TED ANKARA COLLEGE FOUNDATION HIGH SCHOOL

International Baccalaureate

BIOLOGY EXTENDED ESSAY

"How does the different concentrations (0 µM, 100 µM, 300 µM, 500 µM, 1000 µM, 2000 µM) of *ascorbic acid (Vitamin C)* affect the number of the viable A-549 NSLC (non-small lung cancer) cells, that are attached to the cell culture plate; in 24, 48 and 72 hours of treatment durations?"

Supervisor: Ümit YAŞATÜRK MIDILLI

Candidate Name: Elif BORA

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ABSTRACT

There is a statement that Vitamin C has an anticancer activity. The aim of this extended essay was to search how this effect correlates with the concentrations of ascorbic acid, and how the number of A-549 cells change with the increasing concentrations of ascorbic acid. The research question was, "How does the different concentrations (0 μM, 100 μM, 300 μM, 500 μM, 1000 μM, 2000 μM) of *ascorbic acid (Vitamin C)* affect the number of the viable A-549 NSLC (non-small lung cancer) cells, that are attached to the cell culture plate; in 24, 48 and 72 hours of treatment durations?"

The hypothesis states that increased concentrations of ascorbic acid will decrease the number of viable A-549 cells. Therefore, the changing concentrations of ascorbic acid was the independent variable and the number of viable A-549 cells was the dependent variable. This research question is investigated in three treatment periods which were 24, 48 and 72 hours.

When searching for this effect, trypan blue exclusion assay method was used to count the cells. 0, 100, 300, 500, 1000 and 2000 μM of ascorbic acid were put on the A-549 cells and the effect of it on the cell number are observed for the duration of treatments, 24, 48 and 72 hours. This effect is observed in five trials.

The results show that there was a significant difference between the mean numbers of A-549 cells as the concentration of the ascorbic acid is increased in the experiment done for 24 hour treatment duration. However there were not significant differences between the mean numbers of A-549 cells as the ascorbic acid concentration is increased in the experiments done for 48 and 72 hours of treatment durations.

Word Count: 282

TABLE OF CONTENTS

INTRODUCTION

My father is an oncologist. When I visit his office, I see many cancer patients. He tells them a lot of things to do or not to do during the treatment period. This experience, derived me to make some literature search about the prevalence of this disease in the world and I found out that cancer is a disease which has an increasing incidence in the population. According to WHO, cancer is the second cause of deaths after cardiac diseases and it is expected that annual cancer cases will rise from 14 million in 2012 to 22 million within the next two decades.[1] So more people have increasing risk of deaths due to cancer. This facts made me wonder if there was a certain solution for this dangerous disease; and with today's technology, to what extent cancer can be controlled. So I decided to do my extended essay on this topic.

I.CANCER

Formation of a Cancer Cell:

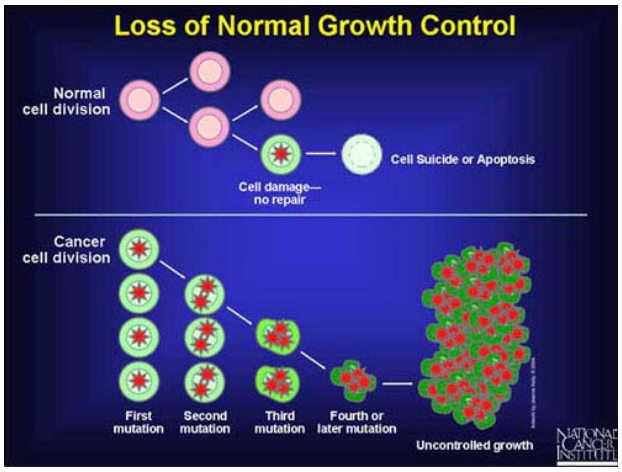

Figure 1: Figure showing the growth control difference between a normal cell and a cancer cell.[2]

In normal conditions, the cells of body grow and divide to produce more cells as they are needed to keep the body healthy. Aged or damaged cells die and are replaced with new cells.

Series of mutations occur as a cell turns into a cancer cell, and this mutations disable the functions that regulate the growth of a tissue. When the DNA is damaged, the regular process of apoptosis (programmed cell death) is also broken. So the cells start to divide uncontrollably and a tumor is formed.

Firstly, mutation inactivates the tumor suppressor gene. So cells start to divide rapidly and increase in number. Then, mutation inactivates DNA repair gene. Then the genes that promote cell growth and reproduction get activated and a tumor starts to be formed.[2]

[1] M.P., Coleman. "Trends in Socioeconomic Inequalities in Cancer Survival in England and Wales." *British Journal Cancer* 1 Jan. 2004: 1367-73. Print.
[2] "What Is Cancer?" *National Cancer Institute*. Web. 12 Jan. 2015. <http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer>.

Not all of the tumor types are harmful for body. Cancer is a malignant tumor which is able to spread to the other parts of the body and damage vital parts of it.

A-549 Non Small Lung Cancer (NSLC):

Malignancy degree are different between the cancer types. In Western world, the most common cancers are lung, breast, skin, gut and prostate gland.[3]

There are three main types of lung cancer according their cells' size and shapes which are known as small cell lung cancer (SCLC), non-small cell lung cancer (NSLC) and carcinoid tumor. About 85% of the lung cancers are NSLC. So, this is the most common and invasive lung cancer type. [3]

II.VITAMIN C (ASCORBIC ACID/AA):

Vitamin C (AA), is an essential nutrient for human body. It is a type of vitamin which is not stored inside the body. Besides of its nutrient property, it is an antioxidant. Thus it can reduce toxic, reactive oxygen species.

Vitamin C and Cancer:

Vitamin C has a primary preventive effect, which means that it prevents normal cells, against becoming a cancer cell. Antioxidants protect the cells against oxidative stress, which is the imbalance between ROS (reactive oxygen species) and antioxidant species. ROS have a mutagenic effect. They may also suppress apoptosis, therefore support proliferation and apoptosis. For this purpose, supplementation of nutrients with antioxidants such as AA are suggested for primary prevention.

Furthermore, there is a secondary, anticancer effect of AA, which means, that it acts against the proliferation and spreading of already existing cancer cells. Several mechanisms of anticancer activity of AA are written below (first three of them are mostly related with cell proliferation in an area; last three, are mostly related with spreading of cancer cells to the other sides of the body (metastasis));

1) It inhibits prostaglandins, which facilitates cell proliferation.[4]

2) During oxidation, AA reacts with a peroxyl radical to yield hydrogen peroxide Hydrogen peroxide is harmful for all cell types. However, normal cells can metabolize it while cancer cells cannot. So AA has a damaging effect on cancer cells[5,6]

3) As the ROS activity may trigger the proliferation of already existing cancer cells, AA is effective against them.

4) It has an effect against the growing of tumor, by helping the collagen formation.

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[3] "Lung Cancer." *Lung Cancer*. Web. 12 Jan. 2015. <http://www.cancer.org/cancer/lungcancer/>.
[4] JR, Beetens. "Ascorbic Acid and Prostoglandin Formation." *International Journal of Vitamin NUtrition Research* 1 Jan. 1983: 131-44. Print.
[5] Y., Mikino. "Induction of Cell Death by Ascorbic Acid Derivatives in Human Renal Carcinoma and Glyostoma Cell Lines." *Anticancer Research* 1 Jan. 1999: 3125-32. Print.
[6] PY, Leung. "Cytoxic Effect of Ascorbate and Its Derivative on Cultured Malignant and Non Malignant Cell Lines." *Anticancer Research* 1 Jan. 1993: 47-80. Print.

5) It strengthens the normal cells around the tumor tissue. Therefore it creates a wall against it.

6) It keeps the ground substance around the tumor to prevent metastasis. [7]

Some studies have suggested the protective roles of AA against cancers at several sites. In one of them, Kassouf et al. tested the effect of AA between 500-2000 μM concentrations on Human Urothelial Tumor. They found that the greatest tumor growth inhibition was observed in the cells treated with 2000 μM AA. [8] In another study, Roomi et al tested the effect of a nutrient mixture that contains AA between 10-1000 μg/ml concentrations, on the proliferation of Bladder Cancer cells. They saw that the total growth inhibition were at cells that are treated with 1000 μg/ml mixture. [9]

After all of the literature search I've done, I have derived a research question as, **"How does the different concentrations (0 μM, 100 μM, 300 μM, 500 μM, 1000 μM, 2000 μM) of ascorbic acid (Vitamin C) affect the number of the viable A-549 NSLC (non-small lung cancer) cells, that are attached to the cell culture plate; in 24, 48 and 72 hours of treatment durations?"**

[7] Head, Kathleen A. "Ascorbic Acid in the Prevention and Treatment of Cancer." *Alternative Medicine Review* 1 Jan. 1998: 174-86. Print.
[8] Kassouf, Wassim. "Vitamin C and K3 Sentisize Human Urothelial Tumors to Gemicitabine." *Journal of Urology* 1 Oct. 2006: 1642-647. Print.
[9] Roomi, M. Waheed. "Antitumor Effect of Ascorbic Acid, Lysine Proline, arginine, and Green Tea Extract on Bladder Cancer Cell Line T-24." *International Journal of Urology* 1 Jan. 2006: 415-19. Print.

HYPOTHESIS

Ascorbic acid's antioxidant property has a preventive effect on the normal cells against becoming a cancer cell. This is the primary preventive activity of AA. In addition to this, AA has a secondary anticancer activity. Regarding at the cell proliferation process, AA does this anticancer activity in two ways. Firstly, high concentrations of AA, inhibit prostaglandins, which causes cell proliferation. Secondly, Vitamin C can generate hydrogen peroxide, which is a toxic substance for all kinds of cells. Normal cells can metabolize this substance, so they protect themselves. However cancer cells does not have this ability. Therefore, hydrogen peroxide may limit their proliferation.

