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The Effect of pH in Proliferation of Endothelial Cells: A Scratch Test Approach

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Abstract

Endothelial cells form a single cell layer on the blood vessels to facilitate transport of materials between tissue and blood. The physiological pH of human blood is found to be close to 7.4 in normal conditions. This experiment aimed to assess how the difference in pH environment may affect cell growth. Proliferation was analyzed through acidic (pH = 6.9) and alkaline media (pH = 7.9), with a positive control (pH = 7.4) through a scratch test technique. This methodology allows for the analysis of progressive growth of cells through the change in width of the scratch. Results showed growth rate within 24 hours of 30% for acidic media, 10% in alkaline conditions and 52% for the control. As coinciding with current literature, alkaline conditions showed significant decrease in healing, followed by acidic, while physiological control was the fastest. The experiment was said to follow the expected trend but may be improved by maintaining optimal laboratory conditions to avoid cell death.

Introduction

Endothelial cells may be found in the inside lining of blood vessels, in which forms a single cell layer that facilitates the transport of oxygen, carbon dioxide, and other material between blood and tissues. This lining separates circulating blood and peripheral organs and is critical in upkeeping the vascular system with the correct supply of nutrients.[3] Consequently, any impairment in endothelial cell function can lead to adverse effects across organs and tissues. Therefore, the intracellular environment is highly controlled through ideal concentration of ions, sugars, and minerals for example. Along with that, the extracellular environment also must be balanced with the ideal temperature as well as hydrogen potential, or commonly known as pH.

Hydrogen Potential (pH) is a measurement of the concentration of hydrogen ion (H^+) in a solution. All cells with enzymes that catalyze metabolic pathways inside the cell have an optimal pH where its activity is the highest. The physiological pH for blood is around 7.4 which would logically be also the optimal pH for the enzymes in endothelial cells hence their location. Therefore, by assessing the growth of these cells, an ideal condition should be found around this physiological pH.

Two previous studies examined the effects on the proliferation of endothelial cells dependent on change in extracellular pH. The studies were performed by Saraina Faes et al. and Mohammad Ali Saghiri et al. According to Faes et al., the study was mainly focused on comparing the growth among acidic media and at physiological pH (treated as control) in which they state: "We found that exposing endothelial cells to acidic extracellular pH resulted in reduced cell proliferation and migration"[2]. In the meantime, Saghiri's group decided to evaluate the proliferation of these cells by comparing acidic and alkaline extracellular pH, in which they found that "Moderately acidic pH values (5.4 and 6.4) enhanced angiogenesis, whereas moderately alkaline pH values (8.4 and 9.4) surpassed angiogenesis"[5]. These studies can give us an approximation on what results we should expect from our experiment.

Considering this, our group decided to combine in this experiment the idea of assessing the proliferation of endothelial cells within two ranges as well as at physiological pH. Therefore, the methodology applied involves a scratch test, in which healing of the scratch will measure proliferation rate. A directional hypothesis taken in this case based on both literature was that the lowest healing rate should occur in the alkaline environment, and a decreased rate in acidic media if compared to physiological conditions.

Methodology

Media preparation

Every media used in this experiment for feeding or experimental conditions was prepared using the same method. All media were prepared to match L-15 10% Fetal bovine serum (FBS)[1] by using 1.23 g of Leibovitz's L-15, 0.6 mL antibiotic, 10 mL of FBS and 89 mL of deionized water. All reagents were stirred until the solution is homogenous. The pH of the media was determined with a calibrated pH meter and adjusted according to its purpose with drops of either 1.0 M HCl to acidify or 1.0 M NaOH to increase the pH as needed. Media for feeding flask, sub-culturing and for control conditions was adjusted to 7.4 pH (following physiological pH); media for the acidic treatment was adjusted to a pH of 6.9; and media for alkaline conditions was adjusted to a pH of 7.9. All media was filtered with a syringe of 0.22 μm and stored in 15 mL falcon tubes in a refrigerated environment. Every falcon tube of media was strictly used twice and discarded to avoid cross-contamination. Proper disposal is ensured by adding 70% ethanol to the media before discarding it.

