Determining Vitamin C's effect on the proliferation and viability of endothelial cells in a high glucose environment

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Biology 3520

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Abstract

Glucose metabolism produces high energy electron carriers that contribute to energy production via oxidative phosphorylation in the mitochondrial membrane. Excess glucose has been shown to increase reactive oxygen species (ROS), causing damage and death to endothelial cells. Vitamin C has antioxidant properties that may mitigate the effects of high glucose content on cell viability and proliferation. This project tested such potential by preparing three differentially treated endothelial cell cultures: a control culture, a high glucose culture, and a vitamin C-supplemented high glucose culture. The viability and proliferation of the cultures were measured via the bicinchoninic acid assay (BCA) and hemocytometry. Cell counts were conducted at three time points (0, 24, & 48h), and protein analysis via BCA was done at 48 hours. Protein concentrations were measured in triplicate using the NanoDrop. The glucose-only flask exhibited heightened cell death, relative to both the control and vitamin C supplemented flasks, supporting that the presence of glucose causes a decrease in cell viability. The glucose + vitamin C flask showed a large increase in living cells relative to the glucose-only flask, suggesting that vitamin C helps mitigate the negative effects of glucose on cell viability. An ANOVA test determined a significant difference in the protein contents of each treatment. The control exhibited the highest protein content, and the glucose-only treatment had the lowest. The vitamin C supplemented flask contained more protein than the glucose-only flask, further supporting its protective function; however, it did not restore protein levels to that of the glucose-absent control. This experiment adds to existing literature supporting that high glucose environments reduce endothelial cell viability and proliferation, and vitamin C can help mitigate these effects.

Introduction

It has been demonstrated that cells subjected to high glucose environments produce excess reactive oxygen species (Hou et al., 2015). Glucose is metabolised in glycolysis and the citric acid cycle, producing the high energy electron carriers, NADH and $FADH_2$. These compounds donate electrons to the electron transport chain in the mitochondrial membrane, employing oxygen as the terminal electron acceptor. This generates the mitochondrial membrane potential (Brownlee, 2001) which drives the oxidative phosphorylation of ADP to ATP: an important process for energy production in the cell (Hulsman et al., 2012). When high glucose concentrations are present, the mitochondrial membrane potential is heightened due to the overproduction of the electron carriers. The electron transport chain becomes inhibited, and the intermediate free radical, ubiquinone, reduces oxygen to the superoxide radical (Brownlee, 2001). When excess reactive oxygen species (ROS) like superoxide radicals are abundant, oxidation of cellular macromolecules propagates, causing eventual cell death (Hulsmans et al. 2012). Superoxide radical has been shown to activate cascade which signal for apoptosis, which will be further detailed in the discussion (Feng et al., 2000). Hou et al. (2015) found that high-glucose environments applied to human vascular endothelial cells increased their ROS concentrations, lowered their viability, and increased apoptosis, dependent on glucose concentration and length of exposure. They suggest that high blood-glucose content, termed hyperglycemia, and its induction of apoptosis of endothelial cells is a significant mechanism to diseases like diabetes (Hou et al., 2015).

Vitamin C, also known as ascorbic acid, is important to endothelial cell health. One of its cellular functions is to act as an antioxidant. At physiological pH, ascorbic acid is ascorbate. Therefore, vitamin C gets transported into the cell as ascorbate, where it can then donate an electron to a ROS, forming the ascorbate radical. More specifically, ascorbate can act in conjunction with the enzyme, superoxide dismutase, to reduce superoxide radical to hydrogen peroxide. This function poses a significant potential in preventing cell death caused by high glucose conditions (May & Harrison, 2013). A previous study did

show that vitamin C successfully inhibited the apoptotic effect of a high glucose environment on endothelial cells. These findings provide potential in treating hyperglycemia with vitamin C supplementation (Feng et al. 2000; Feng et al. 2007).