When forming my hypothesis, I considered the anticancer activity of AA, as long as I am working with existing cancer cells and I formed a hypothesis as, "**Vitamin C prevents proliferation and spreading of A-549 cells. This effect correlates with concentrations of vitamin C. So as the concentration of ascorbic acid is increased, the number of viable A-549 cells will decrease.**" Incubating Vitamin C on A-549 cell line and then calculating the number of the viable cancer cells will provide estimating antitumor effect of Vitamin C and whether the effect is dose dependent or not.

METHOD DEVELOPMENT AND PLANNING

After I decided the topic, I consulted biologist Dr. Zeynep Tokçaer who works in the Laboratory of Ankara University, Department of Biophysics. She told me that I can work in the laboratory which she works in and she got a permission from the director of the laboratory for me to study in.

The Cell Line and the Antioxidant:

I planned to work on A-549 cells because it is one of the most common and invasive cell types. So its death risk is more than many other cancer types. Searching a treatment way for such a cell type was more significant for me.

Vitamin C, is not stored in the body. Therefore, high concentrations of it, can be tolerated easily. Also it has not got any toxic effect on healthy cells. For the other antioxidants (e.g. vitamin E), this condition is not always valid.

Cell Counting Method:

There were two possible standard methods that I could use to count the cells. One of them was MTT assay.[10] The other method was the trypan blue exclusion assay.[11] I decided to use the trypan blue exclusion assay because it was easier for me to do and understand. At the same time, this method shows both viable and unviable cells so it gives more results that I will be able to comment on.

The Concentrations of Ascorbic Acid:

In the other researches, the AA concentration was varying between 50-2000 µM [8, 26] I decided to separate the results into groups as 100 µM, 300 µM, 500 µM, 1000 µM and 2000 µM. The biologist suggested me to add a control group (0 µM of AA) in which we can observe the replication of untreated cells, so that it will be easy to compare.

The Number of Plated Cells:

Before starting the actual experiment, I made a trial experiment, in which I can see some limitations and develop a method for accurate results. So for this trial experiment, I plated 50000 cells per well (into medium of 2 ml).[12] Yet, as the cell number was too small compared to the volume of medium, it was hard to count them. There were big differences between the numbers of cells (between the samples taken from the same tube). In addition, when I observed them under the microscope, the cells were gathering in one area so I could not count them in such a condition. As a result I decided to increase the number to 100000 cell per well.

The Duration of Treatment:

At the beginning I was just going to treat the cells with different concentrations of AA for 24 hours. The biologist, offered that I can observe the effect of AA in other durations of treatment. This was in case of AA might not reduce the number of A-549 cells in 24 hours due to the short duration of treatment. So I decided to set the same experimental design for 48 and 72 hours of treatment durations.

[10] See Appendix 5: Explanation of Terms (MTT Assya)
[11] See Appendix 5: Explanation of Terms (Trypan Blue Exclusion Assay)
[12] See Appendix 3: Formulas That are Used in the Experiment Explained (Calculating the Cell Number per Well)

Using Hemocytometer:

In the literature, I found many methods for counting the cells inside the counting grid of the hemocytometer and one of them was the most suitable for me.[13]

In the trial experiment, I cleaned the hemocytometer with ethanol in case of any cells may stick to the surface of the hemocytometer and would not be wiped away. However, ethanol couldn't be dried even if we used paper towel, and caused the cells to slide between the hemocytometer and the cover slip and prevented taking measurements. So I decided not to clean the hemocytometer with ethanol between each measurement.

In the trial experiment, I loaded the cell-AA-trypan blue solution directly on the hemocytometer and putting the cover slip on this solution. However in such cases the solution may overflow from the counting grid, or some air bubbles may occur, and prevented taking measurements. As a result, I decided to put the cover slip on the hemocytometer first, then loaded the trypan blue-cell solution between them.

Deciding to Count Only Viable and Attached Cells:

At first, I was going to count both of the samples taken from unattached and attached cells[14], so the increase in the number of cells would be observed clearly. However when making the trial experiment, I could not collect any data from the unattached cells. The results were always zero, I did not found any viable or dead cells anywhere. After such a problem occurred, I decided not to count the unattached cells in the real experiment.

The biggest aim of using trypan blue method was to be able to count both the viable and dead cells but in my counting, I generally did not observe any dead cells. When I did, the number was less than 0.5 so I accepted it as zero[15]. I did not add the number of dead cells to my results. Viable cells were enough to make a comment about the increase in the number of cells so the absence of the data of dead cells did not affect my experiment a lot.

[13] See Appendix 6: Explanation of Several Methods Used in the Experiment (Method for Counting with Hemocytometer)
[14] See Appendix 5: Explanation of Terms (Attached-Unattached Cells)
[15] Appendix 6: Explanation of Several Methods Used in the Experiment (Method for Counting with Hemocytometer, Scientific Decimal Rounding Method)

MATERIALS

MATERIAL:[16]	FEATURE:	AMOUNT:
A-549 lung cancer cells		(100000 cell/well 600000 cell/ cell culture plate) 30000000cell/experiment
DMEM	High glucose 4.5g/l	445 ml
trypsin	10x	50ml
penstrep	100x	5ml
trypan blue		100 µl
FBS		
PBS	1x	10 ml
ascorbic acid	176.13g/mole , 0.1M	
ethanol 70%		50 ml
humid cell culture incubator	5% CO_2 , 37 C	n=1
laminar flow		n=1
inverted microscope	10x magnification	n=1
microbalance		n=1
water bath		n=1
filtered flask	75 cm2	n=5
cell culture test plates	six wells (each well can contain 2 ml liquid)	n=5
hemocytometer	0.0025 mm2	n=1
micropipette	1-10 µl	n=1
micropipette	2-20 µl	n=1
micropipette	20-200 µl	n=1
micropipette	100-1000 µl	n=1
tube	2 ml	n=30
filter	2µl	n=1
vortex mixer		n=1
cover slip		n=60
acetate pen		n=1
distilled water		
vacuum pump		n=1
chronometer		n=1

TABLE 1: Shows the materials needed for one experiment done for that duration of treatment. This materials are for five trials. n: number of that material need for five trials of one duration of treatment.

[16] See Appendix 7: Images and the Trades of the Materials Used in the Experiment

METHOD

A. PREPARATION OF MEDIUM AND ADDITION OF A-549 CELLS:

1. Standard cell medium preparation method is used.[17]

B. PREPERATION OF ASCORBIC ACID AND ADDING IT ON THE A-549 CELLS:

2. Measure the 0.1M AA on microbalance, to be 0.17613 g.[18]
3. Solve it in 0.01 L water.
4. Sterile it by passing it through 0.2 µl filter.
5. Put this solution into 6 well plates; each well takes 2 ml of solution so use the calculation M1xV1=M2xV2 [19] to determine the volumes that should be taken for each concentration of AA.
6. Take a cell culture test plate.
7. Label the wells as "control" , "100 µM", "300 µM", "500 µM, "1000 µM", "2000 µM"[20]
8. Take 2 µl of AA solution (using the 1-10 µl pipette) for 100 µM AA and put it into the "100 µM" labelled well in the test plate.
   Take 6 µl of AA solution (using the 1-10 µl pipette) for 300 µM AA and put it into the "300 µM" labelled well in the test plate.
   Take 10 µl of AA solution (using the 1-10 µl pipette) for 500µM AA and put it into the "500 µM" labelled well in the test plate.
   Take 20 µl of AA solution (using the 2-20 µl pipette) for 1000µM AA and put it into the "1000 µM" labelled well in the test plate.
   Take 40 µl of AA solution (using the 20-200 µl pipette) for 2000 µM AA and put it into the "2000 µM" labelled well in the test plate.
9. Add 40 µl of sterile water to control labelled well as AA is dissolved in water.
10. Repeat the processes from 5 to 9 for four more test plates.

C. COLLECTING THE CELLS AND COUNTING THEM WITH HEMOCYTOMETER:

11. Incubate the plates inside the humid cell culture incubator for 24 hours
12. Take six micro centrifuge tubes.
13. Label the tubes as "control", "100 µM", "300 µM", "500 µM, "1000 µM", "2000 µM" by using an acetate pen.[20]
14. Take one of the plates.
15. Aspirate the 2 ml medium from the "control" labelled well.
16. Wash once with 1x PBS and add 500 µl of 1x trypsin. Incubate at 37°C for 5 minutes to detach the cells.
17. Add 500 µl of medium and collect the cells.
18. Transfer them to the corresponding tube.

[17] See Appendix 6: Explanation of Several Methods Used in the Experiment (Preparation Of Medium And Addition Of A-549 Cells)
[18] See Appendix 3: Formulas That are Used in the Experiment Explained (Calculating the Mass of Ascorbic Acid)
[19] See Appendix 3: Formulas That are Used in the Experiment Explained (Calculating the Volume of Ascorbic Acid-Water solution that will be ut on the cels)
[20,,22,23] See Appendix 8:Diagrams and Images:

19. Repeat step 5 for “100 µM”, “300 µM”, “500 µM, “1000 µM”, and “2000 µM” wells.[20]
20. Take the “control” tube.
21. Take 100 µl of trypan blue (by using 100-1000µl pipette) and pour it inside the tube.
22. Mix the solution by using the vortex mixer.
23. Take hemocytometer and put the cover slip on.
24. Take 10 µl of mixture from the 2 ml tube (by using 1-10µl pipette) and load it between hemocytometer and cover slip.
25. Put the hemocytometer under microscope.
26. Count the cells under 10x magnification, inverted microscope, using the method for counting with hemocytometer. [21]
27. Note the results
28. Repeat the processes from 7 to 14 for “100 µM”, “300 µM”, “500 µM, “1000 µM”, and “2000 µM” tubes.
29. Repeat the processes from 2 to 15 for the other 4 test plates.

Repeat the parts A, B and C for the experiments held for 48 and 72 hours.

