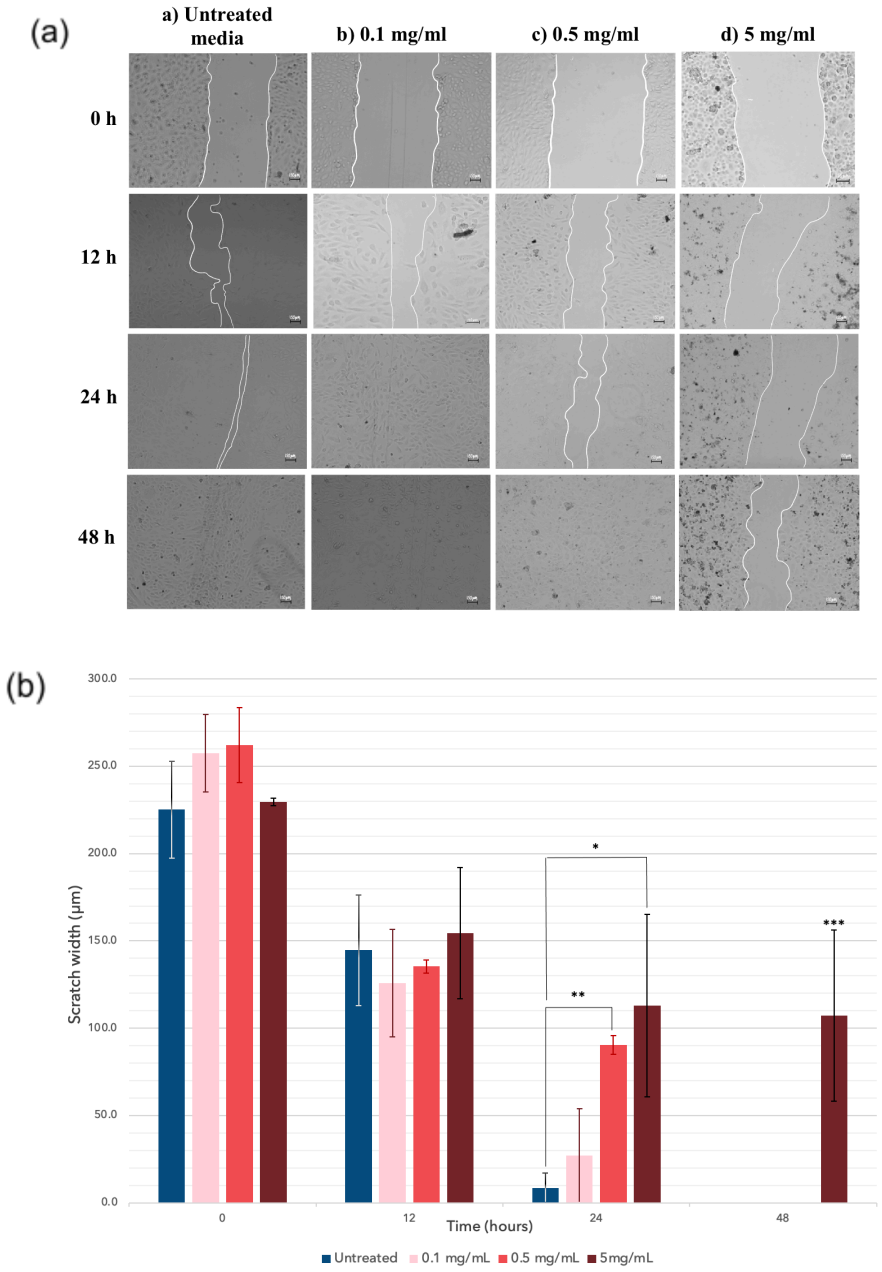

Figure 4. Cell migration of human umbilical vein endothelial cells (Ea.hy926) after wounding (n=3). A) Time lapse microscopical images representing the *in vitro* wound healing nature of beetroot powder. Ea.hy926 cells were incubated in presence or absence of different concentrations of beetroot and images were captured at 0, 12, 24, 48 hrs. (a) Positive control of Leibovitz L-15 + 10% FBS, (b) 0.1 mg/ml of beetroot, (c) 0.5 mg/ml of beetroot, (d) 5mg/ml of beetroot. The boundaries of the scratch wound were determined by the white lines. B) Summary bar graph illustrating wound closure at indicated time points during the scratch wound assay. After 24 and 48 hours, cells treated with 0.5 mg/ml and 5 mg/ml had smaller scratch width compared to control. (*$p<0.05$ versus control (0.0 mg/ml); **$p<0.001$ versus. control, ***$p<0.0001$ versus. control)