Feeding and Sub-Culturing process

Cells were initially provided for the experiment in a T-25 flask and were fed every two days to ensure proper growth prior to applying treatment. The feeding process was performed aseptically and consisted of removing half of existing and used media. With that, a remaining of fresh media was added to a total overall volume of 4 mL of media. Therefore, flasks were then sub cultured once they were deemed with sufficient cell confluency, with around 80% or more of the bottom of the flask.

All media was removed from the flask and cells were washed twice with 4 mL of PBS. Trypsin (2 mL) was added to detach cell culture from the bottom of the T-25 flask followed by addition of 4 mL media to neutralize it. The contents were transferred to a falcon tube and centrifuged at 300 rpm for 10 minutes. The supernatant was discarded, and the remaining pellet was resuspended with 1 mL of fresh media using disposable pipettes. Cells were then distributed among two T-25 flasks. In the case of this experiment, because of high confluence of cells, one subculture process was divided into four flasks instead of one. In addition, once ready, one available flask was sub-cultured into nine well plates to allow the addition of further treatments.

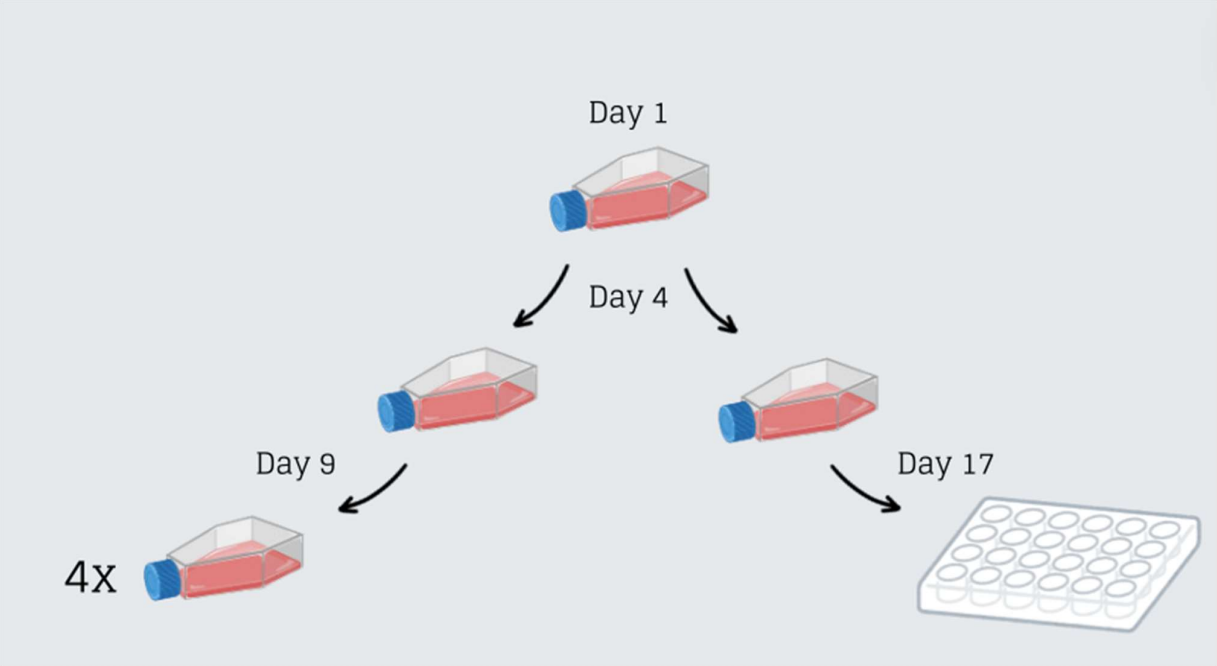

Figure 1. Sub-Culturing process performed in this experiment. Day 1 T-25 flask is subculture into two different T-25 flasks after 4 days of feeding twice a day and confluence of 88%. Subculture process continues upon day 17.