NOTE: Always; Label the wells with concentration values. Wear gloves for the safety of work (any living organism may be transferred inside the cell culture test plates.) and the safety of yours. Spray everything that you get inside the laminar flow with ethanol 70%. Put the cap of tubes and filtered flasks, upside down. Wipe the surface of laminar flow with ethanol 70% before you work there.

[21] See Appendix 6: Explanation of Several Methods Used in the Experiment(Method for Counting with Hemocytometer)

DATA COLLECTION AND PROCESSING

	COUNTED A-549 CELLS IN THE SAMPLES TAKEN FROM EACH PLATE THAT ARE TREATED WITH DIFFERENT CONCENTRATIONS OF ASCOBIC ACID FOR 24, 48 AND 72 HOURS														
TIME (HOUR)	24					48					72				
Concentration of Ascorbic Acid (µM)	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
control (0)	7	2	3	2	4	14	17	16	16	23	7	20	9	20	14
100	2	3	4	1	2	16	21	19	11	17	16	21	17	22	23
300	3	2	2	3	2	21	16	16	19	18	16	12	22	26	25
500	2	2	4	2	3	24	12	16	22	15	27	11	17	28	27
1000	1	0	0	2	3	18	15	15	18	20	27	20	12	21	20
2000	0	0	0	0	0	4	13	11	16	13	16	14	19	21	20

TABLE 2: Shows the number of attached (to the cell culture plate), viable, A-549 cells (inside 10µM sample ejected from the 2ml of cell-AA-trypan blue mixture) counted using the hemocytometer. These are treated with ascorbic acid of concentrations 100, 300, 500, 1000,2000 µM for 24, 48 and 72 hours.

$CELL\ NUMBER = cell\ count \times 10^4 \times DF$

DF: Dilution factor: 1.1 [22]

Example calculation for control well, plate 1, 24 hours;

$7 \times 10^4 \times 1.1 = 77000$

[22] See Appendix 3: Formulas That are Used in the Experiment Explained (Calculating Dilution Factor)

	CALCULATED NUMBER OF A-549 CELLSIN EACH WELL, THAT ARE TREATED WITH DIFFERENT CONCENTRATIONS OF ASCOBIC ACID FOR 24, 48 AND 72 HOURS														
TIME (HOURS)	24					48					72				
Concentration of ascorbic acid (µM)	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	plate1	Plate 2	Plate 3	Plate 4	Plate 5
0	77000	22000	33000	22000	44000	154000	187000	176000	176000	253000	77000	220000	99000	220000	154000
100	22000	33000	44000	11000	22000	176000	231000	209000	121000	187000	176000	231000	187000	242000	253000
300	33000	22000	22000	33000	22000	231000	176000	176000	209000	198000	176000	132000	242000	286000	275000
500	22000	22000	44000	22000	33000	264000	132000	176000	242000	165000	297000	121000	187000	308000	297000
1000	11000	0	0	22000	33000	198000	165000	165000	198000	220000	297000	220000	132000	231000	220000
2000	0	0	0	0	0	44000	143000	121000	176000	143000	176000	154000	209000	231000	220000

TABLE 3: Shows the calculated number (using the standard cell number calculation equation; dilution factor is taken to be 1.1) of viable A-549 cells (that are attached to the cell culture plate) inside the 2ml cell-AA-trypan blue mixture. These are treated with ascorbic acid (of concentrations 100, 300, 500, 1000, 2000 µM) for 24, 48 and 72 hours

Data Analysis

Statistical calculations are done in Microsoft Excel 2013 [23]

STATISTICS FOR THE NUMBER OF A-549 CELLS TREATED FOR 24 HOURS;

Ascorbic Acid Concentration (µM)	**0**	**100**	**300**	**500**	**1000**	**2000**
MEAN	39600	26400	26400	28600	13200	0
MEDIAN	33000	22000	22000	22000	11000	0
SD	22810.08549	12541.9297	6024.94813	9838.6991	14342.2453	0
SE	10200.98035	5608.92146	2694.43872	4400	6414.04708	0
$T_{inverse}$	2.131846786	2.13184679	2.13184679	2.1318468	2.1318468	2.13185
95%CI	21746.92717	11957.3612	5744.13052	9380.1259	13673.766	0

TABLE 4: Shows the mean, median, mode, variance, standard deviation, standard error, $T_{inverse}$ and 95% confidence interval for the number of viable, attached A-549 cells inside the 2ml of cell-AA-trypan blue mixture, which are treated with ascorbic acid (of concentrations 0, 100, 300, 500, 1000,2000 µM) for 24 hours.

STATISTICS FOR THE NUMBER OF A-549 CELLS TREATED FOR 48 HOURS;

Ascorbic Acid Concentration (µM)	**0**	**100**	**300**	**500**	**1000**	**2000**
MEAN	189200	184800	198000	195800	189200	125400
MEDIAN	176000	187000	198000	176000	198000	143000
SD	37625.789	41451.1761	23334.52378	55219.5618	23847.432	49561.0734
SE	16826.7644	18537.5295	10435.51628	24694.9388	10664.896	22164.3858
$T_{inverse}$	2.13184679	2.1318468	2.131846786	2.1318468	2.131847	2.1318468
95%CI	35872.0836	39519.173	22246.92184	52645.826	22735.92	47251.075

TABLE 5: Shows the mean, median, mode, variance, standard deviation, standard error, $T_{inverse}$ and 95% confidence interval for the number of viable, attached A-549 cells inside the 2ml of cell-AA-trypan blue mixture, which are treated with ascorbic acid (of concentrations 0, 100, 300, 500, 1000, 2000 µM) for 48 hours.

STATISTICS FOR THE NUMBER OF A-549 CELLS TREATED FOR 72 HOURS;

Ascorbic Acid Concentration (µM)	**0**	**100**	**300**	**500**	**1000**	**2000**
MEAN	154000	217800	222200	242000	220000	198000
MEDIAN	154000	231000	242000	297000	220000	209000
SD	66456.7529	34259.3053	66183.0794	83773.50416	58723.9304	32070.2354
SE	29720.3634	15321.2271	29597.9729	37464.65001	26262.14	14342.2453
$T_{inverse}$	2.1318468	2.1318468	2.1318468	2.131846786	2.13184679	2.13184679
95%CI	63359.261	32662.509	63098.343	79868.89371	55986.8589	30575.4695

TABLE 6: Shows the mean, median, mode, variance, standard deviation, standard error, $T_{inverse}$ and 95% confidence interval for the number of viable, attached A-549 cells inside the 2ml of cell-AA-trypan blue

[23] See Appendix 4: Formulas That are Used in the Statistical Analysis

mixture, which are treated with ascorbic acid (of concentrations 0, 100, 300, 500,1000,2000 µM) for 72 hours.

ANOVA Calculations:

Anova calculations are done in Microsoft Excel 2013

FOR THE NUMBER OF A-549 CELLS TREATED FOR 24 HOURS;

H_0: There is not a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 24 hours.

H_1: There is a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 24 hours.

Anova: Single Factor

SUMMARY

Ascorbic Acid Concentration (µM)	*Count*	*Sum*	*Mean*	*Variance*
0	5	198000	39600	520300000
100	5	132000	26400	157300000
300	5	132000	26400	36300000
500	5	143000	28600	96800000
1000	5	66000	13200	205700000
2000	5	0	0	0

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4763366667	5	952673333.3	5.62380952	0.0014389	2.62065415
Within Groups	4065600000	24	169400000			
Total	8828966667	29				

TABLE 7: Shows the data from ANOVA(Analysis of Variance): Single Factor test that is made for the cell number of viable, attached A-549 cells, inside the 2ml cell-AA-trypan blue mixture, that are treated with different concentration(0, 100, 300, 500, 1000, 2000 µM respectively) of ascorbic acid for 24 hours. Alfa value is taken to be 0.05. P-value is smaller than Alfa value, so H_0 is rejected and H_1 is accepted.

FOR THE NUMBER OF A-549 CELLS TREATED FOR 48 HOURS;

H_0: There is not a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 48 hours.

H_1: There is a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 48 hours.

Anova: Single Factor

SUMMARY

Ascorbic Acid Concentration (µM)	*Count*	*Sum*	*Mean*	*Variance*
0	5	946000	189200	1415700000
100	5	924000	184800	1718200000
300	5	990000	198000	544500000
500	5	979000	195800	3049200000
1000	5	946000	189200	568700000
2000	5	627000	125400	2456300000

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18730800000	5	3746160000	2.30471464	0.07617568	2.620654
Within Groups	39010400000	24	1625433333			
Total	57741200000	29				

TABLE 8: Shows the data from ANOVA (Analysis of Variance): Single Factor test that is made for the cell number of viable, attached A-549 cells, inside the 2ml cell-AA-trypan blue mixture, that are treated with different concentration(0, 100, 300, 500, 1000, 2000 µM respectively) of ascorbic acid for 48 hours. Alfa value is taken to be 0.05. P-value is larger than Alfa value, so H_1 cannot be accepted.

FOR THE NUMBER OF A-549 CELLS TREATED FOR 72 HOURS;

H_0: There is not a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 72 hours.

H_1: There is a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with different concentrations of ascorbic acid for 72 hours.

Anova: Single Factor

SUMMARY

Ascorbic Acid Concentration (µM)	*Count*	*Sum*	*Mean*	*Variance*
0	5	770000	154000	4416500000
100	5	1089000	217800	1173700000
300	5	1111000	222200	4380200000
500	5	1210000	242000	7018000000
1000	5	1100000	220000	3448500000
2000	5	990000	198000	1028500000

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.3038E+10	5	4607680000	1.28793687	0.3017722	2.62065415
Within Groups	8.5862E+10	24	3577566667			
Total	1.089E+11	29				

TABLE 9: Shows the data from ANOVA (Analysis of Variance): Single Factor test that is made for the cell number of viable, attached A-549 cells, inside the 2ml cell-AA-trypan blue mixture, that are treated with different concentration(0,100, 300, 500, 1000, 2000 µM respectively) of ascorbic acid for 72 hours. Alfa value is taken to be 0.05. P-value is larger than Alfa value, so H_1 cannot be accepted.