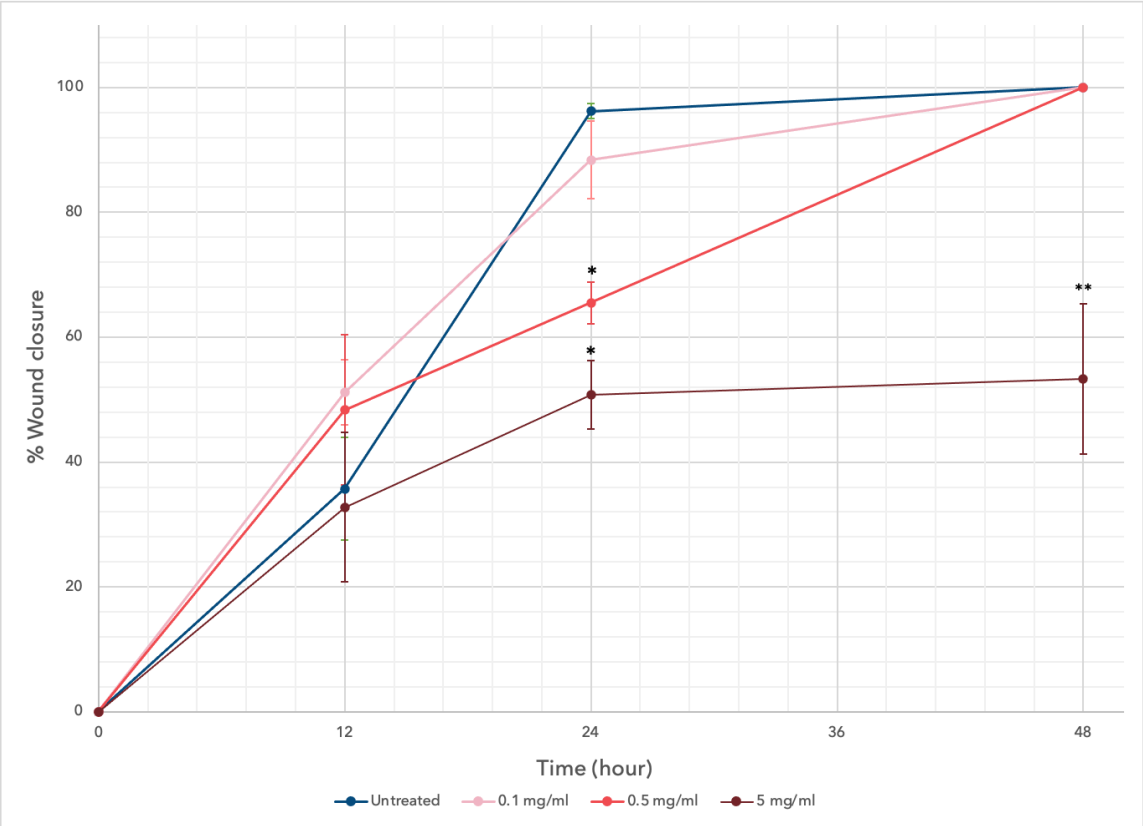

Figure 5. Percentage of wound confluence indicating the rate of endothelial wound closure at 12, 24, and 48 h when comparing different concentrations of beetroot treatment with untreated cells (positive control) (n=3). Blue: cells with culture medium alone; Light pink: 0.1 mg/ml of beetroot powder; Pink: 0.5 mg/ml of beetroot powder; Red: 5 mg/ml of beetroot powder. Cells treated with 0.5mg/ml and 5 mg/ml of beetroot had lower wound closure percentage compared to control at 24 hours. * indicates a significant ($p < 0.05$) different between controls, ** indicates a significant ($p < 0.01$) difference between controls.