With the previous research in mind, this project hypothesized that if vitamin C is supplemented to endothelial cells that are subject to high glucose levels, a reduction of cell death will be observed using hemocytometry and a higher protein content will be measured using the bicinchoninic acid assay, relative to cells without vitamin C supplementation. It aimed to determine if vitamin C positively influences the proliferation and viability of endothelial cells in a high glucose environment.

Methods & Design

Preparation of culture media

Three types of complete medium were prepared: control, just glucose (30mM), and glucose (30mM) with vitamin C (300µM). The control culture medium consisted of 10% Fetal growth serum, Leibovitz's L-15 solution (approximately 1.22 g per 100 mL solution), and an antibiotic/antimycotic (0.6 mL per 100 mL solution). To create the glucose-only medium, 35 mL of control medium was supplemented with 0.2002 g of D-glucose to yield a glucose concentration of 30.24 mM. To create the glucose and vitamin C medium 0.1970 g of D-glucose and 0.0064 g of L-ascorbic acid were added to another 35 mL of control media to yield concentrations of 29.75 mM and 296.6 µM respectively. The altered media were syringe filtered into autoclaved glassware. The target concentrations were derived from a study by Feng, et al. (2007).

Preparation of cell cultures & counting via hemocytometry

A flask (T75) of Ea.hy926 endothelial cells was subcultured to yield two cultures. One week later, each culture was subcultured to yield a total of 4 flasks of cells. Five days later, the experimental conditions were applied to three of the flasks.

The procedure followed for suspending cells into solution for counting is as follows. This was done for three cultures. First, all solutions were brought to room temperature. All the complete media was removed and 5 mL of phosphate buffered saline (PBS) was added to wash off residual medium. The wash was repeated, followed by removal of the PBS and addition of 2.5 of trypsin EDTA. They were incubated for 2 minutes and assessed under the microscope for movement. If the cells did not fully round up, they were incubated for another 2 minutes. Once detached from the flask, 8 mL of complete media was added, and the suspended cell solutions were transferred to 15-mL tubes. They were centrifuged for 10 minutes at 300 rcf. The supernatant was removed to leave the pellet, which was then resuspended in 1 mL of culture medium. The respective altered culture medium was used for resuspension (control, just glucose, or glucose and vitamin C). At this point, 20 µL of cell solution and 20 µL of trypan blue were added to a 0.5-mL Eppendorf tube and mixed for cell counting via hemocytometry using a microscope. Then, 9 mL of the appropriate culture medium was added to each tube. The cell solutions were transferred to a new labelled T75 flask and incubated at 37 ºC in 5% CO_2. This procedure is derived from the Biology 3520 laboratory manual. Hemocytometry was preformed at 0 hr, 24 hr, and 48 hr, meaning this process of suspending the cells was repeated each time. At 48 hours, BCA was preformed after counting.

Protein quantification via bicinchoninic acid assay

Bovine serum albumin standards with concentrations of 0.10, 0.25, 0.50, 0.75, and 1.00 mg/mL were prepared using a 2 mg/mL protein stock and water. The final volume of each standard was 1 mL. The volumes are shown in *Table 1.*

After cells were detached in the previous procedure and resuspended in 1 mL of culture media, the following procedure was done to lyse and isolate the protein from the control, just glucose, and glucose with vitamin C cell cultures. The cells were centrifuged at 2500 g for 5 minutes, the supernatant was removed, and 1 mL of PBS was added. The cells were resuspended via pipette tip, then centrifuged with the same settings as above. This PBS wash was repeated. The supernatant of the second wash was removed. Next, 1 mL of RIPA buffer was added, and the pellet was resuspended again via pipetting. For 15 minutes, the RIPA buffer-cell mixtures were iced and shaken gently each minute. Next, the solutions were transferred to Eppendorf tubes and centrifuged for 15 minutes at 14,000g. The goal of this was to separate the cell debris (pellet) from the protein (supernatant). The supernatant was pipetted into new Eppendorf tubes.