Calculating the mean of the five plates of each concentration;[24]

$$mean\ value\ of\ cell\ number\ for\ each\ concentration = \frac{p1 + p2 + p3 + p4 + p5}{5}$$

$pN = plate\ number\ indicated\ in\ table\ 3$

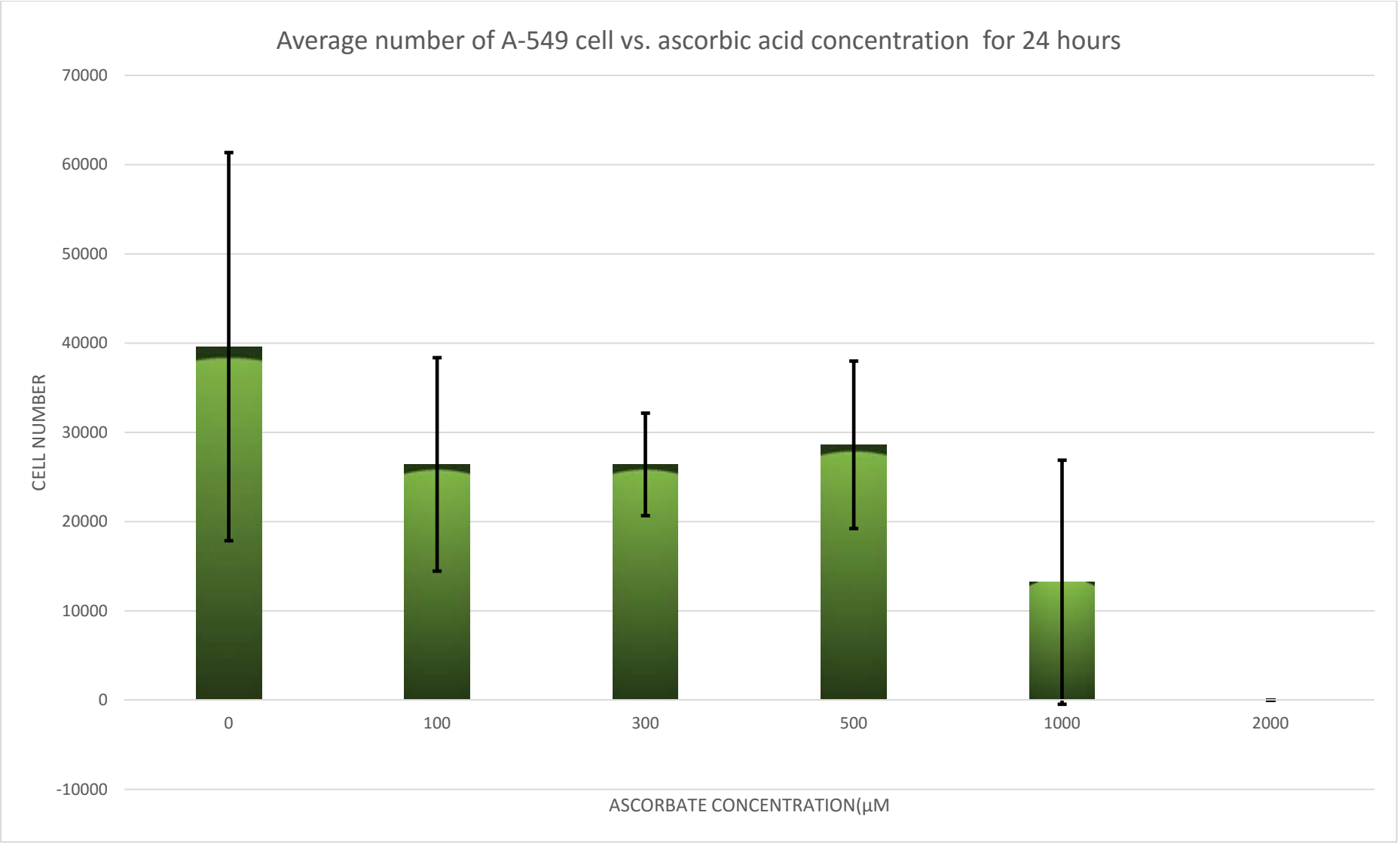
Example calculation for the cells treated with 0 µM ascorbic acid for 24 hours;

$\frac{77000+22000+33000+22000+44000}{5}$ = 39600

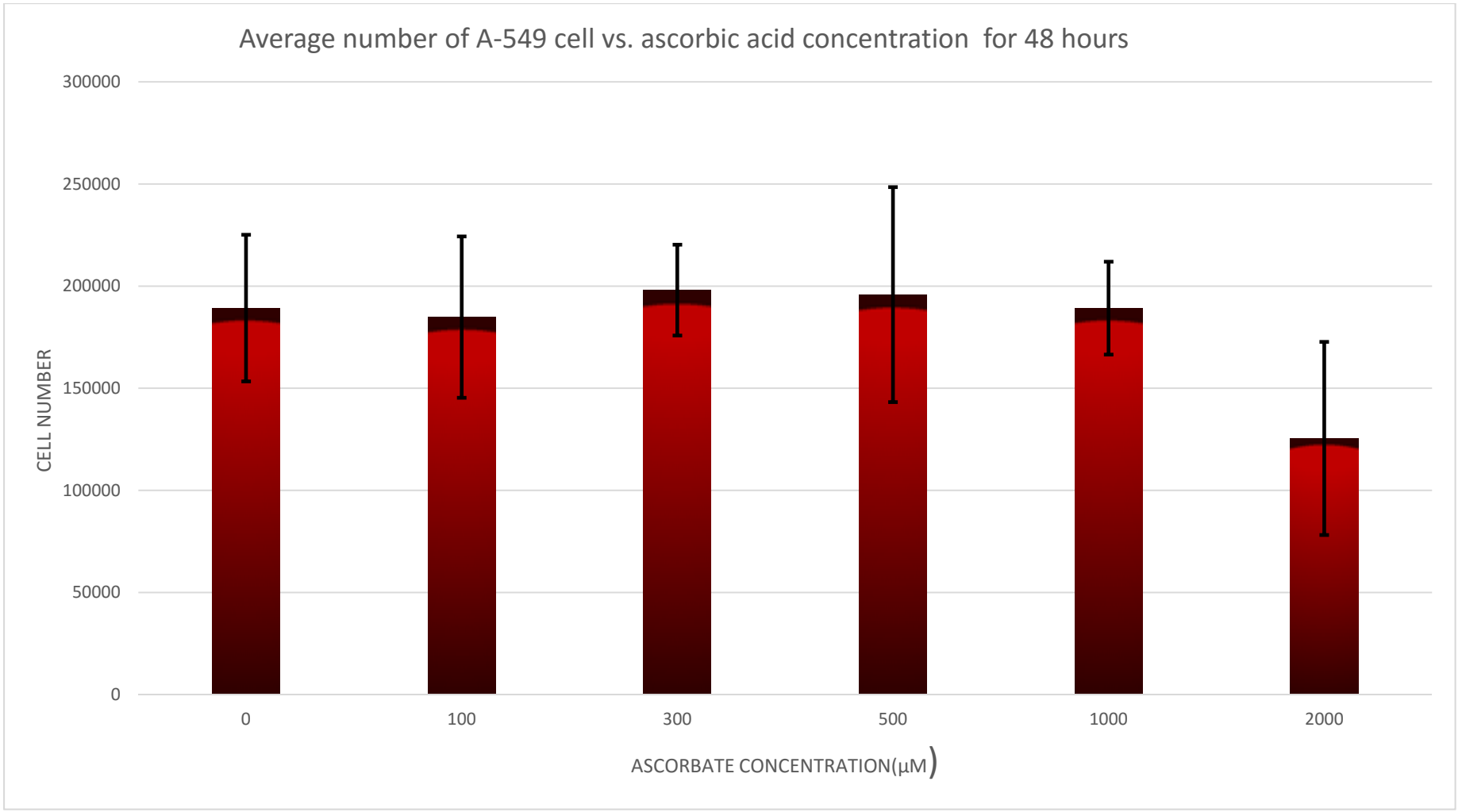
	ASCORBIC ACID CONCENTRATION (µM)					
TIME (Hours)	0	100	300	500	1000	2000
24	39600	26400	26400	28600	13200	0
48	189200	184800	198000	195800	189200	125400
72	154000	217800	222200	242000	220000	198000

TABLE 10: Shows the calculated mean number of (viable, attached) A-549 cells using the data from the five trails (five plates) in table 3. These are treated with ascorbic acid (of concentrations 0, 100, 300, 500, 1000, 2000 µM) for 24, 48 and 72 hours.