4. Discussion

In this study, we investigated the *in vitro* effects of beetroot on cell migration, which is a key process necessary for wound healing. Beetroot effects on cell migration were analyzed with 0.1 mg/ml, 0.5 mg/ml, or 5 mg/ml of beetroot in the culture media. The main findings of the present study do not support the initial prediction that higher concentrations of beetroot increased cell migration in endothelial cells compared to lower concentrations or control conditions. The results found that beetroot significantly inhibits cell migration when treated with 5 mg/ml of beetroot compared to control after 48 h ($p = 0.0291$). The present study also determined the percentage of wound closure. During analysis, the percentage of wound closure was calculated for each of the two conditions. The results of the analysis found that cells treated with 5 mg/ml of beetroot had significantly lower wound closure percentage at 12 h and 24 h ($p = 0.008$, $p = 0.0179$).

These results were contradictory to our expectations, given that previous studies found that dietary nitrates, such as beetroot, increased cell proliferation and migration (Al-Harbi et al. 2021; Hendgen-Cotta et al. 2012). However, Mancini et al., (2021) found that beetroot reduced cell proliferation and migration, contrary to the original prediction. Mahdipuor E., (2022) also found similar results and concluded that treatment of beetroot extract reduced wound healing percentage and therefore overall cell migration. Both of these studies contradict the prediction of this research, that the high concentrations of nitrite in beetroot is related to endothelial cell migration and that cell migration rate would increase after beetroot exposure. In these studies, the reduction in migration potential caused by beetroot was explained by betalains present in

beetroot. Betalains are water-soluble, nitrogen containing-pigments, composed of two structural groups: the red-violet betacyanins and the yellow-orange betaxanthins (Azeredo 2008).

A study found that betalain supplementation is found to significantly reduce the protein levels of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) (Ninfali et al. 2017). TNF-α is a major inflammatory cytokine that is known to influence endothelial cell migration. A study done by Gao et al., (2002), demonstrated that exposure of endothelial cells to TNF-α caused an increase in cell migration. Endothelial cells predominantly express the integrin complexes $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which have both been linked to endothelial migration. Endothelial cells respond to TNF-α by increasing $\alpha_v\beta_3$-integrin activation and ligation and lowering $\alpha_5\beta_1$-integrin activity and ligation to promote cell motility. Reducing the levels of TNF-α in endothelial cells may reduce TNF-α-induced cell migration through this pathway (Gao et al. 2002). This mechanism of betalains in the beetroot decreasing TNF-α may explain the reduction in cell migration seen in our results. Nevertheless, this effect may be due to a synergy of several compounds present in the extract, and not just to betalains. Further research is necessary to understand the exact mechanism of this effect.

The results of the present study also reported a dose-dependent cytotoxicity effect of beetroot extract on the endothelial cells in the first scratch assay. When cells were treated with higher concentrations of beetroot (20 mg/ml-120 mg/ml), most of the cells died (Figure 3 C and D). This may be explained by the high amounts of nitrites in beetroot. It may be possible that the high levels of nitrites found in beetroot powder may have caused unregulated NO production (Murphy 1999). Elevated NO can cause oxidative stress by reacting with oxygen to form peroxynitrite ($ONOO^-$), which is very reactive and can oxidatively damage proteins, lipids, and

nucleic acids in the cell (Murphy 1999; Karwowska and Kononiuk 2020). This can lead to cell death by apoptosis or necrosis, which may explain the increase in cell death in cells treated with higher concentrations of beetroot (Karwowska and Kononiuk 2020).