Next, three replicates were prepared for each standard and samples for protein analysis using the NanoDrop One, along with a blank (water and working reagent). Working reagent consisted of 5 mL of solution A and 0.1 mL of solution B from the BCA kit reagents. In each appropriately labeled 0.5-mL microcentrifuge tube, 200 µL of working reagent and 25 µL of sample/standard was added. The blank and standards were read by the Nanodrop to generate a curve. To do this, 2 µL of mixed solution was pipetted onto the bottom pedestal and the arm was lowered, cleaning between each aliquot. After this, the protein concentrations of the experimental samples were measured and recorded. This procedure is derived from the Biology 3520 laboratory manual.

Table 1. Volumes of BSA stock (1 mg/mL) and water required for a series of BSA standards.

Standard	**[BCA] (mg/mL)**	**Volume BCA (µL)**	**Volume H_2O (µL)**
1	0.10	50	950
2	0.25	125	875
3	0.50	250	750
4	0.75	375	625
5	1.00	500	500

Statistical Analysis

Resulting protein content data was analyzed using a Single Factor Analysis of Variance (ANOVA) to determine the variance between treatments means relative to variation within each treatment. Results were considered significant for p-values below 0.05.

Results

Cell Count

Control Treatment:

The number of viable cells remained relatively stable over the 48-hour period, with only minor fluctuations. The dead cell count peaked at 48 hours but remained relatively stable throughout the experiment. The ratio of live to dead cells varied at each time point, with a notable decrease in dead cells after 24 hours followed by a subsequent increase at 48 hours.

Glucose Treatment:

A significant reduction in live cell count was observed between 0 and 24 hours. By 48 hours, the number of viable cells had decreased more than two-fold compared to the initial time point, as well as in comparison to both other treatments at 48 hours. The ratio of live to dead cells showed a pronounced decline after 24 hours, with an additional slight decrease by 48 hours, indicating a progressive loss of cell viability over time. These findings suggest that glucose induces cellular stress and promotes apoptosis.

Glucose + Vitamin C Treatment:

The glucose + vitamin C treatment maintained a relatively high number of viable cells at all time points. After 24 hours, the number of dead cells increased; however, by 48 hours, this number decreased again. The ratio of live to dead cells remained stable, with only a slight decline observed at 24 hours. These results suggest that vitamin C offers a protective effect, supporting cell viability even in the presence of glucose.

Cell Count Analysis:

The cell count of both dead and alive cells for each treatment at each time point is shown in Figure 1. The ratio of alive to dead cells to account for differences in the starting values is shown in Figure 2. In the control treatment, there was a steady increase in viable cells over time, with only minimal increases in dead cell counts. In contrast, the glucose treatment resulted in a higher dead cell count at 24 hours compared to the control group, and by 48 hours, the number of viable cells was more than two-fold lower than both other treatments at this time. This significant decline in cell survival over time further supports the conclusion that glucose induces cellular stress and apoptosis. Conversely, the glucose + vitamin C treatment showed the highest dead cell count at 0 hours, but by 48 hours it exhibited the lowest dead cell count. This indicates the protective effect of vitamin C, which also resulted in significantly higher survival rates at 24 and 48 hours compared to the glucose-only treatment, further demonstrating its ability to mitigate glucose-induced cell damage.

