Variance in the Proliferation of Mouse Carcinoma Cells Under Varying Glucose Concentrations

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Abstract

In the past fifty years, the field of nutritional therapy has grown and oncology dieticians have become an available resource for cancer treatment. While there are studies studying the link between cancer and sugar, most are studying a low sugar diet's preventative capabilities. There is not a plethora of studies exploring a low sugar diet's impact on already developed cancer cells. The Warburg effect details how cancer cells do glycolysis and fermentation even in the presence of oxygen, also known as aerobic glycolysis. Lower glucose concentrations limit cancer cell's ability to do aerobic glycolysis, therefore a low/no sugar diet will slow cancer proliferation. To test this hypothesis, we cultured cells in three different types of media. The no glucose media, which will be referred to as G- throughout this paper, had 0 mg/mL of glucose. The half glucose media had a glucose concentration of 2025 mg/mL and will be referred to as G1/2. The high glucose media, referred to as G+, had a glucose concentration of 4050 mg/mL. Once cells reached 85% confluence, we performed a WST1 Assay to assess and compare the amount of cell growth by comparing the absorbance readings. Using six absorbance readings per each of the three glucose concentrations, we calculated three means. An ANOVA (Analysis of Variance) test produced an F-score of 40.34 and a p-value of 9.215×10^{-7}. These values demonstrate that the variance seen within the three groups was insignificant compared to the variance between the groups and that the difference found between the means was statistically significant. Through our statistical analysis, it can be concluded that a higher concentration of glucose in growth media increases mouse breast carcinoma cell proliferation. The next step would be to repeat the experiment with an additional set of non-malignant cells to see if variance in glucose would have the same effect. This experiment could determine if the cells were only growing at a faster rate because of the plethora of glucose available. While our findings of slowed proliferation is important to cancer research, more experiments need to be done to explore the effect of sugar deprivation on cell apoptosis. To do this, several apoptosis assays should be performed on the cells after growing in the variant glucose concentrations. Nonetheless, our findings support the idea that a low sugar diet is an effective way to slow the proliferation of cancer cells in the human body.

Introduction

While studies claiming that the consumption of sugar and carbohydrate-rich Western diet meals contribute to cancer cell proliferation have developed exponentially over the past fifty years, there is still much disagreement in the scientific community. In the 1920s, Otto Warburg published the Warburg effect, describing the way cancer cells behave anaerobically, even in aerobic conditions. Since glycolysis yields only 2 ATPs per glucose rather than the 34 ATPs per glucose produced in oxidative phosphorylation, cancer cells need to consume large amounts of glucose to compensate for lost ATP production. This theory was adopted and disputed by other scientists studying oncology, who speculated that starving cancer cells of sugar could be the key to future treatment. Researchers began toying with metabolic pathways in normal and malignant cells, discovering that the type of nutrient and concentration had varying effects on ATP production. Years later, studies suggested that starving malignant cells of glucose lowered their ATP production and induced apoptosis. Researchers Demetrakopoulos et al. (1978) found that when starved of glucose, normal cells were able to maintain previous levels of ATP production for 12 to 24 hours, whereas malignant cells faced a dramatic decrease within the first hour. The difference between the cell's metabolic ATP production inspires the idea that limiting glucose intake could be the key to halting cancer growth and inducing apoptosis in cancer cells. The popular theory that diet has an impact on cancer growth is not new. Nutritional therapy provided by oncology dieticians has been available since the 1990s, however, there are few studies that are cancer-specific examining the effect of varying carbohydrates on cancer cell growth. Most studies on the cancer-sugar link look at diet as prevention. This project aims to look at low-sugar diets as a solution to cancer that has already developed. We will compare the effects of different concentrations of carbohydrates on cancer cell proliferation. To accomplish this goal, we fed NF639 cells, mouse mammary carcinoma, varying amounts of glucose, and then compared the effect of carbohydrate concentration on cancer cell proliferation. To distinguish the amount of cancer cells present, we applied a WST1 assay.