This study had several limitations. First, issues with quantitation and reproducibility limit the scratch assay. Since each well was manually scratched with a pipette tip, varying widths were generated, which could have affected cell migration. The variability in scratch tests is a reflection of how difficult it is to make a cell-free area that is homogeneous in size and location within a microplate well (Gough et al. 2011). Another limitation is that the process of scratching can cause senescent cells (cells in permanent arrest from cell cycle) to produce substances that can impede extracellular signalling, which is necessary to close the monolayer gap. Cellular senescence is a stress response that is implicated in aging. Senescent endothelial cells appear early on in a cutaneous wound, where they accelerate wound-healing by secreting platelet-derived growth factor AA (PDGF-A). PDGF-A promotes myofibroblast differentiation and consequently wound closure (Demaria et al. 2014). The potential variability caused by cellular senescence could have affected our results. Lastly, the act of forming a cellular scratch region may also physically harm the underlying extracellular matrix and/or plate surface, impairing cell migration (Gough et al. 2011; Cormier et al. 2015). The variability of the scratch test may have affected the cell migration of the different groups.

Regarding future research, shorter time intervals should be used to assess the scratch width and cell migration for better representativeness. The rate of cell migration and the dynamics of cell migration over time cannot be accurately determined by checking the cells

every 12 hours. A more in-depth understanding of how beetroot affects cell migration may be obtained by measuring the scratch width at shorter time intervals, such as every 2-4 hours. Additionally, a nitric oxide assay could be performed to determine the levels of nitric oxide in the cells treated with beetroot extract. This would help determine whether the effects of beetroot on cell migration are due to increased production of NO. Additionally, the study only investigated three different concentrations. Future studies should test a larger range of concentrations to determine if other concentrations have the same effect. Lastly, future studies should study the effects of betalains by treating the cells with just betalains to determine if it is a factor in inhibiting cell migration.

5. Conclusion

Our statistical analysis demonstrated that endothelial cells treated with 0.5 mg/ml of beetroot decreased cell migration rate and lowered the percentage of wound closure. The results of the analysis concluded that there was a statistical difference in cell migration between the cells treated with 0.5 mg/ml, 5 mg/ml, and the control condition after 24 hours ($p = 0.0013$, $p = 0.0291$). Similarly, there was no statistical difference in cell migration between the control and the cells treated with 0.1 mg/ml ($p = 0.551$). While the results of the study did not support the prediction that beetroot increases cell migration by increasing nitric oxide production, it is important to take into consideration other confounding factors that may have affected the results of our study.

6. Appendix

Table 1. *Mean scratch widths between untreated and treated cells (n = 3)*

Time	Condition			
	Untreated	0.1 mg/ml	0.5 mg/ml	5 mg/ml
0 h	225.2 ± 45.3	257.5 ± 38.5	262.2 ± 37.1	229.6 ± 2.0
12 h	144.7 ± 55.0	125.8 ± 53.2	135.4 ± 6.5	154.3 ± 37.6
24 h	8.6 ± 14.8	27.0 ± 46.71	90.5 ± 9.4	113 ± 52.2
48 h	0.0 ± 0.0	0.0 ± 0.0	0 ± 0	107.1 ± 49.1

Primary Research Article Abstracts

Al-Harbi et al. 2021:

"The antioxidant capacity of polyphenols and flavonoids present in dietary agents aids in arresting the development of reactive oxygen species (ROS) and protecting endothelial smooth muscle cells from oxidative stress/induced necrosis. Beetroot (Beta vulgaris var. rubra L.; BVr) is a commonly consumed vegetable representing a rich source of antioxidants. Beetroot peel's bioactive compounds and their role in human umbilical vein endothelial cells (HUVECs) are still under-researched. In the present study, beetroot peel methanol extract (BPME) was prepared, and its effect on the bio-efficacy, nuclear integrity, mitochondrial membrane potential and vascular cell growth, and immunoregulation-related gene expression levels in HUVECs with induced oxidative stress were analysed. Gas chromatography–mass spectroscopy (GC-MS) results confirmed that BPME contains 5-hydroxymethylfurfural (32.6%), methyl pyruvate (15.13%), furfural (9.98%), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one (12.4%). BPME extract effectively enhanced cell proliferation and was confirmed by MTT assay; the nuclear integrity was confirmed by propidium iodide (PI) staining assay; the mitochondrial membrane