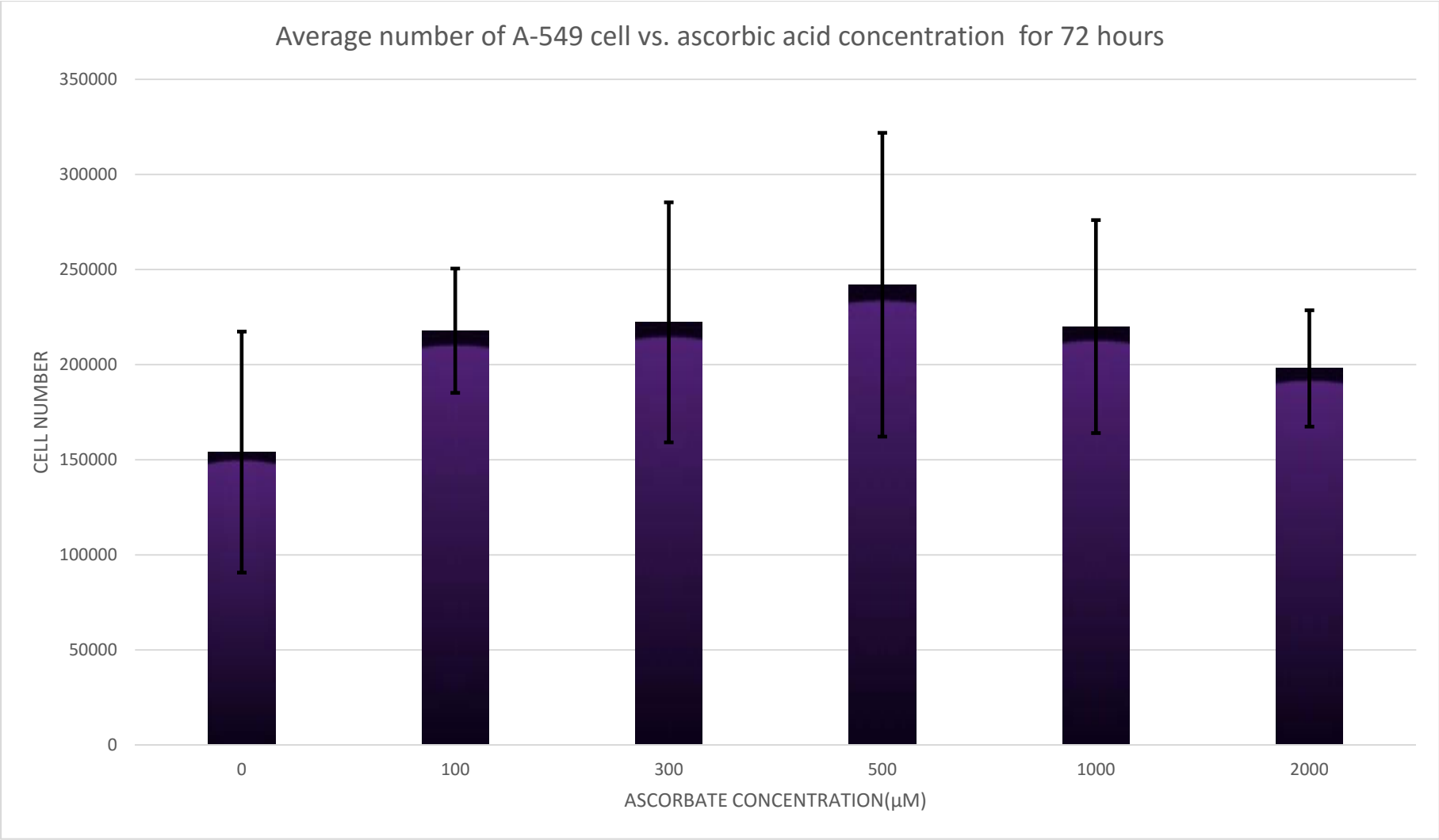
[24] See Appendix 4: Formulas That are Used in the Statistical Analysis(Mean)



GRAPH 1: Shows the mean number of viable, attached A-549 cells, versus increasing concentration values of ascorbic acid (0, 100, 300, 500, 1000, 2000 µM respectively) for 24 hours of duration of treatment.



GRAPH 2: Shows the mean number of viable, attached A-549 cells, versus increasing concentration values of ascorbic acid (0, 100, 300, 500, 1000, 2000 µM respectively) for 48 hours of duration of treatment.



GRAPH 3: Shows the mean number of viable, attached A-549 cells, versus increasing concentration values of ascorbic acid (0, 100, 300, 500, 1000, 2000 µM respectively) for 72 hours of duration of treatment.

CONCLUSION EVALUATION

The research question was “How does the different concentrations of AA affect the number of viable, attached A-549 cells; in 24, 48 and 72 hours of treatment durations?” The aim was to investigate the effect of, increasing AA concentrations on the proliferation A-549 cells, in different treatment durations.

In the experiment, 0, 100, 300, 500, 1000 and 2000 μM of AA is put on the A-549 cells and the antiprolifertive effects of them are observed for 24, 48 and 72 hours duration of treatments.

My hypothesis was “As the concentration of AA is increased, the number of viable A-549 cells will decrease.”

Table 3 shows the calculated total number of cells in the 2ml mixture. At Table 3, in 24 hours, there is a general decrease from the “control” well to the “2000 μM” well. This is clearer in plates 1 and 5. Regarding the data of 48 hours, it is hard to make a general comment. However from “control” well to “500 μM” well there is generally an increase in cell number, then it starts to decrease again. In the data of 72 hours, there is not a certain increase or decrease for each well specifically. There are some abnormally high or low data obtained between the plates, in each time period. In plate 1 of “control” well of 24 hours there is an extraordinary cell number is observed than the other four plates. In plate 1 of “2000 μM” well, plate 5 of “control” well in 48 hours; in plate 5 of “control” well, plate 2 of “300 μM”, plate 3 of “1000 μM” well in 72 hours this problem can be observed again.

Table 10 shows the calculated mean of the data in five plates for each concentration value. It, indicates that; in 24 hours, the maximum number of cells are in “control” well. The number of cells in “100 μM” and “200 μM” well, is much less in number. In the number of cells treated with 500 μM there is observed little increase but then the decrease in number continued. There isn’t any cells found in the “2000 μM” well. In 48 hours, there is a little decrease in cell number from the “control” well to “100 μM” well, but then, the cell number increases in the “300 μM” well. After it the cells number decrease in again. Although, generally there is small increase or decreases in the number of cells, the decrease in the number of the cells treated for 24 hours is clearer (as in the cell number in “2000 μM” well of 24 hours). The data of 72 hours, is the least clear. There is an increase in the number of cells from “control well” to “500 μM” well. The decreases in the number of cells, can be observed after “500 μM” well. In this hour, the cell number in “control” well is less than the number of cells in “2000 μM” well. In general, the decreases in the cell number are observed after the “500 μM”.

Graphs 1, 2 and 3 shows the spread of data in table 10. The uncertain pattern of data until “500 μM” well and the decrease after “500 μM” can be seen in all of them. Especially the distinct decrease in the number of A-549 cells in “2000 μM” well is observed in Graphs 1 and 2.

ANOVA Single Factor test is done on the data obtained from 24 hours duration. P-value was 0.0014389 (Table7) when the Alfa value is 0.05. P-value is smaller than the Alfa value, so H_1 is accepted. Therefore there is a statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with AA for 24 hours. For data obtained from 48 and 72 hours durations, P-value was 0.07617568 (Table8), 0.3017722 (Table9) respectively when the Alfa value is 0.05. P-value is larger than the Alfa value, so H_1 cannot be accepted. Therefore there is not statistically significant difference between the mean numbers of viable, attached A-549 cells, which are treated with AA for 48 and 72 hours.

There are generally high SD, SE and 95% CI values for each treatment duration (Tables 4,5,6). These high values indicate that there are very serious systematic errors done in this experiment. The least of these values are observed in 24 hour duration of treatment (Table 4). So the most accurate results are obtained from this experiment. As the duration of treatment increases, the SD, SE and 95% values also start to increase. So there might be something that should be done in every 24 hours to prevent this inaccurate results. The fact that more extraordinary data observed in Table 3 as the time period increased may be the reason for this inaccuracy.

1) In Graph 2 and 3, the uncertain pattern of the data that is obtained from the cells treated with 0, 100, 300 µM of AA in 48 and 72 hours shows that there is an error. The general decrease in the number of the cells treated with AA for more than 500 µM shows that the effect of AA may be more effective after 500 µM AA. To get more determining results, the AA concentration could chose to be more than 500 µM. High concentrations of AA does not have serious damages to body. So, increasing the concentrations would be an effective solution for this experiment. Another reason of this error might be, I did not use ethanol when cleaning hemocytmeter because of its adverse effects. [25]. Some of the cells from the previous measurement might have stick to the surface of the hemocytometer and made me count more cells in the next measurements. To solve this problem, ethanol may be used to clean the surface of hemocytometer, but it should be waited between each measurement for the ethanol to be dried entirely.
2) In all of the time periods, in "2000 µM" well, there is a significant decrease in the cell number. In 24 and 48 hours the minimum number of cells are in "2000 µM" well. There is not any dead or alive cells found in 24 hours, might indicate that AA has shown a highly toxic effect, and this might be because of hydrogen peroxide comminuting the cell membrane. However in 48 hours cell number in "control" well is less in number compared to "2000 µM" well. Therefore the fact that it is found to be statistically insignificant, could be explained with an error done in control well of 48 hours. To prevent such mistake, more trials could be done.
3) The only decrease in the mean number of cells from "control" well to "2000 µM" well, can be seen in 24 hours. That may be showing that the AA is only effective in a 24 hours period of time. This may be because the cells use up the AA, and the viable cells that did not die in 24 hours start to proliferate again. So it could be better to add a freshly prepared AA solution per 24 hours.
4) The increase of cell number in general, from 24 to 48 hours is more than the increase of the cell number in general, from 48 to 72 hours (Table 10). This can be explained with the cells using up the medium to proliferate. The rate of proliferation decreases in time. The cells have more time to proliferate as the duration increases so a higher mean cell count with increasing duration of treatment is observed. The using up of the medium could be prevented by adding a new medium in every 24 hours.
5) The extraordinary results between the plates in Table 3 , shows there might be some gathering of cells in some parts of the solution and not a totally homogenous sample had been taken to count under microscope; although I used vortex mixer to mix the cell- trypan blue-AA mixture to resuspend the cells. Before I started counting the cells I prepared all of the 60 micro centrifuge tubes. The counting process takes about 3 hours. So the last counted tubes waited more than the others. I always counted the cells treated for 72 hours in the final place. So the cell suspension was more than the others. So in further investigations, the cells should be waited inside cell culture

[25] Method developement

test plates, and it should be transferred to the tubes whenever it is needed to be counted. Also, rather than taking 2 samples from each well, 3 samples can be taken.