Materials and Methods

Cell Line Maintenance

NF639 cells derived from mouse breast carcinoma were cultured in T25 flasks and fed Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) made with different concentrations of glucose and 10% Fetal Calf Serum. The three media types consisted of a high (G) , medium (G1/2), no glucose (G-) concentration. The flasks were kept in an incubator at 37º Celsius and fed three times a week, on Monday, Wednesday, and Friday. When fed, 3.5mL of media was removed from the flask and 3.5mL of fresh media was added. The flasks were kept on their sides at all times.

To support health and prevent culture deterioration, we recorded the confluence, health, and any contamination before each feeding. To do so, we viewed the cells under an inverted, Leica DMi1 400X, microscope. Once the cells reached about 80% confluence, we split into two new flasks. Using a cell scraper to detach the adherent cells, we triturated with a narrow bore pipette in the corresponding media ten times to remove cell clumps. Then, we used a p-200 pipette to seed the cell solution into new flasks at a 1:10 dilution to achieve 10% confluence.

Sterile Procedure

We maintained a sterile environment at all times in order to minimize contamination, which was particularly important as fungizone was not used in our experiment to protect against fungal growth. All procedures were conducted with gloves and apron under the hood in Whitaker lab after it was wiped down with 70% ethanol. We carefully used the ethanol to clean our instruments and hands after each step and cautiously sterilize the media bottles. We practiced and mastered correct use of the auto-pipette to avoid touching the neck of T25 flasks and all instrument wrappers were butterflied open to avoid contamination.

Preparing Growth Media

Three different media with varying glucose concentrations were prepared to compare the effect of the concentration on cell growth. We prepared 75mL of each media in a T25 flask at a time. To prepare the high glucose solution (G+), 45mL of DMEM with the 4050 mg/mL concentration of glucose, using a 50mL pipette, and 5mL of Fetal Calf Serum (FCS), using a 5mL pipette, were added to a pre-labeled flask. Similarly, to prepare the glucose-free solution (G-), 45mL of DMEM with no glucose, using a 50mL pipette, and 5mL of FCS, using a 5mL pipette, were also added to a pre-labeled flask. To prepare the medium-concentrated glucose media (G1/2), 22.5mL of each the high and no glucose DMEM were added to a T25 flask with a 50mL pipette and 5mL of FCS was added using a 5mL pipette. This created media with a glucose concentration of 2025 mg/mL. These flasks were then placed in the fridge.

Seeding the 96-Well Plate

All actions involving the assay components were performed wearing latex gloves for greater protection. We planned each seeding for when the cells reached about 85% confluence, seen in **Figure 1.** When seeding a 96-well plate two days prior to performing a WST1-assay, we labeled the plate properly and performed a split as normal, but before seeding took a hemocytometer count in order to make the