potential (Δψm) was confirmed by JC-1 staining assay. Annexin V assay confirmed that BPME-treated HUVECs showed 99% viable cells, but only 39.8% viability was shown in HUVECs treated with H2O2 alone. In addition, BPME treatment of HUVECs for 48 h reduced mRNA expression of lipid peroxide (LPO) and increased NOS-3, Nrf-2, GSK-3β, GPX, endothelial nitric oxide synthase (eNOS) and vascular cell growth factor (VEGF) mRNA expression levels. We found that BPME treatment decreased proinflammatory (nuclear factor-κβ (F-κβ), tissue necrosis factor-α (TNF-α), toll-like receptor-4 (TLR-4), interleukin-1β (IL-1β)) and vascular inflammation (intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), EDN1, IL-1β)-related mRNA expressions. In conclusion, beetroot peel treatment effectively increased vascular smooth cell growth factors and microtubule development, whereas it decreased vascular inflammatory regulators. BPME may be beneficial for vascular smooth cell regeneration, tissue repair and anti-ageing potential."

Asgary et al. 2016:

"Hypertension is a major risk factor for cardiovascular disease and has a prevalence of about one billion people worldwide. It has been shown that adherence to a diet rich in fruits and vegetables helps in decreasing blood pressure (BP). This study aimed to investigate the effect of raw beet juice (RBJ) and cooked beet (CB) on BP of hypertensive subjects. In this randomized crossover study, 24 hypertensive subjects aged 25–68 years old were divided into two groups. One group took RBJ for 2 weeks and the other group took CB. After 2 weeks of treatment, both groups had a washout for 2 weeks then switched to the alternate treatment. Each participant consumed 250 ml day−1 of RBJ or 250 g day−1 of CB each for a period of 2 weeks. Body weight, BP, flow-mediated dilation (FMD), lipid profile and inflammatory parameters were measured at baseline and after each period. According to the results, high-sensitivity C-reactive protein (hs-CRP) and tumour necrosis factor alpha (TNF-α) were significantly lower and FMD was significantly higher after treatment with RBJ compared with CB (P<0.05). FMD was significantly (P<0.05) increased, but systolic and diastolic BP, intracellular adhesion molecule-1 (ICAM-1), vascular endothelial adhesion molecule-1 (VCAM-1), hs-CRP, interleukin-6, E-selectin and TNF-α were significantly (P<0.05) decreased with RBJ or CB. Total antioxidant capacity was increased and non-high-density lipoprotein (HDL), low-density lipoprotein (LDL) and total cholesterol (TC) were decreased with RBJ but not with CB. Although both forms of

beetroot were effective in improving BP, endothelial function and systemic inflammation, the raw beetroot juice had greater antihypertensive effects. Also more improvement was observed in endothelial function and systemic inflammation with RBJ compared with CB."

Gao et al. 2002:

"Tumor necrosis factor-α (TNF-α), one of the major inflammatory cytokines, is known to influence endothelial cell migration. In this study, we demonstrate that exposure of calf pulmonary artery endothelial cells to TNF-α caused an increase in the formation of membrane protrusions and cell migration. Fluorescence microscopy revealed an increase in αvβ3focal contacts but a decrease in α5β1 focal contacts in TNF-α-treated cells. In addition, both cell-surface and total cellular expression of αvβ3-integrins increased significantly, whereas the expression of α5β1-integrins was unaltered. Only focal contacts containing αvβ3- but not α5β1-integrins were present in membrane protrusions of cells at the migration front. In contrast, robust focal contacts containing α5β1-integrins were present in cells behind the migration front. A blocking antibody to αvβ3, but not a blocking antibody to α5-integrins, significantly inhibited TNF-α-induced cell migration. These results indicate that in response to TNF-α, endothelial cells may increase the activation and ligation of αvβ3 while decreasing the activation and ligation of α5β1-integrins to facilitate cell migration, a process essential for vascular wound healing and angiogenesis."