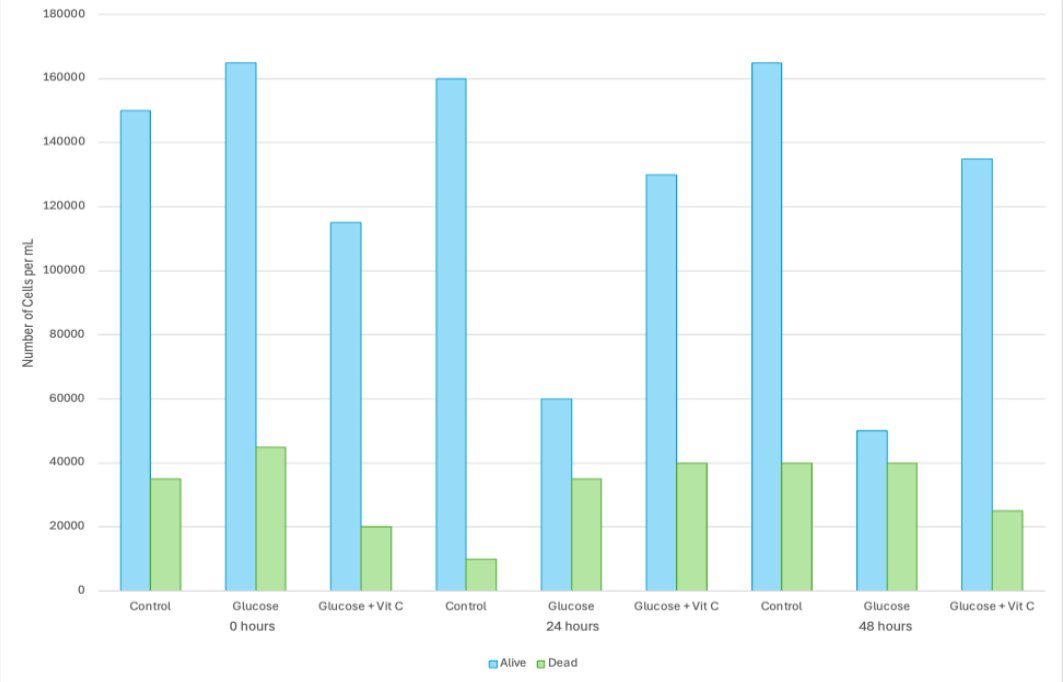

Figure 1. Count of alive and dead cells for control, glucose, and glucose + vitamin C flasks at 0, 24, and 48 hours.

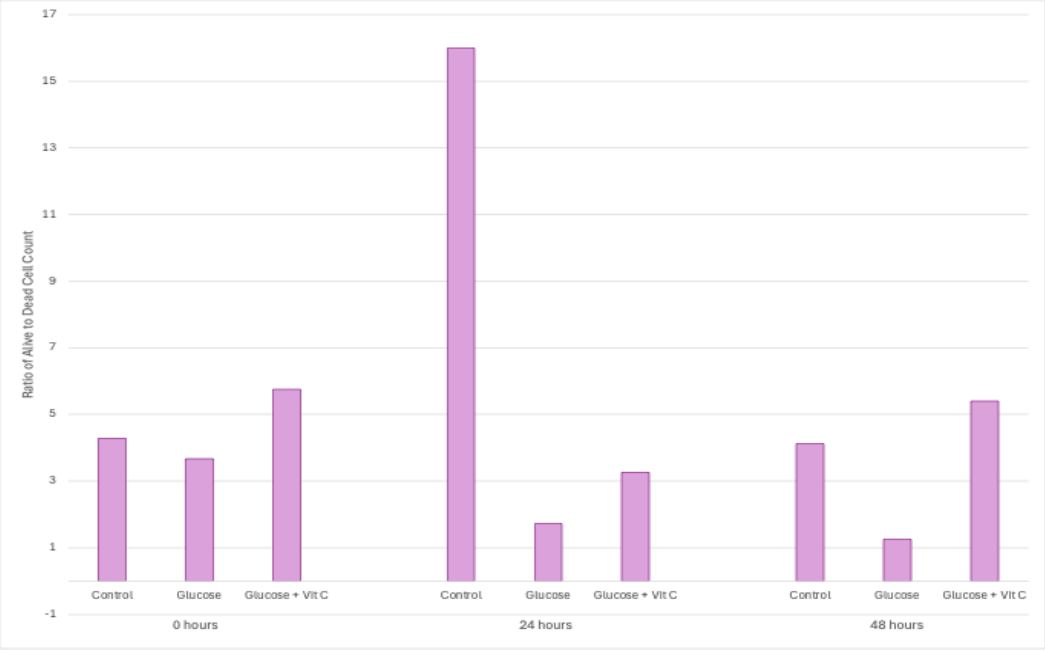

Figure 2. Ratio of alive to dead cells for control, glucose, and glucose + vitamin C flasks at 0, 24, and 48 hours.