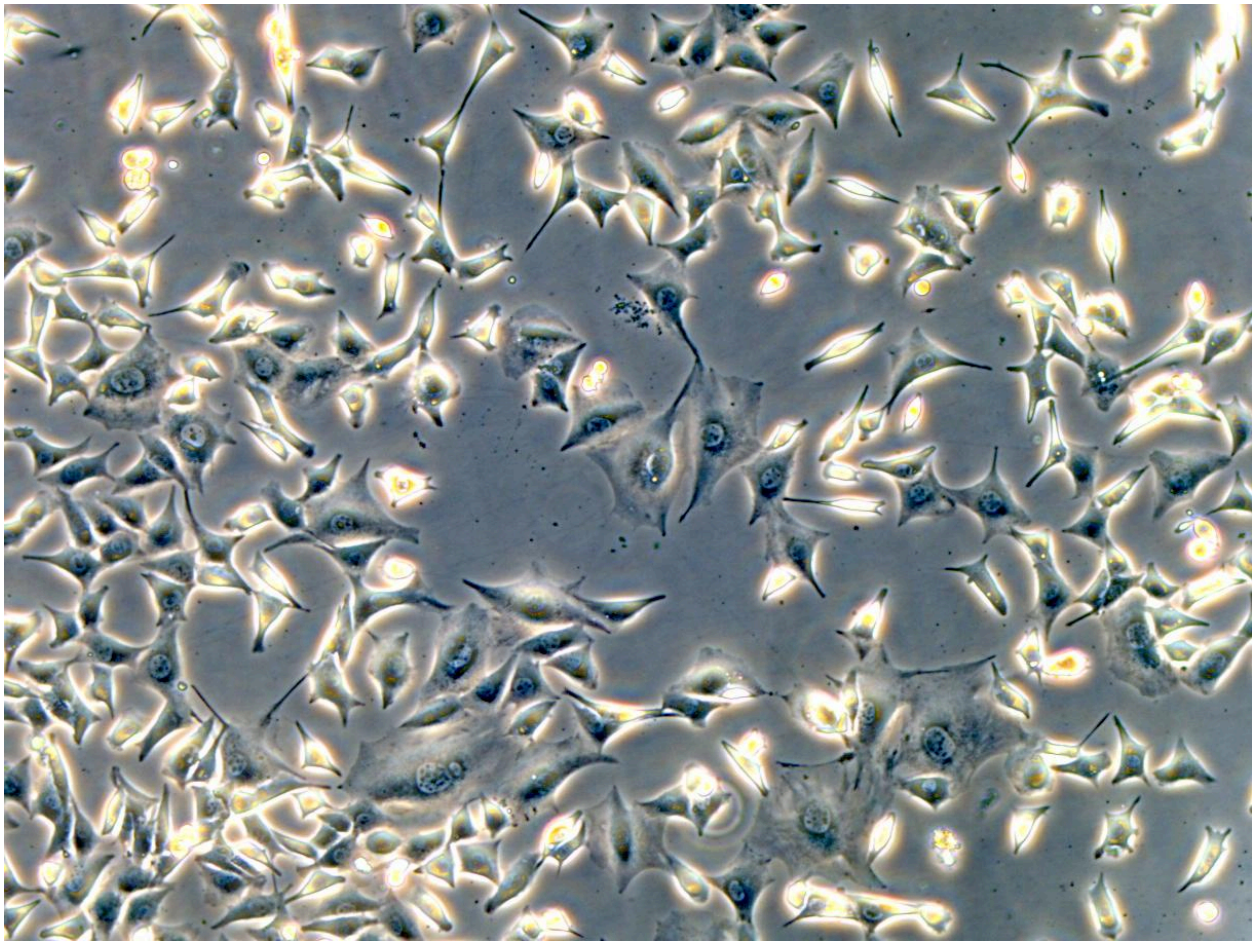
correct calculations. Using the average cell count, that number multiplied by 10^4 equaled the amount of cells in 1mL (this value is what we used for "counted cells" in the calculation below).

Sample calculation:

10^4 cells (desired amount) x 1mL/counted cells x 1000μL/1mL = XμL

This value in microliters was the volume that contained 10^4 cells. The calculated volume of triturated cell solution was added to three wells in a row using a p-20 pipette and the corresponding media was added with a p-200 pipette to reach 200 microliters. The plate was then placed back in the incubator for two days.

Figure 1: Microscopic image of our NF639 Cells



This photo is a microscopic image of NF639 cells at 85% confluence after growing for ten days in the high glucose media.

Performing WST1-Assay

To perform the assay, while wearing latex gloves, we removed the 96-well plate from the incubator and observed under the microscope to check for contamination. The plate was then placed under the hood where the WST1 mixture was created. 50 microliters of WST1 Reagent were added to 50 microliters of Electron Mediator Solution and combined in a sterile tube using a p-200 pipette. The shaft of the p-200 pipette was wiped down with ethanol before, between, and after uses. The WST1 mixture was then manually mixed for one minute. After mixing, 10 microliters of reagent were added to each of the 9 wells using a p-20 pipette. Once the reagent was added, the plate was put back in the incubator for another two hours. After that time had passed, the plate was removed from the incubator and brought up to the classroom with the plate reader (specific name). After the machine had calibrated for a

96-well plate, the experimental plate was placed into the tray and the computer program displayed the absorbance level for each well. This table of values was then saved and printed for later evaluation.