Gopalan & Jadhav, 2021:

"The study on neurodegenerative associated disorders is of immense clinical interest. Thus in the present investigation hydrogen peroxide was chosen as a precursor to induce oxidative cell damage in the cell cultures. The pigment extracted from red beetroot (Beta vulgaris) was analyzed by quantification of betalains by HPTLC, which revealed that the extract was made of two pig-ments, red and yellow. The red pigment was quantified which was 635ng/20µl (31.75mg/ml). The total phenolics and tannic in the extracts were reported to be 0.031µg/ml and 19.232mg/ml. 200µM of H2O2 was used for ROS production. MTT assay was performed showed the H2O2 treatment resulted in a decrease of cell viability percentage by 46.75 ± 0.018. While the beetroot extract at 10mg/ml increases the cell viability percentage to 55.58± 0.020 in 30min of pre-treatment, but no significant change was observed in

post-treatment. Catalase value was highest at 20mg/ml of beet extract (4229.8864µmol of H2O2 consumed/min/mg protein). LDH value 531.67µM/ml in cell lysate, whereas in the LDH exudate highest value recorded was 646.25µM/ml, Catalase exudate activity were higher in both pre and post-treated cells, values were recorded as 4229.88µmol of H2O2 consumed/min/mg protein and 4048.09µmol of H2O2 consumed/min/mg protein respective-ly. Acetylcholine esterase (AChE) activity in post-treated cells was below the H2O2 treatment. In pre-treated cells, all the cells showed lower AChE specific activity in comparison to H2O2 treated cells. The present findings from our work exhibited the protective ability of the red beetroot (Beta vulgaris) against the oxidative stress induced by hydrogen peroxide."

Hendgen-Cotta et al. 2012:

"Revascularization is an adaptive repair mechanism that restores blood flow to undersupplied ischemic tissue. Nitric oxide plays an important role in this process. Whether dietary nitrate, serially reduced to nitrite by commensal bacteria in the oral cavity and subsequently to nitric oxide and other nitrogen oxides, enhances ischemia-induced remodeling of the vascular network is not known.

Mice were treated with either nitrate (1 g/L sodium nitrate in drinking water) or sodium chloride (control) for 14 days. At day 7, unilateral hind-limb surgery with excision of the left femoral artery was conducted. Blood flow was determined by laser Doppler. Capillary density, myoblast apoptosis, mobilization of $CD34^+/Flk\text{-}1^+$, migration of bone marrow–derived $CD31^+/CD45^-$, plasma S-nitrosothiols, nitrite, and skeletal tissue cGMP levels were assessed. Enhanced green fluorescence protein transgenic mice were used for bone marrow transplantation. Dietary nitrate increased plasma S-nitrosothiols and nitrite, enhanced revascularization, increased mobilization of $CD34^+/Flk\text{-}1^+$ and migration of bone marrow–derived $CD31^+/CD45^-$ cells to the site of ischemia, and attenuated apoptosis of potentially regenerative myoblasts in chronically ischemic tissue. The regenerative effects of nitrate treatment were abolished by eradication of the nitrate-reducing bacteria in the oral cavity through the use of an antiseptic mouthwash.

Long-term dietary nitrate supplementation may represent a novel nutrition-based strategy to enhance ischemia-induced revascularization."