Scratch Test Preparation

Fresh Media was prepared the day of performing the scratch test. Sub-culturing was done from one flask containing around 70% confluency and distributed into nine wells in the well-plate (A1 - A3, B1 - B3, C1 - C3). Cells were allowed to grow on 7.4 pH media until cell confluency reached around 66%. Media was removed from wells and washed with PBS. Media with respective treatments were added in triplicates: acidic condition with pH of 6.9 into wells A1, A2 and A3 while alkaline conditions with pH of 7.9 into wells B1, B2 and B3. A control was also set up at physiological conditions with adjusted pH of 7.4 into spots C1, C2 and C3. For reference, before the subculture process, each well was marked with a cross line made with a blade. From then, a scratch was done on the plates using an Eppendorf pipette tip to ensure removal of the cells in that area. Usually, the scratch is performed based on the reference lines previously created. The well-plate was then monitored two to three times a day for 3 consecutive days to assess the healing of the scratch. Images with measurements related to the width of the scratch were taken to ensure proliferation progress.

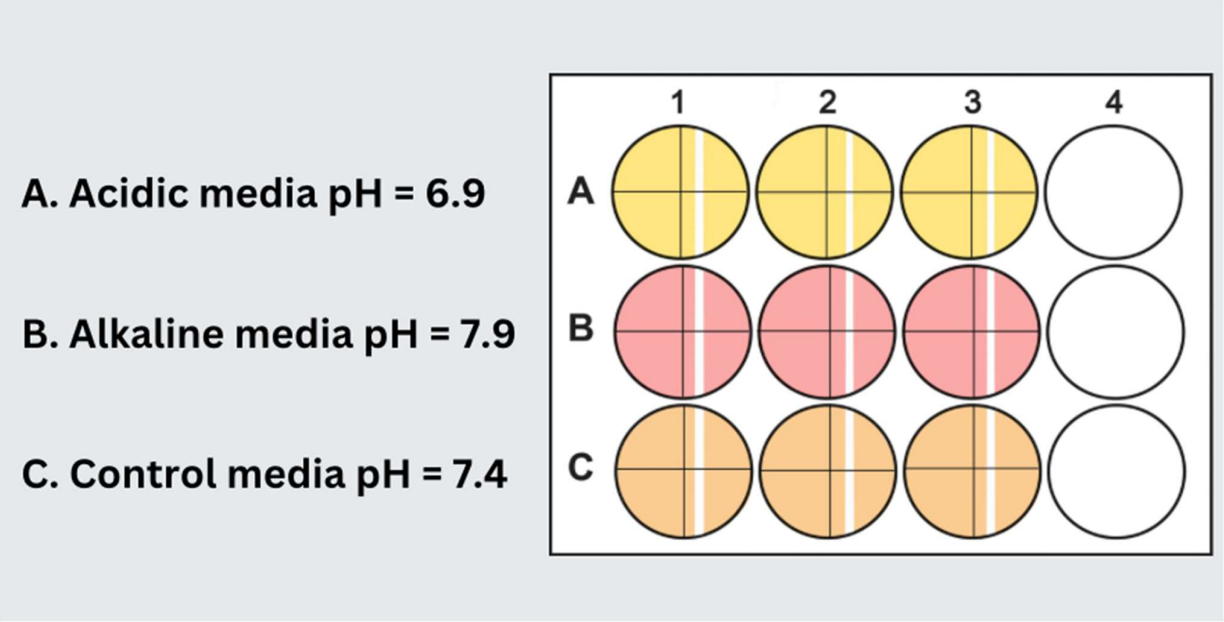

Figure 2. Scratch test layout with triplicates for two treatments and a control. Wells correspond to A - acidic treatment pH = 6.9, B - alkaline treatment pH = 7.9 and C - control pH= 7.4.

Results

Survivability of Cells

Within two days of subculturing, media volume was significantly lowered all throughout the plate. Our group aimed to solve this problem by placing a parafilm around the plate to prevent unnecessary moisture loss. However, on day one after performing the scratch test, three wells (A2, A3, C3) dried off resulting in cell death. Results were still able to be collected due to the triplicates. Despite this intervention, all cells died on the third day within performing the scratch test.