Between the researches I have done, there was one which the effect of AA alone was observed on another lung cancer cell type (NSCLC) cells. Vuyyuri et al. tested that the effect of AA on NSCLC cells. *"Concentrations of AA from 50-500 µM showed a dose dependent decrease in the viability, with 500 µM causing a 40% loss in viability..."* [26] This research gave different results than my experiment. However, there are some similarities. Vuyyuri et al. also found that the effect of AA can be observed clearly after 500 µM. So for further studies, the least concentration of AA could be chosen to be 500 µM.

From literature, I did not find any researches where the concentration of AA is increased to more than 2000 µM. The toxic effect of AA, I observed in 24 hours, shows that these high doses might also be harmful for normal cells. For the further studies, the AA concentrations could be chosen between 500 µM and a larger number than 2000 µM, where the toxic effect of it can be observed. Beside cancer cells, normal cells should be treated with same concentrations. Because, if the same concentrations has a toxic effect on normal cells, it will be concluded that increasing concentrations to more than 2000 µM will not be solution against the proliferation of cancer cells.

[26] Vuyyuri, Saleha B. "Ascorbic Acid and a Cytostatic Inhibitor of Glycolysis Synergistically Induce Apoptosis in Non-small Cell Lung Cancer Cells." *PLOS One* 1 June 2013: E67081. Print.

APPENDICES

APPENDIX 1: ABBREVIATIONS AND UNITS:

- → WHO: World Health Organization
- → ROS: reactive oxygen species
- → AA: ascorbic acid (Vitamin C)
- → NSLC: non-small lung cancer
- → DMEM: Dulbecco's Modified Eagle's Medium
- → FBS: Fetal Bovine Serum
- → PBS: Phosphate Buffered Saline
- → DF: dilution factor
- → CI: confidence interval
- → SD: standard deviation
- → SE: standard error
- → SS: the sum of the squares.
- → df: the degrees of freedom
- → MS: masters- statistics
- → F: F-test
- → ANOVA: Analysis of Variance
- → M: molarity
- → µM: micromole
- → n: mole
- → µl: microliter
- → ml: millilitre
- → l:liter
- → mm^2 : square millimetre
- → cm^3: cubic centimetre
- → g: grams
- → Rpm: revolutions per minute

APPENDIX 2: UNIT CONVERSIONS:

- → µM: 0.001 mM, 1×10^{-6}M
- → µl: 0.001 millilitre,
- → ml: 0.001 litre
- → mm^3: 0.001 cubic centimetre, 0.001 millilitre
- → cm^3: 1 millilitre
- → g: 0.001 kilogram
- → mm^2: 0.01 square centimetre

APPENDIX 3: FORMULAS THAT ARE USED IN THE EXPERIMENT EXPLAINED:

→ **Calculating the Mass of Ascorbic Acid:**

10 ml water = 0.01L water

100 mM = $\frac{\text{Xmole}}{0.01\ L}$

X=10^{-3} mole

Molar mass of ascorbic acid=176.12g/mole

1mol ⟶ 176.12 g

10^{-3} ⟶ 0.17612 g AA should be used

→ **Calculating the Volume of Ascorbic Acid that will be put on the Cells:**

$M_1 \times V_1 = M_2 \times V_2$

2 ml medium=2000 µl medium-cell mixture

100 mM AA =100000 µM AA

For 100 µM AA;

100000 µM × X µl = 100 µM × 2000 µl

X= 2 µl of water-AA solution should be put on the cells.

For 300 µM AA; 6 µl of water-AA solution should be put on the cells.

For 500 µM AA; 10 µl of water-AA solution should be put on the cells.

For 1000 µM AA; 20 µl of water-AA solution should be put on the cells.

For 2000 µM AA; 40 µl of water-AA solution should be put on the cells.

→ **Calculating the Cell Number per Well:**

Cell number = cell count × 10^4 × dilution factor

→ **Calculating Dilution Factor:**

1ml suspension of cells + 100 µl of trypan blue

Total Volume=Dilution Factor= 1.1

APPENDIX 4: FORMULAS THAT AREUSED IN STATISTICAL ANALYSIS

A)Mean: In other words " average". The mean is calculated by adding up all of the values together, then dividing by the number of values.

$$\bar{x} = \frac{\sum x}{N}$$

∑: the summation
X: scores
N :number of scores.

B) Median: The "middle" value in the list of numbers. To calculate the median value, the list should be arranged in the ascending order first, then the formula is stated as:

If the total number of numbers(n) is an **odd number**, then the formula is given below:

$$Median = \left(\frac{n+1}{2}\right)^{th} term$$

If the total number of the numbers(n) is an **even number**, then the formula is given below:

$$Median = \frac{\left(\frac{n}{2}\right)^{th} term \; + \; \left(\frac{n}{2}+1\right)^{th} term}{2}$$

C) Standard Deviation (SD): The amount of variation or dispersion from the average**.**

$$\sqrt{\frac{\Sigma(X - \bar{X})^2}{(n - 1)}}$$

where:

X = each score
$\bar{X}$ = the mean or average
n = the number of values
Σ means we sum across the values

D) Standard Error (SE): An estimate of how far the sample mean is likely to be from the population mean.

E) 95% Confidence Interval: Confidence intervals consist of a range of values (interval) that act as good estimates of the unknown population parameter. This value is represented by a percentage, we express that 95% of the observed confidence intervals will hold the true value of the parameter.

$$\textit{95\% Confidence Interval} = \textit{Standard Error} \times T_{inverse}$$

APPENDIX 5: EXPLANATION OF TERMS:

Tumor Suppressor Gene*:* is a gene that inhibits the cell division and survival.

DNA Repair Gene: provides the future changes of the genes.

Collagen: The main component of connective tissue, it is the most abundant protein in mammals.

Ground Substance: An amorphous (without shape, form) gel-like substance surrounding the cells.

Proliferation: Increase in cell number.

Metastasis: The spread of a cancer or disease from one organ or part to another not directly connected with it.

Attached-Unattached Cells: After plating and incubating, some cells stick to the surface of the filtered flask to proliferate. They are called "attached cells". The others, float inside the medium. These are called "unattached cells". When we aspirate the medium, we also aspirate the unattached cells. However attached cells are not aspirated with the medium. As they are also needed for the research, we detach them from surface by washing the filtered flask with PBS.

Trypan Blue: Trypan blue is a die that cannot pass through the cell membranes of viable cells because of the proteins on their membrane. However, the proteins on the cell membranes of dead cells are no more working, so trypan blue can easily get inside these cells and make them look blue under the microscope. So that it is possible to see both the viable and dead cells at the same time.

Antiproliferative: A substance used to prevent or retard the spread of cells, especially malignant cells, into surrounding tissues.

MTT assay: The viable cells reduce the MTT salt to a blue formazan crystal so that the number of viable cells could be determined.[27]

Trypan Blue Exclusion Assay: Trypan blue is a die that cannot pass through the cell membranes of viable cells because of the proteins on their membrane. However, the proteins on the cell membranes of dead cells are no more working, so trypan blue can easily get inside these cells and make them look blue under the microscope. So that it is possible to see both the viable and dead cells at the same time.[28]

[27]See Appendix 6: Explanation of Several Methods Used in the Experiment (Trypan Blue Exclusion Assay)
[28] Freshney, R. *Culture of Animal Cells: A Manual of Basic Technique*. New York: Alan R. Liss, 1987. 117. Print

APPENDIX 6: EXPLANATION OF SEVERAL METHODS USED IN THE EXPERIMENT:

1. **PREPERATION OF MEDIUM AND ADDITION OF A-549 CELLS:**
    1. take one bottle of DMEM high glucose and remove 55ml out of the bottle. Add 50 ml of FBS and 5ml of pen/strep solution.
    2. A549 cells that are maintained in 75cm filtered flasks in the incubator and their media were changed every 3 days.
    3. take a flask of A549 cells
    4. aspirate the medium with a vacuum pump
    5. wash with 10ml 1xPBS once and aspirate
    6. add 3ml of 1x trypsin (diluted from 10x with 1XPBS) and incubate in the incubator for 5min or till the cells are detached.
    7. add 7ml of medium to inhibit trypsin and collect the cells to a 15ml falcon tube
    8. count the cells with a haemocytometer and plate 100000 cells/well and complete to 2ml with medium

2. **METHOD FOR COUNTING WITH HEMOCYTOMETER:**

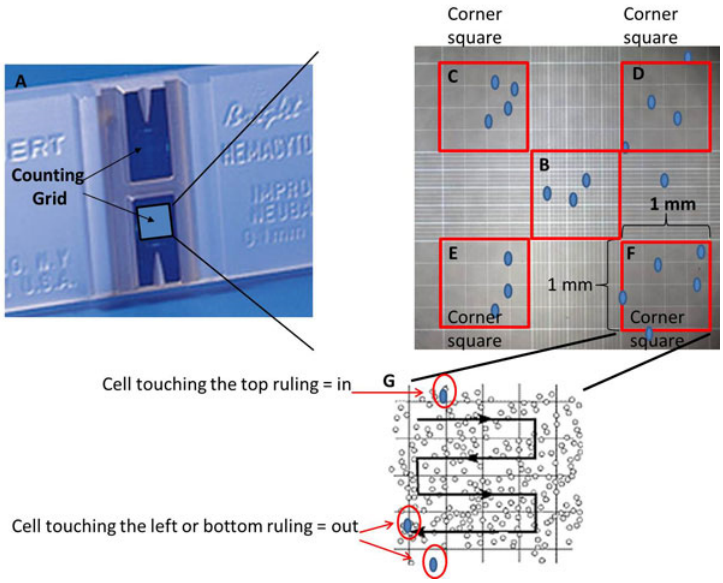


Figure 2:Figure showing the structure of hemocytometer and counting with hemocytometer method.[28]

1. Take 10µM of sample from the 2ml of cell-AA-trypan blue mixture inside the micro centrifuge tube (by using the 1-10 µl micropipette)
2. Load the 10 µM sample on the hemocytometer (between the counting grid and the cover slip).
3. Put the hemocytometer under microscope with 10x magnification.
4. Count the cells inside the four squares at the corners of the counting grid.
5. Inside the first corner square (the most left the most top), count by starting from the littelest square at the left top and count the cells row by row.
6. When passing to the row below, pass under the littlest square from littlest square that you counted least. (shown in figure 2)