Analyzing WST1-Assay Results

The computer program provided a table with the light absorbance levels of each well. The absorbance value of all the empty wells was about 0.050. Using a graph obtained from the Cayman Chemicals WST1 sheet, which plotted cell number versus absorbance, the number of cells per well was determined.

Statistical Test

We conducted an ANOVA test to determine if the variance seen between the cell growth from the three different media was statistically significant. From one assay with two separate plate readings, we had six OD readings for each media type. By taking the average and using a one-way ANOVA test, we calculated the p-value and F score which allowed us to compare the variance between the three groups to the general variance within each group.

Results

Determining Concentration After First WST1

The WST1 assay, with cells having grown 15 days in the growth media, was run through the spectrometer twice and produced two tables with OD readings for each well. **Figures 2 and 3** show these tables with estimated cell count. The absorbance values were used to estimate cell count from the Cayman Chemical example data graph in **Figure 5**. **Figure 4** shows the OD readings for each glucose concentration and the calculated mean that was used later in the ANOVA statistical test.

Figure 2: WST1 Assay First Spectrophotometer Absorbance Readings

Glucose Concentration	Row 1		Row 2		Row 3	
	OD Reading	Est. Cell Count	OD Reading	Est. Cell Count	OD Reading	Est. Cell Count
4050 mg/mL	1.170	40,000	1.436	50,000	1.078	26,000
0 mg/mL	0.316	10,000	0.474	13,000	0.501	13,500
2025 mg/mL	0.819	25,000	0.587	14,000	0.672	22,000

This table shows the first reading of absorbance (OD reading) produced by a spectrophotometer set at 450 nm and optical density where the higher the absorbance indicates a denser viable cell count. The

higher the absorbance indicates a denser viable cell count. These data follow a trend that more glucose in the media produces a higher viable cell count.

Figure 3: WST1 Second Spectrophotometer Absorbance Readings

Glucose Concentration	Row 1		Row 2		Row 3	
	OD Reading	Est. Cell Count	OD Reading	Est. Cell Count	OD Reading	Est. Cell Count
4050 mg/mL	1.335	50,000	0.960	25,400	1.033	38,000
0 mg/mL	0.313	10,000	0.485	13,000	0.482	13,000
2025 mg/mL	0.972	25,500	0.613	15,000	0.692	22,000

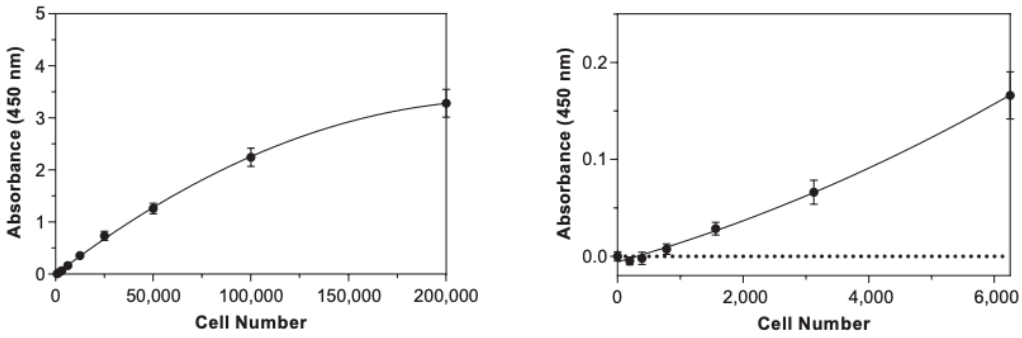
This table shows the second reading of absorbance produced by the spectrophotometer set at 450n m. The higher the absorbance indicates a denser viable cell count. These data follow a trend that more glucose in the media produces a higher viable cell count. These 18 OD readings were used in calculation of the three means.