Scratch test

As previously mentioned, the width of each scratch was measured consistently at the bottom right of the well. In this case, results are only shown for wells A1, B2 and C2, due to being the only available culture of cells with enough confluence. Results show that acidic treatment had a starting width of 587.0 μm, followed by alkaline treatment with 1113.9 μm and 1045.4 μm was the starting point for control. The following picture shows a microscopic picture of each well, in which a scratch is visible. All pictures were collected at the same time and with a consistent scale of 750 μm.

Figure 3. Initial scratch test performed in three well plates. Photos shown represent different treatments applied where A1, B1 and C2, refer to acidic, alkaline and control media, respectively.

Subsequently, microscopic pictures were obtained every three hours. For greater way of distinguishing among scratch width, the following figure shows the progress obtained on day two, around twenty-four hours after the test started. Width obtained in wells A1, B2 and C2 were found to be of 413.3 μm, 1000.6 μm and 498.6 μm, respectively.

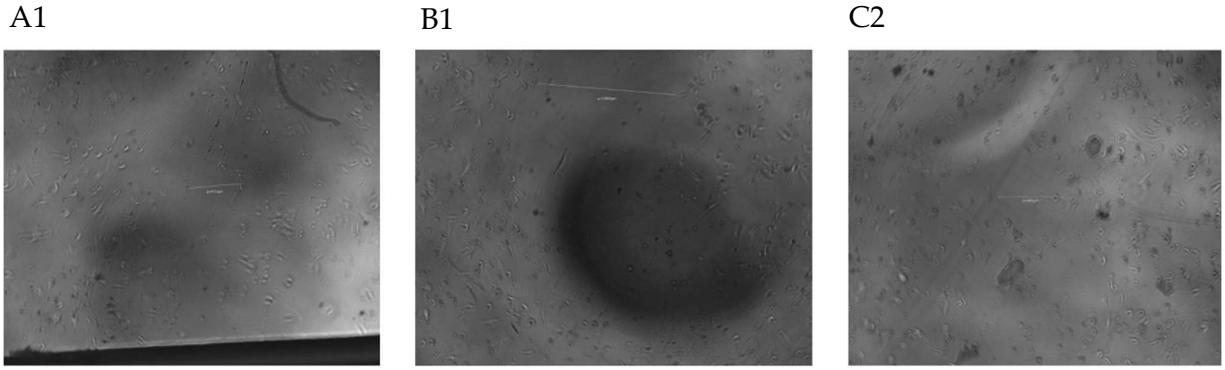

Figure 4. Second day progress of scratch test performed in three well plates. Photos shown represent different treatments applied where A1, B1 and C2, refer to acidic, alkaline and control media, respectively.

Consecutive, on day three, it was not possible to record the scratch distance because majority of the cells were found dead. The following picture shows well plate pictures in which the scratch was undetectable as well as lower cell confluency.

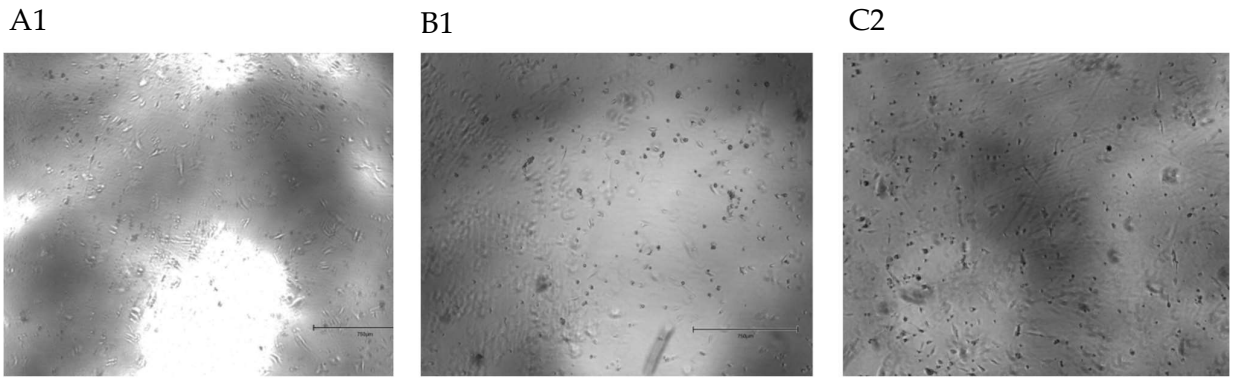

Figure 5. Final day progress of scratch test performed in three well plates. Photos shown represent different treatments applied where A1, B1 and C2, refer to acidic, alkaline and control media, respectively.