Kawasaki et al. 2003:

"To test the hypothesis that the phosphatidylinositol 3-kinase (PI3 kinase)/protein kinase Akt signaling pathway is involved in nitric oxide (NO)-induced endothelial cell migration and angiogenesis, we treated human and bovine endothelial cells with NO donors, S-nitroso-L-glutathione (GSNO) and S-nitroso-N-peni- cillamine (SNAP). Both GSNO and SNAP increased Akt phosphorylation and activity, which were blocked by cotreatment with the PI3 kinase inhibitor wortmannin. The mechanism was due to the activation of soluble guanylyl cyclase because 8-bromo-cyclic GMP activated PI3 kinase and the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-□]quinoxalin-1-one (ODQ) blocked NO-induced PI3 kinase activity. Indeed, trans-fection with adenovirus containing endothelial cell NO synthase (eNOS) or protein kinase G (PKG) increased endothelial cell migration, which was inhibited by cotransfection with a dominant-negative mutant of PI3 kinase (dnPI3 kinase). In a rat model of hind limb ischemia, adenovirus-mediated delivery of human eNOS cDNA in adductor muscles resulted in time-dependent expression of recombinant eNOS, which was accompanied by significant increases in regional blood perfusion and capillary density. Co-injection of adenovirus carrying dnPI3 kinase abolished neovascularization in ischemic hind limb induced by eNOS gene transfer. These findings indicate that NO promotes endothelial cell migration and neovascularization via cGMP-dependent activation of PI3 kinase and suggest that this pathway is important in mediating NO-induced angiogenesis."

Mancini et al. 2021:

"Beet (*Beta vulgaris* L.) has high nutritional value, containing bioactive compounds such as betalains and flavonoids. Scientific evidence points to the use of these natural compounds in the treatment of several types of cancer, such as prostate cancer, one of the main causes of morbidity and mortality in men. Here, we compared beet roots and leaves extracts, and their main compounds, apigenin, and betanin, respectively, in DU-145 and PC-3 prostate cancer cell lines. Both cells presented the proliferation decreased for beetroot and beet leaves extracts. The apigenin treatment also reduced the proliferation of both cell lines. Regarding cell migration, beet leaves extract was able to decrease the scratch area in both cell lines, whereas apigenin affected only PC-3 cells' migration. In colony formation assay, both extracts were effective in reducing the number of colonies formed. Besides, the beet leaves extracts and apigenin presented

strong inhibition of growth-related signaling pathways in both cell lines, and the beetroot extract and betanin presented effects only in DU-145 cells. Furthermore, the extracts and isolated compounds were able to reduce the levels of apoptotic and cell cycle proteins. This study reveals that beet extracts have important anti-cancer effects against prostate cancer cells."

Murohara et al. 1999:

"Endothelium-derived nitric oxide (NO) and its precursor L-arginine have been implied to promote angiogenesis, but little is known about the precise mechanism. The inhibition of endogenous NO formation by Nω-nitro-L-arginine methyl ester (L-NAME) (1 mmol/L) but not its inactive enantiomer D-NAME (1 mmol/L) inhibited endothelial cell sprouting from the scratched edge of the cultured bovine aortic endothelial cell monolayer. Inhibition of endogenous NO release by L-NAME was confirmed by amperometric measurement using an NO-specific electrode. In the modified Boyden chamber, L-NAME (1 mmol/L) significantly inhibited endothelial cell migration, whereas L-NAME did not affect endothelial DNA synthesis as assessed by analysis of [3H]thymidine incorporation. We then examined alteration of endothelial cell adhesion molecule expression after the inhibition of NO by L-NAME in cultured human umbilical vein endothelial cells. In both normoxic and hypoxic conditions, L-NAME (1 mmol/L) inhibited surface expression of integrin αvβ3, which is an important integrin facilitating endothelial cell survival and angiogenesis. However, L-NAME did not affect the expression of platelet endothelial cell adhesion molecule-1, intercellular adhesion molecule-1, vascular endothelial adhesion molecule-1, gap junction protein connexin 43, and VE-cadherin, which have been reported to potentially affect angiogenesis. In summary, inhibition of endothelial NO synthase by L-NAME attenuated endothelial cell migration but not proliferation in vitro. Furthermore, endogenous endothelium-derived NO maintains the functional expression of integrin αvβ3, a mediator for endothelial migration, survival, and angiogenesis. Endothelium-derived NO, thus, may play an important role in mediating angiogenesis by supporting endothelial cell migration, at least partly, via an integrin-dependent mechanism."