[29] Web. 12 Jan. 2015. <http://bitesizebio.s3.amazonaws.com/cellculture/

7. Repeat steps 5 and 6 for the other three squares. (2nd square: the most right the most top, 3rd square: the most right the most bottom, 4th square: the most left the most bottom)
8. After you finished counting the four squares, add the four numbers you found, together.
9. Divide the new number you found to four.
10. In cases where there are too many cells (more than 60) only count the square in the middle and note it. (Do this to prevent the mistakes that might occur when counting the too many cells.)
11. Take another 10µM of sample from the 2ml of cell-AA-trypan blue mixture inside the same micro centrifuge tube (by using the 1-10 µl micropipette)
12. Repeat the steps 2 to 10 for this sample again.
13. Then take the mean of the two number of cells that you obtained fro each sample.
14. If taking mean gave you decimal numbers roll them to whole numbers, by looking at the standard decimal number rounding method.
15. Note the final number you found

NOTE: Always count the cells which are touching to the most top line. Do not count the cells which touches the most bottom and the most left lines. (shown in figure 2)

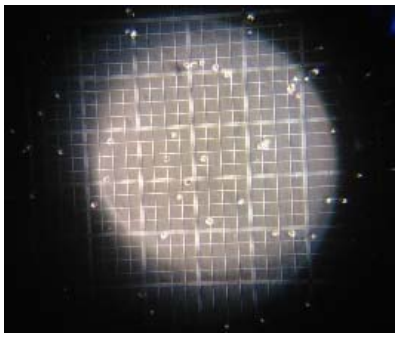


Image 6: Photograph of counting grid under 10x magnification, that is taken while the experiment.

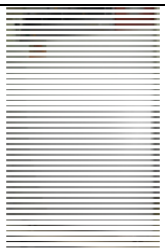
3. STANDARD DECIMAL ROUNDING METHOD :

Rounding decimals is very similar to rounding other numbers. If the hundredths and thousandths places of a decimal is forty-nine or less, they are dropped and the tenths place does not change.

For example, rounding 0.843 to the nearest tenth would give 0.8.
If the hundredths and thousandths places are fifty or more, the tenths place is increased by one. For example, the decimal 0.866 rounded to the nearest tenth is 0.9.

You round a number if the number on the far right is higher than 4.
You leave the number alone if the far right number is 4 and lower.
The far right number will only round the number that's right left of it.
Example: 1.316 rounded 1.32
6 (the far right number) is only going to effect 1 (the number right left of 6)

APPENDIX 7: IMAGES AND TRADES OF THE MATERIALS THAT ARE USED IN THE EXPERIMENT:

MATERIALS:	Figure of the material:	Trade:
DMEM		Lonza®
Trypsin		Lonza®
FBS		Biochrom®
PBS		Lonza®
L-ascorbic acid		Applichem®
5% CO2, 37 C Humid Cell Culture Incubator		Shell Lab®
Laminar flow		LN90®
Centrifuge machine		Nuve®
Inverted Microscope		Leica DMIL
Water bath		Nuve®
Filtered flask		
Micro Centrifuge Tubes		

Cell culture test plates		Orange Scientific®
Hemocytometer		Marienfeld®
Micropipettes		

TABLE 11: Table showing the trades and the pictures taken of the materials used in the experiment.

APPENDIX 8: DIAGRAMS AND IMAGES:

CELL CULTURE TEST PLATES:

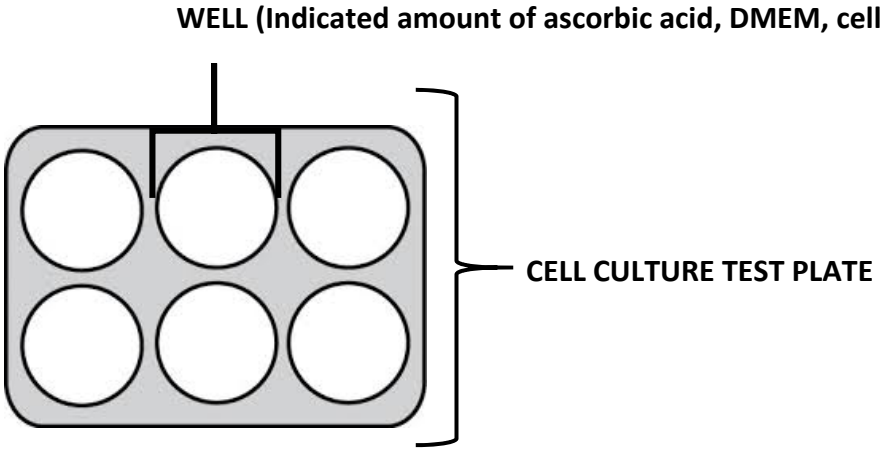


Diagram 2: Diagram indicating the cell culture test plate with six well

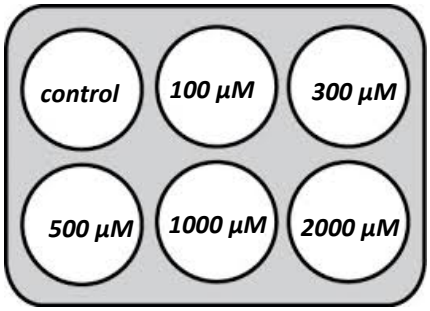


Diagram 3: Diagram showing the labeling I did on each well on a plate. I used 5 plates like this in one experiment. So each plate is a trial. I have five trials in total.

MICRO CENTRIFUGE TUBES:

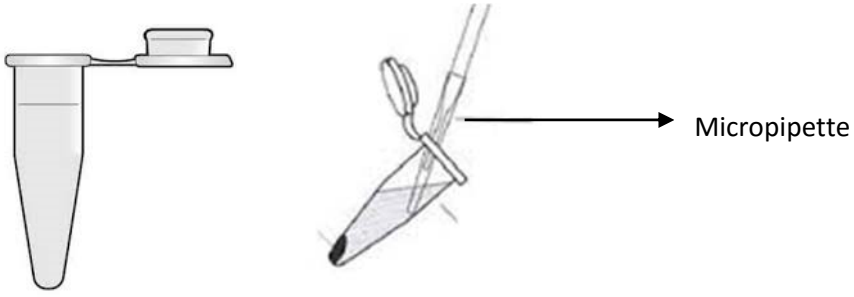


Diagram 4: Diagram showing the 2 ml centrifuge tube that is used in the experiment and the pellet that is formed by the suspended cell. The vortex mixer is used to resuspend that pellet. [30]

[30] Web. 12 Jan. 2015. <http://infopak.co.za/index.php/microcentrifuge-tubes.html>.

MICROPIPETTE:

Diagram5: Diagram showing the structure of a micropipette. [31]

THE TRANSFER OF AA- CELL MIXTURE INTO MICRO CENTRIFUGE TUBES:

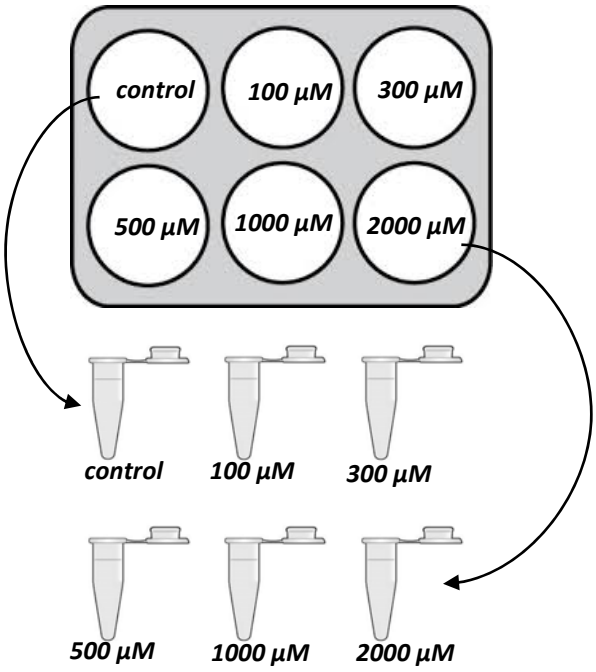


Diagram 6: shows how I labeled the 2ml microcenrifuge tube and the mixture in the corresponding well is poured by the

[31] Web. 12 Jan. 2015. <https://encrypted-tbn1.gstatic.com/images?q=tbn:ANd9GcTmqYhmGbLftJQSKo2UiJ-pzV52aHOs47RRjVSZVVluu4DaabPT>.

FILTERED FLASK:

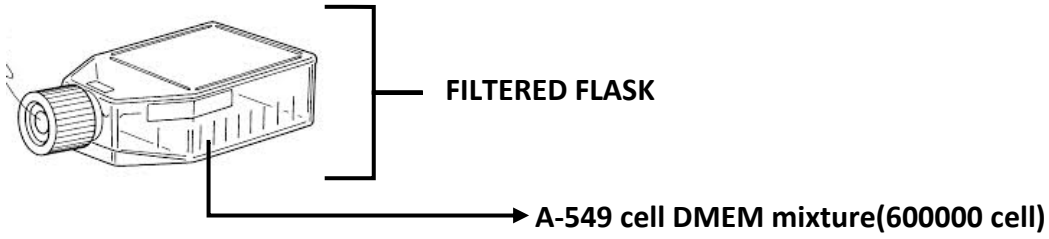


Diagram 7: Diagram indicates the how medium and cells are inside filtered flask. [32]

[32] "Patent EP0890636A1 - Culture Vessel." *Google Books*. Web. 12 Jan. 2015. <http://www.google.com/patents/EP0890636A1?cl=en>.

IMAGES FROM THE EXPERIMENT:

Image 1: Picture showing the process of labelling each plate and each well under laminar flow.