Table 1. Data collected for three days resulting from scratch test in endothelial cells. Distance is reported as the width of the scratch (μm) from a scale of 750 μm.

	Acidic (A1)	**Alkaline (B1)**	**Control (C2)**
Day	**Distance (μm)**	**Distance (μm)**	**Distance (μm)**
1	587.0	1,113.9	1,045.4
2	413.3	1,000.6	498.6
3	Dead	Dead	Dead

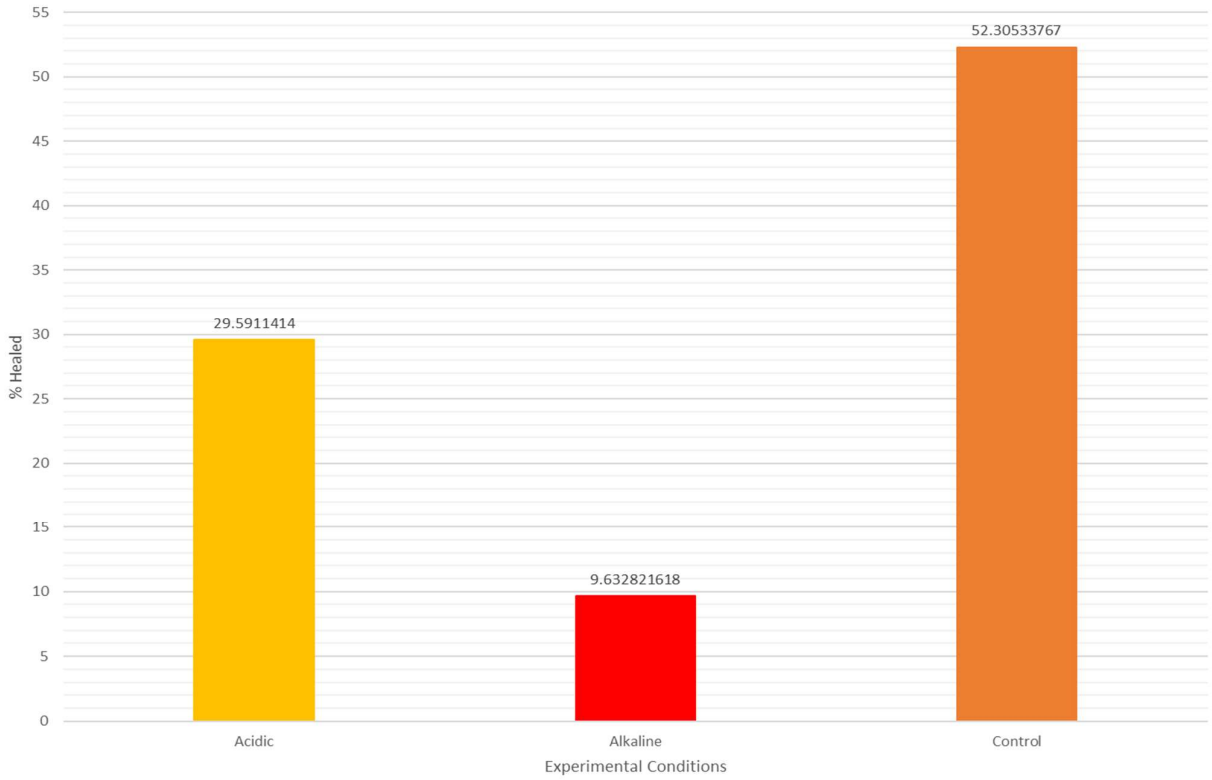

Figure 6. Bar graph displayed the percentage healed based on initial scratch width of scratch test performed in endothelial cells assessing the effect of pH change in its proliferation.