Nogueira Soares et al. 2021:

"Human immunodeficiency virus (HIV) is associated with lower nitric oxide (NO) bioavailability and vascular dysfunction. Nitrate-rich beetroot juice (BJ) has been shown to acutely increase NO availability and vascular function in healthy and individuals at high risk for cardiovascular disease. Thus, we tested the effects of BJ ingestion on flow-mediated dilation (FMD) and pulse wave velocity (PWV) measurements in healthy and HIV-infected patients. Thirteen HIV-infected individuals (age, 36 ± 10 years) and 18 healthy (age, 27 ± 8 years) participated in the study. Individuals were submitted to vascular tests such as FMD and pulse PWV at pre (T0) and at 120 min (T120) after BJ and placebo (PLA) ingestion. The %FMD at T0 of the control group was significantly higher than the %FMD at T0 of the HIV individuals in both interventions. BJ improved the %FMD at T120 when compared with T0 in the HIV and control groups. There was no change in %FMD after PLA ingestion in the control and HIV groups. There were no differences between groups (control vs HIV), time points (T0 vs T120), and interventions (BJ vs PLA) for PWV. Our findings showed that nitrate-rich BJ ingestion acutely improved vascular function in healthy and HIV-infected patients. Clinical Trials Registry no. NCT03485248."

Papapetropoulos et al. 1997:

"Vascular endothelial growth factor (VEGF) is a regulator of vasculogenesis and angiogenesis. To investigate the role of nitric oxide (NO) in VEGF-induced proliferation and in vitro angiogenesis, human umbilical vein endothelial cells (HUVEC) were used. VEGF stimulated the growth of HU- VEC in an NO-dependent manner. In addition, VEGF pro- moted the NO-dependent formation of network-like structures in HUVEC cultured in three dimensional (3D) collagen gels. Exposure of cells to VEGF led to a concentration- dependent increase in cGMP levels, an indicator of NO pro- duction, that was inhibited by nitro-L-arginine methyl ester. VEGF-stimulated NO production required activation of tyrosine kinases and increases in intracellular calcium, since tyrosine kinase inhibitors and calcium chelators attenuated VEGF-induced NO release. Moreover, two chemically dis- tinct phosphoinositide 3 kinase (PI-3K) inhibitors attenuated NO release after VEGF stimulation. In addition, HU- VEC incubated with VEGF for 24 h showed an increase in the amount of endothelial NO synthase (eNOS) protein and the release of NO. In summary, both short- and long-term exposure of

human EC to VEGF stimulates the release of biologically active NO. While long-term exposure increases eNOS protein levels, short-term stimulation with VEGF pro- motes NO release through mechanisms involving tyrosine and PI-3K kinases, suggesting that NO mediates aspects of VEGF signaling required for EC proliferation and organization in vitro."

Suarez-Arnedo et. al 2020:

"In vitro scratch wound healing assay, a simple and low-cost technique that works along with other image analysis tools, is one of the most widely used 2D methods to determine the cellular migration and proliferation in processes such as regeneration and disease. There are open-source programs such as imageJ to analyze images of *in vitro* scratch wound healing assays, but these tools require manual tuning of various parameters, which is time-consuming and limits image throughput. For that reason, we developed an optimized plugin for imageJ to automatically recognize the wound healing size, correct the average wound width by considering its inclination, and quantify other important parameters such as: area, wound area fraction, average wound width, and width deviation of the wound images obtained from a scratch/ wound healing assay. Our plugin is easy to install and can be used with different operating systems. It can be adapted to analyze both individual images and stacks. Additionally, it allows the analysis of images obtained from bright field, phase contrast, and fluorescence microscopes. In conclusion, this new imageJ plugin is a robust tool to automatically standardize and facilitate quantification of different *in vitro* wound parameters with high accuracy compared with other tools and manual identification."

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