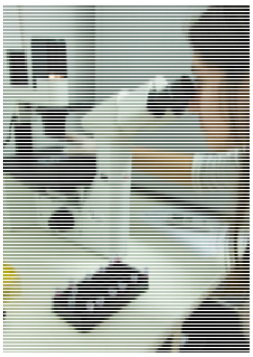


Image 2: Picture showing how I did the counting under 10x magnification inverted microscope, with hemocytometer.

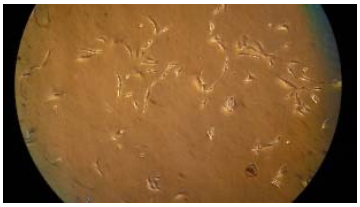


Image 3: Picture taken from the inverted microscope with 10x magnification. This picture shows the attached cells to the cell culture test plate, before the trypsinizing (detaching) and counting. This is the image of the control well of 24 hours.

Image 4: Picture taken from the inverted microscope with 10x magnification. This picture shows the tripsinized (detached) cells from the cell culture test plate, before the counting. This is the image of the control well of 24 hours (the same group of cells in image 3 are now detached).

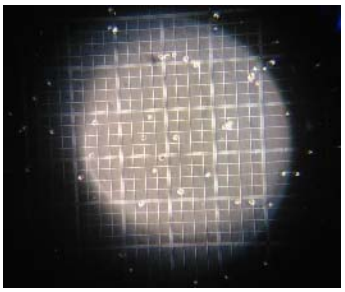
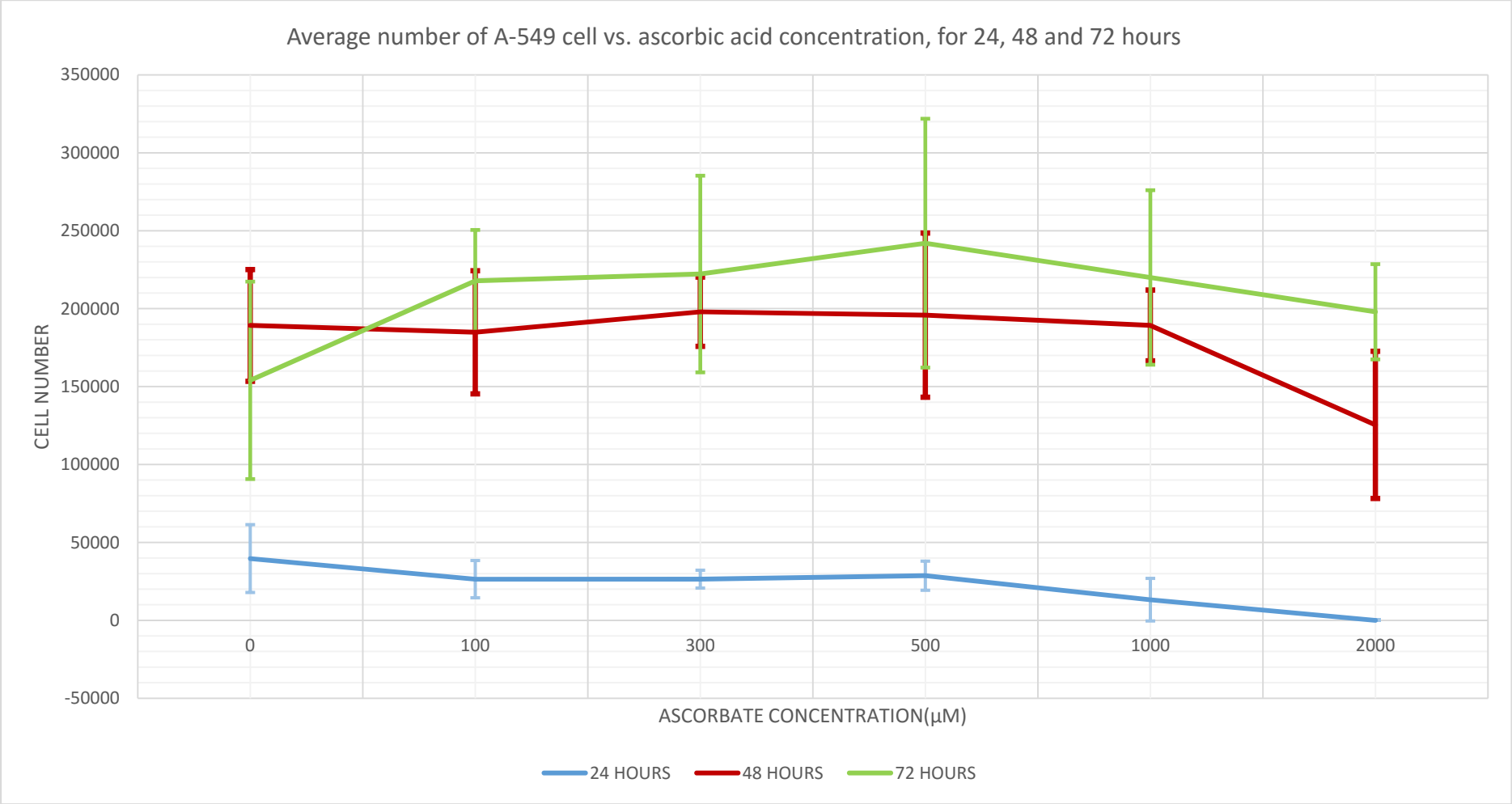
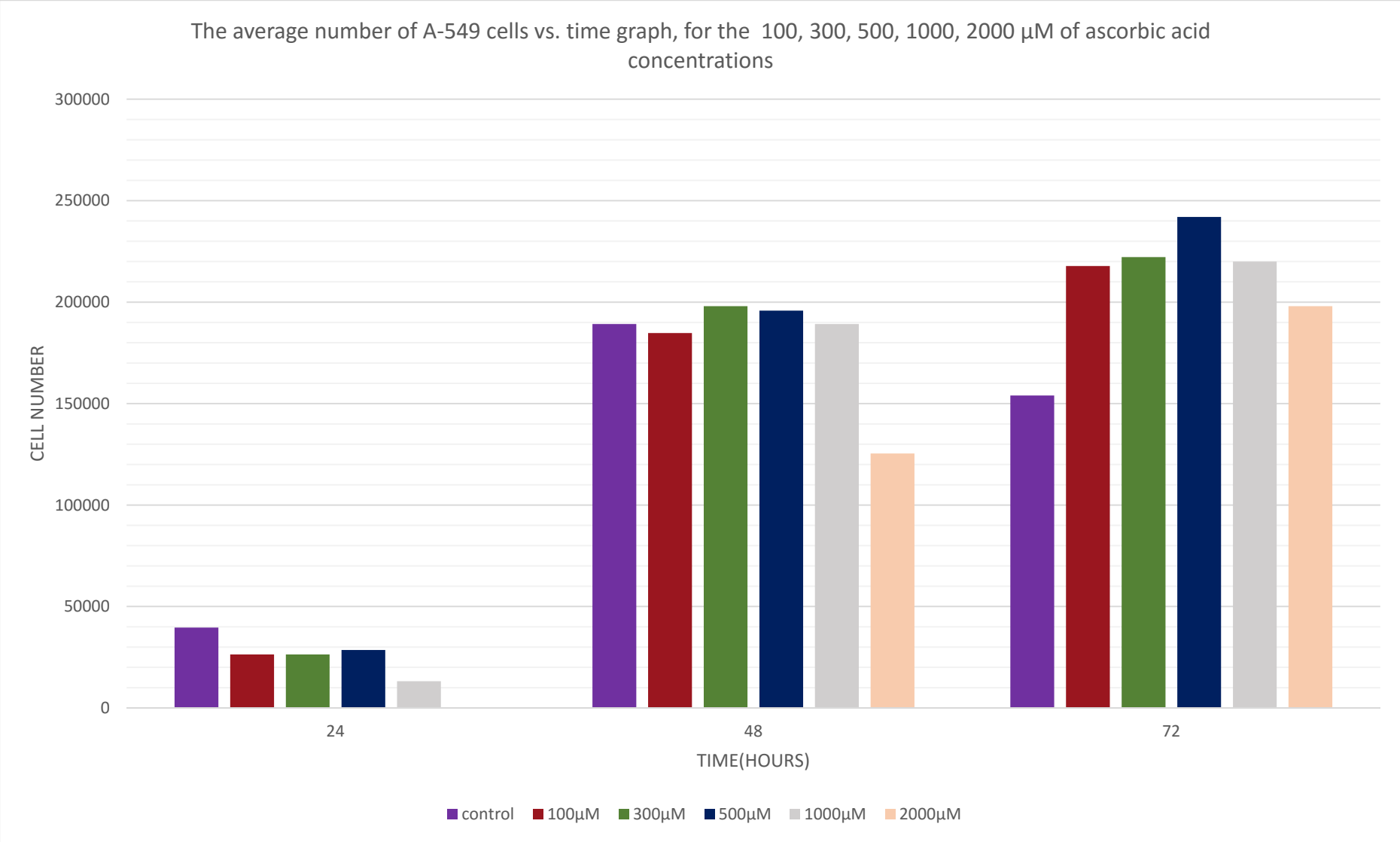


Image 5: Picture taken from the inverted microscope with 10x magnification. This picture shows the cell sample taken from the plate 1, control well in 24 hours (the same group of cells in image 3 and 4) Now, the cell sample is loaded in the hemocytometer to be counted.

APPENDIX 9: OTHER INDICATIONS OF THE EXPERIMENTAL DATA



GRAPH 4: Shows the mean number of viable, attached A-549 cells, with increasing concentration values of ascorbic acid (0, 100, 300, 500, 1000, 2000 µM respectively) for each duration of treatment (24, 48 and 72 hours)



GRAPH 5: Shows the mean number of viable, attached A-549 cells, with increasing duration of treatment (24, 48 and 72 hours respectively) for each concentration of ascorbic acid (0, 100, 300, 500, 1000, 2000 µM respectively).

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