Protein Content

Protein Content Analysis:

The standard curve had an R^2 value of 0.9975, which is very close to 1, indicating a linear curve as shown in Figure 3. A zoomed in image of the standard curve with each treatment plotted is shown in Figure 4. The control treatment exhibited the highest protein content, suggesting that in the absence of glucose, cells maintain optimal protein production and viability. In contrast, the glucose treatment resulted in lower protein concentrations, indicating a detrimental effect on both cell viability and protein synthesis over time. The glucose + vitamin C treatment displayed higher protein concentrations compared to the glucose-only treatment, demonstrating that vitamin C helps to alleviate some of the negative effects of glucose on protein production. However, protein levels in the glucose + vitamin C treatment were still lower than in the control, suggesting that while vitamin C offers protective benefits, it does not fully counteract the detrimental effects of glucose on protein production and overall cell survival.

ANOVA Statistical Analysis:

A Single Factor Analysis of Variance (ANOVA) was conducted to assess the variation between treatment means relative to variation within each treatment, and the results are shown in Figure 5. The null hypothesis was that the mean protein content across all treatments was equal. The F-value is 10.5, which is a high value suggesting that there is more variability between the groups compared to within the groups, indicating the group means are significantly different. Since the F-value of 10.5 is greater than F-critical of 5.1, we can reject the null hypothesis, which confirms that there are significant differences in protein content across the treatments. The p-value of 0.0109, which is less than the significance level of 0.05 confirms a statistically significant result and supports the rejection of the null hypothesis. The ANOVA test demonstrates that there is statistically significant evidence that the protein content of each treatment is different from each other.

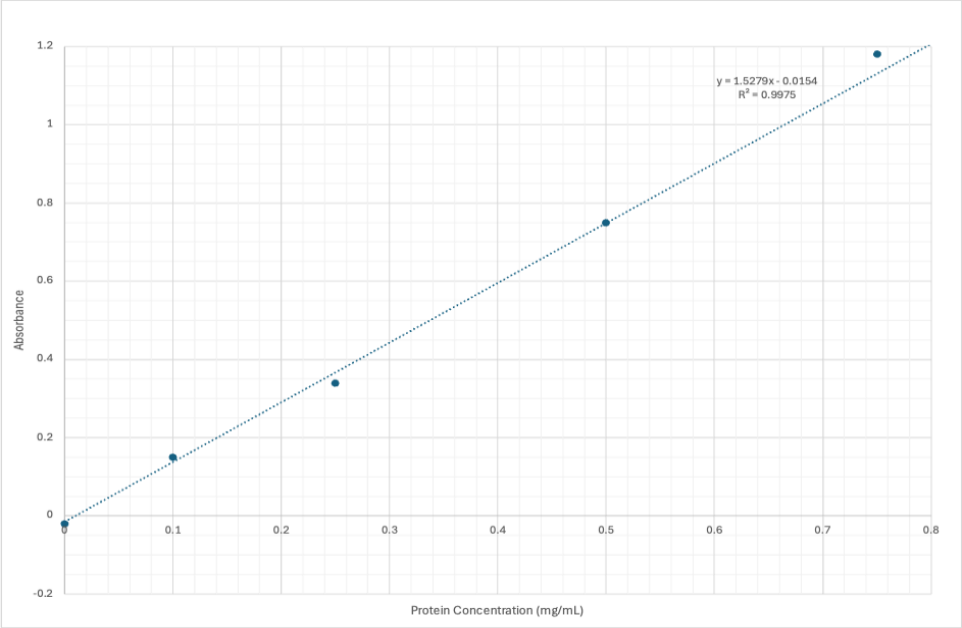

Figure 3. Standard curve for BCA protein content assay generated using Nanodrop (n = 3).