Figure 4: WST1 Assay Absorbance (OD) Readings

G- OD	**G1/2 OD**	**G+ OD**
0.316	0.819	1.170
0.474	0.587	1.436
0.501	0.672	1.078
0.313	0.972	1.335
0.465	0.613	0.960
0.482	0.692	1.033
Mean: 0.425	Mean: 0.726	Mean: 1.169

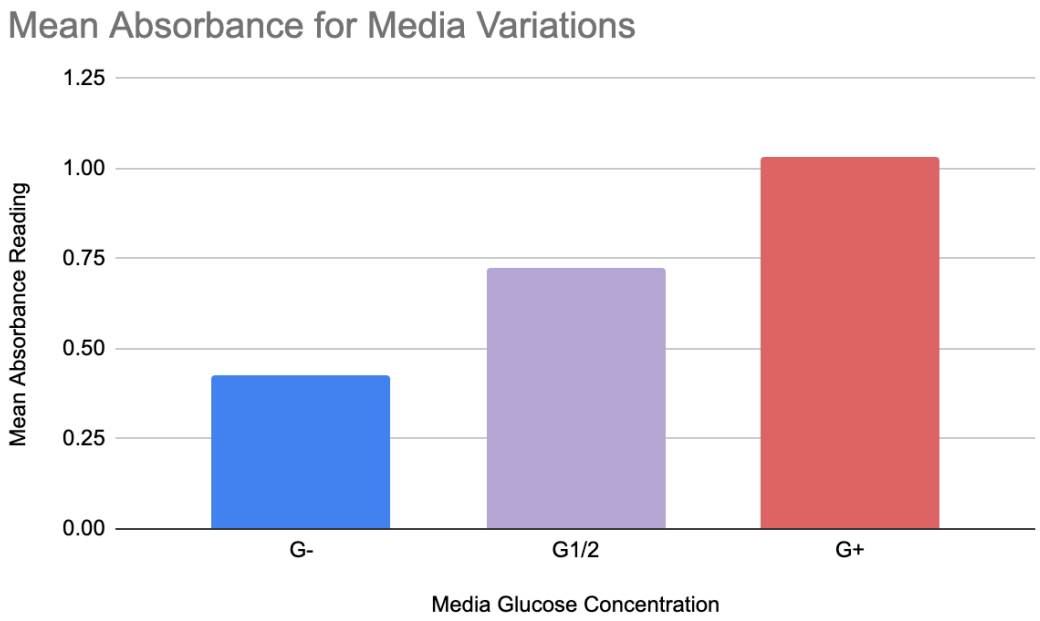
These values are the optical density (OD) readings at 450 nm from each well, which is proportional to the total number of cells in each well. A trend is seen that when the glucose concentration increases, the OD reading increases and therefore the number of cells does as well. G- is no glucose, G1/2 is half concentrated, and G+ is the full concentration of glucose (4050 mg/mL).

Figure 5: Absorbance Reading vs. Cell Number



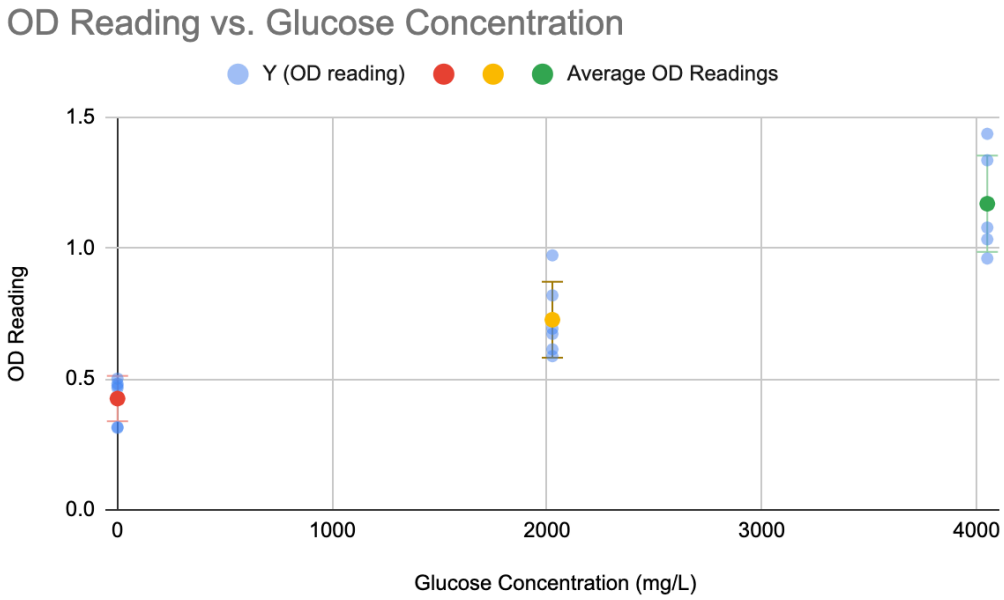
From Cayman Chemicals, these graphs show an estimate of the cell number based off of the WST1 Assay spectrophotometer OD reading created from a typical cell titration experiment using HL-60 cells.