Discussion

The growth and healing rate of endothelial cells was assessed through a scratch test in two environments as well as a control vial. In this case, alkaline and acidic media with pH of 7.9 and 6.9 were prepared as comparison treatments. An additional media at physiological pH of 7.4 was treated as control for the growth baseline. In this case, the scratch test was performed in the morning in nine wells, having all treatments in triplicates. Vials were monitored three times a day and the width of the scratch was measured at the same spot, on the bottom right of each well. Despite the efforts of the group monitoring the plate, wells A2, A2 and C3 dried on the second day they were subcultured. However, data was still able to be collected and the experiment was carried upon the third day.

To prevent other wells from dying, parafilm was used to prevent loss of humidity in the well-plate. Despite these extra precautions the cells in all the wells died on the third day of measurements. Results showed in Table 1 report the width observed in wells, with a constant scale of 750 μm. In this case, it is possible to see the decrease in width of the scratch in all three wells. This is expected hence the pH used in the media was not intended to induce apoptosis but affect the growth rate. Additionally, based on these results, Figure 1 shows the healed percentage from the first to the second day. In this case, based on the width obtained

it was possible to see that the control healed around 52% within 24 hours, while alkaline had the slowest healing rate, of only 9.63%. As expected, the acidic media healed cells at a rate of 29.6%.

In this case, it is important to mention that the original scratch was not consistent among all treatments. The acidic media had originally a smaller scratch then any other wells. This is due to inconsistent in scratch pressure. Usually, in a scratch test, the same size of pipette tips is used to avoid discrepancy and ideally the same amount of pressure is needed. This leads to a consistent width and proliferation may be assessed with more accuracy. On top of that, the drying of wells may also have affected the results. Once monitored, more than half of the wells had low volume of media and three had completely dried off. This could have been due to the lack of control over the incubators since it was shared by multiple groups performing different experiments. This could have led to loss of humidity and to the wells drying out.

Additionally, cell confluence is also decreased within the progression after 24 hours. For comparison, it is easier to noticed in the control well. Figure 3-C2, which demonstrates the initial scratch, cells are seen almost uniformly distributed among the well, in which makes it easy to identify the scratch performed. On the other hand, Figure 4-C2 shows significant decrease in cell density. This was already an indication of non-ideal external conditions, that were directly impacting cell proliferation. Note that this trend is seen in all figures, in which the cell density is negatively impacted despite the treatment applied.

However, the directional hypothesis was still able to be proven correct and that the experiment was directed towards success. In this case, even though the comparison is being made within one day and more accurate predictions could have been made with more data points, the expected trend is still being followed. Wells exposed to media at physiological pH had the fastest growth among treatments and showed the most confluency. Alkaline treatment was the slowest, indicating greater impact in endothelial cell proliferation. Lastly, in accordance with Faes et al., acidic media did in fact reduce proliferation.

For future work, this experiment could have been better performed and may be taken further in controlled environment by, for example, having a single incubator dedicated exclusively for this experiment in which cell conditions are maintained at least 90% of the time. Another way the experiment could be improved on is to remove the time constraints the group experienced while performing the experiment. Because the experiment needed to be completed in a specific amount of time, sub-culturing process had to be performed before the cells reached 80% confluency. This affected the experiment's reliability and may be the reason the scratches were difficult to find when doing the scratch test, leading to fluctuation of measurements.

Conclusion

Endothelial cell proliferation rate in different media pH levels was evaluated through a scratch test approach. It was found that growth was faster on acidic media than the alkaline media. In this case, acidic media showed a healed scratch with 29.6% growth from the initial measurement, while in alkaline media cells only healed 9.6% of the scratch. However, cell growth in both media is relatively slower compared to the control in which the cells healed 52.3% of the scratch. These results are aligned with the discoveries of the previous studies mentioned before in which it was proved that cells grow the fastest on physiological pH, while slower in alkaline environments. Lastly, future work indicates stricter sterile protocols as well as isolation of wells in the incubator to ensure proper growth.

References

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