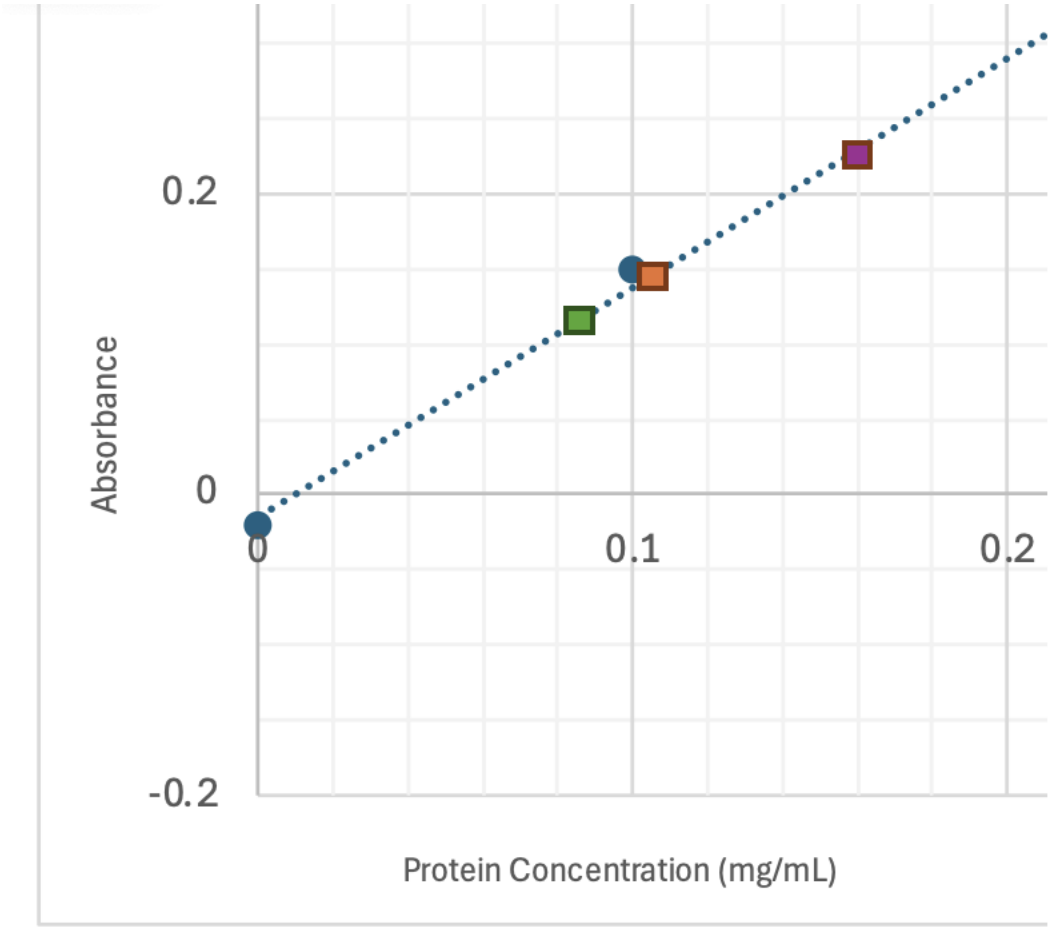

Figure 4. Zoomed in image of standard curve for BCA protein content assay showing the protein content of the control flask in purple, glucose flask in green, and glucose + vitamin C flask in orange.

SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Control	3	0.478	0.159333333	0.000101333		
Glucose	3	0.252	0.084	0.000651		
Glucose + Vit C	3	0.319	0.106333333	0.000529333		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.008982889	2	0.004491444	10.51313394	0.01094197	5.14325285
Within Groups	0.002563333	6	0.000427222			
Total	0.011546222	8				

Figure 5. Single Factor Analysis of Variance (ANOVA) of the protein content of each flask measured using the BCA assay and Nanodrop.