Figure 6: Distribution of Mean Absorbance for Media Variations



This graph shows the linear growth in absorbance reading as the glucose concentration increases from 0 mg/L (G-), 2025 mg/L (G1/2), and 4050 mg/L (G+).

Figure 7: OD Reading vs. Media Glucose Concentration



This graph shows three means surrounded by their data points to demonstrate the low variability within groups and lack of overlap between groups.

Statistical Analysis

The first WST1 assay was used to conduct a one-way ANOVA test. Using the six absorbance values for the high, medium, and no glucose media groups, the three means were calculated (Figure 4**)**. The distribution of these means of G-, G1/2, and G+ respectively are 0.425, 0.726, 1.169, showing a linear increase (Figure 6**)**. By conducting the ANOVA test, we produced an F value of 40.34. The F value is a ratio of the distance between the three groups to the variance between the 18 individual measurements. We produced a very large F value because the spread between the three groups is much more significant than the deviances between each value. At the 0.05 significance level, we got a p-value of 9.215×10^{-7}, which provides statistical evidence that the difference seen in the means was not due to sampling variability. In other words, the variance between our three means was not due to chance. This finding allowed us to reject the null hypothesis that there is no difference in cell growth based on the concentration of glucose in the media. These data were plausibly normally distributed for the project we conducted. We then plotted the graph of OD Reading vs. Glucose Concentration (Figure 7). The purpose of this graph was to show the three mean OD readings surrounded by the individual readings to show the precision within each group and the lack of overlap between groups. Most points are within the error bars which show one standard deviation from the mean, meaning the values are fairly consistent. Contrastingly, the error bars for the three different media variations do not overlap, demonstrating significant distinction between the amount of cell growth for each media.

Discussion

With a p-value of 9.215×10^{-7} we can conclude that cancer cell proliferation slows with the decrease of glucose concentration in their environment. In addition to the WST1 assay that led us to our p-value, we also conducted two other WST1 assays which we chose to exclude. One featured a comparison of three wells grown in positive glucose concentrations and three wells grown in negative glucose concentrations. The other was a comparison of six wells of cells grown in a ½ glucose concentration. We chose to exclude both assays from our results for several reasons. Firstly, with the positive and negative concentrations, our seeding was mishandled. Since the seeding was mistakenly done at a cell number of $1/10^5$ instead of 10^5 like previously done assays, we cannot compare the cell numbers across assays. The WST1 done with a ½ glucose concentration cannot be compared across assays either as there is no way to ensure that the environment was the same. For the most accurate analysis, we chose to only include the two spectrophotometer runs of the second WST1 assay. If allotted more time, we would incorporate more assays into our experimental design to ensure our results were more statistically accurate. We would also compare the production of L-lactate and lactic acid across cancer cells grown in different glucose concentrations with a Lactate dehydrogenase (LDH) colorimetric assay. The use of this assay would enable us to look at both lactic acid production and cell number side by side and we could see if depriving cells of the ability to do aerobic glycolysis had an effect on their proliferation. We would expect to see a large amount of lactic acid production in the cells with access to more glucose as they would be able to do more aerobic glycolysis. Further, we would want to do an assay to measure cell apoptosis as previous studies have found that glucose deprivation has induced cell death in cancer cells (Ganesh Kumar Raut et al. 2019).With more time, it would also be necessary to perform this same experiment again for more trials and also with non-malignant cells as a control in order to see if the glucose concentration truly has an effect on cancer proliferation rather than just cell proliferation.

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