Discussion

The results of our study support the hypothesis that high-glucose levels induce apoptosis in endothelial cells, and that this process is inhibited by ascorbic acid. These findings are consistent with existing literature. Previous research has demonstrated that when human umbilical vein endothelial cells are exposed to high-glucose environments, they undergo apoptosis and cell death. This cell death was attributed to elevated glucose levels resulting in an increase in the production of reactive oxygen species (ROS). The ROS activate the JNK pathway, which in turn triggers capase-3 activation, leading to apoptosis. Ascorbic acid was shown to suppress high-glucose-induced apoptosis by reducing ROS production, thereby inhibiting both JNK and capase-3 activation and preventing apoptosis (Feng et al., 2000).

A more recent study on the protective effects of ascorbic acid in corneal endothelial cells exposed to oxidative stress found that ascorbic acid not only protected against apoptosis but also preserved corneal function. The study suggested that ascorbic acid exerts its protective effects by promoting the activation of the PI3K/Akt signaling pathway, a pathway that enhances cell survival and supresses apoptosis (Hsueh et al., 2020). This suggests that the protective mechanism of ascorbic acid may be linked to the PI3K/Akt pathway (Hsueh et al., 2020).

Another study by Feng et al. (2007) reported that high glucose levels lead to ROS-induced activation of matrix metalloproteinase-2 (MMP-2), an enzyme that breaks down cellular structures, and a reduction in tissue inhibitor of metalloproteinase-2 (TIMP-2) expression in human endothelial cells. This imbalance of MMP-2 and TIMP-2 leads to apoptosis. The study further found that ascorbic acid protects endothelial cells from apoptosis by reducing oxidative stress, restoring the normal levels of MMP-2 and TIMP-2, and preventing the associated cell death (Feng et al., 2007).

Limitations

This study faced several limitations. Firstly, there were limited time points. Cell growth was only measured at 0, 24, and 48 hours. Additional time points could provide a more complete picture of how glucose and vitamin C impact endothelial cell viability over time. Secondly, the cells were suspended multiple times, meaning they were repeatedly being handled and bothered. This could have stressed the cells and impacted their growth and protein production. Third, the study only used one type of endothelial cell, limiting its generalizability. Different types of cells may have reacted differently to glucose and vitamin C. Additionally, this study could have benefited from a more precise method of counting, such as MTT or flow cytometry to provide a more detailed measure of cell viability. Another limitation of our study is that the addition of glucose to the complete medium may have increased the solution's tonicity, potentially creating a hypertonic environment that led to cell shrinkage. Additionally, the protein analysis likely gave lower results for all three conditions because a pellet did not form after adding 1mL of PBS and centrifuging. Finally, there was no negative-control flask to observe the effect of vitamin C-only on endothelial cell viability and protein production, so it is unknown whether vitamin C was mitigating the negative effects of glucose, or whether vitamin C alone also encourages cell viability.

Future Research Directions

One potential future direction for this study is to explore the long-term effects of vitamin C supplementation on endothelial cell apoptosis under high-glucose conditions. Investigating the effects of chronic vitamin C exposure over extended periods of time could provide valuable insight into whether the protective effects are sustained, or if a tolerance to vitamin C develops. This could help determine the practical utility of vitamin C supplementation in managing glucose-induced cellular damage in the long run.

Additionally, future studies could focus on investigating the effects of vitamin C supplementation specifically in human endothelial cells. By examining the impact of vitamin C on endothelial cells derived from individuals with diabetes or other metabolic disorders, researchers could gain a deeper understanding of its effectiveness and applicability in human vascular health. This would provide more relevant data for clinical applications and help translate findings from model systems to human physiology.

Another important area of future research would be to examine whether vitamin C supplementation can protect other cell types, such as smooth muscle cells or fibroblasts, in vascular tissue from apoptosis induced by high glucose. Since these cells also play critical roles in vascular function and integrity, determining if vitamin C has broader protective effects could reveal its potential for improving overall cardiovascular health in diabetic patients and other individuals affected by high glucose levels.

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